Plant Cell Monographs

Peter Nick
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Applied Plant Cell Biology

Cellular Tools and Approaches for Plant Biotechnology

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Preface

The pronounced developmental and metabolic capacities of plant cells provide the base of agriculture and green biotechnology and have been studied very intensively with respect to its genetic aspects. However, it becomes increasingly clear that the potential of applied plant science can only be fully exploited when we grasp the underlying cellular mechanisms such as compartmentalisation, intracellular transport, cell differentiation, and communication. To understand biotechnologically relevant molecular mechanisms for stress tolerance and accumulation of interesting compounds, we need a strong background in molecular cell biology.

The so-called Green Revolution has ensured a reliable and cost-efficient supply of a growing population with food resources. A major part of this success story is based on three factors: advances in plant nutrition, advances in plant protection, and advances in plant genetics. The challenge of the coming years will be to reconcile increased agricultural productivity with sustainability. The potential of plant nutrition and chemical plant protection has been basically exploited; what remains, are advances in generating new genotypes with improved productivity, tolerance to biotic and abiotic stresses, and improved or even novel metabolic potential. The book is therefore divided into four parts that deal with the control of development, the control of stress tolerance, the control of metabolic activity, and novel additions to the toolbox of modern plant cell biology.

A general theme subtending all fields of contemporary plant biotechnology, often in an implicit manner, is the regenerative ability of plant cells, which is much more pronounced as compared to animals. The book will therefore begin with a *prologue* describing how the current concepts on the "totipotency" of plant cells has evolved in the historical context. Interestingly, already the beginnings of plant cell biology were linked with application, namely the intention to understand the pronounced regenerative ability of plants and the mechanisms of their vegetative propagation. The prologue of this volume will therefore recapitulate this development that began in the second half of the nineteenth century in Central Europe and has been extremely fruitful since. From the plant-specific concepts created at that time, meanwhile a plant-specific concept of stem cells has emerged that is progressively supported by detailed molecular mechanisms. In the field of application,

these concepts have helped to lead plant regeneration as central strategy for green biotechnology from the realm of pure empiry into a technology that can be controlled and designed on a scientific base. This is of particular importance for so-called recalcitrant systems such as particular cell strains, organs or organism that do not respond to conventional techniques of vegetative propagation.

Control of Growth and Development

The first part of the book will address the cellular base of growth and development. The highly plastic plant cells can respond to biotic and abiotic factors and integrate information over the state of their environment with developmental programs through signalling pathways converging at the chromatin level. Signal-dependent changes of gene expression, in turn, will be expressed as adaptive changes of growth and morphogenesis that help to cope with environmental challenges.

One of the central players for this adaptive morphogenesis is the plant cytoskeleton. A plant-specific theme is the so-called cortical cytoskeleton that, in interphase cells, is intimately linked to the cell wall, whose physical properties define the turgor-driven growth of plant cells, providing versatile tools for biotechnological manipulation of plant architecture. However, during the last years, a novel function of this cortical cytoskeleton has emerged: it not only acts as downstream effector for the environmental response, but conveys a second function situated upstream in the sensory process itself. Cortical microtubules participate in the sensing of abiotic and biotic stress factors, which opens new possibilities for application, for instance, in the context of cold tolerance of crop plants.

The plasticity of plant development is organised and balanced by a dynamic "hormonal status," which is a process integrating the current cellular content of a phytohormone (depending on synthesis, transport and metabolic inactivation), and the activity of perception and signalling in response to this cellular hormonal content. The central players are auxins and cytokinins, and, for both hormones there is a long tradition of practical application – it is not exaggerated to say that green biotechnology would have been impossible without the discovery of auxins and cytokinins. Two chapters therefore summarize the current state of the art, but also applications based on new synthetic analogues as well as transgenic approaches targeting hormonal transport, metabolism, and local maxima/minima providing a whole plethora of tools and strategies for biotechnological manipulation.

A further plant-specific target for application is the manipulation of programmed cell death for development, addressed in the two final chapters of the first part. It seems that specific cell lineages have to be assigned for cell death in order to activate the developmental potency of other lineages to turn into stem cells. To get control of the astounding regenerative capacities of plants will therefore require cell-type specific manipulation of programmed cell death. The signalling underlying this fatal decision of individual cells between "Life" and "Death" is described in

one of the two chapters in the context of somatic embryogenesis, a field that provides the technological choice for industrial propagation of economically important species, especially conifers. Breeding has been the crucial factor for the success of the Green Revolution. The final chapter is therefore dedicated to pollen development as key target for breeders. It is the pollen that, in nature, ensures the sexual motility required to sustain efficient gene flux supporting the genetic diversity of a species. Because many crop plants are self-fertile, breeders need to suppress selfing in order to maintain heterozygosity. Since the days of Gregor Mendel, this is achieved by manual removal of the stamina in the receiving flower of a cross – a technique as cumbersome as it is expensive. A comprehensive overview on the cellular events regulating pollen genesis, maturation and development, and their underlying molecular mechanisms has opened numerous venues to control and manipulate male fertility in numerous important crop plants which are of high relevance for breeders.

Stress Tolerance

Since plants cannot run away, they have to adapt to environmental challenges to a much stronger degree as compared to animals. Stress tolerance is progressively seen as the major factor for the future of agriculture and its sustainability. Desertification, urbanisation and the ongoing increase of the world population require agriculture to be extended to so-called marginal lands that are often challenged by undesired chemical elements released from acidic soils. To safeguard yields, at present, extensive plant protection is required, progressively arising public concern. Thus, not only the tolerance to abiotic stress, but also cellular mechanisms of innate immunity have shifted into the focus of application.

The part starts with a chapter on the cellular responses to Zn and Cd as central heavy metals in polluted soils. Interestingly, the role of Zn is a bit scintillating since it is also required as trace element, however, in very low concentrations. To assess the toxicity of these elements, it is relevant to understand, not only on a cellular but on a systemic level, how Zn and Cd actually enter the root of a plant, and which barriers have to be crossed on their path from the root to the aerial organs. The detailed knowledge of the molecular and cellular mechanisms relevant for uptake have, again, stimulated novel applications to manipulate this uptake, whereby the aim can be diverse – on the one hand, it is beneficial to prevent these metals from accumulation in those parts of a crop plant that actually will enter the food chain. On the other hand, it can also be rewarding to stimulate uptake, when heavily contaminated soils are to be sanitized by so-called phytoremediation.

Also the second chapter deals with an element that can be either noxious or beneficial depending on its dose: Uptake and metabolism of selenium is closely related with the compartmentalisation of sulphur. Again, molecular information on transporters and the mechanisms of intracellular sequestrations likewise allows to prevent excessive accumulation of Se in crop plants, or to safeguard a minimal

daily uptake of Se in regions depleted from this exotic element. The synthesis of sulphur-rich secondary compounds, which is also reviewed in this chapter, not only bears on redox homeostasis crucial to control otherwise destructive oxidative burst triggered in response to abiotic stress, but also connects with the field of secondary metabolism not only highly relevant for biomedical applications of plant products, but also for the field of defence against pathogen attack.

Tolerance against biotic stress is in the focus of the third chapter of this part: the plant flagellin receptor as central player of plant innate immunity has been identified as crosspoint that allows to tune defence with signalling from hormonal or developmental signalling. Defence represents a considerable investment that impinges on the resources available for growth, development and agronomical yield. As the human immune system must be strictly controlled in time and amplitude, also plant immunity has to be confined. The flagellin receptor, as worked out in this chapter, undergoes endocytosis, and this phenomenon emerges as a switch that not only allows the plant to tune the amplitude of the defence response with the amplitude of the pathogen challenge, but also to decide which type of defence response is selected. No wonder that this event is also target for microbial effectors that have evolved to quell plant immunity. Again, detailed cellular and molecular insights into defence signalling have allowed to design novel strategies to manipulate plant defence as central element of more sustainable approaches to plant protection.

Plant Metabolism

Plants produce a vast array of natural products (primary and secondary metabolites), many of which have evolved to confer adaptive advantages against biotic and abiotic stresses in natural environments. Often, certain species produce and accumulate particular metabolites. The transcription factors controlling plant metabolic pathways leading to biosynthesis of flavonoids, glucosinolates, lignins, and of terpenoid indole alkaloids have been isolated and characterised. This information can now be used to decipher the molecular mechanisms responsible for coordinate induction of transcriptional networks in particular cell types. But it can also be used to engineer plant metabolism. This is exemplarily demonstrated in the three chapters of the third part.

The cytoskeleton represents an attractive target for therapeutical compounds, because via the cytoskeleton, unregulated cell divisions underlying the growth of cancer can be controlled. Classical anti-cancer drugs such as colchicines, vinca alkaloids, taxanes, and podophyllotoxins are of plant origin. Due to their complex chemical structure, they cannot be synthetised technically, but have to be extracted directly from plant cells. The market for anti-cancer compounds is tremendous, and the need for more specific compounds has stimulated high-throughput screening of secondary plant products based on bioassays with animal cells. The first chapter not only surveys the fascinating field of biomedically active plant compounds, but also

describes a very innovative strategy, how new anticytoskeletal plant compounds can be isolated and identified.

In addition to the search for novel compounds, also the existing knowledge on the medical effect of traditional plant ailments is progressively mined by comprehensive approaches. Especially India and China have established very elaborated traditional healing systems that are based on the experience from several thousands of years and await to be merged with contemporary science on molecular modes of action and cellular responses triggered by specific secondary compounds. In one of the probably most comprehensive approaches available so far, reviewed in the second chapter of the third part, the traditional medical plant Ashwagandha (*Withania somnifera*) has been thoroughly analysed with respect to the developmental and tissue-dependent profile of medically active metabolites, the chemistry of the underlying pathways, the enzymes driving the steps of these pathways, the regulation of the genes coding for these enzymes, and the diversity of chemically different genotypes (so-called chemovars) within the species.

Wood represents the most important biomaterial on this planet, and this biomaterial comes in a huge multitude of versions differing in mechanical, esthetical and biological properties. In contrast to other plants used by mankind, domestication of wood plants is still in an early phase. Humans exploit wood as if they still were in the hunter-gathering phase prior to the neolithic revolution. This cannot go on for long, since non-sustainable exploitation of wood has wiped out most of the rain forests on our planet. However, the secondary metabolism of lignin is quite well understood, and the last chapter of the third part describes novel strategies for genetical engineering wood with preset properties. It is to be hoped that a smart biotechnological use of the molecular and cellular mechanisms defining wood quality will allow for sustainable alternatives to the wild deforestation in developing countries.

The Cell Biology Toolbox: New Approaches

The last two decades were mainly shaped by breakthrough technologies of molecular biology, often linked to high throughput "-omics" approaches. The renewed interest in epigenetic phenomena has reconfirmed the importance of spatial organisation, for instance, when gene activity is linked with the spatial organisation of chromatin. Stimulated by this shift in concept, several techniques and experimental models have experienced a renaissance, which is now firmly rooted to the molecular base of epigenesis. It is to be expected that the postgenomic era will see a rising impact of cell biology. However, the toolbox for cellular manipulation is still to be developed – here it is not sufficient to make specific molecules appear or disappear in a global manner. In cell biology, it is space and time that matter. Therefore, the last part of the book is dedicated to the methodological base of applied plant cell biology.

Flow sorting, widely used in medicine, has been successfully transferred to plant cells and developed into a very powerful approach to collect and purify specific chromosomes. The success of this technology, as pointed out in the first chapter of the final part, depends on the methodological details – the secrets behind the success story of this technology are revealed and discussed as well as the versatile application of this tool. By far the most important application in plant science is the sorting of nuclei to determine nuclear DNA content. The information derived from this approach is of crucial importance for the design and management of genome projects, but also has advanced diverse fields of plant research as from understanding regulation of the cell cycle till detection of incipient speciation in evolutionary studies.

Concepts of the cell have been shaped by the available technologies: the advent of biochemistry and enzymology in the 1950s and the concept of the cell as a "bag of enzymes" was followed by a fascination for the internal structure of cells made visible by the brand-new technology of electron microscopy, and the concerted effect of green fluorescent proteins and advanced fluorescence microscopy revealed a surprisingly dynamic cellular structure – the term "cytoskeleton" coined from (fixed and sectioned) electron microscopical images was replaced by a dynamic equilibrium of rapidly cycling microtubules and actin filaments. As shown by the second chapter of the final part, photoconvertible reporters might become the next methodological advance with prospective conceptual consequences: These reporters not only allow to see specific molecules in living plant cells, but to visualise their history and compartment-specific reporters allow to get valuable insight into the dynamic compartmentalisation characteristic and essential for the metabolic proficiency of plants.

Cell biology has profited tremendously from cellular models. As pointed out in the prologue chapter, the race for immortal cell lines was initially won by the animal field. However, after auxins and cytokinins had been identified as reprogramming factors for the developmental potency of plant cells, the plant fields caught up immediately. Plant cell strains, reviewed in the final chapter, have become a unique experimental model to study the cellular aspects of cell division, cell expansion, cell morphogenesis, senescence and cell death, which are often not experimentally accessible in the context of an organ composed of complex tissues. Especially for the widespread tobacco cell line BY-2, fluorescently tagged markers for different organelles and proteins have been established such that non-invasive life-cell imaging has been thoroughly integrated providing powerful tools for molecular cell biology. However, against the conventional prejudice that cell lines are just chaotic masses of rapidly dividing cells, a closer look reveals that these cell strains undergo a defined, albeit strongly reduced, developmental programme. By this property they turn into unique experimental models for plant cell phenomics allowing integrated molecular, biochemical, cytological and morphological analysis in living, intact plant cells in the absence of the correlative (especially mechanical) constraints of the complex organism, such that the primordial, basal morphogenetic potential of individual plant cells becomes accessible to analysis. Thanks to their enormous reproductive ability, some cell strains (with

tobacco BY-2 being the most prominent example) have been successfully adapted to molecular farming. Especially for products tailored to specific medical applications, where diversity and versatility of molecular farming is more relevant as compared to large yield, plant molecular farming is economically superior as compared to animal or bacterial models. The considerable potential of this accession to the cell biology toolbox still provides space for exploitation.

Cell biology has been traditionally perceived as a "pure", "fundamental" field of science striving to understand, the building blocks of all living beings. It has been overlooked that cell biology, by its very essence, subtends and supports all biotechnological applications. This is prominently true for plants, where organisation is much deeper routed in the versatility of individual cells. The mission of this book is to show that plant cell biology evolved from the continuous and intensive dialogue between fundamental research and application – a non-interrupted line of tradition that has been successfully pursued over almost more than 150 years. The next step for this fruitful liaison will be to integrate the challenge to develop sustainable solutions for the agriculture of the twenty-first century.

Karlsruhe, September 2013 Peter Nick
Prague, September 2013 Zdeněk Opatrný

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From Němec and Haberlandt to Plant Molecular Biology

Zdeněk Opatrný

Abstract The high regenerative capacity of plants is a crucial feature of their life strategy. It is an essential part of the mechanisms that both allow these sessile organisms to repair injury caused by pathogens, herbivores and abiotic factors and to undergo rapid vegetative reproduction, so allowing them to dominate in particular environmental niches. Furthermore, various forms of natural regeneration contribute to techniques that are widely used in plant propagation and plant breeding. The biological nature of plant regeneration has been studied since the very beginnings of plant physiology as a science. Research on regeneration of intact plants in vivo was conducted by Bohumil Němec, and early studies of in vitro regeneration in plant tissue cultures were carried out by Gottlieb Haberlandt. At this stage, however, suggestions that somatic plant cells possessed a regeneration "totipotency" were in practice often not acknowledged. Nevertheless, real experiments demonstrated that the regenerative ability of particular cells and tissues is clearly determined by the specific interplay of both genetic (or epigenetic) and physiological factors. This makes some systems "nonresponsive" to the standard regeneration procedures. This regenerative recalcitrancy hampers both the routine vegetative propagation of various plant species and the construction of genetically modified crops. This chapter addresses the basic historical background of studies on plant regeneration and discusses both the results and ideas acquired by means of classical anatomical and morphological studies in the light of our current state of information obtained using modern molecular techniques. The present knowledge of plant regeneration is also viewed in the light of studies of structure and function of the "stem cell niches" of multicellular organisms, examining their role in the ontogenesis of intact plants and in the processes of embryogenesis and organogenesis in vitro. With reference to other chapters in this book, the role of genetics for the realisation of these processes as well as the role of various regulatory factors, of

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both exogenous and endogenous nature – especially phytohormones – is also examined. The importance to classify regenerative processes unambiguously using exact terminology (in the context of the allied field of regenerative medicine) as a prerequisite for the formation and validation of appropriate working hypotheses is discussed. Finally, this chapter summarises the main problems of current research on regenerative processes in plants and outlines possible directions for solving problems of recalcitrant materials in the context of their use for application.

1 Cell Biology in Plant Propagation and Breeding

1.1 Němec and Haberlandt: Early Contributions

Self-repair of individual somatic cells is an almost universal property of multicellular organisms, both plants and animals. This ability is necessary to allow continuous replacement of cells lost through senescence or damaged by wounding. In both lower animals and most plants, the regeneration process can lead to the formation of new organs. In plants particularly, various regeneration strategies have culminated in mechanisms of vegetative propagation that either complement or even entirely substitute sexual propagation.

Empirical knowledge of regeneration in various plant species had accumulated over thousands of years of practical gardening and agriculture. However, the knowledge of the cellular and physiological mechanisms underlying these processes is relatively recent and directly linked to the recognition of plant physiology as a new research discipline in the mid-nineteenth century, in the development of which Central European scientists played a prominent role.

From this group of scientists, at least the name of Julius von Sachs (1832–1897) is probably familiar to most recent plant physiologists. "At an early age he showed a taste for natural history, becoming acquainted with the Breslau physiologist Jan Evangelista Purkyně. . ." to quote the recent Wikipedia page. But the role of Jan Evangelista Purkyně (1787–1869), both in the life and education of young Julius Sachs and in the establishment of cellular physiology as a new science, was much more pronounced (see Žárský 2012 for details). In general, Purkyně is best known for his discovery of *Purkinje cells* in 1837, the large neurons with many branching dendrites found in the cerebellum; for the discovery of *Purkinje fibres*, the fibrous tissue that conducts electrical impulses from the atrioventricular node to all parts of the heart ventricles in 1839; and for his discoveries of Purkinje images, reflections of objects from structures of the eye, and the Purkinje shift, the change in the brightness of red and blue colours as light intensity decreases gradually at dusk. He introduced the scientific term *plasma* for the component of blood left when suspended cells have been removed and *protoplasma* – the substance found inside the cells. Purkyně was the first to use a microtome to make wafer-thin slices of a tissue for microscopic examination and was among the first to use an improved version of the compound microscope.

Purkyně was born in Bohemia, then part of the Austrian monarchy, now Czech Republic. In 1818, he graduated from the Charles University in Prague with a degree in medicine and was appointed there as professor of physiology. In 1839, he established the world's first department of physiology at the University of Breslau in Prussia (now Wrocław, Poland) and in 1842 the world's first official animal physiology laboratory.

He returned to Prague in 1849 to become professor at the Charles-Ferdinand University, where he personally continued mainly his animal studies. However, through his students and assistants, he also investigated the structure and function of both anthers and pollen grains and strongly promoted plant physiology as a research discipline.

In 1851, Purkyně's family living in Prague took care of a 19-year-old student from Breslau, Julius von Sachs (1832–1897), whose parents had recently died. Sachs entered the Charles-Ferdinand University, became Purkyně's assistant, and in 1856 received a degree of Doctor of Philosophy. He established himself as "Privatdozent" of plant physiology at the same university, where he remained for 3 years, carrying out research and lecturing to students. Here, and in the following years in Germany at the Agricultural Academy at Poppelsdorf, now part of the University of Bonn, he wrote the first world textbook of experimental plant physiology, published in 1865.

Sachs played a leading role in the development of plant physiology in the second half of the nineteenth century. He was an extraordinary teacher and scientist and greatly influenced the progress of botany and horticulture, not only in Central Europe but also in Britain and America. Among other achievements, he is noted for laying the foundation of microchemical methods. He proposed numerous methods for plant culture and research, including hydroponics as a tool to study plant nutrition. He described the morphological and physiological details of seed germination. In connection with his photosynthetic studies, he described the appearance of starch grains in plants as the first visible product of this process, and during work on plant flowering, he indicated the role of plant growth substances in its regulation – until then unknown. In this way, he further elaborated the pioneering discovery of Charles Darwin (Darwin 1880) on the "influences capable of regulating phototropic plant growth" (see also chapter by Skůpa et al., this volume).

The pioneering work of Sachs on the study of plant growth and development was carried forward by two other well-known Central European scientists: Gottlieb Haberlandt (1854–1945) and Bohumil Němec (1873–1966).

To students of plant physiology, their names are mainly known from basic textbooks which describe the starch-statolith hypothesis of gravity perception by columella cells of plant root apices. The authorship of this hypothesis can be assigned to either of them – or to both – since they published it independently in two separate communications not only in the same year (1900) but even in the same issue of Berichte der Deutschen Botanischen Gesellschaft (Němec 1900; Haberlandt 1900). With exception of this coincidence, their earlier and later careers

differed not only in their research priorities but also in the methodology they applied to study the processes of plant growth and development.

Haberlandt, later denominated worldwide mainly as the "father of plant tissue culture" for his ideas summarised in his publication "Kulturversuche mit isolierten Pflanzenzellen" (1902 – see also the compendium published by Laimer and Rucker 2003), was better known in his time as a plant physiological anatomist – the first edition of his "Physiologische Pflanzenanatomie" appeared in 1884, followed by six further editions.

Němec was 20 years younger than Haberlandt and is generally less well known. Between 1892 and 1896, he studied both zoology and botany at the Charles-Ferdinand University in Prague. In 1899, he defended his habilitation thesis titled "Contributions to Plant Cell Physiology and Morphology". In 1901, Němec laid the foundation for the new university Institute of Plant Physiology which has, in various forms, under different names and with only a small interruption during World War II, existed till now. He was appointed its leader and later, in 1907, became full professor in plant anatomy and physiology (1907).

Němec is one of the founders of experimental plant cytology. Much of his work was devoted to the influence of various factors on cell division. He also studied polyploidy, plant fertilisation, physiology of growth and irritability, tropisms, regeneration, mycology, phytopathology, the role of trace elements and the history of botany. Both his name and experimental work, characterised by his perfectionism, enjoyed high esteem in the research community of the last century. After Jan Evangelista Purkyně, he was the only other Czech scientist to become an honorary member of the Linnean Society of London.

1.2 Haberlandt's Dogmatic Dream and Its First Realisation

As vividly described in the review by Vasil (2008), the alleged friendship between the botanist Matthias Jakob Schleiden (1804–1881) and the animal physiologist Theodor Schwann (1810–1882) stimulated, among others, the formation of the "cell theory". Schleiden (1838) was the first to formulate the hypothesis that all plant or animal structures are composed of cells (or their derivatives) that preserve the complete functional potential of the organism.

Half a century after the cell theory had been formulated, both plant and animal biologists started to verify it experimentally. Initially, attempts were made to demonstrate the "immortality" of animal and plant somatic cells by means of their in vitro culture. The next challenge – only successful for plants – was to regenerate functionally complete organisms from these cultured tissues.

Against the preconceived expectation that the high functional autonomy of autotrophic plant cells would make it easier to cultivate them in vitro, it was an "animal" researcher who won the first round of this race when Ross Granville Harrison, working at Johns Hopkins Medical School and later at Yale University, published his results of experiments from 1907 to 1910, which established the

methodology of animal tissue culture (Harrison 1907). It took a further quarter of a century before comparable results were achieved for plants, by the independent work of White (1939), Nóbecourt (1939) and Gautheret (1939).

The possible reasons for Haberlandt's persistent failure to persuade isolated plant cells to divide in vitro have been extensively discussed in various critical reviews. Maybe the most objective conclusion is that reached by Gautheret (1985):

> Unfortunately, he (Haberlandt) was influenced by Schleiden and Schwann's cell theory rather than by experimental expectation. And he neglected both Duhamel's results as well as Vöchting's and Rechinger's experiments (Note: experiments with plant parts containing various meristematic tissues, thus generating calli or regenerating buds; cf. Gautheret). His dogmatic attitude and the ignorance of the past explain the failure of his own attempts. But he appreciated very clearly that, when the technical difficulties were removed, the method of cultivating isolated cells in nutrient solution should make possible the experimental study of many outstanding problems from a new point of view. He, therefore, chose to work with single cells. Appreciating the importance of photosynthesis he presumed that green cells would be the best material. However, he neglected the fact that green cells of phanerogams are relatively differentiated and cannot recover meristematic competence without stimulating substances which were unknown at the time. He worked with palisade cells of Lamium purpureum, pith cells from petioles of Eichhornia crassipes, glandular hairs of Pulmonaria and Urtica, stamen hairs of Tradescantia, stomatal guard cells of Ornithogalum, and many other materials.
>
> At this time it was recognized that asepsis was absolutely necessary when culture media were enriched in organic substances metabolized by microorganisms. Haberlandt's media contained glucose and peptone, he carefully avoided contamination and his cultures remained free of microorganisms. The results, however, were disappointing. The cells survived for several weeks. They were capable of synthesizing starch and enlarging, but they were never dividing. Fifty six years passed before the realization of Haberlandt's dogmatic dream. . ..

Undoubtedly, it was mainly thanks to the use of these phytohormones that Muir et al. (1954, 1958) succeeded in obtaining new cell colonies from isolated cells of *Nicotiana tabacum* and *Tagetes erecta*. Application of a synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), in the culture medium allowed Steward et al. (1966) not only to obtain a well-growing suspension culture of carrot callus cells and their aggregates but also to regenerate from them somatic embryos of potentially unicellular origin. Eventually, as a result of the long-term competition between the Skoog and Steward teams to isolate and identify the active compound in the plant "fetal serum", i.e. coconut milk (for a review, see Amasino 2005), cytokinins were first isolated and became a universal component of the plant tissue culture media. Vimla Vasil and Hildebrandt (1965a, b) convincingly documented the whole procedure "from a somatic cell to a regenerated plant" by means of time-lapse photography of individual cells of the hybrid *Nicotiana glutinosa* × *Nicotiana tabacum*. From a recovered single-cell-based colony, they regenerated the entire flowering plants, exploiting the results by Skoog and Miller (1957) on the influence of auxin to cytokinin ratio on regeneration.

These experiments, however, failed to prove the validity of Haberlandt's vision "from individual somatic cells to complete plants"; the problem was that the primary explants used were multicellular and thus the original callus colonies

from which the so-called single-cell clones were derived. Later technology, however, finally confirmed Haberlandt's original dream in all its details. It was shown that when single cells were stripped of their cell walls to produce the so-called protoplasts (Cocking 1960, 1972, 2000; Takebe et al. 1971), these protoplasts were able to regenerate not only into walled cells (Nagata and Takebe 1970; Opatrný et al. 1975, 1980) but also into complete plants (Takebe et al. 1971; Schumann et al. 1980). Isolated protoplasts, cultured separately in microchambers, could regenerate real "protoclonal plants".

Through the alternative methodology of pollen culture, developed in the late 1970s, new (even haploid) plant organisms can be generated directly from plant spores.

But, in spite of these discoveries, experimental knowledge remained limited by the mostly empirical approach to regeneration. Moreover, the use of regenerative techniques in both fundamental research and practical applications repeatedly encountered the problem of "recalcitrance" of a particular model material. This recalcitrance depended either on species, variety or genotype; the origin of cuttings or explants used; the age or composition of the material; and a variety of other features. As a rule, further detailed studies on the factors affecting plant developmental and regenerative abilities and mechanisms were – and still are – necessary to solve such problems.

1.3 Němec's Studies on Regeneration Processes in Plants

In contrast to Haberlandt, Němec did not focus his experiments on the cultivation of isolated somatic cells or tissues but concentrated mainly on the studies of their regenerative abilities in vivo and *in planta*. His research strategy made use of the broad empirical experience of practical gardeners, farmers and foresters. Their experience encompassed information on plant growth and development, plant nutrition and, of course, plant propagation, both generative and vegetative. For vegetative propagation, based on the observations of plant life under natural conditions, various techniques of cultivation of the isolated plant parts were applied – either with the desire to root shoot or leaf segments or in effort to induce de novo regeneration of complete plant bodies.

The formation of the key, now almost universally accepted rules for "plant clonal propagation", is, among others, closely associated with the foundation of the American Society for Horticultural Science in 1903. Its members proposed various strategies of plant propagation for typical groups of plant cuttings including "(stem) hardwood, softwood, semi hardwood, herbaceous and leaf and root". The following "century of progress in vegetative plant propagation" comprised a series of methodological and thematic phases which paid attention either to the effects of culture conditions (type of substrate, moisture, temperature, light) or to the biological characteristics of the primary cuttings, including their age and the appropriate strategies for their rejuvenation (for a review, see Preece 2003).

The broad and diverse field of plant regeneration abilities and various strategies for their vegetative propagation became also one of the favourite research territories of Bohumil Němec. In his Memories (2002) (p. 184), he made the following statement (translated from Czech): "The work of the American biologist Jennings on the recovery of Turbellarias gave me the impetus to attempt the regeneration of root apices. I did a lot of experiments on this subject as well. Out of them grew a great work called Studien über Regeneration which was published in 1905 by Dr. R. Horst, the owner of Gebrüder Borntraeger, a renowned publishing house in Berlin. The book was beautifully presented on excellent paper, with 387 pages and 180 pictures in the text. But this book was not sufficiently appreciated in the plant physiology field. . .".

What a pity that professor Němec died many years before the Internet boom provided a real globalisation of science. Today, he could find his book digitalised by Google in the library of University of Michigan – see http://archive.org/details/studienberdiere00nmgoog. Readers fluent in German can enjoy Němec's results and comments and also the numerous drawings illustrating the responses of plant objects, cultured hydroponically or in wet sand – from his favourite horse bean to conifers and ferns towards microsurgical injuries of their roots (either as single cuts or in combination in various parts of their root apex). Returning to Němec's statement above, it is difficult to determine how well this book has really been assessed. But in any case, it is known in the recent Anglo-Saxon research community and now freely accessible worldwide.

A complex categorisation of the regeneration mechanisms in plants was, unfortunately, only published by him almost 40 years later and then only in Czech (Němec 1943). "Jak rostou rostliny (How plants grow)" is the title of the fourth volume of the so-called Aventine Botany (Aventinský rostlinopis), a cult compendium systematically issued in Czechoslovakia since 1930.

Němec distinguished three main mechanisms for the regeneration of new organs (or individuals) from the plant body.

The first, called *restitution*, was based on the results of the above-mentioned experiments with injured root apices. New functional organs recover or regenerate as a result of the continued activity of the pre-existing, partially injured meristem – consequently at the same place, without any developmental or differential change of the preprogrammed local cell population (Fig. 1).

Where total loss or damage of the apical meristem (either shoot apical meristem (SAM) or root apical meristem (RAM)) occurs, a second alternative mechanism is initiated, termed *reproduction* by Němec. Again, the process is based on the function of the pre-existing meristems but is localised outside of the injured area. Dormant meristems of axial stem buds or silent plant root primordia are, in accordance with the model of "apical dominance", automatically activated, and their "products" – i.e. new branches and roots – substitute for the previous main stem or root.

Of course, under natural conditions, plants are often not only injured but fragmented into pieces lacking SAM or RAM tissues. Therefore, to survive, plants need to regenerate a new body not only from shoot but also from leaf or root

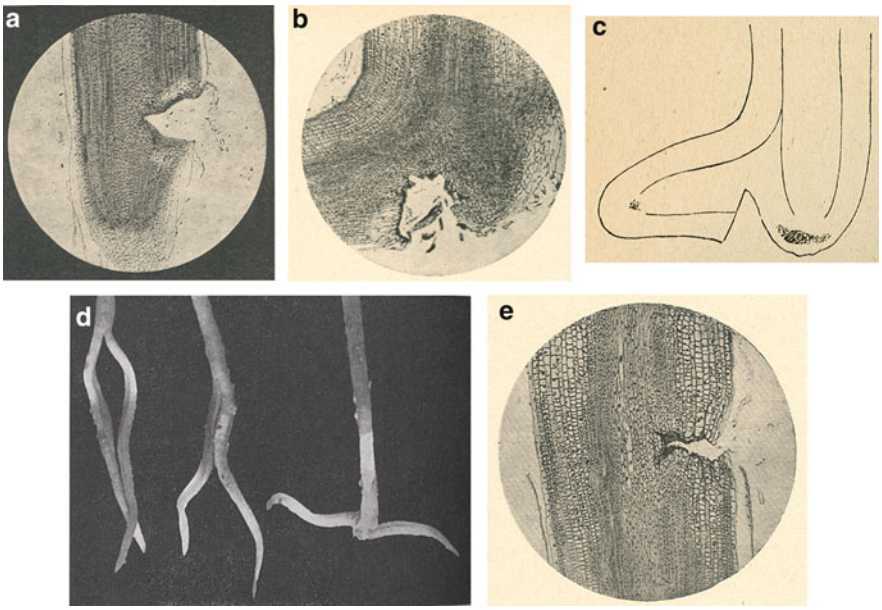

Fig. 1 Restitution of a new root apex from a pre-existing apex by mechanical injury (transverse or longitudinal razor incisions) in seedlings of *Vicia faba* (From Němec 1943). (**a**) Transversal incision in 1 mm distance from the root tip, response of 24 h after treatment. (**b**) Dto., situation 3 days after treatment. A new apical meristem has partially regenerated from the upper side of the incision. (**c**) Schematic illustration of statolithic starch grains occurring both in columella cell of the original root apex and in differentiated tissue of the restituted apex. (**d**) After median, longitudinal razor incision, two new root tips restituted. (**e**) Transversal incision in 3 mm distance from the root tip, 3 days after treatment. No new apex has restituted

segments. For this specific situation, plants have developed a mechanism termed *regeneration* de novo (*Rdn*). Němec studied the various forms of *Rdn* on a broad spectrum of plant cuttings, including herbaceous (like chicory, carrot and dandelion root segments) and woody (like chestnut tree) objects, and described both the morphological and anatomical characteristics of this process.

Němec noted a pronounced variability of the regenerative responses of various experimental objects and effects of both internal ("phylogenetic" origin, physiological and developmental state of a particular organ or its explant) and external (humidity, temperature) factors on these processes. Stimulated by the global development of plant physiology, he started to experiment with predicted, but still unidentified, hormonal compounds. His photographic documentation from the late 1920s to 1930s depicts various forms of *Rdn* in calli, roots or shoots from the surface of the explant, covered by either living or dead microorganisms. Indeed, half a century before the development of the *Agrobacterium*-based plant transformation, Němec studied various forms of neoplasia and/or organogenesis induced in plants by *Agrobacterium (Bacillus) rhizogenes* (Němec 1943; Fig. 2).

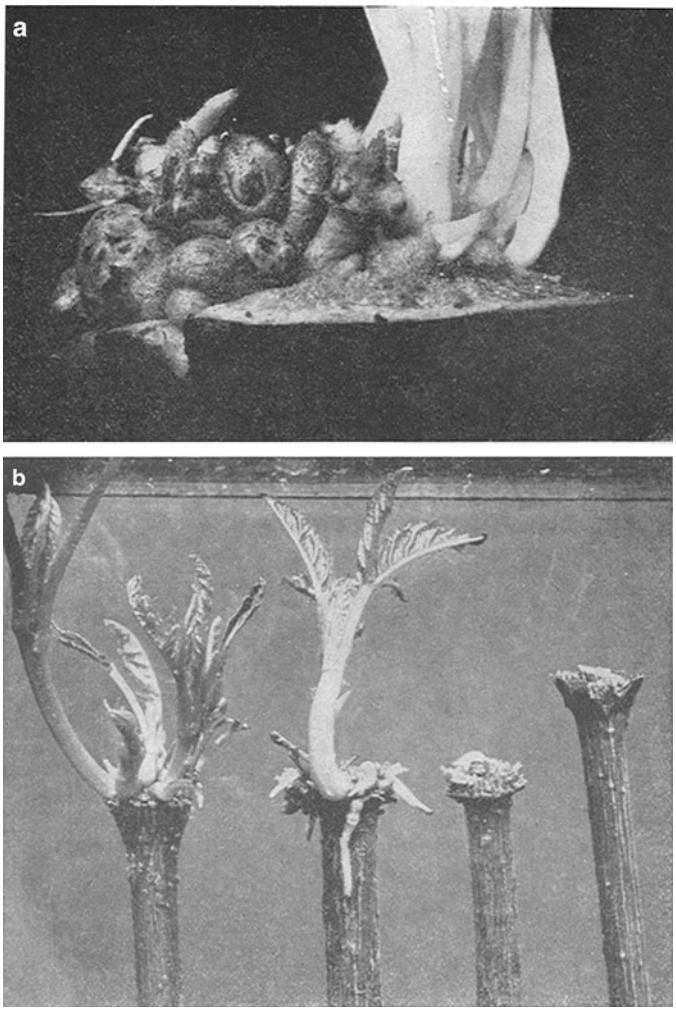

Fig. 2 Morphogenetic effect of treating root or shoot cuttings with living bacterial cultures (From Němec 1943). (**a**) Root cutting of *Cichorium intybus*. Buds and shoots regenerated from upper cut surface after cultivation in wet sand (*right half*). On the contrary, regeneration of roots or formation of tumours from the left half treated by *Pseudomonas (Agrobacterium) rhizogenes* culture was observed. (**b**) Shoot (hypocotyl) cuttings of *Aesculus hippocastanum*. From left to right: control, untreated, shoot/bud regeneration from the entire cut surface; shoot regenerates from untreated half, roots from the half treated by bacterial culture; only tumours and root primordia were formed from the cut surface completely covered by bacterial culture

All three of these basic mechanisms of regeneration, especially reproduction and de novo regeneration, have been broadly applied and are still in use today in practical gardening, agriculture and forestry. Since the 1960s, these mechanisms have provided the biological foundation for the technology of in vitro

"micropropagation" for a wide spectrum of cultured plants. However, for an extended period, the biological mechanisms underlying these technologies were only poorly known and understood.

Systematic studies of these mechanisms were reinitiated in the 1970s and 1980s using two model systems: either intact plants grown in vivo or cells, tissues and organs cultured in vitro. In both cases, scientists repeatedly were faced with the phenomenon of regenerative nonresponsiveness of plant segments, cuttings and explants. In vivo, the high regenerating ability of roots and shoot buds of some species (both herbaceous species such as various weeds and ornamental plants and woody plant species such as willow) contrasted markedly with that of others (not only woody such as oak, pine or walnut but also herbaceous models). In vitro, this responsiveness differed both between species and even between varieties and cultivars.

Apart from the convincing genotype-specific "determination" of the actual regeneration ability of either cutting or explants, we should answer following set of key questions:

- To what extent do cells of the adult plant body really remain "totipotent", preserving the morphogenetic competency of the zygote, and to what extent are they able to express it?
- What other cells are able to revert to this stage, through the processes of "dedifferentiation and re-differentiation"?
- What genes and transcripts underlie these processes?
- What hormonal or nonhormonal factors regulate them?
- To what extent are we able to use and apply this knowledge in practical situations, in, for example, agriculture, horticulture and forestry?

2 Plant Developmental and Regenerative Potency: Recent Classification

Before we can assess either the Schleiden-Schwann cell theory or Haberlandt's belief in the massive totipotency of somatic plant cells, it is necessary to define "totipotency". In the literature relevant to plant tissue culture, a variety of terms appear, including omnipotency, multipotency or pluripotency, sometimes wrongly regarded as synonyms. Consequently, as a first step of a complicated discussion on the mechanisms of regeneration in multicellular organisms, a clear definition of the key terms is necessary.

2.1 Terminology of the Levels of Plant and Animal Regenerative Abilities

As it is generally accepted, the developmental and regenerative ability of both animal and plant multicellular organisms is based on the existence of "stem cells". Stem cells originate, as a rule, during early zygotic embryogenesis and are thought to maintain an initial state whereas their neighbours undergo a sequence of cell and tissue differentiation, histogenesis and organogenesis. Mostly, stem cells are not isolated, but located in particular territories called "stem cell niches" with various functions and functional potency. What are the recent, relatively universally accepted characteristics of stem cell behaviour in the animal field?

2.1.1 Totipotency

Wikipedia and the Regenerative Medicine Glossary 2009 (Mitalipov and Wolf 2009) define totipotency as "[. . .the ability of a single cell to divide and produce all the differentiated cells in an organism, including extra embryonic tissues. Totipotent cells include spores and zygotes. . .]". Consequently, the human zygote is totipotent, and its totipotency is preserved also in all daughter cells of the morula. However, after having reached the 16-cell stage, the morula cell clones differentiate into two "subpopulations". One will become the inner mass of the blastocyst, the prospective source of "stem cells", which will later develop into any of the three human germ layers (endoderm, mesoderm and ectoderm). The second subpopulation will develop into the trophoblast – and later into the tissues of the placenta.

2.1.2 Pluripotency

These subpopulations – both inner cell mass and trophoblast cells – are no longer totipotent but remain pluripotent only. They are able to differentiate further into the relevant specialised tissues, but they are no longer able to regenerate a new individual by themselves.

2.1.3 Multipotency

During further natural histogenesis and ontogenesis, the regenerative potency of human cells decreases even further. Thus, various progenitor cells can develop into several cell types but only into those of a similar fate – e.g. hematopoietic cells can generate various blood cells, but not neural cells. These cells are no longer pluripotent but remain multipotent only.

Even in humans, some of these processes seem to be not irreversible, since modern medicine is starting to use various techniques of "transdifferentiation" by

means of somatic expression of combined transcription factors, a discovery awarded by the Nobel Prize in Medicine for 2012 jointly to Sir John B. Gurdon and Shinya Yamanaka for "the discovery that the mature cells can be reprogrammed to become pluripotent". But how realistic is such "regenerative comeback" of totipotency? Undoubtedly, regenerative totipotency is preserved in various adult somatic cells of some lower animals (like *Hydra* spp.) and also in both lower and higher plants. Shall we also become able to clone higher mammalians or even primates via transdifferentiation from "post-totipotent" stem cells? Will the limits of these technologies be set by biological constraints or by ethical limitations only?

In plants, the regeneration of a new individual can be achieved by two alternative routes – either through organogenesis or through embryogenesis. Traditionally, both routes are thought to begin with the activation of particular totipotent cells. In the framework of the terminology defined above, the term totipotency should remain confined to specific forms of in vitro embryogenesis (zygotic, somatic and pollen derived) that would meet the criteria of "real" cellular totipotency. In contrast, any regeneration de novo via organogenesis should be classified simply as the expression of regeneration ability of originally "only" pluripotent cells, although "transdifferentiated" under various conditions and by means of various factors.

2.2 *Where Are You from and What Is Your Regenerative Potency? Evolution and Function of Plant Stem Cell Niches*

Not surprisingly, research on animal or plant stem cell niches has been mutually inspiring, and, because of this, recent original papers and reviews try to confront plant and animal stem cell characteristics from traditional model organisms, such as *Arabidopsis*, *Drosophila* and mouse (Sablowski 2004; Somorjai et al. 2012).

2.2.1 What Are the Historical Roots of This Research in Plants?

Our current knowledge of molecular aspects of the function of stem cell niches was preceded by some key cytological and histological studies conducted more than half a century ago. Let us briefly review some of them.

Satina et al. (1940) treated *Datura* seedlings with colchicine and traced the localisation of daughter clones from individual polyploid cells in the shoot apex. Based on their observations, the behaviour of the shoot meristem mimics that of the human zygote: "[. . .] shoot meristem consists of clonally separate layers of cells: an outer L1 layer, from which the epidermis is derived, a sub epidermal L2 layer, and an internal L3 layer. This three-layer organization is typical of the shoot meristems

of dicotyledonous seed plants, but varies in monocotyledons (two layers), gymnosperms (one layer) and more basal species (mosses, ferns) where all cells originate from a single apical cell. . .” (quoted from Aichinger et al. 2012).

But to what extent – and by what mechanisms – is the subsequent morphogenetic programme of the individual layers fixed and progressively put into reality?

The morphological and histological studies of Stewart and Dermen (1970) documented, in mericlinal chimeras of various plant species, the phenotypical homogeneity of the cells for a given shoot cell layer. The cells in approximately one-third of the shoot circumference originated from a single stem cell in the shoot meristem. These descendants of the original stem cell can be viewed as pluripotent. As the authors say: “Importantly, marked sectors occasionally broadened from one third to two-thirds and even to the entire circumference at the expense of the progeny of non-marked stem cells and vice versa, indicating that stems cells can be replaced and act as stem cells only as long as they are in a specialized environment. Finally, when an L1 stem cell was displaced into the sub epidermal layer by an occasional periclinal division, it gave rise to L2 cell types, suggesting, that the fate of its progeny is not determined by the stem cell’s history but rather by the position of the differentiating offspring. . .”.

This clear statement can be verified using recent technical innovations such as laser ablation microscopy, allowing us to follow the behaviour of particular somatic cells intentionally transferred to a different environment.

Combining the knowledge gained from the plant and animal models, we arrive at a general concept of the stem cell niche for multicellular organisms (Aichinger et al. 2012, based on van den Berg et al. 1997):

(a) Cells that are protected from differentiation by signals from neighbouring cells in specific niches can divide and thus function as stem cells,
(b) Cells that leave the niche are bound to differentiate. Differentiation of the daughter cell leaving a niche appears to be dictated by the environment rather than by cell origin.

In this context, the term “environment” means the “internal environment” of a particular organ or tissue, the complex of surrounding cells in a particular place and time and their interactive communications. This environment is defined by a broad spectrum of both physical and chemical factors (nonhormonal and hormonal), which affect or tentatively determine local gene expression and modify gene activity, transcription or even translation profiles by means of various epigenetic tools such as gene methylation/demethylation and acetylation, via changing structure and function of nuclear histones or through several types of iRNAs.

2.2.2 Formation of Embryonal Stem Cell Niches

In both animals and plants, the formation of stem cells niches is connected with the first developmental activity of the zygote and therefore takes place in early embryogenesis. General features of this process in plants are known basically from the studies of *Arabidopsis thaliana* models.

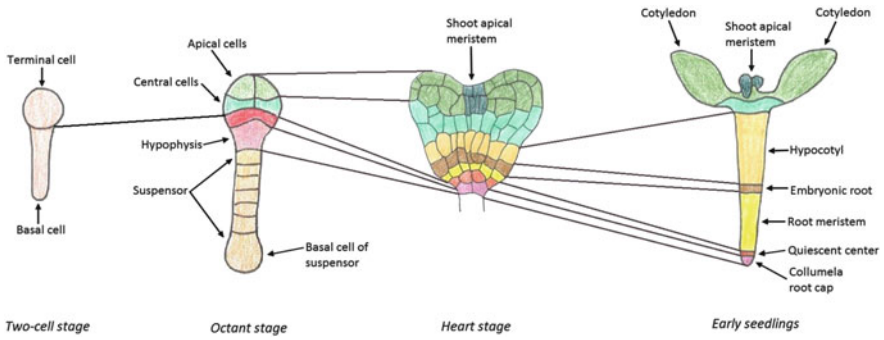

Fig. 3 Zygotic embryogenesis of *Arabidopsis thaliana*, successive differentiation of future SAM and RAM stem cell niches (Modified from Taiz and Zeiger (2002))

The formation of the stem cell niche is linked to the asymmetry – and polarity – of both the division of the first zygote and the differentiation of the daughter cells (Somorjai et al. 2012; Hu et al. 2011; Xiang et al. 2011; see also the chapter by Smertenko and Bozhkov, this volume, for some details). The small, cytoplasmically dense apical daughter cell gives rise to the embryo itself, and the shoot apical meristem, cotyledons and hypocotyl are specified during its subsequent divisions (Weigel and Jürgens 2002; Fig. 3).

In contrast, the larger, vacuolated basal daughter cell gives rise to the suspensor, the extra embryonic tissue that connects the embryo with maternal tissue. Also, the suspensor progenitor cell originates the cell lineage leading to the hypophysis, the initial cell of the primary root meristem. The hypophysis later divides asymmetrically to generate a lens-like structure that will become the quiescent centre (QC), the prospective organising centre for root stem cells (see Mayer et al. 1993; De Smet et al. 2010 and the chapter by Skůpa et al., this volume, for details).

Asymmetry of cell divisions in the prospective RAM gives rise to the complex differentiation of the root tissues. Again, the role of phytohormones (in particular auxin) in these processes is convincing, especially for both division and specification of the hypophysis via the auxin response factor MONOPTEROS, and the transcription factor TARGET OF MONOPTEROS 7 (TMO7). In response to auxin, MONOPTEROS activates the transcription of TMO7 in cells adjacent to the hypophysis. TMO7 then diffuses into the root stem cells, underlining the importance of auxin responses for asymmetric cell division (Schlereth et al. 2010; Weijers et al. 2006).

A second organisation centre (OC) is differentiated stepwise in the future SAM – it maintains local stem cells in their niche and under control of numerous factors (including phytohormones, mainly auxins and cytokinins; see the chapter by Skůpa et al.) coordinates the activities in the various zones of the SAM.

2.2.3 In Plants, Four Key Stem Cell Territories Exist

In plants, four stem cell niche territories exist. The shoot apical meristem (SAM) and the root apical meristem (RAM) function as the key producers of indispensable new cells and tissues (Fig. 4). Moreover, two additional meristems are involved in stem thickening (radial growth), both in trees and herbaceous plants: the cambium of the vasculature, responsible mainly for the transport and mechanical support of the stem, and the phellogen (cork cambium), producing new tissues for the outer layer (bark).

The germ lines of these meristems can work for hundreds to thousands of years in extreme cases. During this enormous time, they have to preserve their morphogenetic potency and also genotypic stability without accumulating mutations which otherwise could stepwise disturb their original biological identity.

2.2.3.1 The Positioning of SAM and RAM Stem Cell Niches

It is generally accepted that the identity of the SAM stem cell niche is maintained by a signal conveyed by the WUSCHEL gene product (a homeodomain transcription factor, TF) produced by the cells subtending the SAM. The descendants of the stem cells are displaced to the periphery of the meristem and therefore no longer a target of this signal, such that they can be recruited into new organs. The innermost region of the SAM, called the "rib" meristem, produces the internal tissues of the stem. WUSCHEL (WUS, from the curly appearance of the meristem in the *wuschel* mutant of Arabidopsis which lacks a shoot meristem; Laux et al. 1996; Mayer et al. 1998) encodes a plant-specific homeodomain protein and is the founding member of the WUSCHEL-RELATED HOMEOBOX (WOX) gene family, which regulates diverse aspects of plant development (van den Graaf et al. 2009). WUS expression in the SAM defines the OC and maintains the "undifferentiated" nature of the stem cells.

As mentioned above, the role of the custodian for stem cell identity in the RAM is played by the cells of the quiescent centre (QC). The QC has been known for a long time both as the cell reservoir necessary for the repair of injured RAM and as the main local producer of cytokinins. The positioning of the QC is affected by the complex interaction of overlapping transcription factors: SCARECROW (SCR) and SHORT ROOT (SHR) on one side and PLETHORA (PLE) on the other. Expression of the PLE transcription factor is, in turn, controlled by the apical-basal gradient of auxin maintained by PIN transporters. Stem cell maintenance in the RAM is, similarly to SAM, controlled by the WUS homologue WOX5, which is required to prevent QC stem cells from differentiation into columella cells. All these regulatory genes respond to either near-range or far-range intercellular signalling. Together, these genes control the rate of cell growth and division, thus establishing the size of SAM/RAM territories and also controlling the entry of the meristem descendants into the typical differentiation pathways. However, the

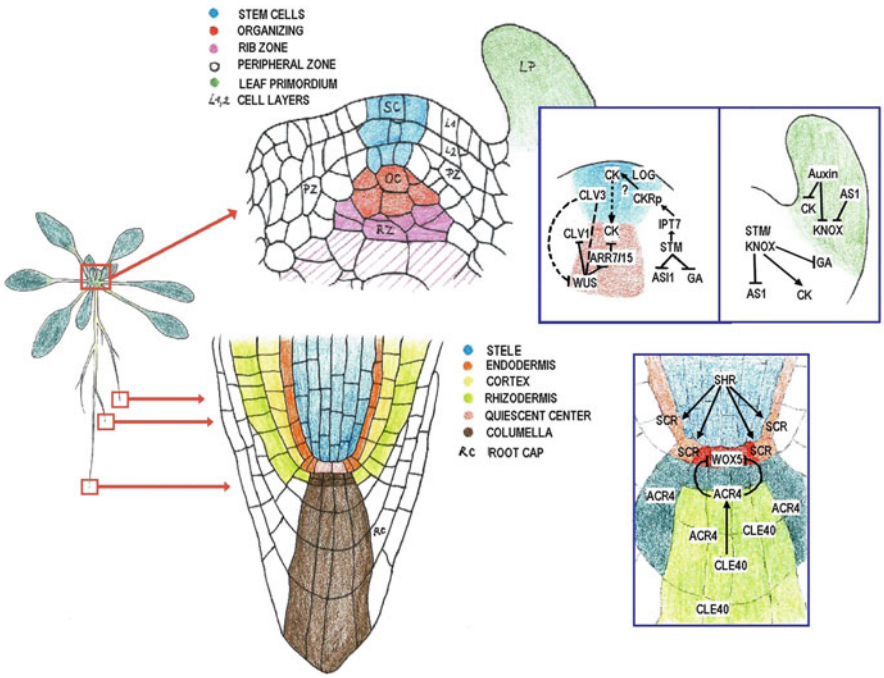

Fig. 4 Plant stem cell niches: histological zonation and mutual cell communication in shoot apical meristem (SAM) and root apical meristem (RAM) territories of *Arabidopsis thaliana* during vegetative growth:
Model for *SAM* positioning:
LP leaf primordium, *OC* organising centre, *PZ* peripheral zone, *RZ* rib zone, *L1,2* cell layers. *Blue* regions of CLAVATA 3 expression; *red* regions of WUS/AHK4 expression. Auxin and AS1 *(ASYMMETRIC LEAVES1)* repress the meristem-promoting activities of *KNOX* genes and cytokinin (CK) in the leaf primordium, cooperating *STM (SHOOTMERISTEMLOSS)*, and *KNOX* genes repress *AS1* in the meristem, activate CK biosynthesis and repress biosynthesis of gibberellic acid (GA). A pool of pluripotent stem cells (*blue*) is maintained by a WUS/CLV3 negative feedback loop. *STM* activates *IPT7* enzyme, which catalyses cytokinin biosynthesis. Alternatively or in parallel, the gene product of *LOG (LONELY GUY)* gene product, detected in the L1 of rice, might convert cytokinin riboside 5′-monophosphates into free, active cytokinins. Higher sensitivity to CK in the OC is achieved by localised expression of *AHK4* (cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4) and repression of the cytokinin signalling inhibitors *ARR7* and *ARR15*
Model for SHR/SCR and CLE40/ACR4 action in *RAM* positioning:
SHR expressed in the stele moves to the surrounding endodermis and quiescent centre. There, SCR (SCARECROW, a transcription factor) is required for nuclear localisation of SHR (SHORT ROOT, transcription factor), and, vice versa, SHR activates SCR expression. CLE40 peptide is expressed in columella cells, and counteracting stem cell-promoting QC signals allow distant columella cells to differentiate. Its activity is mediated by the interaction with the receptor-like kinase ARABIDOPSIS CRINKLY 4 (ACR4) (Adapted from Aichinger et al. (2012) and Taiz and Zeiger (2002))

molecular details of how cells are channelled from a stem cell fate to differentiation are still only partially understood.

A variety of hormonal or nonhormonal signals are known to participate in the regulation of SAM or RAM function. Among the hormonal signals, auxin and cytokinins are the most important. Within the group of nonhormonal signals, the "best understood intercellular signal used in meristem maintenance" (Ito et al. 2006) is the glycosylated dodecapeptide CLAVATA 3 (CVL3), produced by shoot stem cells and interacting with the above-mentioned transcription factor WUS. CLV3 belongs to a family of 32 small proteins called CLV3/EMBRYO SURROUNDING REGION (CLE) named after mutants in *Arabidopsis*, where both vegetative and floral meristems progressively enlarge (Cock and McCornick 2001; Ito et al. 2006). Consequently, these mutants produce more organs than wild-type plants (Clark et al. 1993, 1995). The phenotype of *clv* mutants is to some extent under the control of WUS expression. This control is bidirectional – overexpression of CLV3 results in repression of WUS transcription and phenocopies *wus* mutants (for a review, see Aichinger et al. 2012; Brand et al. 2002; Lenhardt and Laux 2003).

In the SAM, cytokinin and WUS activity reinforce each other through multiple feedback loops which is reflected in the localised expression pattern of cytokinin receptors (ARABIDOPSIS HISTIDINE KINASE 2 and 4) in the rib meristem. We know that not only auxin but also cytokinin is produced in the apical region of the SAM. The WUS expression domain might be continuously specified in the position, where a sufficient amount of apically produced cytokinin reaches its receptors in the rib meristem.

2.2.3.2 RAM and SAM Maintenance and Differentiation

Apart from their role in the original positioning and subsequent maintenance of the stem cell niches, auxin and cytokinin are necessary to regulate the balance between cell division and differentiation. Their synergistic effect in SAM differentiation is illustrated by auxin activation of MONOPTEROS (MP), which directly represses ARR7/15, negative regulators of cytokinin response (for details, see Skůpa et al., this volume), that, in turn, inhibit meristem functions at least partly by activating CLV3 (Zhao et al. 2010).

In case of the RAM, auxin and cytokinin traditionally exhibit antagonistic effects. Here, auxin flow promotes cell division, whereas cytokinin promotes cell differentiation. Molecular studies have revealed the regulatory base for this antagonism: the cytokinin response regulator ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) activates expression of SHORT HYPOCOTYL 2 (SHY2), which represses auxin signalling, and the expression of the PIN auxin transporters. Conversely, auxin causes the degradation of SHY2, allowing PIN expression and recovery of the auxin flow required to maintain the RAM (Dello Ioio et al. 2008a, b).

2.2.3.3 The Vascular Stem Cell Niche

In addition to SAM and RAM, all cormophytic plants possess vascular systems composed of xylem and phloem. In gymnosperms and dicotyledonous angiosperms, this vascular system is endowed with a cambium, which is absent in monocotyledonous angiosperms that are therefore not capable of secondary lateral growth. Although the participating cell types are shared, the details of vascular architecture are very variable. Despite this variability, the current opinion is that the cambium contains stem cells with phloem mother cells on one side and xylem mother cells on the other (reviewed in Elo et al. 2009), a concept that is supported by transcriptional profiling in *Populus* (Schrader et al. 2004, see Aichinger et al. 2012 for details).

To determine the regulatory factors of xylem tracheal differentiation, in vitro models, in particular the system of direct differentiation of trachear cells from palisade parenchyma in *Zinnia elegans*, have been used. In this system, Ito et al. (2006) purified the CLE peptide TDIF (tracheary element differentiation inhibitory factor), which simultaneously inhibits xylem differentiation and promotes cell proliferation. The *Arabidopsis* TDIF homologues, called CLE41 and CLE44, are expressed in the phloem and are able to induce stem cell proliferation in the neighbouring procambium of hypocotyls and mature shoot (see Aichinger et al. 2012 for details). The differentiating phloem daughter cells thus provide stem cell-promoting signals and also interact with the SAM OC and the RAM QC.

On the basis of this stem cell pattern, subsequently more distinct boundaries are formed – the cell types in the plant vasculature become organised mostly owing to the sophistically regulated flows of cytokinin and auxin. In the shoot, the effects of these hormones can be synergistic. In contrast, they are mutually inhibitory in the root, which determines boundary formation between procambium stem cells and protoxylem (Bishop et al. 2011).

Correlative communications between all types of stem cell niches, i.e. SAM, RAM and the various "intercalary types" (such as cambial meristems), are crucial for the body plan during normal growth and development. But are they also necessary for the regeneration of injured or lost plant organs, either in vivo or in vitro?

2.2.4 RAM Regeneration and Cellular Transdifferentiation

Similarly to Němec's pioneering microsurgery experiments on root tip regeneration (1905; 1943), Sena et al. (2009) characterised the regenerative response of the RAM to mechanical injury. Of course, their experimental system had been modernised: they used *Arabidopsis*, rather than the roots of faba bean (*Vicia faba*) or conifers, and instead of ablations by a razor or a razor blade, they inflicted tiny tissue ablations with a sterile dental needle under a dissecting microscope, and, to characterise the consequences of this treatment, they did not rely on classical plant anatomy but made use of live cell cytology and microarray analysis to analyse

patterning of both original and regenerated tissues. They also precisely documented the dynamics of the regeneration process – which could be classified as "regeneration de novo". Their results are very impressive and worth of a detailed description.

They successfully performed standard excisions at 130 μm from the root tip, resulting in the complete removal of the quiescent centre (QC) of the RAM, all surrounding stem cells along with several tiers of daughter cells, and the root cap, including all of the columella and most of the lateral root cap.

They followed the regeneration of a new, functional root tip at 5, 13 and 22 h and 7 days after excision by both direct microscopical observation and expression profiling for different cell types. To determine the particular cell identity, they compared global and cell-specific transcriptional profiles of the regenerated tissues (more than 100 markers for each cell type).

Although neither hormones nor other exogenous factors were administered, the first cell divisions and the subsequent recovery of cell molecular identities are initiated very rapidly and had begun within 5 h after cutting. For the columella, the molecular recovery had reached 55 % in 22 h. As early as one day after complete excision of the columella, a new set of cells was not only expressing columella markers, but 13 % of these cells also had produced starch and exhibited a clear gravitropic response. Undoubtedly, Němec and Haberlandt would have loved this model system.

All these regenerative processes obviously occurred in the absence of a functional RAM stem cell niche. On the contrary, as authors postulate: "What distinguishes these regeneration-competent cells from the stereotypical stem cells of the niche? For the Arabidopsis root, the stem cell niche has been shown to be critical for indeterminate growth. This suggests that the unique feature of the stem cell niche is the ability for continuous growth without differentiation, while organogenesis might be a general feature of plant cells."

These results confirm the classical findings by Němec – in the tissue context, of in vivo cultured plant and without any additional application of growth substances, the "full functional restitution" of a damaged RAM is only possible through original stem cells.

2.2.5 SAM Regeneration and Cellular Transdifferentiation

Compared to the RAM, the behaviour of the SAM seems to be different. Already, Loiseau (1959), through mechanical destruction of the shoot meristem centre in *Impatiens roylei*, was able to induce new meristems from the shoot peripheral zone (PZ). Obviously, the repression of the developmental potential of the PZ by lateral inhibition was interrupted by this treatment.

More than 40 years later, Reinhardt et al. (2003) used laser ablation to study regulatory mechanisms in the tomato shoot apex. Among other results, they traced the transdifferentiation of various zones using the activity of the key "shoot determining gene complex" *WUSCHEL* as marker and elucidated the interplay of

stem cells (SC), the subtending cell layer of the organising centre (OC), the broader central zone (CZ) and the basal rib zone (RZ). To eliminate the influence of the PZ from the "restitutional programme", it was necessary to ablate not only the stem cell layer but also the OC. After this, a rapid activation of WUS in the PZ was observed.

Alternatively to microsurgical treatments, various forms of molecular manipulations can be used. For example, Reddy and Meyerowitz (2005) affected the cells of the CZ in the *Arabidopsis* SAM by inducible RNA interference-based downregulation of *CLAVATA3* (*CLV3*). As a result, SAM cells close to the CZ reverted back to a stem cell fate (manifested as derepressed *WUS* expression), and the more remote cells increased their division activity.

The superior role of the WUS complex in mediating stem cell homeostasis by "[. . .] regulating stem cell number and patterns of cell division and differentiation of stem cell progenitors" was further substantiated by results of Yadav et al. (2011). They observed that downregulation of *WUS* in the OC was also accompanied by a gradual shift of the auxin maxima marking lateral organ *Anlagen* towards the CZ.

As Aichinger et al. 2012 summarised: "[. . .] control of WUS gene expression and multiple levels of lateral inhibition contribute to robustly maintaining the boundaries between pluripotent stem cells and differentiating descendants".

Undoubtedly, similar mechanisms participate in the regulation of regenerative processes in the in vitro cultures of various plant explants – starting from isolated cells and finishing with the cell complexes and/or organs.

3 Plant Regeneration In Vitro

Vegetative plant multiplication, generation of virus-free plants and plant breeding including GM technologies – all these fields of plant biotechnology are more or less connected with our ability to regenerate new plant individuals under in vitro conditions. Not surprisingly, tens of substantial monographs have been devoted to this key procedures (e.g. George and Sherington 1984; Vasil 1984, 1985, 1986). Moreover, thousands of articles on "regeneration" of various herbaceous and woody plants, crops and weeds have been published worldwide in specialised plant biotechnological journals. To summarise these miscellaneous bits of information from the practical point of view, we can conclude:

- No universal technical protocol exists.
- Synthetic phytohormones – in particular auxins and cytokinins – are key regulators of these processes.
- The auxin to cytokinin balance, as elaborated by Skoog and Miller, seems to be crucial but seems to vary with respect to quantity, quality and timing for organogenesis, and so far, there is no unifying theory, just empiry.
- 2,4-D represents a key tool to induce or modulate somatic embryogenesis (again, in combination with other hormones like cytokinins, abscisic acid or brassinosteroids).

- Almost all these protocols are empirical, and only during the last few years have data on their molecular background been accumulating.

3.1 Hormonal Regulation of In Vitro Regeneration

Numerous recent reviews have illustrated the interplay between the genes or gene families maintaining a SAM and RAM cell fate, their products (including tens of various transcription factors) and key phytohormones such as auxins and cytokinins (but also others, like gibberellic acid, brassinosteroids or even jasmonates). A good overall picture is offered in the review by Sablowski (2011). Not surprisingly, such reviews start their historical introduction with a citation of the pioneer Skoog and Miller (1957) paper. Also typical are the following comments (see Moubayidin et al. 2009): "[. . .] little is still understood about the in vivo significance of these tissue culture experiments and of the molecular mechanisms. . .". But the problem is not caused by the "tissue culture" model. The review precisely summarises the state of the art concerning cytokinin and auxin biosynthesis, transport and signalling in intact plants, their effect on cell division and cell differentiation and the crosstalk between various stem cell niches and tissue domains; but the nature of the real "molecular masters" – if they exist at all – remains unknown.

In the more recent studies, some "master genes" overlapping, controlling or coordinating stem cell gene expression have been described, such as the *POPCORN* (*PCN*) gene, which participates in the early embryogenesis in *A. thaliana* (Xiang et al. 2011). Its mutation causes pronounced malformations in early zygotic embryogenesis, but its transcript (WD-40 protein) is also expressed postembryonically both in the RAM and SAM territories. In the *pcn* mutants, meristem-specific expression of *WUSCHEL (WUS)*, *CLAVATA 3 and WUSCHEL-RELATED HOMEOBOX5* is perturbed. *SHOOT MERISTEMLESS, BODENLOS (BDL) and MONOPTEROS (MP)* are misexpressed. Several findings link *PCN* to auxin signalling and meristem function.

The paramount complexity of auxin to cytokinin signalling has recently been treated in the review of Hwang et al. 2012. They describe the interaction of auxin and cytokinins – with occasional intervention of other factors like gibberellins – in the "*organ proliferation and differentiation*" of four typical plant body complexes and/or situations: formation and function of the primary root meristem; origination and development of lateral root meristems; formation of shoot and root vasculature; and participation in root nodule organogenesis. The authors conclude their excellent publication with a summary of fifteen "points and future issues", in which the last one states: "The comprehensive understanding of cytokinin signalling networks will require elucidation of the single-cell-based, genome wide cytokinin responses by integrating transcriptome, proteome, interactome and metabolome in kinetics and physical contexts. . .".

As mentioned above, under in vivo conditions, the capacities for regeneration (based on the pluripotency and tissue/organ patterning) appear to be separated from

the narrowly confined capacity for indeterminate growth secured by the existence of stem cell niches (Sena et al. 2009). Only the methodology of in vitro cell, tissue or organ cultures, isolated from the "mother" (or father) organism and inoculated into media enriched with a broad spectrum of growth and differentiation affecting compounds, will allow to connect both processes.

The famous Skoog-Miller (1957) model demonstrates how organs can regenerate from cells in G_0 or G_1 cells of tobacco (*Nicotiana tabaccum cv. Wisconsin 38*) stem pith stock parenchyma tissue. Increasing the cytokinin versus auxin ratio induces the formation of shoot buds; a balanced level of both phytohormones results in callus formation; and a high proportion of auxin induces root formation. After the transfer of young shoots into CK free medium, complete plants can be obtained. The whole process is thus an example of the chain leading a cell through transdifferentiation from multipotency or pluripotency to potential totipotency".

So far, however, the original morphological description has not been supplemented by more precise cytological or molecular analyses. Tobacco is a nice material for various in vivo or in vitro physiological studies, e.g. on pollen biology or early embryology, but its genetics remains too complicated. As a rule, in recent studies on molecular mechanisms of de novo regeneration, *Arabidopsis* has been favoured over tobacco.

3.2 Callus Tissues: Dedifferentiated or Transdifferentiated?

Experiments involving callus tissues generally follow a classical strategy: various types of primary explants are first exposed to "callus"-inducing medium (CIM), which contains both an auxin (usually 2,4-D) and a cytokinin (kinetin) in various concentrations; second, they are transferred to media with different hormonal composition to induce regeneration of organs (Valvekens et al. 1988; Sugimoto et al. 2010; He et al. 2012). A representative example for the current state of the art can be found in Sugimoto et al. (2010). These authors used cotyledon, root and petal explants of seedlings from a collection of *A. thaliana* ecotype "Columbia" marker lines cultured on CIM. Subsequently, various concentrations of auxin and cytokinins in shoot- or root-inducing medium were used to force calli to differentiate into shoot or root tissues. The authors not only precisely documented the morphology and histology of callus formation and early regenerative responses but also analysed transcriptome changes during callus formation using microarrays. The gene expression profile of explants cultured on CIM for 10 days was compared to that of the original tissue. They concluded that callus, regardless of the organ of origin, is a root-like tissue, and they document and compare callus formation with the formation of lateral roots from specialised pericycle cells. As they conclude, the process can be interpreted as a transdifferentiation of possibly specific somatic cells in the explants. In any case, their data are not supporting the notion of "callus" cells as anonymously "dedifferentiated", but the transcriptome rather supports a cell state of pluripotent, pericycle-like cells.

In harmony with other authors (De Smet et al. 2006; Atta et al. 2009; Parrizot et al. 2008), Sugimoto et al. (2010) also assign a shoot-forming capacity to some of the callus cells, but the molecular mechanisms remain unclear: "[. . .]This leaves opened the question, how root meristem-like callus tissue has the ability to form aerial shoots in the next stage of the regeneration process (Gordon et al. 2007). Soon after transfer onto shoot induction medium gene expression patterns in callus start changing dynamically and shoot genes initiate expression (Banno et al. 2001; Cary et al. 2002). Callus forming cells are partitioned into regions of different gene expression, and a small number of progenitor cells, found in small patches, initiate development of new shoot meristems (Gordon et al. 2007). Thus, among the shoot forming explants, there are multiple types of cells, and only limited numbers of cells contribute to new shoot formation. These cells may derive from multiple root-like cell types found in callus, or from only one, in which case one of the root-type cell types found in the callus is itself another type of plant stem cells, much like the pericycle-like cells. These and many other questions remain unanswered."

4 Molecular Mechanisms of Regeneration and Their Practical Consequences

Compared to the pioneering studies by Němec and Haberlandt, our overall picture of the mechanisms of plant growth, development and regeneration has now dramatically advanced. Understanding the function of relevant genes and their transcripts and knowledge of both the metabolic pathways and the mode of action of various phytohormones as well as nonhormonal signals allow us to re-evaluate the traditional view of the key regenerative processes defined by Němec's classification from their theoretical aspects and also to assess their importance for practical plant propagation.

4.1 Organogenesis

First of all, we should reassess traditional views on the technologies based on organogenesis.

With regard to *restitution* (currently massively used mainly for in vitro cloning of various ornamental plants, in particular orchids), it is plausible that it is mainly caused by the repetitive activation of pre-existing apical or axillar totipotent "SAMs" – although transdifferentiated pluripotent cells originating from the tissue complex of the repeatedly cut (segmented) protocorms with high probability participate as well.

Micropropagation based on *reproduction* represents possibly the most frequently used in vitro technique of recent horticulture. In the early 1950s, the French

scientists Morel and Martin (1952, 1955) published their results on the cultures of isolated shoot apices from potato and *Dahlia* which were of high value for plant pathologists, mainly virologists: by culturing very small shoot apex explants, it was possible to obtain virus-free plants – a discovery that has been later massively used in numerous crop plants. Additionally, they also observed a phenomenon called "multiple shoot formation", in which the development of the main shoot bud is, as a rule, accompanied by the differentiation of new lateral buds. In vivo, their later outgrowth would be automatically inhibited by auxin-dependent apical dominance. But in vitro, even under the simultaneous influence of the cytokinins added to the culture medium, lateral buds are progressively activated as well. As a result, a small "forest" of new stems of almost the same developmental stage can be obtained from one original apical bud.

For years, this phenomenon was believed to be based just on the repetitive activation of new, sequential, "naturally" differentiating sets of SAM that had escaped from control by auxin-dependent apical dominance in response to the cytokinin applied to the culture media. If this hypothesis were valid, the multiple shoots should be genetically identical, a prerequisite for the "true-to-type character" of commercially used regenerates. But, taking into account the recent molecular studies on the hormonal and nonhormonal interplay in the developing SAM, we should accept also the possibility of various transdifferentiations in both the original explants and the following sub-clones. These transdifferentiations can be connected not only with various epigenetic changes but also with the risk of an increased mutation rate and subsequent increase in the somaclonal variability of the regenerates. This, of course, represents an undesirable risk for the micropropagation industry requested to produce uniform, genetically almost identical seeds or seedlings and, to some extent, also for the application of the "shoot tip technique" in plant virus eradication and "germ line storage" in gene banks.

This is also one of the main reasons why the third "organogenic" in vitro technology, i.e. *regeneration* de novo, is pronouncedly used in plant breeding, but not for plant propagation (neither the technique nor its key factors – i.e. growth regulators). In contrast to the long and frequent use of various "rooting compounds" (see chapter Skůpa et al., this volume, for details) in practical gardening, no relevant "shoot regenerative preparations", containing both auxins and cytokinins, are commercially applied to improve standard regenerative effectiveness for in vivo technologies.

Simply put, the "callus phase", as the traditional first stage of the regeneration de novo process in vitro (and maybe in vivo as well), is perceived as a "genetically risky period", which either has to be shortened as much as possible or, better, eliminated altogether. The results on the stability of stem cell niches support this point of view. As Fulcher and Sablowski (2009) observed, both shoot and root meristem cells in Arabidopsis are hypersensitive to DNA double-strand breaks – a phenomenon that is common in any intensively dividing cell but rare in the noncycling cells. In native SAMs or RAMs, cells with damaged DNA are mostly discarded via programmed cell death (PCD). However, under in vitro conditions, the effectiveness of PCD-based quality control seems to be pronouncedly

decreased. Consequently, both in case of canonical "cell dedifferentiation" and "transdifferentiation", e.g. primary callus formation or regeneration of pluripotent cells with shoot- or root-like characteristics (Sugimoto et al. 2010), we encounter "somaclonal variability". Although this is mostly undesired, it can be useful as source of genetic variation that can later be selected by breeders (Larkin and Scolcroft 1981). Massive modifications of the cell differentiation programme and severe changes at the epigenetic level (Tanurdzic et al. 2008; Li et al. 2011; Sugimoto et al. 2010; He et al. 2012) probably also lead to an increase in the mutation rate.

Undoubtedly, the long-term empirical experience of numerous "plant tissue culturists", summarised in the rule that "the longer in callus form, the higher subsequent somaclonal variability", has its background in the molecular features described above. In its "free-range" callus state, plant cells offer maximal freedom to develop various deviant cell subpopulations that survive and multiply – and, consequently, can regenerate into variously aberrant organisms.

4.2 Embryogenesis

The technology of in vitro cultivation of various "explanted" plant parts has contributed enormously to our knowledge of the various forms of plant embryogenesis – not only zygotic (ZE) but also somatic (SE) and pollen-derived (PE) types of embryogenesis. These phenomena, relatively rare in nature, proved to be a frequent event under the conditions of the transfer of "mother tissue" into a modified in vitro environment. Consequently, this regenerative procedure could be realised in numerous plant species and used practically for their propagation and breeding, but also for other biotechnological applications (such as in the pharmaceutical industry).

By the way, the positive results of in vitro cultivation demonstrate again the universality of the general stem cell niche concept for multicellular organisms mentioned above:

(a) Cells that are protected from differentiation by signals from neighbouring cells in specific niches can divide and thus function as stem cells,
(b) cells that leave a niche are bound to differentiate. Differentiation of the daughter cell leaving a niche appears to be dictated by the environment rather than by cell origin. (Aichinger et al. 2012, based on van den Berg et al. 1997)

4.2.1 Somatic Embryogenesis

Regeneration of embryos from somatic cells of intact plants (somatic embryogenesis – SE) is, in fact, if taken in sensu lato, a relatively frequent phenomenon, only hidden from "non-professional eyes". One can meet it, for instance, as "polyembryony", i.e. formation of several embryos in a single seed. Such embryos may arise

in a single embryo sac (true polyembryony) or from different embryo sacs (false polyembryony). With true polyembryony, several embryos develop from a single zygote as a result of irregular zygotic division (e.g. in some tulips) or when the proembryo is delaminated from proembryonic apical cell (as in the European yellow water lily). Ectopic embryos may also develop from cells of the suspensor (in *Lobelia*). Often in true polyembryony, the embryo arises from one or two synergids (as in iris, lily and mimosa) or antipodal cells (as in *Allium odorum*). Additional embryos may arise even without fertilisation from cells of the nucellus and the integuments. With false polyembryony, the embryo is formed either from several embryo sacs in the ovule (as in strawberry or *Pyrethrum*) or when more than one of the four megaspores develop (as in lilies and lady's mantle). Additional embryos may also form owing to the development of supplementary aposporous embryo sacs (from vegetative cells) and a normal embryo sac (as in hawkweed and wormwoods).

These cell complexes can thus also be perceived – and classified – as a kind of stem cell niche, with the common developmental commitment towards formation of embryogenic structures. In situations in which the "master" zygotic embryogenesis programme fails (or sometimes, even in parallel with canonic embryogenesis), other neighbouring cells are subjected to the signals of the "local niche environment" and take over the function and realisation of the embryogenic programme.

In contrast, natural (in vivo) macroscopically detectable formation of somatic embryos from leaf, stem or even root tissues is generally supposed to be a very rare phenomenon and known only in very few instances such as the embryogeny from leaf margin cells in *Bryophyllum calycinum* (Yarbrough 1932) or in the orchid *Malaxis paludosa* (Taylor 1967). In these cases, both the apical and basal poles (apex root complex) are differentiated simultaneously, in the same place and at the same time. Numerous other, seemingly identical, cases where vegetative propagules are formed – like the tiny plantlets on the leaf margins of various *Crassulaceae* – are in fact caused by organogenesis in which earlier differentiated buds later form roots.

Somatic embryogenesis (SE) in vitro was originally described almost simultaneously by Reinert (1958a, b, 1959) and by Steward (Steward and Pollard 1958; Steward et al. 1958a, b). As vividly described in the review of Vasil (2008), these groups first of all succeeded in the selection of a suitable model, i.e. carrot tissue, the convenience of which for in vitro studies had already been established by Gautheret (1939) and Noubécourt (1939). As we know meanwhile, various members of the *Umbelliferae* family proved to be very convenient for the regeneration of somatic embryos under in vitro conditions. Moreover, both the Reinert and Steward groups used as auxin the synthetic 2,4-D rather than IAA, and their media also contained coconut milk. This liquid endosperm provides, among other factors, a natural cocktail of phytohormones, in particular cytokinins.

In his review, Reinert (1973) assumed that the coconut milk factor is essential for the initiation and development of the embryos, particularly in case of suspension-cultured somatic cells. Its effect could be later attributed mainly to cytokinins. 2,4-D itself seems to be essential for the initial or early phase of the

embryogenic process in order to induce formation of the first proembryos and/or to secure long-term cultivation of the "proembryonic mass". This synthetic auxin can be either applied alone but, in case of some materials, like conifers, has to be accompanied by cytokinins. Under these conditions, the embryos mostly do not develop further than the globular phase ("proliferative phase of the culture"), but their transition to the subsequent maturation phase can be enabled by omission of 2,4-D. Besides, other growth regulatory factors, in particular abscisic acid (ABA), are added (for details, see Skůpa et al., this volume; Smertenko and Bozhkov, this volume).

Why is 2,4-D, which after all is not a naturally occurring auxin, so efficient? Is the morphoregulatory effect of 2,4-D connected with specific target genes – or merely a consequence of its altered transport or metabolic stability? At present, there is no evidence neither for specific genes activated by 2,4-D during the processes of cellular reprogramming nor for alternative direct effects of 2,4-D on the modification of a chromatin structure (see Orkin and Hochedlinger 2011 for a review).

As summarised in the chapter by Skůpa et al. (this volume), 2,4-D differs from other commonly used auxins, such as IAA or NAA, by its transport characteristics. Active influx carriers mediate a strong import. Once it has entered the cell, it undergoes little metabolism, and there seem to be no carrier systems to facilitate its efflux, which is therefore slow. Consequently, relatively high concentrations of 2,4-D accumulate inside the target cells. Furthermore, 2,4-D has much more pronounced effects on endocytosis than IAA. Taken together, these characteristics mean that compared to other relevant, either "natural" or "synthetic" auxins, 2,4-D has a much more pronounced effect on the treated tissue, especially with respect to promoting cell division.

It is also possible that the interaction of 2,4-D with particular auxin receptors differs from that of other auxins. For example, compared to IAA itself, the affinity of 2,4-D to the TIR1 family of auxin receptors (which encode for the F-box subunit of the ubiquitin ligase complex SCF^{TIR1}) is somewhat lower (Dharmasiri et al. 2005; Kepinski and Leyser 2005). In this way, 2,4-D ensures the establishment of the time-limited endogenous auxin maxima necessary to initiate somatic embryogenesis; this is followed by maturation of somatic embryos after 2,4-D is removed.

During the last 40 years, somatic embryogenesis has been successfully induced in hundreds of plant species, from herbs to trees and from cruciferous plants to legumes. In some species, like cereals or conifers, it has even become the "method of choice" – based on the fact that it is very difficult to get micropropagation via organogenesis. As a rule, some hardwood trees, in particular conifers, are currently preferentially propagated only by means of either zygotic or somatic "seeds". However, the problem of "practical regenerative recalcitrance" of particular experimental models remains, caused by unknown genetical or physiological factors.

Paradoxically, up to now, somatic embryogenesis has never been convincingly demonstrated for the traditional organogenic model *Nicotiana tabaccum*. In this aspect, it differs from most other members of the *Solanaceae* family, exhibiting

relatively high flexibility of the forms of in vitro regeneration – from organogenesis to SE (*see* Konar et al. 1972, as one of the earliest examples).

In contrast, simultaneously with the genotype-based limitations, either physiological or epigenetic mechanisms block SE formation in explants taken from various adult and/or mature organs of perennial plants, in particular woody species. In fact, for perennial plants only nonmature zygotic embryos yield explants that are embryogenically totipotent (see Smertenko and Bozhkov, this volume). The difference between annual and perennial plants is worth to be studied intensively to advance our understanding of the mechanisms of plant ontogenesis, maturation and senescence in general. Such an understanding is of tremendous practical value in the context of real cloning of the valuable tree genotypes: their in vivo propagation by means of conventionally cuttings is mostly unsuccessful – and in vitro propagation by means of zygotic embryos is in fact "pseudocloning", preserving only a heterogenous population of F1 progeny.

But how do we classify examples of clearly "de novo embryogenesis" originating from or initiated in both structurally and functionally "differentiated" somatic cells from "mature" leaf, root, or stem? Should we explain these examples as de novo *organogenesis*, as the result of specific transdifferentiation of various pluripotent somatic cells, or as the activation of latent islands of embryogenic descendants of the original zygotic ancestors? What key stem cell niches (see Sect. 2.2.3) are participating in this process?

Taking into account just the general phenomenology of numerous experimental models which have been used to study somatic embryogenesis since the time of Reinert/Steward, it is possible that vascular stem cell niches are favourite candidates in this competition. But maybe there are alternatives for this classical view.

In this context, Verdeil et al. (2007) proposed that the plant stem cell concept could be extended by inclusion of single embryogenic cells as "totipotent stem cells based on their capacity to regenerate or develop into an embryo under certain conditions", and based on this extension, they offer a rather ambitious list of "cellular, physiological and molecular similarities and differences between pluripotent and totipotent plant stem cells". They build their classification system on the traditional "dedifferentiation-differentiation" hypotheses, according to which the cells of primary explants should "dedifferentiate" first, after being excised from the donor plant and transferred to in vitro conditions to acquire their new fate. The following "re-differentiation" into new subjects, like continuous callus culture (with no regeneration ability, embryogenesis or organogenesis), roots, shoots or embryos, occurs either via the "dedifferentiated" callus or "directly". The authors admit that, in contrast to the current knowledge of the mechanisms of pluripotent stem cell initiation and maintenance, little is known of those mechanisms that enable a somatic cell to become a totipotent stem cell which can give rise to a somatic embryo. They further express the hope in a quick development of the relevant research, not dissimilar to the recent studies on formation and function of plant stem cell niches.

4.2.2 Pollen Embryogenesis

As an alternative to "polyembryony" based on regeneration of somatic cells, plants also developed regenerative programmes based on the formation of complex bodies from macro- or microspores, or their "atypical" descendants. Again, these processes are only rarely observed in nature. Almost 100 years ago, Blakeslee et al. (1922) published their observation of the occurrence of haploid "mutant" plants of *Datura stramonium*. Even earlier, in 1898, Němec described the formation of "pollen embryo sacs" in petaloid stamens of *Hyacinthus* (Fig. 5). This peculiar pollen behaviour, linked with repeated microspore cell division, was later observed in some other members of the *Liliaceae* family as well as in *Ornithogalum* – and called *the Němec Phenomenon* (De Mol 1923; Stow 1930, 1934). According to Němec, the number of nuclei in the sac can reach 4–16, they may or may not be organised and they mostly originate from the vegetative nucleus, while the generative nucleus degenerates.

The development of in vitro culture made it possible to study both the mechanism of gynogenesis (embryogenesis from the female gametophyte) and androgenesis (embryogenesis from the male gametophyte) at various levels, moving from general phenomenology to molecular analysis. It also enabled the complete development of these mostly haploid embryos – and of the plants arising from them.

The first successful *pollen embryogenesis* was achieved by Guha and Maheswari (1964, 1966). Their regenerants of *Datura innoxia* anther cultures were haploid – i.e. of microspore origin. The methodology was successfully repeated with numerous plant species ranging from herbs to woods, motivated by either theoretical or applied aims (Opatrný 1973; Opatrný et al. 1977; Vagera et al. 1979). Later on, the technology of pollen cultures, in particular in *Brassica*, allowed both the precise characterisation and the practical application of this phenomenon, in its complexity absent under in vivo conditions.

Both the studies of pollen biology and of the early phases of pollen embryogenesis offer us numerous cytological and molecular data comparable with the research on developmental biology of the stem cell niches. But simultaneously, the empirical observations provided interesting information in the context of the ancient "cell theory", markers of functional totipotency and its regulatory factors. For example, immature microspores of some species (e.g. *Nicotiana tabacum* or *Brassica napus*) are able to realise alternatively two "developmental" programmes in vitro: either to differentiate into mature pollen grain or into an embryo. The "switch" from a pollen to an embryonal programme can be induced, at least in case of tobacco, simply by isolation of anthers from flower buds and their transfer to agar media containing only water and iron. No special external morphoregulatory factors, like phytohormones, are necessary. In this case, early pollen embryos reached the globular stage (Vagera et al. 1979), but their further development was blocked.

These observations are in good agreement with more general observations on the mostly "autonomous" origin of somatic embryogenesis and the proembryonal differentiation up to the globular phase, whereas the continuation of development

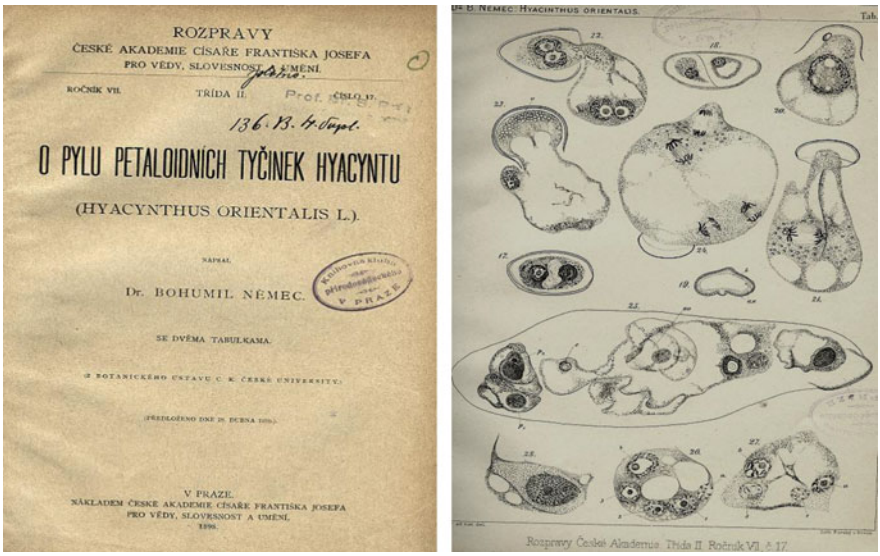

Fig. 5 Observation of "pollen embryo sacs" in petaloid stamens of *Hyacinthus* – the "Němec phenomenon". The documentation of atypical repeated microspore division in vivo, leading to the formation of the structures similar to the "female" embryo sac (From Němec 1898)

requires a supply of various exogenous, either hormonal or nonhormonal, factors (Smertenko and Bozhkov, this volume).

Undoubtedly, at least the main structure of some ancient embryogenic programme seems to be preserved in the memory of various plant cells, both sporophytic and gametophytic, and is expressed more strongly than we should expect. Furthermore, in the case of pollen, this expression is limited to some "open windows" during microspore ontogenesis and is controlled by various epigenetic factors.

5 From Tradition to Reality: Concluding Remarks

The study of regenerative mechanisms in multicellular plants forms an important part of experimental plant physiology and began more than a 100 years ago. Early in this work, the "cell theory" postulated permanent functional and morphogenetic "totipotency" of somatic plant cells. Totipotency is crucial not only for the different types of plant recovery from the effects of abiotic and biotic stresses but also for the rapid and accurate genetic (clonal) propagation of the plant species. Fundamental research on totipotency was aimed not only to understand the mechanisms of plant growth and development but also to use this knowledge in practical plant propagation, preservation and breeding.

The technology of in vitro cultivation of plant cells, tissues, organs, isolated embryos or seedlings has been significantly extended through the use of chemical "morphogenic factors" and a detailed analysis of their effects at the molecular level. However, problems persist, connected mainly with the low or absent "regeneration ability" of particular plant tribes, species and genotypes as well as the loss of the regeneration capacity of plant organs/tissue already in the early phases of their ontogenesis. This limits the potential of these techniques either in "normal" plant cloning (adult conifers, beech, oak, walnut) or in the generation of genetically modified plants (routine de novo regeneration of GM buds from tobacco leaves versus no or very rare regeneration in cereals such as wheat or maize). The molecular background of such problems remains unclear. A possible solution may lie in a more sophisticated use of known or novel synthetic growth regulators or known natural plant hormones.

Intensive research on "plant stem cell niches" over more than half a century has provided considerable insights into these problems. Advancement in ablation techniques, particularly the use of directed laser "surgery", has confirmed and added to the early findings of Němec. Original findings based on anatomical and cytological studies have been supplemented through the use of "molecular markers", enabling us to detect the "histogenic identity" of newly formed cells or tissues. Taken together, the new information has contributed to a clearer understanding of the type of regeneration and maturation processes that occur in plants and thus their proper classification. An increasing number of authors have become aware of the necessity of precise terminology to characterise these events and the ambiguity caused by loose and inaccurate use of terms like "totipotency" or "dedifferentiation" that has to be replaced by logical and accurate descriptions of the path of cell transdifferentiation, various types of pluripotency and their interplays in time and space. Even the term "callus" is no longer to be used in the sense of a chaotic group of confused cells but as a dynamic complex of "molecularly determinated" cells, exhibiting certain functional/morphogenic competencies.

These studies also increasingly use highly accurate analytical methods for a number of phytohormones, particularly auxins and cytokinins. They allow us to detect dynamic changes in their contents at the level of individual cells and on a timescale as short as one second. Alternatively, or simultaneously with phytohormone analyses, a broad spectrum of tissue-specific molecular markers, e.g. transcription factors, can be determined to trace individual and sequential phases of various regenerative processes, based on the key mechanism of either organogenesis or embryogenesis. In this context, practical solutions to the problems of "non-regenerative responsiveness/recalcitrance" of some materials remain a focus of interest. However, it is probable that solutions to such problems may only be found using an integrated approach which includes knowledge and application of new types of synthetic hormones (see chapters by Skůpa et al., and Šmehilová and Spíchal, this volume). We need to understand more precisely the dynamic balance between "pro-life" and "pro-death" mechanisms in plant organisms (see chapters Smertenko and Bozhkov and Opatrný et al., this volume). Only

such integrated knowledge will provide effective tools to influence cellular morphogenesis of plants for efficient application.

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Part I
Control of Growth and Development

Why to Spend Tax Money on Plant Microtubules?

Peter Nick

Abstract Plant microtubules have evolved into a versatile tool to link environmental signals into flexible morphogenesis. Cortical microtubules define the axiality of cell expansion by control of cellulose orientation. Plant-specific microtubule structures such as preprophase band and phragmoplast determine symmetry and axiality of cell divisions. In addition, microtubules act as sensors and integrators for stimuli such as mechanic load and gravity but also osmotic stress, cold, and pathogen attack. Many of these functions are specific for plants and involve unique proteins or the recruitment of proteins to new functions. The review aims to ventilate the potential of microtubule-based strategies for biotechnological application by highlighting representative case studies. These include reorientation of cortical microtubules to increase lodging resistance, control of microtubule dynamics to alter the gravity-dependent orientation of leaves, the use of microtubules as sensitive thermometers to improve adaptive cold tolerance of chilling and freezing sensitive plants, the reduction of microtubule treadmilling to inhibit cell-to-cell transport of plant viruses, or the modulation of plant defence genes by pharmacological manipulation of microtubules. The specificity of these responses is controlled by a great variety of specific associated proteins opening a wide field for biotechnological manipulation of plant architecture and stress tolerance.

1 Motivation: Plant Architecture Defines Yield

Plant architecture represents a target with high potential for plant biotechnology. When leaf angles can be manipulated, this will allow the sunlight to penetrate deeper into the canopy (Zheng et al. 2008). When internodes can be shortened, this will increase lodging resistance. When the formation of new tillers is suppressed,

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this will promote the filling of grains on the main ear (Sakamoto and Matsuoka 2004). These important traits for breeding of high-yielding cultivars have been modelled numerically to assess the impact of genetic traits on plant morphology and expected yield on a quantitative base (Xu et al. 2011). Plants with 'ideal architecture' would show reduced shoot length, reduced tiller number, and increased grain weight as traits (Jiao et al. 2010). Microtubules, as central regulators of plant growth and development, provide an important target for biotechnological applications aiming to change plant architecture. However, the potential of microtubules for plant biotechnology, so far, is still to be exploited motivating the current chapter.

Yield losses by lodging and windbreak are considerable: In rice, for example, they are estimated to range up to 40 % (Nishiyama 1986). Control of plant height has therefore been a major topic in cereal crops, because the resistance of a plant to lodging and windbreak is inversely related to the square of plant height (Oda et al. 1966). This means that a reduction of internode elongation by 50 % will reduce lodging to 25 %. Thus, the agronomic importance of reduced shoot length cannot be overestimated, shifting the control of plant height into the centre of interest, especially for cereal crops. In fact, a central factor in the green revolution of cereals has been a mutation in the so-called DELLA regulators of gibberellin-dependent shoot elongation (Peng et al. 1999), and recently it was uncovered that during the domestication of japonica rice a semidwarf mutation linked to gibberellin synthesis had been selected (Asano et al. 2011).

However, the impact of plant architecture is not confined to lodging resistance. For instance, the resistance of crop plants to wind depends on the angle between main and branch roots (Stokes et al. 1995), and the marketable yield very often depends on the partitioning of biomass. As already pointed out in the 1920s as 'Law of Homologous Series' established by the Russian geneticist Vavilov (Vavilov 1922) for the domestication of crop plants, apical dominance represented a central factor. Instead of numerous, small axes bearing numerous, but smaller fruits, one main axis was established during domestication of many crops. This change of architecture is impressively illustrated by the transition from the ancestral teosinte to modern maize linked to mainly one locus controlling the formation of side branches (Doebley et al. 1995). Production of fewer but larger fruits, tubers, or grains facilitates processing, whereas in other cases, such as breeding of potatoes or tulips, the advantage might be on the side of more but smaller structures. The morphogenetic events involved in the formation of tubers, fruits, or side branches might thus be manipulated to recruit biomass optimally between product quantity, size, and quality, without the need to interfere with source–sink relations or photosynthetic efficiency in general.

Microtubules control plant architecture at two levels: (1) They act downstream as effectors. Microtubules control the axis of cell division and cell expansion and therefore link the output of signalling triggered by environment and development to a response of plant architecture. (2) They act upstream as sensors. Microtubules can integrate the mechanic load resulting from growth and architecture and feed this

information into the deposition of load-bearing elements. Both functions will be discussed in more details in the subsequent sections.

2 Plant Microtubules as Morphogenetic Tools

Plant microtubules pass through a series of different arrays during the cell cycle. These arrays are not only morphologically distinct but convey different cellular functions. Most of these functions are linked with cell axiality. Since cellular migrations, as central element of animal development, do not play a role in the walled plant cells, the spatial control of both cell expansion and cell division are the only mechanism at hand to control plant morphogenesis. The dynamically interchanging arrays of plant microtubule act as central morphogenetic effectors in the induction, maintenance, and perpetuation of cellular axiality.

During interphase, microtubules form arrays of parallel bundles oriented perpendicular to the axis of preferential cell expansion. These cortical microtubules define the biophysical properties of the yielding cell wall and thus the geometry of expansion. In fact, 'microtubules' were predicted to exist from merely biophysical considerations on expanding plant cells (Green 1962) and only later actually discovered exactly half a century ago by electron microscopy (Ledbetter and Porter 1963). The classical model assumes that cortical microtubules define the orientation in which newly synthesised cellulose microfibrils will be laid down (reviewed in Geitmann and Ortega 2009; Nick 2008a). In cylindrical cells, where isotropic action of turgor pressure is predicted to produce only half of the strain in the longitudinal direction relative to the transverse direction, a transverse orientation of cellulose microfibrils maintains the lateral reinforcement needed to drive elongation (Green 1980).

Cortical microtubules can change their orientation in response to a broad range of signals, both exogenous and endogenous, and thus allow to tune plant morphogenesis with the challenges of the environment. This signal-dependent reorientation is transformed into altered deposition of cellulose microfibrils, a mechanism that allows to adjust the direction in which the cell wall yields to the turgor pressure exerted by the expanding protoplast and eventually alters the proportionality of cell expansion in response to the stimulus (for review, see Nick 2008a). Except for cells that undergo tip growth, the cell wall is formed by apposition of textured cellulose layers to the inner surface of the cell wall. The cellulose-synthesising enzyme complexes are integrated into the membrane by fusion of exocytotic vesicles and are thought to move within the fluid membrane leaving a 'trace' of crystallising cellulose. The movement of the enzyme complex will determine cellulose orientation and thus the anisotropy of the cell wall. It is the direction of this movement where cortical microtubules interfere with the mechanical anisotropy of the expanding cell wall.

The direct contact between cortical microtubules and newly emerged cellulose microfibrils has been demonstrated by electron microscopy but is also supported by

a wealth of data where signal responses of cell expansion were preceded by a corresponding reorientation of cortical microtubules. As to be expected from a microtubule-based mechanism for cellulose orientation, elimination of cortical microtubules by inhibitors produces a progressive loss of ordered cellulose texture. The resulting loss of axiality causes lateral swelling and bulbous growth. The mode of action of several herbicide classes, including the phenyl carbamates or the dinitroanilines, is based on the microtubule dependency of cell-wall texture.

The striking parallelism between cortical microtubules and newly deposited cellulose microfibrils stimulated two alternative models: The original 'monorail' model proposed that motor proteins moving along cortical microtubules pull cellulose synthetases (Heath 1974). In contrast, the latter 'guardrail' model assumed that microtubules induce small protrusions in the plasma membrane constraining the movement of the enzyme complexes, whereas the actual movement is driven by the crystallising cellulose itself. This 'guardrail' model was stimulated by observations where the cellulose synthase complexes were found 'in gap' between adjacent microtubules (Giddings and Staehelin 1988). However, electron microscopy observation is prone to artefacts of chemical fixation, and great luck is required to locate the right section where the topological relation between microtubules and cellulose synthases can be assessed. Therefore, the results left space for controversial interpretations. The situation was further complicated by situations where the orientation of microtubules and cellulose microfibrils differs (for review, see Wasteneys 2004), casting doubt on microtubule-guided cellulose synthesis in general.

During the last decade, the classical 'monorail' model has recovered by evidence for a central role of kinesins and microtubule-binding proteins in cell-wall deposition (recently reviewed by Cai and Cresti 2012). A screen for mutants with reduced cell-wall integrity recovered the mutant *fragile fiber 2* with stunted and swollen cells. This phenotype was caused by a mutation in the microtubule-severing protein katanin, also affected in the mutant *botero* (Bichet et al. 2001). A second mutant, *fragile fiber 1*, was mutated in a kinesin-related protein belonging to the KIF4 family of microtubule motors. The phenotype of this mutant suggested a role of the FRA1/KIF4 motor in the movement of cellulose synthases (Zhong et al. 2002). In fact, fluorescently tagged cellulose synthases could later be shown to move in tracks adjacent to the subtending cortical microtubules (Paredez et al. 2006), and a cellulose synthase (CSI1) binds directly to microtubules (Li et al. 2012).

Based on situations where a transverse cellulose orientation persisted although microtubules had been eliminated by drug treatment or temperature-sensitive mutations, a self-organisation of cellulose has been proposed. During cell elongation, microtubules would sustain cellulosic self-organisation by constraining the secretion of noncellulosic polysaccharides (Fujita et al. 2011). Irrespective of the underlying mechanisms (that are not mutually exclusive), the cell axis seems to be linked to microtubules rather than to actin filaments.

However, the 'monorail' model suffers from a couple of 'chronic problems' that call for extensions and modifications. In polylamellate walls, layers with differing microfibril orientation coexist. This phenomenon could be plausibly explained by a

rotary movement of groups of microtubules (for review, see Lucas and Shaw 2008). A second 'chronic problem' arises from situations where a transverse cellulose orientation persisted although microtubules had been eliminated by drug treatment or temperature-sensitive mutations. These observations were explained by cellulosic self-organisation sustained by microtubules during cell elongation by constraining the secretion of noncellulosic polysaccharides (Fujita et al. 2011). A third problem is the observation that cellulose microfibrils are often observed to be intertwined (Preston 1988), again pointing to the self-organisation of cellulose synthesis. The microfibrils that are already laid down could act as templates for the synthesis of new microfibrils (for review, see Mulder et al. 2004). As a consequence, microtubules would not be required throughout all stages of cellulose deposition.

Last but not least, the relation between cell wall and cortical microtubules is bidirectional. Through transmembrane proteins, cortical microtubules are connected with the extracellular matrix. The molecular nature of these transmembrane proteins has remained elusive, but they share analogies with animal integrins (for review, see Pickard 2008). This link seems to stabilise cortical microtubules, because removal of the cell wall renders microtubules more cold sensitive in tobacco cells (Akashi et al. 1990). Moreover, cobtorin, a compound identified from a screen that specifically disturbs the parallelity of microtubules and microfibrils (Yoneda et al. 2007), has meanwhile been found to affect cell-wall pectins (Yoneda et al. 2010).

Thus, although often discussed in this manner, there is no reason why the 'monorail' model and cellulose self-organisation should be mutually exclusive. The 'chronic problems' of the original 'unified hypothesis' (Heath 1974) can be easily remedied by adding two aspects: (i) The deposition of cellulose microfibrils not only depends on microtubules but also on geometrical input from pre-existing microfibrils, and (ii) the link between microtubules and cellulose is not a one-way street, but bidirectional, i.e. the orientation and dynamics of microtubules depend on input from the cell wall.

Upon cell division, cortical microtubules are replaced by a rapidly changing sequence of diverse arrays: radial microtubules, preprophase band, spindle, and phragmoplast. In preparation for mitosis, the nucleus moves into the cell centre and somehow commits the site where the prospective cell plate will be formed (for review, see Nick 2008a). At the same time, radial microtubules are nucleated at the nuclear envelope and connect with the cortical cytoskeleton driving and stabilising nuclear migration. Once the nucleus has reached the cell centre, it will organise the preprophase band, a broad band of microtubules girdling the cell equator. The preprophase band predicts site and orientation of the prospective cell plate, although this will become manifest only much later, when mitosis has been completed. The preprophase band disappears when the division spindle forms, always orthogonal to the plane of the preprophase band. In late anaphase, the microtubular phragmoplast array is organised at the site that had been predicted by the preprophase band. The phragmoplast, a double ring of interdigitating microtubules, guides the growth of the expanding cell plate. The eclipse of the

preprophase band that nevertheless defines orientation and position of phragmoplast and cell plate has been a major enigma of plant cell biology. This mystery had been resolved by the discovery of an endosomal belt laid down adjacent with the preprophase band and persisting through mitosis (Dhonukshe et al. 2005). This endosomal belt is recognised during late anaphase by exploratory microtubules emanating from the cell poles throughout the dividing cell. Those microtubules that hit the endosomal belt defined by the preprophase band are stabilised, whereas those that fail to find their target will undergo catastrophic decay.

Microtubules are therefore used to establish and maintain the axis of cell expansion and division. This is important to adjust plant development with the variable challenges of the environment. In other words, microtubules are tools by which plants can exert control over their morphogenesis.

3 Plant Microtubules as Morphogenetic Sensors

The preceding section dealt with the classical role of microtubules as part of the response machinery that links signalling with cellular morphogenesis. However, microtubules play a second role that is more upstream and linked with morphogenetic signalling itself. This sensory function is linked to the high stiffness of microtubules (Gittes et al. 1993). The combination of mechanic rigidity with high dynamics of assembly and disassembly renders allows to integrate mechanic load even across the borders of individual cells.

Mechanical tension is important to integrate the architecture of the expanding plant. In terrestrial plants, the considerable lever forces from branches and leaves are not compensated by buoyancy and require compensatory deposition of supportive structures. Mechanic force can reach instantaneously even the remotest parts of a tree and therefore provides an ideal signal to integrate compensation with mechanical load. Unlike the metazoan cell that is surrounded by a strictly regulated isotonic environment, the cells of multicellular plants are faced with a hypotonic environment leading to considerable turgor pressures of the expanding protoplasts upon the counteracting cell wall. The turgor of individual cells accumulates to considerable tissue tension on the organ level. It is this hydraulic principle that is used as signal to integrate the body plan of a plant (Niklas and Spatz 2004). When new organs are laid down, this will change the pattern of tissue tension, which in turn will guide the development of additional organs in a manner that a state of minimal energy is established. This has been intensively studied and modelled for phyllotaxis by Paul Green and co-workers (for review, see Green 1980).

Their work demonstrated that the buckling of the pre-existing older primordia altered the stress–strain pattern in the growing apical meristem and that the positions of incipient primordia could be predicted as the sites of the local energy minima. As to be expected from this model, local release of tissue tension by softening the cell wall using beads coated with expansin (Fleming et al. 1997) induced appendices that resembled primordia.

The first cellular event of primordial initiation is a reorientation of cortical microtubules that reorient perpendicular to the microtubules of the noncommitted neighbour cells. This difference is first sharp but later smoothened by a transitional zone of cells, where microtubules assume intermediate orientations. Eventually, a gradual, progressive change in microtubule reorientation is produced that extends over several tiers of cells (Hardham et al. 1980).

This phenomenon has been revisited using a combination of live-cell imaging with fluorescent microtubule markers modelling of stress–strain patterns in *Arabidopsis thaliana* (Hamant et al. 2008). Again, cortical microtubules were found to align in the direction of maximal mechanical stress in a transcellular pattern. In the next step, the outer meristem layer was removed locally by laser ablation, and the resulting changes of stress pattern were modelled. In fact, microtubules were then observed to reorient as predicted by these pattern, leading to a compensatory bulging of the apex. The impact of cortical microtubules is further corroborated by recent evidence for a role of the microtubule-severing protein katanin for meristem patterning (Uyttewaal et al. 2012). However, mechanic load can not only align cortical microtubules but also division-related microtubule arrays. Already three decades ago it could be demonstrated that new cell plates (oriented by phragmoplast microtubules) align with the force vector when a callus was subjected to compression forces (Lintilhac and Vesecky 1984). Furthermore, a mild centrifugation can align cell divisions parallel with the force vector, and this alignment requires microtubules to be present at the time, when this mechanic stimulus is administered (Wymer et al. 1996).

The ultimate reason, why plant microtubules are mechanosensitive, is gravity. Terrestrial plants must arrange force-bearing elements such that mechanic load by gravity is minimised. The pattern of mechanical strains (due to tension of the turgescent tissue) is used to guide the arrangement of supportive structures. As is valid for any physical stimulus, gravity sensing requires a transformation of the physical stimulus into a different type of energy that can be perceived by a biological receptor, a process termed *susception* (Björkman 1988). When a plant is misoriented with respect to gravity, its flank will be subjected to the same gravitational field. This means that a gradient in the strength of the terrestrial gravitational field strength would not work. Gravitation as stimulus must therefore be first transformed into mechanical force by acting on heavy particles, the statoliths. In higher plants gravity susception is brought about by the amyloplasts as proposed simultaneously, but independently by Nemec (1900) and Haberlandt (1900) more than a century ago (see chapter by Opatrný, this volume), and finally proven in ingenious experiments using high-gradient magnetic fields by Kuznetsov and Hasenstein (1996). Of course, the statoliths (as well as their accessory structures) are not gravisensitive; they merely assist gravity sensing by acting as *susceptors*.

Since gravity is sensed by individual cells, the maximal energy available for stimulation is the potential energy of the sensing cell. This energy must be focussed upon small areas to exceed thermal noise. Microtubules as rigid, elongate structures would be ideal levers for gravitropic perception. In fact, gravitropism can be

blocked by antimicrotubular drugs but also by taxol (for review, see Nick 2008b), indicating that microtubules not only have to be present but have to undergo dynamic turnover. A role of microtubules in the perception of gravity has also been identified for the gravimorphosis of germinating fern spores (Edwards and Roux 1994).

Since microtubules participate also in growth (see previous section), it is not trivial to discriminate their function in gravity sensing from their role in gravitropic curvature. Cortical microtubules reorient during the gravitropism of both shoots (Nick et al. 1991) and roots (Blancaflor and Hasenstein 1993) consistent with a model, where gravitropic stimulation causes a transverse flux of auxin from the upper to the lower flank of the organ (see chapter by Skůpa et al., this volume). Auxin depletion in the upper flank (shoots) or auxin accumulation in the lower flank (roots) will cause a microtubular orientation resulting in altered cellulose deposition and thus culminating in differential growth (Nick et al. 1990). When microtubules are eliminated, this will affect the mechanism of differential growth. The absence of gravitropic curvature therefore does not prove a role of microtubules in gravity sensing – when a prisoner without legs does not leave the prison, although the door has been opened for him, this does not mean that he cannot see that the door has been opened.

To pinpoint the sensory function of microtubules and their role in executing gravitropic bending, the lateral transport of auxin can be used because this response is upstream of differential growth (Godbolé et al. 2000; Gutjahr and Nick 2006). Using rice coleoptiles as experimental system, whereas the gravitropically induced lateral transport of radioactively labelled auxin can be easily measured, the elimination of microtubules suppressed auxin transport. Interestingly, taxol that acts as stabiliser of microtubules by suppressing their dynamic turnover blocked lateral auxin transport leaving longitudinal transport untouched. This not only unequivocally demonstrates a gravity-sensitive microtubule function that can be separated from the growth response but also suggests that microtubules have to be dynamic to sense gravity.

Not surprisingly, microtubules as mechanosensitive structures also participate in osmoadaptation. Osmotic stress induces massive bundles of microtubules termed macrotubules (Komis et al. 2002) that confer osmotic adaptation (Komis et al. 2006). It may appear less straightforward that the mechanosensitive nature of microtubules can also convey sensitivity to cold and pathogens. For instance, pharmacological manipulation of microtubules can be used to control cold hardiness (Kerr and Carter 1990; Abdrakhamanova et al. 2003). Since several events of plant defence can be triggered by localised mechanostimulation (Gus-Mayer et al. 1998), it is also possible to induce defence genes in the absence of elicitors by mere pharmacological manipulation of microtubules (Qiao et al. 2010).

The molecular details of this sensory role of plant microtubules are still to be explored. Generally, two paradigms are used to explain mechanosensing: Stretching of proteins will change their conformation and create new binding sites for the recruitment of associated proteins (for review, see Janmey and Weitz 2004). Alternatively, forces from the lipid bilayer can be directly transduced to

mechanosensitive ion channels. Such channels will open when the plasma membrane is deformed or when the channel is pulled by a tether (for review, see Kung 2005). In plants, both mechanisms seem to be integrated into a so-called 'plasmalemmal reticulum' (for review, see Pickard 2008). This network might focus mechanic force upon stretch-activating membrane channels and simultaneously might transduce forces into conformational changes that can result in differential decoration with associated proteins triggering signalling. Microtubules could act and focus mechanic stress upon mechanosensitive channels, similar to the set-up found in touch-sensitive cells of *Caenorhabditis elegans* (Savage et al. 1989). This would be a classical susceptor function. However, microtubules might be mechanosensors themselves: A growing microtubule is subjected to considerable mechanic tension. This tension is caused by transition of the tubulin dimers into a kinked conformation when the GTP residue of newly inserted dimers is progressively dephosphorylated into GDP with increasing distance of the dimer from the growing tip (Akhmanova and Steinmetz 2008). Microtubule plus-end tracking proteins (+TIP proteins) form complexes that counteract this innate tension and thus stabilise the growing microtubules. One of these proteins, EB1, binds to microtubule plus ends at the seam that joins the tubulin protofilaments (Sandblad et al. 2006) and is therefore a good candidate for a conformational mechanosensor. During microtubule catastrophe, the protofilaments bend outwards, which means that they have to be actively tied together in order to sustain microtubule growth. The +TIP complex, in general, and EB1, in particular, are therefore subject to mechanic tension and must be considered as primary targets for mechanic strains on microtubules. In fact, mutation of EB1 genes renders *A. thaliana* touch insensitive (Bisgrove et al. 2008). Imaging of tobacco protoplasts expressing fluorescently tagged cytoskeletal markers by total internal reflection microscopy (TIRF) shows that the microtubules adjacent to the membrane emanate in a starlike manner from specific focal points that are also subtended by actin filaments (Hohenberger et al. 2011). It remains to be elucidated whether these foci contain ion channels that might be rendered mechanosensitive by a microtubule-based accessory system.

4 Microtubules and Green-Revolution Architecture

Shorter plants are more resistant against windbreak and lodging. Reduction of stem length was therefore a central factor for the success of the green revolution. Lodging is inversely related to plant height by the relation

$$L = \frac{W M}{l^2 w}$$

with W fresh weight, M bending momentum at breaking, l shoot length, and w dry weight of the shoot (Oda et al. 1966). Lodging will therefore decrease parabolically with decreasing shoot length. A very efficient strategy to increase lodging

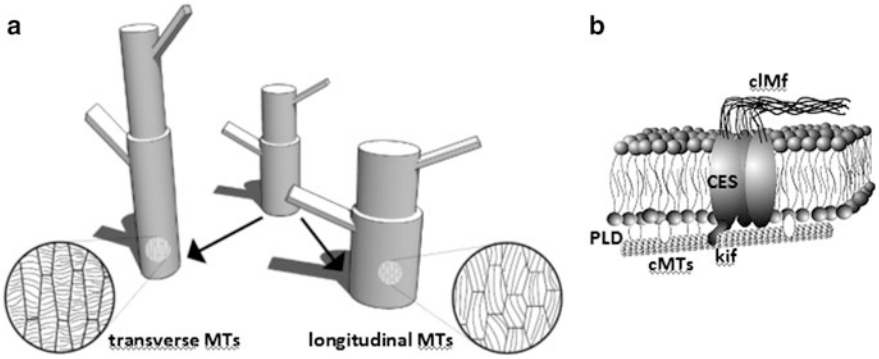

Fig. 1 Microtubules and lodging sensitivity. Elongation of internodes is promoted for transverse orientation of microtubules (MTs), whereas longitudinal microtubules repartition growth from elongation to produce thicker and shorter stems that are more resistant to lodging (**a**). The underlying mechanism is the movement of cellulose synthetases (CES) along cortical microtubules driven by the activity of specific kinesins (kif), such that cellulose microfibrils (clMf) are laid down parallel to cortical microtubules that are anchored to the plasma membrane by switchable linker proteins such as phospholipase D (PLD)

resistance is to repartition shoot growth from elongation to thickening, keeping fresh weight W constant (Fig. 1a). The conventional practice to use chemical growth regulators such as chlormequat chloride or ethephon (Luib and Schott 1990) follows exactly this strategy but is limited by undesired side effects on fertility. More relevant for the success of the Green Revolution was a genetic strategy based on semidwarf varieties (Wang and Li 2006). These varieties are either deficient in gibberellin synthesis (such as the green revolution rice *sd-1*) or constitutively repress gibberellin-responsive genes by dominant-negative mutation of DELLA genes (as in the case of the green revolution wheat *Rht-B1*/*Rht-D1*, Peng et al. 1999). Not only in cereals, reduced plant height is a desirable trait (Luib and Schott 1990). Shorter plants help light to penetrate into the canopy (rapeseed), improve the access of insecticides to the lower parts of the plant (cotton), or facilitate mechanical picking (fruit trees).

However, modern agriculture creates an environment that stimulates internode elongation through high nutrient influx and high canopy densities. Plants can sense competing neighbours through an increase of reflected far-red light using the phytochrome photoreceptor system (Smith 1981) and respond by activation of auxin synthesis through a non-canonical tryptophan-dependent pathway (Tao et al. 2008) promoting cell elongation in the internode. This shade-avoidance response protects plants against overgrowth by competitors but at the same time increases the risk of lodging. The increased levels of auxin produced by the phytochrome-triggered shade-avoidance response will sustain a transverse orientation of cortical microtubules in the outer epidermis (Nick et al. 1990). Since cortical microtubules guide the movement of cellulose synthases in the plasma membrane (Fig. 1b), the transverse microtubules will induce a transverse orientation of the

inner cellulosic layer of the epidermal cell wall. Since the epidermis mechanically constrains the expansion of the subtending tissues, the entire internode will become longer (Fig. 1a).

Can the desirable semidwarf trait be achieved through altering microtubular orientation? In fact, a screen for rice mutants that were resistant against ethyl-N-phenylcarbamate (EPC) (a traditional potato anti-sprouting agent acting on plant microtubules) yielded a mutant, where the microtubular reorientation in response to auxin was interrupted by mutation (Nick et al. 1994). A similar situation has been reported for the hypocotyl of tubulin mutants in thale cress (Matsumoto et al. 2010). In this mutant, cortical microtubules were arranged in oblique or even longitudinal arrays and uncoupled from auxin. As to be expected, this resulted in reduced cell length and a semidwarf phenotype of leaves and culms. Recently, a similar observation was made with respect to gibberellins, a second central regulator of elongation growth in rice. Here, a mutant termed *gibberellin-deficient dwarf 1 (gdd1)* was isolated from a T-DNA mutant collection. The mutant was completely rescued by exogenous gibberellin (Li et al. 2011) pointing to a defect in gibberellin synthesis. Surprisingly, the mutation was located to a kinesin-like protein (BRITTLE CULM12) that controls the formation of secondary cell walls (Zhang et al. 2010). It turned out that this protein fulfils dual functions and also acts as transcriptional regulator of *ent*-kaurene oxidase, a key enzyme of gibberellin synthesis. Again, the orientation of cortical microtubules was altered into oblique and longitudinal arrays accounting for the observed semidwarf phenotype.

In addition to internode elongation, the inclination of leaves is crucial for yield, for it determines how far light can penetrate into a canopy and therefore limits the maximal density of planting. In cereal crops, leaf inclination is defined by the angle between leaf sheath and blade and by the inclination of the leaf sheath in the pulvinus (Fig. 2a). In fact, the pulvinus can respond to canopy density, with leaf sheaths becoming more erect, when canopy density increases (Gibson et al. 1992). This response is brought about by differential cell expansion at the two flanks of the pulvinus. The apical region of the upper flank of the leaf-sheath pulvinus does not elongate in contrast to the remaining regions, and this asymmetry is enhanced by antimicrotubular herbicides such as isopropylphenylcarbamate or dichlorobenzonitrile, suggesting that the movement is driven by gravity-triggered microtubule orientation (Dayandanan and Kaufman 1984). The inclination of leaves is also adjusted by the collar region, delineating leaf sheath and blade (Fig. 2a). The angle between sheath and blade can be actively regulated by cell divisions in the adaxial epidermis of the collar (Zhao 2010), controlled by brassinosteroids. In fact, a classical bioassay for brassinosteroids makes use of this phenomenon and uses the inclination of the leaf lamina in rice for quantification (Takeno and Pharis 1982). Mutants of brassinosteroid synthesis in rice have steeper leaf blades and produce higher yields even in the absence of fertiliser (Sakamoto et al. 2006). Interestingly, the leaf-blade collar is not exhibiting a gravitropic response. However, it is able to sense gravity and to respond by a preformed gravinastic movement (Maeda 1965). In the so-called *lazy* mutants, where gravitropic responses are impaired, inversion of plants causes a curious stimulation of leaf-blade growth and elevated levels of gibberellins

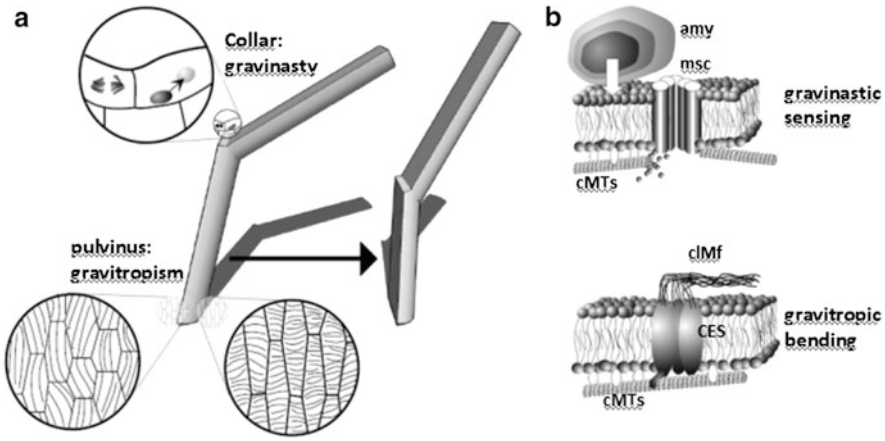

Fig. 2 Microtubules and leaf inclination. In cereals leaf inclination depends on a gravitropic bending (by inhibition of cell expansion at the adaxial flank of the pulvinus) of the pulvinus and a gravinastic orientation of the leaf blade (by inhibition of cell division at the adaxial flank of the leaf collar) (**a**). Both processes are controlled by microtubules (**b**). Gravitropic bending of the pulvinus depends on a gradient in the orientation of cellulose microfibrils (clMf) caused by the movement of cellulose synthetases (CES) along cortical microtubules (cMTs). The role of microtubules in gravinasty is sensory. Microtubules modulate the response of mechanosensitive channels (msc) to the pressure exerted by the sedimenting amyloplasts (amy)

(Abe et al. 1998), demonstrating that the inclination of the leaf blade represents an active gravinastic response. Again, microtubules seem to be involved because in rice mutants with reduced microtubule dynamics, the inclination of the leaf blade is significantly increased resulting in a fan-like appearance of the plant (Nick 2000). The underlying mechanism has to be sought in the modulation of gravity sensing by microtubules (Fig. 2b).

Thus, biotechnological control of microtubule dynamics could be used to control leaf inclination (Fig. 2), whereas control of microtubule orientation can constrain internode elongation and thus lodging resistance (Fig. 1). Microtubules represent therefore a crucial target to tune two central factors deciding yield especially in cereals as most important staple crops.

5 Microtubules and Cold Tolerance

Temperature limits crop yield in most temperate regions. Attempts to increase photosynthetic rates by conventional breeding programmes, although pursued over a long period, have not been successful, indicating that evolution has already reached the optimum (Evans 1975). Optimal photosynthesis requires that leaves are fully expanded, but the cold sensitivity of growth is much more pronounced than that of photosynthesis. This means that it is leaf growth which constrains

productivity (Watson 1952; Monteith and Elston 1971), a conclusion supported by the finding that in cool climates the production of biomass is not source but sink limited (Warren-Wilson 1966). The velocity of shoot development depends on the cold response of roots (Atkin et al. 1973), and cooling of the root can trigger adaptive responses in the shoot (Suzuki et al. 2008). However, the issue of cold sensitivity in agriculture is not confined to temperate regions. Many tropical and subtropical plants suffer severely when they are exposed to cool temperatures that are even still far above the freezing point. This poses extreme problems when fruits have to be harvested and cooled for transport and processing, because these fruits rot rapidly as soon as they return to warmer temperatures. This phenomenon has been known for a long time and was originally termed *Erkältung* (chilling damage) by Molisch (1897) in distinction from actual freezing damage. In extreme cases, even very moderate cooling can irreversibly damage a plant when it hits a very sensitive period of development. For instance, rice can lose fertility when temperature drops below 18°C during flower development. The economical consequences of this phenomenon can be drastic – for instance, according to estimates of the Japanese Ministry of Agriculture, Forestry and Fishery, during the cool summer of 1993, the rice yield was reduced by around 25 %. Insight into the mechanisms of cold sensitivity is therefore of high agronomical impact.

Since microtubules disassemble in the cold, they can limit the cold tolerance of a species. Despite the relatively high conservation of tubulin, cold sensitivity of microtubules is not constant but variable and thus subject to evolutionary change: Whereas mammalian microtubules disassemble already at temperatures below +20°C, the microtubules from poikilothermic animals maintain integrity at much lower temperatures (Modig et al. 1994). The cold stability of plant microtubules is generally more pronounced as compared to their animal counterpart, as to be expected from the higher temperature plasticity of plants. The critical temperature where microtubules disassemble varies between different plant species, and this is correlated with differences in chilling sensitivity (Jian et al. 1989).

The link between microtubule stability and cold hardiness is corroborated by the following observations:

- Treatment with abscisic acid that can stabilise microtubules against low temperature (Sakiyama and Shibaoka 1990; Wang and Nick 2001) also promotes cold hardiness (Irving 1969).
- Pharmacological manipulation of microtubules leads to corresponding changes of cold hardiness (Kerr and Carter 1990).
- Tobacco mutants where microtubules are more cold stable due to expression of an activation tag show cold-resistant leaf expansion (Ahad et al. 2003). Destabilisation of microtubules by assembly blockers such as colchicine or podophyllotoxin increased the chilling sensitivity of cotton seedlings, and this effect could be rescued by addition of abscisic acid (Rikin et al. 1980).
- Gibberellin, an inhibitor of cold hardiness (Rikin et al. 1975; Irving and Lanphear 1968), renders cortical microtubules more cold susceptible (Akashi and Shibaoka 1987).

Cold-resistant species are able to sense low temperature and to respond by adaptation. It is possible to increase the cold resistance of an otherwise chilling-sensitive species by precultivation at chilling, but not freezing temperature (Fig. 3a). Cold sensing is generally ascribed to a reduced fluidity of membranes that will alter the activity of ion channels or the balance of metabolites (Lyons 1973). For instance, overexpression of desaturases reducing membrane fluidity can modify chilling sensitivity in plants (Murata et al. 1992). Cold hardening can be detected on the level of microtubules as well. Microtubules of cold-acclimated cells persist even during a freezing shock (Bartolo and Carter 1991a in spinach mesophyll; Pihakaski-Maunsbach and Puhakainen 1995 in roots of winter rye; Wang and Nick 2001; Abdrakhamanova et al. 2003 in roots of winter wheat). The development of acclimation was suppressed by taxol (Kerr and Carter 1990; Bartolo and Carter 1991b), indicating that microtubules have to disassemble to a certain degree in order to trigger cold hardening. Interestingly, abscisic acid, a well-known inducer of cold hardiness, has recently been found by live-cell imaging to trigger a transient disassembly of microtubules that is only at later stages followed by stabilisation (Seung et al. 2013). Thus, microtubules have to yield first to persist later.

How to explain this microtubule-based thermometer function? Cold perception is triggered by a loss of membrane fluidity (Los and Murata 2004). For instance, the input of low temperature can be mimicked by chemical compounds that decrease fluidity, such as dimethyl sulfoxide, whereas fluidity promotors such as benzyl alcohol can block cold signalling (Sangwan et al. 2001). The fluidity change triggers a spike of intracellular calcium as shown in classical experiments with tobacco plants expressing the bioluminescent aequorin reporter (Knight et al. 1991). This calcium spike is not only a by-product of the cold response but necessary to trigger cold acclimation as demonstrated by pharmacological data (Monroy et al. 1993). Using a cold-responsive reporter system, it could be demonstrated that disassembly of microtubules by oryzalin or treatment with the calcium ionophore A23187 could mimic the effect of low temperature, whereas the calcium channel inhibitor gadolinium or suppression of microtubule disassembly by taxol prevented the activation of this promotor by low temperature (Sangwan et al. 2001). These data are consistent with a model where microtubules constrain the permeability of mechanosensitive calcium channels that are triggered by membrane rigidification (Fig. 3b).

As to be expected from this model, the activity of cold-triggered calcium channels is negatively modulated by pharmacological stabilisation of microtubules but amplified by microtubule elimination (Mazars et al. 1997). The resulting signal cascade will activate cold hardening as an adaptive response to cold stress. Interestingly, microtubules will be rendered cold stable as a consequence of this cold hardening (Pihakaski-Maunsbach and Puhakainen 1995; Abdrakhamanova et al. 2003), which in turn should reduce the activity of the calcium channels that respond to membrane rigidification. Thus, microtubules would not only mediate cold sensing with high sensitivity but, in addition, trigger adaptation by downregulating sensitivity upon prolonged stimulation, a key requirement for any biological sensory process.

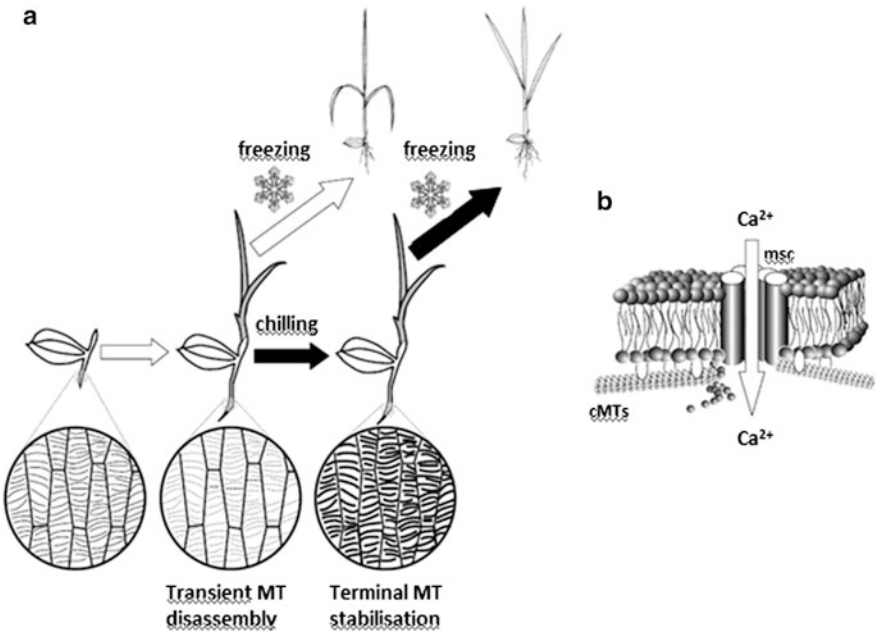

Fig. 3 Microtubules and cold hardening. Precultivation at chilling but nonfreezing temperature renders seedlings resistant against freezing. (**a**) Microtubules disassemble transiently during chilling but are replaced by stable microtubules that sustain vigorous growth even after freezing. The transient disassembly of microtubules is necessary and sufficient to trigger cold hardening and microtubule stabilisation and thus represents a 'thermometer function'. (**b**) Proposed mechanism for the microtubular 'thermometer': cold-sensitive calcium channels (msc) are gated by cortical microtubules (cMTs). The cold-induced reduction of membrane fluidity and the calcium influx cause a self-amplifying decay of microtubules that in turn will amplify calcium influx. This strong and sudden rise in cytosolic calcium will trigger the signalling culminating in cold hardening and stabilisation of microtubules (which in turn will also dampen the activity of the calcium channel)

Is it possible to use this microtubular thermometer function to improve cold tolerance (Fig. 3a, b)? This question was followed in a proof-of-principle experiment in three cultivars of winter wheat of different freezing tolerance (Abdrakhamanova et al. 2003). When these cultivars were exposed to 4°C, the growth rate of roots recovered at a rate that correlated with the degree of cold tolerance. In parallel, the roots acquired progressive resistance to a challenging freezing shock (−7°C) that would impair growth irreversibly in non-acclimated roots. When microtubules were monitored during cold hardening, a rapid, but transient and partial, disassembly was observed in cultivars that were freezing tolerant but not in a cultivar that was freezing sensitive. However, a transient treatment with the antimicrotubular herbicide pronamide was able to confer freezing tolerance in the sensitive cultivar. This demonstrated that a transient, partial disassembly of microtubules is necessary and sufficient to trigger cold hardening. By engineering microtubule dynamics, it should be possible to induce more efficient cold hardening and thus to render crops more independent of climatic fluctuations.

6 Microtubules and Viral Resistance

Viruses exploit functions of the host for their own propagation cycle. Since viruses have to move from cell to cell, the cytoskeleton as a central element of motility represents an ideal target for this viral usurpation. In fact, many animal viruses spread through interaction with host microtubules (Greber and Way 2006; Leopold and Pfister 2006; Radtke et al. 2006) – the cellular function they use for this purpose is probably the transport of mRNA, a central element of developmental signalling (reviewed in Martin and Ephrussi 2009). Signalling through RNA transport is also common in plants (reviewed in Lucas et al. 2001). Actually, it was in the green alga *Acetabularia* where for the first time mobile signals were discovered that later turned out to be RNA. During regeneration of the hat, a morphogenetic signal (untranslated mRNA) is transported from the nucleus into the stalk (Hämmerling 1934). Since actin and tubulin are highly conserved, also plant viruses might use the cytoskeleton to spread from the initial infection site through the rest of the plant (Fig. 4a).

Viral transport has been most intensively studied in the case of tobacco mosaic virus (TMV) moving by virtue of a virus-encoded movement protein (TMV-MP). The complex of viral RNA and TMV-MP assembles near the endoplasmic reticulum, probably anchored to microtubules, and is then translocated to the plasmodesmata by a mechanism dependent on the ER and microtubules (reviewed in Heinlein 2008).

The interaction of the TMV-MP with microtubules is based on molecular mimicry of the TMV-MP with a motif in α-tubulin involved in lateral interactions of microtubule protofilaments. Transmission of TMV-MP viral RNA has been shown to be closely linked to the ability of MP to interact with microtubules (Boyko et al. 2000). The microtubule-dependent transport might be caused by two possible mechanisms (Fig. 4b): Either microtubule might serve as tracks for translocation driven by molecular motors (Heinlein et al. 1995) or the viral particles bind to the treadmilling microtubule and are released at their destination (Sambade et al. 2008).

In order to discriminate motor-driven versus assembly-driven movement, viral spread was analysed in tobacco mutants with reduced microtubular turnover. These plants had been generated by T-DNA activation tagging and selected for their tolerance to EPC, a traditional inhibitor of potato sprouting that sequesters tubulin dimers and therefore eliminates microtubules depending on their innate turnover (Ahad et al. 2003). Principally, resistance of a mutant to antimicrotubular compounds could be caused by altered binding sites as it has been found for mutants of goosegrass (*Eleusine indica*) resistant to microtubule-eliminating dinitroaniline herbicides (Anthony et al. 1998). The binding site of EPC has been located to the carboxy-terminus of α-tubulin (Wiesler et al. 2002; Morettini et al. 2013). However, since in activation tagging, any insertion of the tag into an exon would result

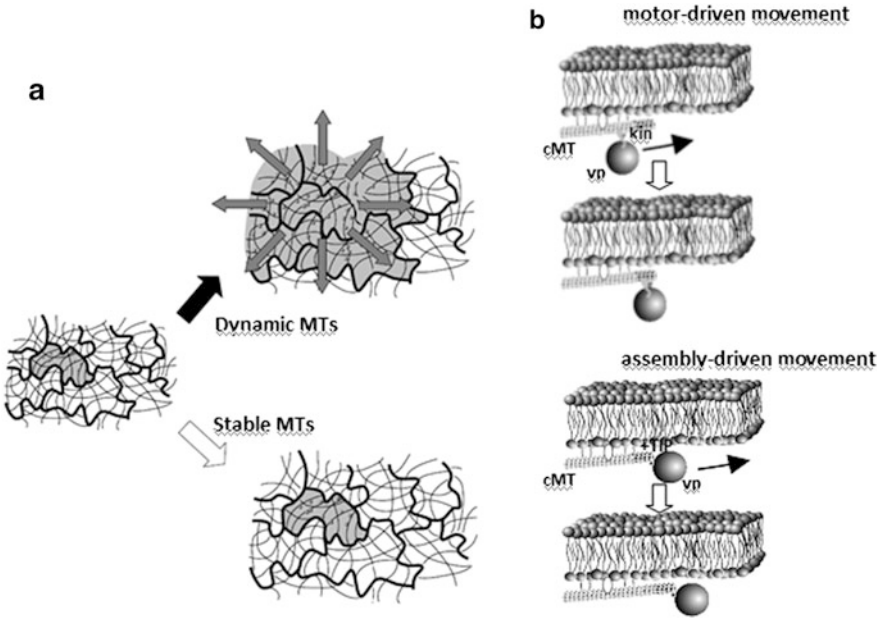

Fig. 4 Microtubules and viral movement. Many plant viruses usurp the microtubular cytoskeleton of their hosts to spread from the infected cell all over the tissue (**a**). This spread is correlated with microtubule dynamics. Stabilisation of microtubules can block viral movement. (**b**) Proposed mechanisms for microtubule-dependent viral movement. Cortical microtubules (cMTs) might serve as tracks along which viral particles (vp) move pulled by kinesin motors (kin). Alternatively, cortical microtubules that are anchored to the plasma membrane might push viral particles with their growing plus ent (+TIP). Reduction of microtubule dynamics should promote motor-driven movement, whereas assembly-driven movement should be impaired. In addition to movement itself, microtubules might participate in viral spread by controlling the release of viral particles to the ER or by constraining the aggregation of overexpressed proteins

in a knockdown of the gene function, the tolerance of these plants to EPC is rather expected to be caused by reduced microtubular dynamics (Ahad et al. 2003). If viral movement is brought about by a turnover-dependent mechanism (Fig. 4b), it should be impaired in these mutants. In one of these mutants, *ATER2* (for *activation-tagged EPC resistance*), the activation tag was inserted into an intron of *CYP87A3*, a gene encoding a cytochrome P_{450} found to be induced by EPC. The insertion by the tag resulted in a tenfold upregulation of this transcript in the *ATER2* mutant. The biological function of the tagged gene is not fully understood, but the rice homologue of *CYP87A3* had been isolated originally by fluorescent differential display based on a rice mutant that had been recovered from a screen for EPC resistance (Wang and Nick 1998). This gene might act as a regulator for synthesis or activity of microtubule-associated proteins that control the dynamic equilibrium between assembly and disassembly of microtubules. Microtubule lifetimes are increased in the *ATER2* mutant as evident from increased resistance of growth to EPC and

oryzalin, increased ratios of detyrosinated tubulin monitoring elevated activity of tubulinyl-tyrosine decarboxylase (an enzyme that binds preferentially to assembled microtubules), and reduced movement of the microtubule plus-end marker EB1 (Ouko et al. 2010).

Based on the evidence for reduced microtubule turnover, it was possible to use *ATER2* as a tool to assess the role of reduced microtubule treadmilling in the movement of TMV using MP-GFP-tagged viruses. In fact, the cell-to-cell movement was reduced in the *ATER2* mutant by about 25 %. This reduced cell-to-cell movement was accompanied by a strongly reduced expression of systemic disease symptoms. Thus, although the reduced microtubule turnover did not prevent viral infection per se, it did impair cell-to-cell movement (Fig. 4a). What are the consequences of this slower viral spread on the level of the whole plant? The SR1 line used as background for the mutants is susceptible to TMV because it lacks a functional N resistance gene (Dinesh-Kumar et al. 2000). Following TMV infection, the virus is capable of replication and systemic spread culminating in terminal necrosis as final stage (not caused by a systemic hypersensitive response). This necrotic response was strongly reduced in the *ATER2* mutant as compared to the wild type (Ouko et al. 2010).

The link between host microtubules and viral spread is more complex though – since, during infection, they seem to play multiple roles: Microtubules tether and later release viral replication complexes adjacent to the endoplasmic reticulum in early infection, and later they anchor the maturating virus factories and eventually, in the centre of an infection site, bundle microtubules through their movement protein. Thus, there exist several specific targets for containment of plant viruses based on microtubular manipulation. Microtubular interference is not confined to TMV but is also found for other plant viruses such as cauliflower mosaic virus (Martinière et al. 2009) or the grapevine fanleaf virus (Laporte et al. 2003); the genetic or pharmacological manipulation of microtubule dynamics might be an efficient strategy to control viral spread in other crop plants as well.

7 Microtubules as Switches for Stress Resistance

Life is full of challenges, and there are basically two ways to cope with this: run away or adapt. Animals prefer to run away; plants are sessile and therefore have to go for the more heroic approach: adaptation. The developmental flexibility of plants allows them to overcome adverse environmental conditions. To achieve this, plants must integrate the signalling evoked by different stress factors into a balanced and appropriate response. For instance, osmotic adaptation requires transport of ions into the vacuole, whereas the most efficient way to encounter attacking biotrophic fungi is programmed cell death (Fig. 5a). The specificity of stress signalling might be brought about by specific molecular players and events that convey the signal to the downstream targets. Alternatively, signalling might utilise common, quite

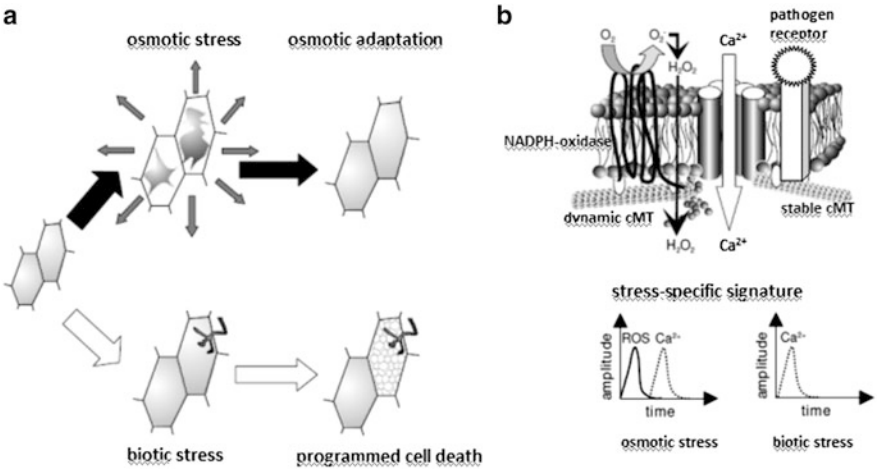

Fig. 5 Microtubular decoding of stress signatures. Many stress factors overlap in the signalling events they induce. Microtubules assist in the decoding of stress quality essential to select the appropriate cellular response. (**a**) Osmotic stress and biotic attack both induce an influx of calcium. The adaptive response must be different – osmotic adaptation has to restore the full turgescence of the cell achieved by transport of ions into the vacuole. In contrast, for biotic attack programmed cell death of the infected cell is the most efficient way to block the intruder. (**b**) Proposed mechanism for microtubule-dependent decoding of stress signatures. For osmotic stress, deformations of the membrane result in a transient decay of dynamic cortical microtubules (cMTs) that will be transduced by the microtubule-bound phospholipase function (*white ellipses*) upon the NADPH oxidase that then generates reactive oxygen species (ROS) that enter the cytoplasm. Cytoplasmic ROS species cause a decay of microtubules releasing the constraint of stable microtubules on calcium channels. As a result, an early peak of cytoplasmic ROS is followed by a transient peak of calcium. In case of biotic stress, a pathogen receptor binds a pathogen-derived elicitor and through accessory microtubules of the calcium channels triggers an early calcium peak (that *can* be followed by a later peak of cytoplasmic ROS in cases where a pathogen affects the integrity of the membrane)

general elements that are specifically recombined to produce appropriate outputs. A short survey on stress signalling yields a limited number of fairly general players including reactive oxygen species, calcium, or jasmonate. How can signalling be specific when the signals are so general? Specificity must derive from the context; the 'code' of stress signalling must be embodied in the spatiotemporal pattern (the so-called signature) rather than in the molecular nature of the signals. For instance, using transgenic plants expressing the aequorin reporter, it could be demonstrated that different stress factors induce different calcium signatures (Knight et al. 1991, for review, see McAinsh and Hetherington 1998). For reactive oxygen species, it is their subcellular distribution that confers specificity towards drought and salinity signalling (for review, see Miller et al. 2010) or towards programmed cell death (see chapter by Smertenko and Bozhkov, this volume). For the jasmonate pathway, it is the crosstalk of different transduction chains (i.e. the signalling history) converging at the proteasome that channels signalling into specific outputs (for

review, see Kazan and Manners 2008). Hence, specificity of stress signalling seems to rely on specific combinations of relatively general primary signals. A code requires a decoder, and there is evidence that microtubules can act as decoders of stress signals.

Hyperosmotic stress causes a strong and transient response of microtubules: Microtubules first disappear, but soon are replaced by massive bundles, the so-called macrotubules (Komis et al. 2002). Formation of macrotubules can be suppressed by oryzalin, which at the same time blocks osmoadaptation, demonstrating that this microtubule response is not a by-product of adaptation but represents an essential event. Inhibitors of phospholipase D, such as *n*-butanol or N-acetylethanolamine, suppress both macrotubule formation and osmotic adaptation (Komis et al. 2006). The product of phospholipase D, phosphatidic acid, can rescue the inhibition by *n*-butanol. As to be expected, T-DNA insertions into the PLD locus impair drought adaptation in thale cress (Hong et al. 2008).

Interestingly, the relationship between cortical microtubules and phospholipase D is bidirectional – on the one hand, microtubules can be detached from the membrane upon inhibition of phospholipase D; on the other hand, microtubules bind phospholipase D and thus might modulate enzymatic activity (Chae et al. 2005). Phospholipase D had originally been identified as membrane linker of plant microtubules (Gardiner et al. 2001), suggesting phospholipase D as signalling hub controlling the interaction between plasma membrane and cytoskeleton. Membrane deformations, for instance, imposed by osmotic challenge might render membrane lipids more accessible to phospholipase D, providing a mechanism to transduce mechanical load on the membrane into changes of cytoskeletal dynamics. The hub model is supported by the fact that phospholipase can trigger different signal pathways.

An attractive possibility, to be explored, would be a modulation of the phospholipase D signalling hub depending on interaction with microtubules. For instance, salt stress was shown to detach a plant-specific microtubule-associated protein, SPIRAL1, from microtubules followed by proteolytic degradation of this protein (Wang et al. 2011). This detachment renders microtubules unstable, which might then simultaneously activate phospholipase D-dependent signalling culminating in osmotic adaptation causing, among other responses, the formation of stable macrotubules. This mechanism would explain why microtubules have to yield in order to persist.

Microtubule decoding can be used not only to sense membrane load in the context of osmotic stress but also to sense the attack of pathogens. The role of microtubules in defence has been traditionally seen in their role for the formation of cell-wall papillae around sites of attempted pathogen penetration. The formation of these papillae is preceded by a reorganisation of the cytoskeleton causing redistribution of vesicle traffic and cytoplasmic aggregation towards the penetration site (for reviews, see Takemoto and Hardham 2004; Kobayashi and Kobayashi 2008) and a somewhat slower migration of the nucleus (for review, see Schmelzer 2002). These responses could also be evoked by a localised mechanic stimulation in the parsley cell model and were accompanied by the formation of reactive oxygen

species and the induction of defence-related genes (Gus-Mayer et al. 1998). Mechanic stimulation could thus mimic several aspects of a pathogen attack and was equivalent to treatment with the corresponding chemical elicitor pep-13. These observations indicate a possible role of microtubules as decoders also in the context of defence.

If this link exists, it should be possible to manipulate defence responses through microtubules. This idea was tested using two cell lines from grapevine that differ in their microtubular dynamics and their susceptibility to Harpin elicitors (Qiao et al. 2010). In fact, pharmacological manipulation of microtubules could induce expression of defence genes in the absence of elicitor. This response was more pronounced in the cell line, where the elicitor triggered a transient elimination of microtubules – similar to the situation in cold adaptation (Abdrakhamanova et al. 2003) and osmotic adaptation (Wang et al. 2011), microtubules have to yield first in order to persist later. Similar to cold acclimation, it is a sensory role of microtubule that provides a promising target for biotechnological manipulation of plant defence.

A signature decoder must discriminate the history of a signal rather than its actual amplitude. To read history, some kind of feedback of downstream signalling upon perception is required. The microtubular stress decoder is endowed with these properties (Fig. 5b): Reactive oxygen species as those generated in response to osmotic stress would, through phospholipase-activated NADPH-oxidase activity (Guo et al. 2012), destabilise microtubules (Livanos et al. 2012) closing a self-referring signalling circuit, because microtubules modulate in turn the activity of phospholipase D. Disassembly of microtubules gating calcium channels (Ding and Pickard 1993; Mazars et al. 1997) would subsequently result in calcium influx. In contrast, biotic attack, through membrane-based receptors binding pathogen-derived elicitors, activates calcium influx more rapidly (Jeworutzki et al. 2010). Depending on the stimulus quality, microtubules therefore create different signal signatures that allow to activate the appropriate adaptive response.

8 New Tools for Cytoskeletal Manipulation

This chapter ventures to demonstrate that microtubules and their accessory proteins provide attractive targets to optimise plant architecture and stress tolerance. However, what tools and approaches do we have at hand to manipulate microtubules?

The most straightforward strategy is genetic engineering of tubulins. This has been actually employed already to engineer tolerance to dinitroanilines (Anthony et al. 1998) or aryl carbamates (Nick et al. 2003; Ahad et al. 2003) that bind to specific motives on α-tubulin. Since tubulins are very general players of the cellular lifecycle, unwanted side effects of mutated tubulins are an issue. This can be addressed by making use of the specific and versatile regulatory features of innate tubulin promotors (Breviario and Nick 2000). Nevertheless, the relative evolutionary conservation of tubulins has led to a highly efficient design of protein structure

that leaves only limited flexibility for engineering without impairing the core functions of the protein. Alternatively, those proteins that have specifically evolved in higher plants and fulfil more confined tasks could be useful. Among those, the highly diverse and apparently functionally flexible kinesins are certainly key targets as exemplified by the newly discovered gdd1-kinesin (Li et al. 2011).

A complementary route would be chemical engineering using microtubule-directed compounds. Antimicrotubular compounds have been traditionally used for growth control as potato sprouting suppressors or as herbicides (reviewed in Vaughn 2000). Screening of chemical libraries has identified new promising compounds such as cobtorin that specifically interferes with the microtubule guidance of cellulose synthesis (Yoneda et al. 2007). A novel approach, described in the chapter by Sadot in this volume, is based on bioactivity screening cytoskeletal responses of mammalian cells as readout. A further promising development in chemical engineering are designed peptides that can be tailored to interfere with specific targets in the host cells. A drawback of chemical engineering through peptides is the difficulty of membrane passage. So-called cell-penetrating peptides (CPPs) provide an attractive tool to overcome this bottleneck. They share common structural features such as short size and a positive charge usually stemming from multiple lysine or arginine residues (Su et al. 2009). Several CPPs such as transportan, pVEC, arginine-rich peptides, or BP100 have already been introduced into plant cells (for instance, Mizuno et al. 2009), but without the attempt to introduce a functional cargo. Recently, we were able to fuse the novel actin-binding peptide Lifeact with BP100. This fusion was imported rapidly, efficiently, and specifically into tobacco BY-2 cells that successfully labelled the phragmosomal actin cables that tether the nucleus in the cell centre (Eggenberger et al. 2011). We are presently adapting this approach to plant tubulin as a target by titration of specific domains on the tubulin heterodimer with ectopic peptides.

The plant microtubular cytoskeleton conveys a broad range of very specific functions that are not known in animal cells. Some of these functions are structural; others are of a sensory nature. This provides ample space for biotechnological manipulation that is subtle, specific, and safe (because these functions are not relevant for animals and man). The success of this approach will depend on the precision of our tools. This precision can only be reached when application is underlaid by solid pure science.

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Auxin Biology: Applications and the Mechanisms Behind

Petr Skůpa, Zdeněk Opatrný, and Jan Petrášek

Abstract This chapter describes the state of the contemporary knowledge of auxin action reflected in its applications in agriculture and biotechnology. We summarise the current understanding of the mechanism of action for endogenous and major synthetic auxins highlighting their morphogenic character that modulates numerous aspects of plant development. Various auxins and auxin-like compounds are used in techniques of plant vegetative propagation, in vitro culture and regeneration, and they play also a role as important herbicides. We discuss potential applications of auxins in commercially relevant procedures used in the context of plant generative and fruit development, abscission, apical dominance and tropisms. These technologies are based rather on the phenomenology of auxin applications, and the molecular mechanisms behind are still not fully uncovered.

1 Introduction

The study of the intriguing group of plant hormones known as 'auxins' (from the Greek word *auxein* meaning 'to grow') had its origins in the investigations of Charles and Francis Darwin (Darwin and Darwin 1881) into the bending responses of grass coleoptiles towards a unilateral light source. In an elegant series of experiments, they demonstrated that the directional light stimulus was perceived by the tip of the coleoptile, while the growth response leading to reorientation of coleoptile growth, dependent on differential elongation of either side of the coleoptile, occurred in some distance from the coleoptile tip in response to a 'signal' conveyed from the tip itself. Later work on *Avena* coleoptiles demonstrated that the

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'signal' was a chemical substance transported asymmetrically from the tip (Went 1928). Although still unidentified, the substance involved could be isolated by diffusion into agar blocks which, if placed laterally on *Avena* coleoptiles, would induce growth curvature by stimulating differential elongation. Indole-3-acetic acid (IAA) isolated from human urine (Kögl et al. 1934) was very effective in the *Avena* curvature test and was eventually found to be the predominant endogenous auxin in plants (Davies 2004). Efforts to find other substances with auxin activity led to the discovery of various compounds, both synthetic and natural. The most frequently used synthetic auxins in basic research and applications, 2,4-dichlorophenoxy acetic acid (2,4-D) and naphthalene-1-acetic acid (1-NAA), do not act completely identically as the native IAA. Only some of the compounds identified as auxins, namely, indole-3-butyric acid (IBA) (Zimmerman and Wilcoxon 1935), phenylacetic acid (PAA) (Koepfli et al. 1938) and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter and Thimann 1965), are actually synthesised by some plants and could be understood as 'endogenous auxins'. Their roles and mechanisms of action are still not satisfactorily described (Simon and Petrášek 2011).

From the very beginning of auxin research, the definition of the term 'auxin activity' was based on the competence of such substances to promote elongation growth in coleoptiles and to stimulate rooting. Indeed, the ability of auxins to stimulate rooting gave rise to their original name of 'rhizocalines' (Went 1934). On the cellular level, auxin influences both cell division and cell expansion (Skoog and Miller 1957; Perrot-Rechenmann 2010), two major cellular processes that shape the sessile plant body. Together with the effect of auxin on cell differentiation and the determination of cell fate, auxins influence literally all aspects of plant development (Vanneste and Friml 2009) displaying a morphogenic character (Bhalerao and Bennett 2003) modulated by other phytohormones and the environment and defined by dynamic changes in the machinery of auxin signal transduction. Therefore, auxin concentration gradients are important for the coordination of plant growth and development (Leyser 2011) and have had impact also on the evolution of plant body (Finet and Jaillais 2012).

The knowledge on auxin biosynthesis, metabolism, transport and mechanism of action contributes vastly to all fields of plant biology. Following this reasoning, auxin could be understood as a tool for studying many aspects of plant developmental and cell biology and consequently also as a tool for agronomy, horticulture and biotechnology applications. In application, the interpretation of 'auxin activity' is somewhat different and reflects the phenomenology of exogenous additions of auxins to plants or plant organs. This applies to auxins as compounds used in procedures for in vitro cultures, regeneration, organogenesis and vegetative propagation as well as compounds with herbicide effects. In addition to summarising these procedures in the current chapter, we also want to point to the fact that our understanding of the mechanisms that collectively regulate responses to auxin is not widely exploited in its field applications and might therefore represent good opportunity for the future.

2 Auxins and Their Mechanisms of Action

2.1 Auxin Concentration Gradients in Plant Morphogenesis

The habitus of plants is largely set through the complex balance of cell division and expansion in various plant tissues. Auxin is (in addition to other plant hormones, namely, cytokinins) a central substance that influences both of these processes (Skoog and Miller 1957). It is the concentration of auxin that is very often crucial for the resulting response, and therefore auxin concentration gradients are instructive during literally all phases of plant development. This fact is a prerequisite for a plethora of practical applications. Already from the very early phases of embryogenesis, auxin concentration maxima are generated in developing embryos marking sites for future development of cotyledons and root apical meristems (Friml 2003). During postembryonal development, auxin concentration maxima regulate root and shoot apical meristem patterning and lateral organ development (Benková et al. 2003; Sabatini et al. 1999). They are also important for vasculature development (Mattsson et al. 2003; Scarpella et al. 2010), root and shoot bending responses (Friml et al. 2002), flower and fruit development (Sundberg and Ostergaard 2009) and also during senescence (Ellis et al. 2005) and plant-pathogen interaction (Kazan and Manners 2009). For all of these developmental events, auxin concentration gradients are collectively generated by processes of auxin biosynthesis and metabolism as well as by inter- and intracellular auxin transport. Finely tuned auxin gradients determine how much of auxin will be triggering specific downstream responses that might, but not necessarily needs to, include gene expression.

2.2 What We Know About the Mechanism of Action of Endogenous and Synthetic Auxins

As mentioned in the introduction, the most frequent natural auxin is IAA. However, for numerous applications, various endogenous and synthetic auxin-like compounds are used, inadvertently taking advantage of differences in their biosynthesis, transport and mechanism of action.

As reported for *Arabidopsis thaliana*, IAA is synthesised to various levels literally by every cell (Ljung et al. 2001), predominantly in young tissues. Several pathways from the IAA precursor L-tryptophan (L-Trp) have been described, and also aL-Trp-independent biosynthetic pathway has been postulated (for review see Ljung 2013). The physiologically active pool of IAA is balanced by a complex conjugation and degradation enzymatic machinery, where IAA conjugation to sugars and amino acids and oxidative degradation are the most important (Ljung 2013; Ludwig-Müller 2011). However, for IAA, directional cell-to-cell auxin transport through integral plasma membrane carriers seems to be the major

mechanism establishing auxin gradients during plant development (for review see Petrášek and Friml 2009). This machinery includes auxin influx carriers from the AUX1/LIKE AUX1 (AUX1/LAX) family (Bennett et al. 1996), auxin efflux carriers from the PIN-FORMED (PIN) family (Gälweiler et al. 1998) and ATP-binding cassette subfamily B (ABCB) (Noh et al. 2001). Directional influx by the influx carriers acts in concert with non-directional uptake of IAA from the apoplast through an ion-trap mechanism (Rubery and Sheldrake 1974) which is important for auxin canalisation (see Sect. 3.3). Moreover, some PINs (Mravec et al. 2009; Ding et al. 2012; Dal Bosco et al. 2012) and PIN-LIKE (PILS) proteins (Barbez et al. 2012) that are localised on endomembranes are supposed to maintain intracellular IAA homeostasis by transporting IAA or perhaps even IAA conjugates between ER (or its derivatives) and cytoplasm. It is hypothesised that cellular IAA homeostasis includes mechanisms that coordinate IAA transport and metabolism (Rosquete et al. 2012). Depending on its actual concentration, IAA binds to a nuclear receptor belonging to the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) family of F-box proteins, subunits of the SCF E3-ligase complex (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005). Upon binding, degradation of transcriptional repressors of the Aux/IAA family releases auxin response factors (ARFs) that activate or suppress specific gene expression. It was shown that various combinations of TIR1/AFBs and Aux/IAAs determine the specificity of this 'co-receptor' system to IAA (Calderón Villalobos et al. 2012). In addition, two additional IAA receptors have been described, AUXIN BINDING PROTEIN 1 (ABP1, actually the first identified auxin receptor) and S-phase kinase-associated protein 2A (SKP2A). ABP1 affects predominantly events at the plasma membrane linked with cell expansion, e.g. the activation of plasma membrane ATPase and K^+ channels, clathrin-mediated endocytosis and cytoskeletal rearrangements (for review see Sauer and Kleine-Vehn 2011). SKP2A, another F-box protein, regulates cell division through the proteolysis of cell cycle transcription factors and is degraded upon binding of IAA (Jurado et al. 2010).

While metabolism and mode of action are relatively well understood for IAA, there is significantly much less information for three other naturally occurring auxins, 4-Cl-IAA, PAA and IBA. 4-Cl-IAA is the auxin predominantly found in developing seeds of legumes, including agronomically important species like pea, alfalfa or lentil. A typical activity of 4-Cl-IAA is the stimulation of pea pericarp growth (Reinecke 1999). This auxin is produced by enzymatic conversions of 4-chlorotryptophan to 4-Cl-IAA (Tivendale et al. 2012) and can also be conjugated with amino acids (Ludwig-Müller 2011). Auxin transport competition assays showed that 4-Cl-IAA might be transported by auxin influx and efflux carriers (Simon et al. 2013). It might also share some mechanisms of action with IAA, as indicated by high competition of 4-Cl-IAA with IAA in binding displacement assays (Zažímalová and Kutáček 1985), by the ability of 4-Cl-IAA to trigger ABP1-mediated events on the plasma membrane in maize (Karcz and Burdach 2002) and, interestingly, also by 4-Cl-IAA-stimulated interaction of TIR1 with the Aux/IAA repressor (Yu et al. 2013). Another endogenous auxin, the phenyl-

derivative PAA, was detected in a number of plant species (Wightman and Lighty 1982; Korasick et al. 2013). It is synthesised by the nitrilase pathway (Ludwig-Muller and Cohen 2002) and rather poorly transported by auxin influx and efflux carriers (Simon et al. 2013). It is not known whether PAA is a good substrate for TIR1/AFB, but it seems likely that the mechanism of action of PAA involves both TIR1 (Simon et al. 2013) and ABP1 (Napier and Venis 1990). The fourth endogenous compound that is often classified as endogenous auxins is IBA. Although recent reports from Novák et al. (2012) using novel analytical approaches in various tissues of *A. thaliana* (liquid chromatography-multiple reaction monitoring-mass spectrometry) failed to detect IBA at all, its presence in a wide range of plants including *A. thaliana* has been documented in multiple previous reports (Korasick et al. 2013). As summarised in Strader and Bartel (2011), IBA represents an important IAA precursor that is formed by β-oxidation, thus regulating the active IAA levels during plant development. IBA conjugates with glucose were reported to increase resistance to water stress (Tognetti et al. 2010). In addition, it seems that IBA is transported by a transport machinery that differs from that for IAA and includes the ABCB36 and ABCB37 auxin efflux carries (Strader and Bartel 2011). So far, it does not seem to be likely that IBA directly triggers TIR1- or ABP1-mediated responses. The finely tuned metabolic conversion of IBA to IAA might actually be the reason why IBA is often better suited for various applications producing a remarkably stable auxin activity that even excels that of IAA or its synthetic analogues.

Since IAA has been reported to be less stable in culture media, where it is photodegraded within several days (Yamakawa et al. 1979; Nissen and Sutter 1990), numerous experiments and applications use more stable synthetic structural and functional auxin analogues. The most frequently used compounds are 1-NAA and 2,4-D. Metabolism, transport and mechanism of action of these molecules share many features with those of IAA, but as described in next few lines, there are also some specific points that need to be considered. 1-NAA and 2,4-D are more stable when compared with IAA in terms of their slower metabolic conversions as documented for 2,4-D (Delbarre et al. 1996; Hošek and Kubeš et al. 2012) and 1-NAA that is reported to be not as readily converted by some IAA auxin-conjugating enzymes (Peat et al. 2012). Radioactively labelled trace amounts of 1-NAA and 2,4-D turned out to be suitable tools for selective measurements of auxin transport, i.e. carrier-driven auxin influx (2,4-D) and carrier-driven auxin efflux (1-NAA) (Delbarre et al. 1996; Petrášek et al. 2006). Interestingly, the evidence accumulated from these assays, and supported by mathematical modelling (Hošek and Kubeš et al. 2012), shows that for auxin efflux, 2,4-D, in contrast to the literature, is transportable to a certain degree, however, less efficiently. Lower cellular efflux of 2,4-D together with 2,4-D-specific auxin influx through the ABCB4 transporter (Kubeš et al. 2012) might thus be responsible for the high herbicidal activity of this synthetic auxin (see later). Both 1-NAA and 2,4-D can activate the TIR1/AFB-Aux/IAA co-receptor system (Calderón Villalobos et al. 2012), but their affinity to TIR1 is somewhat lower in comparison with IAA (Dharmasiri et al. 2005a; Kepinski and Leyser 2005). It is important to mention that

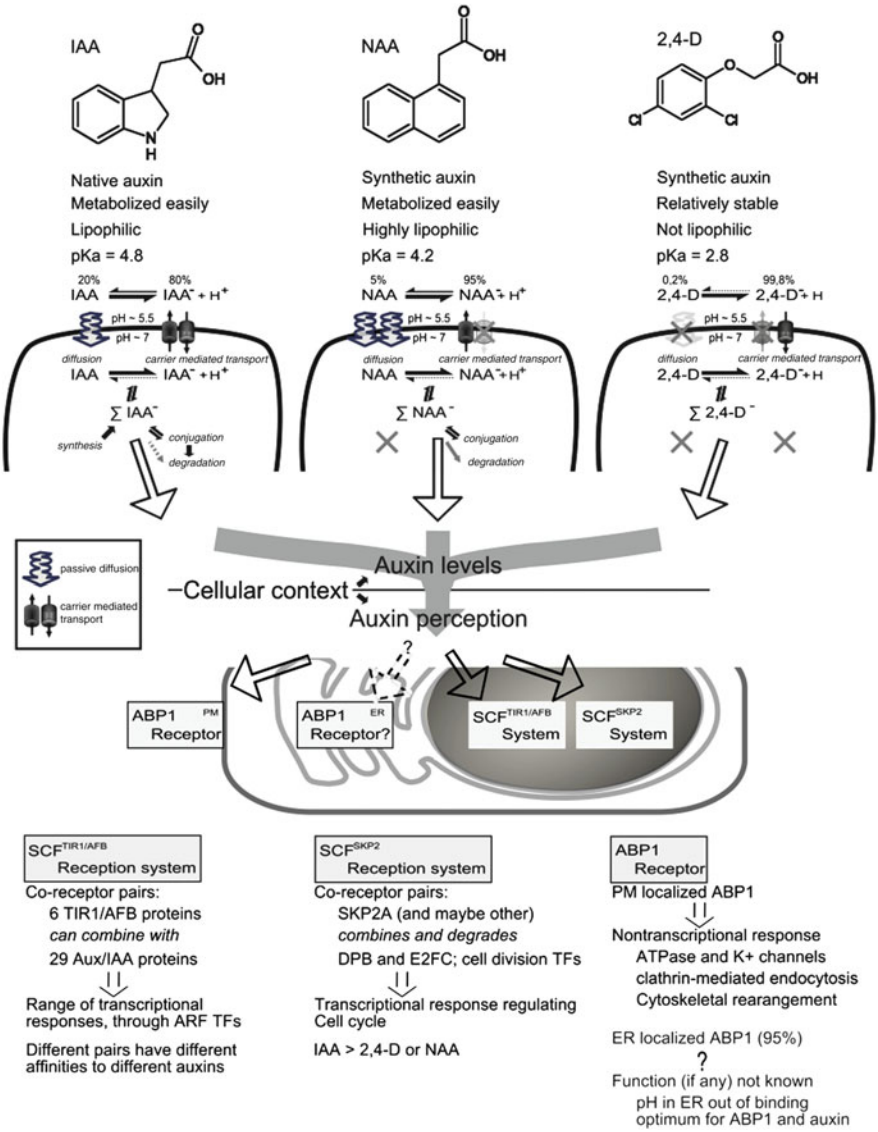

Fig. 1 Schematic depiction of the mechanisms involved in the timely and spatially restricted tuning of endogenous levels of native auxin IAA and the two synthetic auxins, 1-NAA and 2,4-D. Both free diffusion and carrier-mediated transport have been described for auxins. The amount of the dissociated form of the particular auxin depends on the pH of the compartment. As calculated based on the dissociation constant, the amount of the non-dissociated form is 20 % for IAA, 5 % for NAA and 0,2 % for 2,4-D. Based on this dissociation rate and also on the lipophilicity, 2,4-D is uptaken almost exclusively by carriers, IAA by both carriers and diffusion, and NAA almost exclusively by diffusion. Within the cell, the pH is higher and under this situation, for all three auxins, carrier-mediated transport predominates. Here, while IAA and NAA are very good substrates for the described auxin carriers, 2,4-D is much less transported. Moreover, the metabolic conversions that deactivate the pool of auxin are the slowest for 2,4-D, keeping its concentration high for downstream transcriptional control that is based on the proteasome-mediated degradation

picloram, an auxinic compound with herbicide effects, is selectively bound by the AFB5-Aux/IAA co-receptor (Calderón Villalobos et al. 2012). This indicates that individual TIR1/AFBs and their combinations with Aux/IAAs might determine the sensitivity of the particular tissue to auxins or auxin-like compounds. In addition to TIR1/AFBs, 1-NAA and 2,4-D can also trigger responses through the activities of auxin receptors ABP1 (Löbler and Klämbt 1985) and SKP2 (Jurado et al. 2010). It seems that there might exist in general two pathways for the control of cell division and cell elongation that are triggered by 2,4-D (cell division) and 1-NAA and IAA (cell elongation) (Campanoni and Nick 2005; Simon et al. 2013). A schematic summary of the current knowledge on transport and signalling is given in Fig. 1.

2.3 *Mechanisms of Co-operation Between Auxins and Cytokinins: Setting the Plant Morphogenesis*

The morphogen-like effect of auxin is regulated by crosstalk between numerous signal transduction pathways triggered by external stimuli like gravity, light quality and length, temperature or nutrient availability. With respect to the applications of auxin, crosstalk with other phytohormones is of particular importance. Among them, cytokinins are the most frequently applied phytohormones that act in concert with auxin in basic morphogenic processes, i.e. cell division and cell growth/differentiation. The importance of mutual co-operation between auxin and cytokinins for organogenesis was elegantly shown in the pioneering experiments with regenerating tobacco stem pith explants (Skoog and Miller 1957; see Sect. 3.2 and chapter by Opatrný, this volume). However, it is only recently that the molecular mechanisms of auxin-cytokinin interplay were at least partially understood. Cytokinins (see chapter by Šmechilová and Spíchal, this volume) repress auxin signalling through the activation of the positive (type B) cytokinin response regulator ARR1, thus activating the transcription of the Aux/IAA auxin signalling repressor IAA3/SHY2. This co-operation was reported to regulate root growth and root meristem size, where auxin supports meristem activity and cell division, whereas cytokinin supports differentiation (Dello_Ioio et al. 2008; Moubayidin et al. 2009). In contrast, the establishment and maintenance of the embryonic root pole

Fig. 1 (continued) of ubiquitinated transcriptional regulators upon binding of auxin to F-box proteins from the TIR/AFB family. This mechanism is common for all three IAA, NAA and 2,4-D, albeit with differential affinity of the particular auxin receptor for the particular auxin that is further regulated by the interaction with specific transcriptional repressors from the Aux/IAA family. Other inputs, not shown here, modulate on various levels auxin effects through the regulation the 'cellular competence', i.e. they might be instructive for a plethora of developmental processes. The processes represented in the figure are depicted in a schematic way, for the sake of lucidity. Therefore minor actions (such as some small metabolisation of 2,4-D) were disregarded in the scheme

(hypophysis) is regulated in inverse manner. Here auxin upregulates the negative (type A) cytokinin response regulators ARR7 and ARR15 (Müller and Sheen 2008). As nicely summarised in recent reviews (Bishopp et al. 2011; Su et al. 2011; Bielach et al. 2012; Vanstraelen and Benková 2012), programming of cell division and growth/differentiation through the interaction of auxin and cytokinin is critical for key developmental processes involving meristematic activity. The mechanism includes a number of regulatory loops and signalling components that are under the transcriptional control of both phytohormones, e.g. the genes for auxin efflux carriers, auxin and cytokinin biosynthetic and metabolism enzymes as well as upstream transcription factors. Moreover, non-transcriptional crosstalk has been described recently for the cytokinin-stimulated vacuolar degradation of auxin efflux carrier PIN1 (Marhavý et al. 2011).

3 Auxin Applications

3.1 Auxin as a Tool

Auxin became incorporated as a tool in the hands of plant breeding from very early on. The early applications included already most of applications used nowadays, namely, prevention of fruit and leaf drop, fruit thinning, induction of parthenocarpic fruits, stimulation of rooting of cuttings and auxin herbicidal effects (Preece 2003; Loach 1988).

As mentioned in the introduction, auxin was discovered through its ability to mediate plant responses to the environment. With continuing theoretical research, it became evident that auxin is of fundamental importance for a wide spectrum of vital processes, of which growth responses to environmental stimuli represent only a small, albeit important, subset. In fact, the capacity of this single molecule to cause a very diverse range of seemingly unrelated effects in different body parts, different time or under different conditions is one of the fundamental, yet most puzzling, characteristics of auxin. Each cell in the plant body may respond to auxin differently depending on its position, ontological and/or positional context, with a range of different possible cellular responses (Trewavas 1982; Kieffer et al. 2010; see also Sect. 2.2, and also Opatrný, this volume).

The plant body as an entity is shaped through physiological response of the tissues to particular pattern of auxin gradients distributed over the plant parts. As the auxin gradients stimulate auxin signalling pathways differentially along the gradient, various growth responses and cell differentiation are triggered (see Sect. 2.1). These processes may result in the change of cell specifications based on the positional information provided by the gradient and subsequently in the formation of new cell patterns, tissues and organs and also in responses of existing plant cells, tissues and organs to the environment (Friml 2003; Vanneste and Friml 2009). This morphogen-like character of auxin action in plant tissues contributes to

the control of large array of essential developmental processes (specified in Sect. 2.1 and Opatrný, this volume).

Therefore, auxin may be seen as signalling molecule, as a mobile unit conveying information of either endogenous or exogenous origin. All auxin molecules, irrespective of their origin, are relocated by the plant organism, and informational value of this distribution is translated with regard to the responsiveness (competence) of the receiving local tissue. And in that regard, embryo formation either from the zygote or from various somatic cells (see chapter by Smertenko and Bozhkov in this volume), as well as subsequent morphogenesis of the plant body, represents probably the most common applications of auxin signals in plant life. Alternatively, this informational wealth is employed for numerous regenerative events, as background for either wound healing or plant vegetative propagation (see Sect. 3.3 and the chapter by Opatrný in this volume). Another 'systemic' application of the auxin signal is utilised frequently in the case of auxinic herbicides. Synthetic auxins are applied as potent species-selective herbicides and defoliants (described in Sect. 3.4). The partially related abscission effect is also smartly used to cause fruit drop in apple orchards in order to regulate the number of fruits per tree. Several diverse applications related to fruit development have been conceived, with induction of parthenocarpy being probably the most interesting. Moreover, there exist also some indirect applications, where auxin metabolism, transport or perception is targeted, complementing the exogenous application of auxin per se (Sect. 3.5).

3.2 Auxin and In Vitro Cultures

Propagation of plants using in vitro techniques has become an important commercial technology, where auxin represents a fundamental component for the large-scale propagation of cell mass, organs or whole plants, or, to get rid of innate pathogens, in the generation of virus-free plants (Preece 2003).

The crucial importance of adequate endogenous levels of phytohormones has been demonstrated already during early stages of in vitro techniques. This hormone dependence is considered to be one of the main reasons why the pilot attempts of Gottlieb Haberlandt to cultivate a variety of functionally differentiated cells in vitro failed (Haberlandt 1902, see also Opatrný, this volume). In contrast, root cultures of tomato (White 1934) were the first plant entities growing in vitro without limitations, thanks to the production of IAA and cytokinins in the root apical meristem. Moreover, the first plant tissue cultures were callus cultures supplied with auxin either thanks to their tumour (e.g. auxin producing) origin (White 1939; Smith 1988) or due to the external application of IAA into the culture media (Nobécourt 1939; Gautheret 1939). While some cultured plant cells or tissues retain the capacity to synthesise auxin on their own, in most cases, however, external auxin has to be added into the culture media. The reason may partially be a so-called elution effect that is also known to contribute to losses of other essential

metabolites, typically after using subcritical density of culture inoculum (see also Opatrný et al., this volume). These days, plant tissue or cell cultures are propagated only rarely with the addition of the natural IAA itself, and the dominant auxins for technical applications are 2,4-D or 1-NAA, administered either alone or in combination. This applies also for the most frequently used cell lines/strains in basic research, i.e. Arabidopsis (Menges and Murray 2002), tobacco BY-2 (Nagata et al. 1992) and VBI-0 (Opatrný and Opatrná 1976).

In extreme cases, auxin autonomy can be induced by genetic and epigenetic changes that accompany in vitro propagation of plant tissue and cell cultures. The process of acquired auxin autotrophy, originally noticed by Gautheret (1942) and designated as 'anergy', leads to progressive insensitivity of long-term cultures to auxin even to a level where exogenous auxin can cause growth inhibition (Gautheret 1942, 1955, 1985). For such gradually acquired hormone autotrophy, the term 'habituation' had been coined by F. Meins (Meins 1982, 1989), originally for cytokinins. The mechanism of habituation is still not fully understood, and it is even not clear whether it is of genetic or rather of epigenetic background (Pischke et al. 2006). However, it has been reported to include up-regulation of some auxin ABC-type transporters in tobacco cells (Shimizu et al. 2006). Irrespective of the underlying mechanisms, habituation has significant implications for auxin applications. Hormonal substances are generally indispensable and one of the most expensive components of culture media. So, the possibility of their elimination is appealing and even more in the context of large-scale cultivation technologies for industrial purposes. On the other hand, relevant spontaneous phenotypic changes of production lines (or strains) which are associated with their (sudden or gradual) habituation may have adverse economic consequences.

Both natural (endogenous) and exogenously applied auxins participate in regenerative responses of various types of plant explants. In general, two main alternatives of regenerative procedures are employed to receive new organisms from the somatic cells of the donor plant: organogenesis and somatic embryogenesis (see Opatrný, this volume). For each of these procedures, rather specific regenerative protocols ('cookbooks') exist, both based on the empiric experience of their first users.

All recent protocols for the induction of organogenesis are variations of the original procedure of Skoog and Miller (1957), performed on the in vitro cultures of tobacco Wisconsin 38 stem pith primary explants modulated by various combinations of IAA and the cytokinin kinetin (See also Sect. 2.3). These parenchymatic tissue cultures sensitively responded to exogenously applied kinetin and IAA by rapid cell division and massive formation of callus. The addition of IAA alone or the massive predominance of IAA over kinetin stimulated pronounced root formation. Inversed ratios with higher kinetin concentration promoted the generation of shoot meristems that developed into shoot buds on medium with decreasing concentration of exogenous IAA. Our recent understanding of auxin-cytokinin interaction during de novo organogenesis has significantly profited from studies of the 'stem cell niches' in *A. thaliana* and the regenerative capacity of primary explants from roots, leafs, hypocotyls or cotyledons (Opatrný, this volume). These primary

explants are regenerated on media containing optimised ratios of auxin and cytokinin for callus induction (callus induction medium, CIM), shoot induction (shoot induction medium, SIM) or root induction (root induction medium, RIM). According to Valvekens et al. (1988), induction of callus with CIM needs the establishment of tissue-specific auxin concentration maxima by addition of higher concentrations of 2,4-D, the auxin that is very stable, because it is less exported from the cell and less metabolised (see Sect. 2.2). Subsequent shoot induction with SIM is then initiated by lowering the concentration of external auxin by use of the transportable (but instable) IAA. Depending on genotype and type of primary explant, the length of cultivation on SIM or RIM (medium with IAA only) has to be adjusted to the particular experimental system and the respective goal of regeneration. Based on this knowledge, a huge number of combinations of auxins, cytokinins and other phytohormones have been used in the propagation of broad spectrum of plant species (George and Sherrington 1984; Vasil 1986). However, organogenesis in some species has remained unsuccessful to variable degrees, a phenomenon known as 'recalcitrance'. To explain the reasons for these failures is, of course, a problem of both high theoretical and applied impact (see Opatrný, this volume).

As shown by gene expression profiling and the analysis of cell-type-specific protein markers in Arabidopsis, increased concentration of auxin in CIM applied to primary explants either in the form of less transportable 2,4-D or in the form of higher concentrations of IAA or 1-NAA triggers the ectopic activation of the developmental programme for lateral root initiation, one of the processes induced *in planta* by local auxin maxima (Sugimoto et al. 2010). This programme is initiated in the population of pluripotent, pericycle-like cells that might be derived not only from roots but also from cotyledons and petals. Therefore, a common mechanism characterised by the activity of specific sets of transcription factors and signalling components has been proposed (Atta et al. 2009; Sugimoto et al. 2010). During the initiation of pluripotent cells, auxin-stimulated expression of negative cytokinin response regulators ARR7 and ARR15 (see Sect. 2.3) prevents shoot formation (Buechel et al. 2010). Moreover, as shown on hypocotyl explants, auxin-induced root organogenesis involves also tissue-specific activation of cytokinin signalling that subsequently negatively regulates auxin transport through the inhibition of PIN auxin efflux carriers (Pernisová et al. 2009). Shoots are formed after transfer to SIM from shoot progenitor cells that appear upon a transcriptional switch triggered by combination of cytokinin and decreased concentrations of auxin (Gordon et al. 2007).

In addition to de novo organogenesis, auxin is also involved in the regeneration via the process of somatic embryogenesis (see chapters by Smertenko and Bozhkov, and Opatrný, this volume). This process can be initiated in vitro, either directly from primary explants or from callus derived from these explants. Typically, high concentrations of 2,4-D (Raghavan 2004) are applied to initiate embryogenic callus on immature zygotic embryos during early phases (globular and torpedo phases), while 2,4-D applied to immature embryos in the late cotyledonary stage initiates direct embryogenesis (for a protocol in Arabidopsis, see Gaj 2011).

Subsequent transfer of somatic embryos to auxin-free media initiates the development of plantlets. The technique of in vitro plant regeneration and propagation via somatic embryogenesis has been successfully used in a broad range of plant species (Raghavan 2004). In some systems, like cereals or conifers, it has even became the method of choice since for these species micropropagation via organogenesis has not been very successful, due to unknown genetic or developmental factors. As a rule, some hardwood trees, in particular conifers, are recently preferentially propagated only by means of either zygotic or somatic 'seeds'. Here, 2,4-D is the preferentially used auxin in the induction and proliferative media. The main reason is perhaps similar to the case of de novo organogenesis: 2,4-D generates stable and effective auxin concentration maxima that are not disrupted by excessive auxin transport and metabolism.

3.3 Auxins and Vegetative Propagation

In agriculture, horticulture and forestry (plant-propagating industry), vegetative propagation is used to produce large numbers of plants of equal genotype, and it is extensively used for multiplication of elite plant clones (Hartmann et al. 1990). Vegetative propagation is often the only option for plant growers, because only by this strategy, they are able to preserve all desirable traits of the parent plant (Salaš et al. 2012). Vegetative reproduction as a method of choice is exploiting natural competences of plants, both their innate ability to reproduce clonally and the general phenomena of plants to regenerate. A key step and essential part of most protocols for vegetative propagation is the formation of adventitious roots at some stage on the plant propagule. That is notably true for plant propagation by cuttings. In contrast to other forms of vegetative plant propagation, propagation by cuttings is frequently used as particularly efficient and attractive technique for industrial propagation (Ford et al. 2002; Salaš et al. 2012), and is making use of a long history of rooting stimulants based on auxins, and therefore will be our main focus here.

Development of the main root and the formation of lateral roots from the main axis of the root are meanwhile relatively well understood; the biology of adventitious root formation has received less attention, however. Adventitious roots are formed postembryonically in a de novo process either on stems or leaves or on already lateralised root axis or on any other plant organ (Chriqui 2008; Barlow 1994; Taiz and Zeiger 2002). Many plants, as part of their strategy for reproduction and survival, possess the ability to form adventitious roots naturally or under specific circumstances such as environmental stress or after mechanical damage. While some species, such as willow, might be grown simply by placing their cutting into a moist soil, the majority of species requires special attention, and there are many plant species on the other side of the spectrum, which are, for various reasons, very difficult to propagate even with the help of auxin-containing 'rooting substances'. The ability to root (without the help of rooting stimulants) is severely limited both with regard to the range of species amenable to spontaneous rooting

and also in the yield of those species that root easily (Ford et al. 2002). With the help of auxin regulators, plant growers can now go far beyond the original capacity of plant parts to produce new plants. With the proper use of these agents, cuttings will form better and more uniform roots in a shorter length of time. Cutting is a technique in which a piece of stem (rhizome, root or leaf) is excised from the parent plant and encouraged to grow into a plant that is independent of the parent. While narrowed to only certain species, conditions and with various efficacies, the natural disposition of plant parts to form roots has been exploited by gardeners for centuries.

Before describing the whole process of adventitious root formation (ADRF), it is necessary to differentiate between adventitious root primordia that are induced de novo and those that are already preformed on the stem (see also the classification by Němec recapitulated in the chapter by Opatrný in this volume). Preformed root initials are produced during the normal development of some plants; they are present mostly in woody plants and tend to develop slowly. If root primordia have yet to develop de novo in response to a triggering event, they are most conveniently formed in response to wounding (Blakesley et al. 1991). Actually, cuttings may be understood as a wounding event. Based on experiments with apple microcuttings, a sequence of successive phases has been defined by De Klerk et al. (1999) for formation of adventitious roots, namely:

(i) Callus induction(0–24 h)
(ii) Root induction (24–96 h)
(iii) Root outgrowth from the stem (96–120 h)

Endogenous IAA plays a central role for ADRF in vivo. Auxin is predominantly produced in shoot apical meristems or, more specifically, in the youngest leaves nearest to the meristem and, from there, is transported down through the adjacent stem parts (Ljung et al. 2001). Since auxin moves downwards, it is progressively loaded into the phloem (Berleth et al. 2000), its main transport route on longer distances (Baker 2000). When parts of the stem vasculature are wounded (severed), auxin would accumulate, in response to such wounding, in high concentration at the upper border of the wound (Sachs 1991). In concert with wound response factors, accumulation of auxin triggers leads to initial reprogramming of the cells into callus cells. When auxin is transported through this callus further downwards, to the lower parts of the plant, this leads to differentiation of new vascular tissue along the route of transported auxin, as described by canalisation hypothesis proposed by Sachs (1969). In a case of a cutting wound, endogenous auxin may move naturally downwards (rootwards) until it arrives at the position of incision, and it may be safely assumed that, after auxin has hence accumulated there, its rising levels help to induce a first wound response. Accumulated auxin leads then to transdifferentiation of several cell layers near this cut (see also Opatrný, this volume). This event would correspond to the first phase of the rooting as proposed by De Klerk et al. (1999).

Different roles for auxin during those phases of root initiation were shown by Ludwig-Müller et al. (2005). In the first phase, callus is formed preferentially from

cambium cells. According to Sugimoto et al. (2010) and also other authors (see Opatrný, this volume), these primary callus cells exhibit molecular markers of pericycle-like pluripotent stem cells. Under in vivo conditions, under long-term effect of exogenously applied IAA or NAA, they further differentiate into root apical meristems. The team of Ludwig-Müller has shown on *Arabidopsis* segments that both IAA and synthetic 2,4-D are comparable in their 'auxin' potency during this phase. Considering that neither 2,4-D nor PAA are ever used as rooting stimulants, it is interesting that both 2,4-D and even PAA were shown to be effective in this stage (Sugimoto et al. 2010), where the role of auxin is to induce the cells to proliferate into callus, from which roots may arise during the following phase. The shift from the first to the second phases of ADRF seems to be related to different gene expression during the two phases. The chromatin-remodelling component PROPORZ1 (PRZ1) mediates auxin effects on gene expression through chromatin remodelling. Noteworthy for the effect of PRZ1 is the differential auxin response of the mutant to auxin: whereas application of auxin promotes formation of lateral roots in wild type, it only triggers formation of tumorous callus-like tissue on *prz1-1* roots (Sieberer et al. 2003).

The process of adventitious root formation proceeds after some 24 h to the next phase, rhizogenesis, lasting from the second to approximately the fourth day. This process of root initiation might be roughly compared to the root initiation on RIM during in vitro rhizogenesis (see Sect. 3.2) and requires the continuous presence of a strong auxin signal (De Klerk 1999 and literature therein, Ludwig-Müller et al. 2005). During this stage, only NAA, IAA or IBA are effective, but not 2,4-D or PAA. This functional partitioning of the auxins is quite noteworthy (see also Sect. 2.2): 2,4-D and PAA (which cease to be effective just after callogenesis) are not well transported polarly, because they are rather poor substrates for auxin efflux carriers (Simon and Petrášek 2011). In contrast, IAA, NAA and, in a way, IBA (see later) are effective during both the first and second phases of ADRF and are substrates for cellular efflux such that they can participate in the organisation of auxin flow. Several studies confirm the importance of endogenous, basipetal IAA transport for adventitious rooting (for review see Ford et al. 2002). For instance, Jarvis and Shaheed (1986), by application of the polar auxin transport inhibitors TIBA and a morphactin on cuttings of *Phaseolus aureus*, also inhibited ADRF, even when a basal level of IAA was supplied exogenously. This finding insinuates the importance of organised auxin transport for this phase of root primordia establishment. If so, this would draw analogies to general non-restorative organogenesis, where auxin gradients, created by coordinated auxin transport, are crucial (Benková et al. 2003; Friml 2003, see also Sect. 2.1). Proper redistribution of the auxin signal over the neighbouring cells in space and time is most readily achieved by directional transport. Intercellular auxin transport and mainly auxin cellular efflux mediated by PIN proteins are driving this coordination, and it is well documented that not all auxins are equally good substrates for PIN proteins (see Sect. 2.2). The third and last phase of ADRF is marked by the outgrowth of roots from their primordia in the stem, and during this last part of the process, auxin can actually act already even inhibitory. Thus, the promoting role of auxin for ADRF is

mainly confined to the first 4 days (i.e. the first two of three distinct stages) of the process.

The process of rooting is dependent on the condition of the regenerating organ, on the condition of the maternal plant, on the season of the year, on the position of the tissue within the maternal plant and on the technique used (Bojarczuk and Jankiewicz 1975). Each of these parameters has impact on the available endogenous IAA or its transport routes, for example, from outgrowing apical buds, as well as on the responsiveness of the tissue to auxin. Most cuttings used lack their own shoot tips and so the original major source for natural auxin. Therefore, in many of these cases, application of exogenous auxin is required to achieve rooting (Salaš et al. 2012; Diaz-Sala et al. 1996; Blazkova et al. 1997). The practical application of auxin became amenable, when it was discovered that auxin can also induce root formation when applied on the surface of the basal cut (Hitchcock and Zimmerman 1936). Some plants regenerate roots on cuttings spontaneously as described above, but these are plants, where endogenous auxin is produced in the apex (or outgrowing lateral buds in spring) and transported basipetally to the cut surface in sufficient amounts to act as trigger: removal of the apex reduces both the level of endogenous auxin in the basal portion of a cutting and, consequently, the number of regenerated roots (Nordström and Eliasson 1993). Moreover, even in these plants, application of exogenous auxin strongly increases the number of roots (Salaš et al. 2012; Nordström et al. 1991; Liu and Reid 1992a).

Application of rooting stimulants is performed by several but similar methods. In spite of efforts to develop new rooting treatments for commercial operations, new methods have not emerged (Salaš et al. 2012; Hartmann et al. 1990): nowadays almost all auxin applications are conducted through a short dip (for a few seconds) of the cutting base into gel, solution or powder containing active auxin. Thus, the available auxin has to be absorbed via the base of plant cuttings before ADRF is initiated, and the auxin taken up during that period has to suffice for the whole process of rooting. The choice of suitable auxins became eventually narrowed to 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), with IBA being strongly preferred over NAA (Salaš et al. 2012). The limited spectrum of auxins suitable for rooting provokes the question why? Form and circumstances under which auxin must be applied onto the plant probably predetermine, to the highest degree, the potency and choice of different auxin analogues for the task. The benefits of IBA and NAA in this regard had been discovered soon after the identification of auxin (Preece 2003 and references therein), and even today, IBA is still the predominant auxin of choice for rooting stimulators. IBA is by far the most used, active component of rooting substances on the market. The suitability of NAA (as second most used compound in rooting stimulants) in comparison to IBA or even IAA has been summarised as follows (Salaš et al. 2012): ‘NAA is not that effective as IAA, and its main advantage on the market was just its lower price’. The dominance of IBA on the market might be further illustrated by the decision of the biggest European producer of rooting stimulants – the Rhizopon Company: as of 2012, the company informed their customers that product lines based on any other active compound than IBA would be withdrawn and that, based on their

rooting guide, this actually will not bring any harm to the plant growers, because well-adjusted treatment with product lines based on IBA will replace well all non-IBA-based stimulant lines (recommended before for about 5 % plant-species-dependent treatments by Rhizopon). This decision of Rhizopon relates to the future commercial unavailability of IAA and NAA on the European market, since NAA and IAA will be most probably taken from Annex (Annex 1) of the European Crop Protection Directive list. In Europe, the responsibility of collecting information on properties and safety aspects of substances manufactured or imported at or above one tonne per year lies with the companies. Since the industrial demand for NAA- and IAA-based products was so low, the economic interest was not sufficient to make up for the costs of the evaluation process under the European legislation, such that the company decided not to have them relisted in the Annex 1 of the Directive.

We will try to sum up here by which qualities IBA has been able to outclass all the other auxins for commercial applications of rooting. As written above, auxin stimulators may only be applied through a pulse treatment at a beginning. This time frame is located just at the beginning of the process, but actually auxin is required during the two subsequent phases of root initiation (see above). Unlike the situation for in vitro rhizogenesis, the plant material in commercial rooting will not experience prolonged contact with auxin-containing media, which would continuously supply the plant tissue with the necessary auxin throughout the whole length of the rhizogenetic process. Moreover, a higher concentration (activity) of auxins is needed during the second as compared to the first phase. Thus, if the plant tissue is treated with auxin at the beginning of the process running for several days, chemical and biological instability of different auxins will be a limiting factor. Some auxins, among others 2,4-D and IBA, are stable, with a low rate of metabolism. However, while IBA is valued highly, 2,4-D seems to be the least used and ineffective auxin in root stimulation. This seems to be a further key to determine the requirements posed on an active root stimulator. As Ludwig-Müller et al. (2005) have shown, both IAA and 2,4-D are comparable in their potency during the first phase, whereas the second phase required high concentrations of polarly transported NAA and IAA or (in higher concentration) of IBA. Based on the substrate preference of efflux carriers alone (giving polarity to auxin flow; see Sect. 2.2), possibly the best major auxins for the rhizogenetic stage of ADRF would be IAA and NAA, along with the rare 4-Cl-IAA (Simon et al. 2013). 4-Cl-IAA has not been used as rooting substance for commercial applications so far. Interestingly, this auxin was found by Katayama et al. (2010) to be exceptionally strong as root stimulator, at least on cuttings of *Vigna mungo*. Both IAA and NAA have been successfully employed as rooting substances as soon as root stimulants were commercialised. However, the native auxin IAA is very vulnerable and chemically instable if applied externally. In addition, it is metabolised rapidly. If applied on the root cuttings at the beginning of the process, supply with IAA will run out, presumably already during the first stage of ADRF. So we might expect that even a strong auxin signal delivered by IAA to the cutting would be relatively short lived and with high probability the remaining auxin levels on the beginning of the second stage would be not sufficient for efficient rhizogenesis. This is highlighted by the

differences of rooting potential of cuttings with growing tip from the apical part of shoot compared to those from the basal shoot lacking a tip. Cuttings without shoot tip are much more dependent on the external application of auxin in comparison to the upper shoot parts, as the growing tip in the cutting is able to provide stable, continuous supply of natural auxin necessary for both callogenesis and rhizogenesis underlying ADRF. Most of the cuttings created lack a growing tip, such that the continuous supply of auxin becomes the limiting factor. As found by Correa et al. (2012), the efficacy of different auxins administered during 2–4 days via solid media to sustain ADRF was comparable. Thus, the superior suitability of IBA as rooting substance must be linked to the mode of its application in practice, i.e. the pulse manner, at the beginning of the rooting on the cutting basis: IBA is slowly and steadily converted into polarly transportable IAA that is steadily released into the plant tissue. (Ludwig-Müller et al. 2005). It would be interesting to test if new rooting stimulators might be developed based on forms of actively transported but stable forms of auxin (including 4-Cl-IAA), which might provide the qualities of IBA described above.

3.4 Auxinic Herbicides

Almost 70 years ago, when auxins started to be used as herbicides (Hamner and Tukey 1944), a new era of modern weed control in agriculture started, due to the advantages those herbicides offered for the first time. Even now, 2,4-D remains to be the most widely used herbicide in the world (Szmedra 1997; Monaco 2002; Industry Task Force II on 2,4-D Research Data). 2,4-D and other auxinic herbicides offer the advantage of having well-defined crop selectivity, i.e. susceptibility of dicot plants and substantial resistance of monocot crops, with low cost and high efficacy of application, systemic effect of the herbicides on plants (Monaco 2002; Kelley and Riechers 2007) and minimal development of herbicide-resistant weeds over time (Heap 1997; Mithila et al. 2011). To the date 2006 more than 1,500 products were registered declaring 2,4-D as their active ingredient (Industry Task Force II on 2,4-D Research Data).

Auxinic herbicides mimic both effects of auxin application and its phytotoxicity at superoptimal concentration (Monaco 2002). In lower concentration, they stimulate cell divisions, elongation and the growth responses typical for auxins. With increasing dose, they develop symptoms of phytotoxicity as observed by overdoses of IAA, such as disturbed growth and signs of herbicidal injury. Typically, a dicot weed will get at least 100 μg of 2,4-D in a typical field application, which is exceeding the level of endogenous auxin in a plant by at least 1,000 times (Cobb and Reade 2010). Monocots and in particular grasses respond differently to these high levels of exogenously applied auxins. Lack of phytotoxicity in monocots is attributed to a spectrum of different reasons, such as processes involved in auxin management and auxin response (Kelley and Riechers 2007; Grossmann 2007, 2010), but also to the fact that the apical meristem before heading is hidden in the

interior of the plant, where it is protected by the leaf sheaths. Within minutes of auxin application, profound changes in membrane permeability to cations can be discerned. The first phase, over the first hours after application, is stimulatory in susceptible plants, gene expression patterns are changed, and growth and metabolic activity are abnormally stimulated. Some typical growth responses can be seen within hours after auxin herbicide application, such as leaf epinasty (cuplike downward bending of leaves), stem curling and tissue swelling. Later, growth of malformed leaves, often with ectopic, parallel veins, leaf abscission and abnormal elongation of roots can be observed. Meristematic tissues in the stem tend to undergo excessive cell proliferation, with cambium, endodermis, pericycle and phloem parenchyma being most sensitive (Leopold 1955; Dnyansagar and Khosla 1969; Monaco 2002). Often, depending on the species, adventitious roots are formed (see also Sect. 3.3) at the basal parts of stems. Those phenomena are later followed by chloroplast damage, by foliar senescence with progressing chlorosis, and by the destruction of membrane and vascular system integrity, plant withering and, eventually, tissue necrosis and decay (Cobb 2010; Grossmann 2000), accompanied by many secondary symptoms (Monaco 2002).

While the phenotypic response to auxin-like herbicides is well described and decades of research have been dedicated to the development of new auxinic herbicides, many aspects of their mode of action and the basis for crop selectivity of auxinic herbicides have remained unclear (Kelley and Riechers 2007). Countless results from the rapidly growing body of knowledge on the role of auxin in plant development need to be connected with application to resolve the puzzle of selective toxicity of applied auxins and the primary mechanism of their herbicidal action. Over the years, several theories, all connected with the concepts and findings on auxin action, have emerged (Leopold 1955; Kelley and Riechers 2007). While several phytotoxic mechanisms in susceptible plant species have been proposed, the question of the ultimate reason for plant death has remained open; so far, no single mechanism was identified as the exclusively deciding factor in herbicide activity. Probably several effects in concert attribute to the herbicidal impact, and their relative contribution to the global effect of herbicidal treatment remains to be properly resolved.

The actual amplitude and character of an auxin response at a particular point depends on the integration of two major factors in any target cells: first, the pool of the signalling molecules present in the cell and, second, the tuning of the signal perception in the cell. There are two possible basic processes which both contribute to the establishment and modulation of the pool of the signalling molecule: metabolism and transport. While the concentration of natural auxins is a matter of tight regulation as described above in Sect. 2.2, synthetic compounds selected as auxins for herbicidal formulations significantly differ in their stability and persistence in plant tissues and evade normal homeostatic control (see Sect. 2.2). As a consequence, they may more easily trigger overinduction of auxin response in susceptible plants. Differential potential to metabolise 2,4-D and other active auxin herbicides was often proposed as one potential reason for monocot resistance towards these herbicides (Gauvrit and Gaillardon 1991; Monaco et al. 2002). IAA conjugation

with sugars and amino acids and oxidative degradation are the most frequent form of auxin deactivation (see also Sect. 2.2). IAA-glucose conjugates participate significantly in the deactivation of IAA. However 2,4-D is not a substrate for IAA-glucosyl transferase (Jackson et al. 2001), and, consequently, overexpression of this enzyme failed to cause 2,4-D tolerance (Jackson et al. 2002). Six IAA-amino acid-conjugating enzymes from the GH3 gene family have been isolated from *Arabidopsis*, and all six conjugate IAA to multiple amino acids in vitro (Staswick et al. 2005). Interestingly, the expression of these enzymes is induced in response to exogenous auxins, including 2,4-D or dicamba (Staswick et al. 2005) suggesting they may play an important role in inactivating excess auxin to support auxin homeostasis (Kelley and Riechers 2007). However, as auxin substrates for these GH3 enzymes, in addition to IAA, indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPA), phenylacetic acid (PAA) and α-naphthaleneacetic acid (NAA) are metabolised as well. In contrast, Trp, the active halogenated natural auxin 4-chloroindole-3-acetic acid (4-Cl-IAA) and the herbicides 2,4-D and dicamba are not substrates for GH3 enzymes (Staswick et al. 2005). Hormonal imbalance due to application of persistent auxinic herbicides inducing strong conjugation (and consequently inactivation) of native auxins is also suggested as possible mechanism of their phytotoxicity. All those factors may explain the particular choice of auxinic compounds based on their chemical structures. While at the beginning of the research NAA was in 1944 also successfully used as selective agent with herbicidal action (Gauvrit and Gaillardon 1991; Cobb 2010), its effects were much weaker, much higher doses had to be applied, and it was abandoned as soon as the potency of 2,4-D was discovered.

All current auxin herbicides are weak acids with pK values ranging from 2 to 4 (Monaco 2002). Structurally, their dissociated molecules share weaker positive charges on the planar aromatic ring in about 0.5 nm distance from the strong negative charge of the carboxyl group (Grossmann 2003) and can be further divided into four classes based on their particular chemical structures with slightly differing crop selectivity: phenoxycarboxylic acids (e.g. 2,4-D), benzoic acids (e.g. dicamba), pyridines (e.g. picloram) and the newer group of quinoline-carboxylic acids (e.g. quinclorac). Crop selectivity and maybe partly mode of action of the last group differ more from the other groups. Some quinolone carboxylic acids are able to control monocot weeds in the background of a monocot crop, and others target some dicotyledonous weeds in resistant dicot crops (Grossmann and Kwiatkowski 2000). The fact that 2,4-D is less toxic to monocots is often explained by differences in susceptibility for metabolism and degradation. Gauvrit and Gaillardon (1991) actually proposed that selectivity of auxin herbicides might be based on differences in auxin homeostasis, as 2,4-D is rapidly degraded in maize (Gauvrit and Gaillardon 1991; Monaco et al. 2002). Different induction of GH3 enzymes in response to 2,4-D application is also debated as possible factor contributing to the 2,4-D resistance of monocots (Kelley and Riechers 2007). The principal routes for the metabolism of phenoxyalkanoic acids are conjugation, hydroxylation and side-chain cleavage (Cobb 2010), depending on the respective species. A strategy of metabolic deactivation was utilised recently by Dow

AgroScience Company, which successfully developed new transgenic line of corn (DAS-40278-9) resistant to 2,4-D and other phenoxy-auxin herbicides. Resistance was based on expression of aryloxy-alkanoate dioxygenase (AAD-1) from *Sphingobium herbicidovorans*, a gram-negative soil bacterium (Tagliani 2011; Wright et al. 2010). AAD-1 is able to cleave phenoxy acids, and AAD-12 acts on pyridyl-oxyacetate auxin herbicides such as triclopyr and fluroxypyr (pyridines) (Wright et al. 2010).

The type of herbicidal formulation modulates the effects of auxinic compounds significantly. The choice whether auxins will be applied as free auxins in their salt (mostly the amino salts) or in their ester form will impact the permeability of auxinic herbicides into the plant through the cuticular layer and also affects transportability through the plant and between different tissue layers. Esterised auxins enter into the plants more easily, yet they are less readily transported through the plant body (Leopold 1955). The choice of the respective auxinic chemical should also consider their stability *in planta* and the above-mentioned systemic effects. Transportability of herbicide through the plant is a precondition for a systemic action, which allows to kill the plant as a whole including its underground part. For instance, 2,4-dichloropropionic acid has a strong local effect, yet it does not enter the vascular system and therefore is not transported across plant body (Leopold 1955). As a result, it fails to kill regrowing weeds. While endogenous auxin is transported through the plant by combination of polar auxin transport and passive flow in the phloem, auxinic herbicides mostly do not participate in polar auxin transport and therefore must at least enter the vascular system to be then carried by the passive flow through the plant body. This has been demonstrated for 2,4-D and other auxinic herbicides that are transported well both downwards to the root in the phloem and up from the roots to the stem and leaves in the xylem, with speed being in both cases concentration dependent (Leopold 1955; Monaco 2002).

Auxins and auxin-like compounds affect different plant tissues and organs in different manner. Thus, auxin phytotoxicity and symptoms of herbicidal injury differ between stems, roots and leaves. According to McCarthy-Suárez (2011), stems of pea plants did not show elevated levels of reactive oxygen species (ROS), whereas ROS do accumulate in both foliage and roots of auxin-treated pea plants in harmful levels with relevant herbicidic injury, as described later in this section in more detail. Nevertheless, stems of affected plants display also injury, but of a different type. The stems undergo morphological changes depending on the concentration of auxins in different stem tissues (Leopold 1955 and references therein) and different sensitivity of different tissue layers. While the phloem is generally one of the least sensitive tissues in plants towards auxin in general (Leopold 1955), the cells of phloem and its companion cells are the most affected stem tissue after application of auxinic herbicides, which is explained by their close contact with the high concentrations of active auxinic compounds accumulated in the phloem stream (Eames 1950; Dnyansagar and Khosla 1969). As a result, the continuity of the vascular system is affected leading to withering of dicotyledonous plants. Distorted cell division and expansion leads to deregulated growth with collapse of the correlating plant growth structure (Cobb 2010). Except for the phloem, the

strongest radial proliferation was reported in auxin-sensitive tissues with high division rates – especially cambium – followed by endodermis and pericycle (listed in Leopold 1955; Dnyansagar and Khosla 1969) that tend to proliferate and further degrade (Leopold 1955 and references therein). Conversely, the more the cells are differentiated, the stronger their resistance towards auxin-like compounds. It was actually proposed by Struckmeyer (1951) that the selective action of auxinic herbicides against dicotyledonous plants might be based on the different morphology of the stem vasculature in monocotyledonous and dicotyledonous plants. For the grasses being unaffected by herbicide action do not possess neither cambial layers nor weakly differentiated cells adjacent to phloem. Auxin preferentially acts on cells with high division rate, and therefore, meristematic cells are most vulnerable. It is known that severe distortion of patterns of auxin gradients, especially in the apical meristems, may be lethal for the plant (Weijers and Jürgens 2005).

Despite the statement by F.A. Gilbert (1946) that auxin herbicides cause susceptible plants 'to grow themselves to death' and the fact that auxinic herbicides are not good substrates for polar auxin transport, the possibility that the severe detrimental effect may be due to disrupted patterns of auxin transport in apical meristems, with subsequent failure of meristem organisation, has been neglected in the recent literature. Yet, the synergistic effect of the auxinic herbicide dicamba and the auxin transport blocker diflufenzopyr was successfully utilised in field application (Wehtje 2008) and thought to result from elevated concentration of dicamba in meristematic tissues.

Further insight into herbicidal auxin action and auxin herbicidal injury was provided by the observation that strong oxidative damage by excessive levels of reactive oxygen species (ROS) to leaves and roots of affected plants (Schopfer and Liszkay 2006; Pazmiño et al. 2012) is caused by auxin overdose. ROS are particularly responsible for the toxic effects of 2,4-D and other auxinic herbicides, at least in foliage and roots (McCarthy-Suárez et al. 2011). 2,4-D exerts failure of the detoxifying cell mechanisms, causing oxidative damage, fragmentation of nuclear DNA and cell death (Pazmiño et al. 2012). Auxins are also known to induce a programmed and cell-specific generation of ROS and to regulate the level of antioxidants. Onset of 2,4-D-induced leaf senescence is marked by overproduction of H_2O_2 and O_2^- and stimulation of enzymatic and nonenzymatic antioxidative systems (Karuppanapandian et al. 2011). This study also showed that changes produced in ROS metabolism by 2,4-D treatment can cause chlorophyll degradation and lipid peroxidation as typical for leaf senescence. Auxin-related ROS induction is probably conditioned by the activation of phosphatidylinositol 3-kinase activity, and ROS production is considered necessary for some auxin-regulated processes such as gravitropism (Joo et al. 2005).

Induction of ROS species in high doses by 2,4-D is probably mediated by high levels of abscisic acid (ABA Grossmann et al. 1996; Hansen and Grossmann 2000; Zhang et al. 2009) and related stress reactions (Hansen and Grossmann 2000). ABA contributes to the mode of action underlying the late auxin herbicide effects in sensitive dicots, especially the induction of tissue decay and cell death (Grossmann 2000). It was shown that ABA levels are profusely increasing in treated plants due

to cleavage of xanthophyll to xanthoxal, a critical step in ABA biosynthesis (Taylor et al. 2000) catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED; Hansen and Grossmann 2000). The abundance of NCED enzymes is strongly and rapidly upregulated by high levels of several auxinic compounds as observed in several plant models (*AtNCED1*, Raghavan et al. 2006; *AtNCED3* Raghavan et al. 2005; Gleason 2011; *AtNCED5* Gleason 2011; *GaNCED1* Hansen and Grossmann 2000). NCED overexpression in plants is associated with ABA accumulation in the leaves (Taylor et al. 2000). Induction of NCED in the shoots will subsequently lead to ABA transport through the plant to the leaves, where it causes stomatal closure, impinging on carbon assimilation and, consequently, biomass production and growth (Scheltrup and Grossmann 1995; Grossmann et al. 1996; Grossmann 2000; Grossmann and Kwiatkowski 2000; Hansen and Grossmann 2000). During this process, high ABA levels are linked with high levels of ROS (Grossmann 2000; Zhang et al. 2009). Grossmann (2000) has proposed that both auxin and auxinic herbicides primarily induce ethylene, which is then the trigger of an increase in the biosynthesis of abscisic acid (Hansen and Grossmann 2000; Grossmann 2003, 2007). Importantly, strong ethylene biosynthesis starts very early after application of IAA or synthetic auxins through induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase – the rate-limiting factor in ethylene biosynthesis (Woeste et al. 1999). Hansen and Grossmann observed that it was the auxin-induced ethylene that stimulated xanthophyll cleavage. Quinclorac-induced phytotoxicity in several susceptible grasses has been considered to be due to the induction of the ethylene precursor ACC-dependent cyanide (Grossmann and Kwiatkowski 1995). However, the role of ethylene in general 2,4-D toxicity is disputed (Pazmiño et al. 2012), and several authors have demonstrated that while ethylene may participate in the 2,4-D-induced plant senescence (Karuppanapandian et al. 2011), it does not participate in other 2,4-D-dependent symptoms as does leaf epinasty (Keller and Van Volkenburgh 1997; Pazmiño et al. 2012).

It is generally accepted that 2,4-D and IAA share a common signalling pathway (e.g. Taiz and Zeiger 2002), yet there are some specific differences with respect to auxin-triggered cell division and signalling between 2,4-D from 1-NAA and IAA (Campanoni and Nick 2005; Simon et al. 2013; Rahman et al. 2006) that might be potentially relevant to herbicidal action. A detailed analysis of the transcriptome revealed that IAA and NAA induce mainly similar genes, clustered in one group, whereas 2,4-D, in addition to the gene induction shared with IAA and NAA, also induces a subset of genes that cluster in a unique group (Pufky et al. 2003). Some differences between 2,4-D and IAA with respect to regulation and signalling of homeostasis have already been described in Sect. 2.2 of this chapter. Walsh et al. (2006) suggest that some degree of synthetic auxin selectivity and potency may also be based on differences within the auxin reception machinery through the SCF/AFBs complex. The TIR1 homolog AFB5 has been found to confer resistance particularly to auxin herbicides from pyridinecarboxylic acid -type (Picloram; Walsh et al. 2006) and benzoic acid-class auxin herbicides (dicamba; Gleason 2011), with only minimal cross-resistance to 2,4-D or IAA (Walsh et al. 2006). According to Calderon-Villalobos et al. (2012), picloram is selectively

bound by AFB5-Aux/IAA co-receptor pairs. While most attention has now shifted towards the reception through the TIR1/AFBs signalling system, decreased sensitivity of ABP1 to auxinoids is also considered as a potential source of resistance to auxinic herbicides (Mithila and Hall 2005). In the evaluation of auxinic herbicide effects, it seems that several mechanisms contribute to herbicidal injury in parallel, and their contribution and overall importance has to be clarified yet. Further elucidation of the separate pathways triggered by 2,4-D or other classes of auxin herbicides is expected to promote new strategies for the future development of new herbicidal solutions.

3.5 Auxin in Other Biotechnological Applications

Aside from the three major uses of auxinic compounds described in the sections above, auxin is and has been used from the beginning in wide variety of different scenarios to reach a range of diverse practical objectives. Many different practical applications are described in available literature, whereas other practical objectives seem to be, at least theoretically, possible. Nevertheless, many of such applications described in the older literature or the Internet were abandoned later in practice, due to economic or environmental limitations of the technology. Due to legislative constraints, many prospective compounds had to be withdrawn from the market. Therefore, this short review does not intend to encompass full range of applicable solutions in practical use. This section merely intends to review currently often used practices of auxin application in a variety of biological and practical settings and interpret the auxin biology behind them. The choice of a given auxinic substance for a particular application is often a compromise between the desired maximal physiological effect and the avoidance of unwelcome side effects, which present naturally a high threat, symptoms of herbicidal injury after spraying being the most perilous (see also Sect. 3.4).

Auxin had been used to initialise flowering. In 1942, Clark and Kerns found that flowering could be induced in pineapple by auxins (NAA), through evolution of ethylene within 1 day after application (Burg and Burg 1966). Polar auxin transport, driven by PIN1 into subapical tissues, is necessary for correct floral development (Kuhlemeier and Reinhardt 2001). On the other hand, some of the polar auxin transport inhibitors had been used to decrease the degree of apical dominance in pot flowers in order to increase the number of lateral branches bearing flowers. Transgenic increase of auxin synthesis in the ovule epidermis of cotton plants had been the key in developing plants producing higher yield of quality cotton lint fibres – highly elongated cells derived from the ovule epidermis (Zhang et al. 2011).

It was shown that auxin administered to the ovary may cause development of flowers into fruits even in the absence of pollination. Successful pollination initiates ovule growth – known as fruit set. Auxin is normally produced in vicinity of developing seeds and, along with gibberellins, may act primarily to induce fruit set, which trigger endogenous auxin in some fruit tissues (reviewed in Ruan

et al. 2012). This was the base for strategies, where transgenic overproduction of auxin in ovules using the tissue-specific promotor *DefH9* driving the auxin-synthesis gene *iaaM* in tobacco and tomato plants produced parthenocarpic fruits (Ficcadenti et al. 1999). Alternatively, tomatoes with SlPIN4 being silenced in their ovaries produced the same outcome (Mounet et al. 2012). Redistribution of auxin by PIN4 within the ovary is important as also demonstrated by parthenocarpic effects of NPA on tomatoes (Serrani et al. 2010). Application of auxin as sprays can be used in some instances to start parthenocarpic development of fruits in field routine (a common practice for tomatoes and strawberries, figs, watermelons and spiny gourd; Leopold 1955; Rasul et al. 2008; Maroto et al. 2005). Also fruit ripening can be regulated by impairing ripening-related ethylene and auxin metabolism and signalling as used in young developing peach fruits (Torrigiani et al. 2012).

Fruit growers also benefit from auxin applications to regulate different forms of abscission on fruit trees. Application of auxins may influence abscission of leaves, flowers or fruits from plants in a dual manner. In some cases, application of auxins is meant to cause the abscission and shedding of the organs (application of defoliants or fruit-thinning agents), whereas in others it prevents abscission (e.g. premature drop of flowers or maturing fruits). Detachment of abscising organs from the plant body is taking place in a zone of specialised cells – the abscission zone (AZ), which is usually preformed at the base of the petiole or fruit stalk. In some cases, more than one AZ may be present. When cell walls within the abscission layer of the AZ are digested, they become weak, and the connected organ breaks free and falls to the ground. Ethylene is considered to play a crucial role in fruit abscission, by activating new gene sets competent to digest those cell walls, and presence of ethylene receptors in the AZ might define sensitivity of the AZ to the ethylene. The currently accepted model is reviewed in Estornell et al. (2013) and Xie et al. (2013).

In some applications, auxin can prevent abscission in cases where abscission is normally senescence-triggered. As long as endogenous or applied auxin is transported from the organ through the AZ of the plant organ, differentiation of an abscission layer and, consequently, abscission of the organ are prevented. Senescent fruits, leaves or flowers export only small amounts of auxin into stem through the AZ, probably because senescence-born ethylene decreases auxin flow from the organ. The reduced supply of auxin to the AZ together with a loss of its transport polarity enhance the sensitivity of the AZ to ethylene and promote the activation of cell wall-degrading enzymes there (reviewed in detail by Xie et al. 2013). While this process is mainly controlled by environmental factors, auxin, sprayed on plants, can, despite such conditions, prevent undesirable flower or preharvest fruit drop. For example, in potted plants like *bougainvillea*, a spray with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; from the class of phenoxycarboxylic acids) precludes abscission of bracts and keeps plants in a marketable shape (Meir et al. 2007). Interestingly, the use of picloram application was derived by Goldschmidt and Leshem (1971) to prevent the abscission of floral parts from etrog fruits (*Citrus medica*) on Israeli markets. The shape and condition of *etrogs*

have cultural significance for the Jewish holiday of *Sukkot*. Styles and stigmas still intact on top of the citrus fruits are called *pitam* in Jewish practice, and *etrogs* with an intact *pitam* is considered especially valuable. Spraying with 3 ppm (i.e. 12 μM) solution of picloram on trees at anthesis resulted in almost complete prevention of pistil abscission. It seems that one of the most apparent differences to the abscission process described above is the absence of a priori developed abscission layer tissue capable to separate the organ. Consistently, application of ethylene cannot stimulate abscission under those circumstances (Goldschmidt and Leshem 1971).

Different mechanisms have to be invoked to explain the abscission of young growing fruits which do not show symptoms of senescence yet, and the difference in mechanism also requires a different practice of auxin application. Angiosperms usually produce much less fruits than the initial flower number (Klein et al. 2007), and autoregulation of developing fruits in orchards is a naturally occurring phenomenon relevant to many fruit trees with heavy flowering (apples, citruses, avocado) and is distinct from the later shedding of older fruits, regulated by the mechanism described above. External application of regulators, so-called fruit thinning, is meant to enhance this mechanism in commercial orchards in order to decrease the number of developing fruits, which leads to increase fruit size and improved colour of the remaining fruits, improved plant vigour and annual bearing. Bangerth (2000) offered an explanation in the conceptual framework of correlative dominance, where shoots and fruits and fruits in clusters compete for assimilate relocation. The fruits in a cluster show a clear ranking, and their relative position within the cluster has a direct causal effect on their potential for precocious abscission. The more fruits, the stronger the mutual competition, such that the fruits of the lowest rank are doomed to abscission and precocious shed (Bangerth 2000). The rank of a fruitlet depends on auxin flow: the stronger its auxin export to the phloem of the fruit stalk, the more dominating its position in the competition. Auxin application on leaves and fruitlets leads to amplification of these differences in correlative hierarchy. In the best-studied model, during the so-called June drop of apple trees occurring 3–4 weeks after flowering (Losada and Herrero 2013), NAA and naphthalene acetamide (NAD) as common thinning agents act promotive when sprayed at full bloom or before flower drop. Some non-auxinic compounds such as 6-benzyladenine (BA) can be applied successfully as thinners as well, as they target correlation of plant organs based on different mechanisms (Botton et al. 2011). This mutual competition of auxin flow resembles the mechanisms of apical dominance as described by Balla et al. (2011) for lateral buds dominated by basipetal auxin flow from the apical tissue. In stems of pea, lateral buds are prevented from exporting their own auxin into the stem, due to impaired directional auxin efflux. To overcome the dominance by the apical bud, the axillary bud has first to establish directional auxin export by subcellular polarisation of PIN auxin transporters (Balla et al. 2011). Only after auxin flow has been connected with the main axis, this auxin transport route may start to differentiate into vascular bundles, securing nutrition to the lateral bud. Conversely, if external auxin is applied to and absorbed by the growing tip of the shoot, the conservation of basipetal PIN polarisation in the stem is stronger, and a smaller number of lateral buds are able to escape from apical

dominance. This control of apical dominance by exogenous auxin is utilised in practical applications as well. Tre-Hold, an NAA-containing product, is marketed as tree sprout inhibitor and used to control sprouts and sucker growth on apples, pears, olives and ornamental woody plants and trees. Apical bud dominance is maintained even after pruning, such that lateral bud outgrowth is minimised. This can be used to control branch growth in orchards, residential areas and areas where tree branch growth may pose a hazard, such as power lines.

4 Future Prospects for Auxin Biotechnology

Auxins have been famous and popular chemicals in plant production circles for already extended period of time. A vast amount of practical applications have been attempted, and numerous real-life applications have been developed based on the successful strategies, whereas a multitude of them had to be omitted later, not due to methodological failure but in response to economic or environmental challenges connected to them. The practical use of auxins succeeded to a degree that it is often very challenging for fundamental biology to decipher the biological mechanism behind this application. Under such circumstances, it is rather daring to propose prospective application of auxins.

However, advanced and more detailed understanding of the biological mechanisms can still provide new improvements and chances for fine-tuning of existing practices. However, to successfully transfer this scientific knowledge from the laboratory to the field, sufficient economic payoff is required, allowing to fund the environmental and toxicological tests necessary for the registration of the compounds into the databases of chemicals which are meanwhile strictly regulated in all developed markets.

Other yet unexploited opportunities may come from the field of genetically modified plants, provided that they will be allowed to enter the market. Considering our growing knowledge of auxin-related biology, future plants may be able to utilise many of the mechanisms described above, such as parthenocarpic fruiting, resistance to auxinic herbicides or better regulation of fruit set. More visionary prospects would be to direct artificial shaping of plant architecture through transgenic tuning of auxin-related mechanism. Theoretically, altered plant architecture (see also the chapter by Nick in the current volume), such as changes in branching in the root system or shape and habitus of canopy or change of morphological features such as shape of leaves, flowers and other body parts, should be possible in the future.

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The Biotechnological Potential of Cytokinin Status Manipulation

Mária Šmehilová and Lukáš Spíchal

Abstract Cytokinins are highly conserved plant hormones with a long evolutionary history that regulate various aspects of plant growth and development. The genetic background of the mechanisms involved in the regulation of plants' cytokinin status has recently been elucidated. Studies on transgenic plants with altered cytokinin biosynthesis, metabolism, degradation, or signaling revealed interesting consequences of cytokinin deficiency or disruption of cytokinin perception that can be applied in plant biotechnology and agriculture. Cytokinin levels and the sensing thereof can be manipulated using transgenic approaches and by treating plants with novel exogenous compounds in order to promote root system development, biomass formation, yield-forming traits, nutrient uptake, and tolerance of biotic and abiotic stresses. This chapter provides an overview of recent findings concerning the molecular basis and genetic background of cytokinin signaling and metabolism and the ways in which they can be manipulated to tailor plants' traits to meet specific requirements.

1 Introduction to Cytokinins

It has only been 100 years since Gottlieb Haberlandt suggested that there are diffusible factors within the phloem of potato tubers that stimulate cell division and around 60 years since the identification of the first molecule with such activity, kinetin, by Miller and Skoog (Miller et al. 1955). Because of their ability to promote cell division, *cytokinesis*, kinetin and the diverse subsequently discovered natural and synthetic compounds with similar activity were termed cytokinins. Miller and Skoog demonstrated that the ratio of auxins to cytokinins plays a key role in

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controlling organogenesis in vitro (Miller et al. 1956), suggesting that the manipulation of cytokinin levels might have considerable utility in plant biotechnology.

More recently, it has been demonstrated that in addition to cell division and organogenesis, cytokinins (CK) also regulate seed dormancy and germination, senescence, the release of buds from apical dominance, and bud formation de novo and stimulation of leaf expansion (reviewed by Mok 1994). Furthermore, they control the growth and development of plant organs, mediate plant responses to extrinsic factors such as the light conditions in the shoot, and the availability of nutrients and water to the roots, and play important roles in responses to biotic and abiotic stresses (reviewed by Werner and Schmülling 2009). Cytokinins are not unique to plants: they are also present in phylogenetically diverse organisms ranging from bacteria to humans, where they are involved in RNA translation (reviewed by Spíchal 2012). In addition, certain plant-interacting organisms such as bacteria, fungi, nematodes, and insects produce CKs or otherwise manipulate the CK status of plants in order to control their development (reviewed by Spíchal 2012).

In chemical terms, natural cytokinins are N^6-substituted derivatives of adenine. The main determinant of cytokinin function is the side chain, which may be of isoprenoid origin (e.g., zeatin) or aromatic (e.g., N^6-benzyladenine, BA). The activity of a cytokinin is further influenced by structural changes or interconversions at the side chain and the adenine moiety (Fig. 1). The relationships between the structure and activity of natural and synthetic adenine- and urea-type cytokinins (e.g., thidiazuron, TDZ, Fig. 1) have been elucidated using classical cytokinin bioassays based on their physiological effects (for a review, see Mok 1994) and more precisely by investigating their ability to activate cytokinin signaling pathways by interacting directly with cytokinin receptors (Yamada et al. 2001; Spíchal et al. 2004).

Another important determinant of cytokinin activity is their local concentration inside individual plants and in their organs, cells, and organelles. Cytokinins are present in plants at very low concentrations (pmol.g^{-1} fresh weight). Investigations into the biological functions of CKs have therefore been greatly facilitated by the rapid development of certain analytical techniques, particularly with respect to their sensitivity, throughput, and the amount of material required for analysis. For example, high throughput analyses played a key role in a recent comprehensive investigation into the abundance of *cis*-zeatin (cZ) throughout the evolutionary history of land plants. This zeatin isomer, which was long considered to be biologically inactive, was shown to be ubiquitous within the plant kingdom and is the most abundant cytokinin in some plant families such as the *Poaceae* (Gajdošová et al. 2011). Based on these findings and previously reported results concerning the metabolism and ontogenetic occurrence of cZ in plants, it has been proposed that cZ-type CKs may function as delicate regulators of CK responses in plants under growth-limiting conditions (Gajdošová et al. 2011). In addition, the results presented by Kudo et al. (2012) suggest that cZ has physiological effects on growth and development in rice.

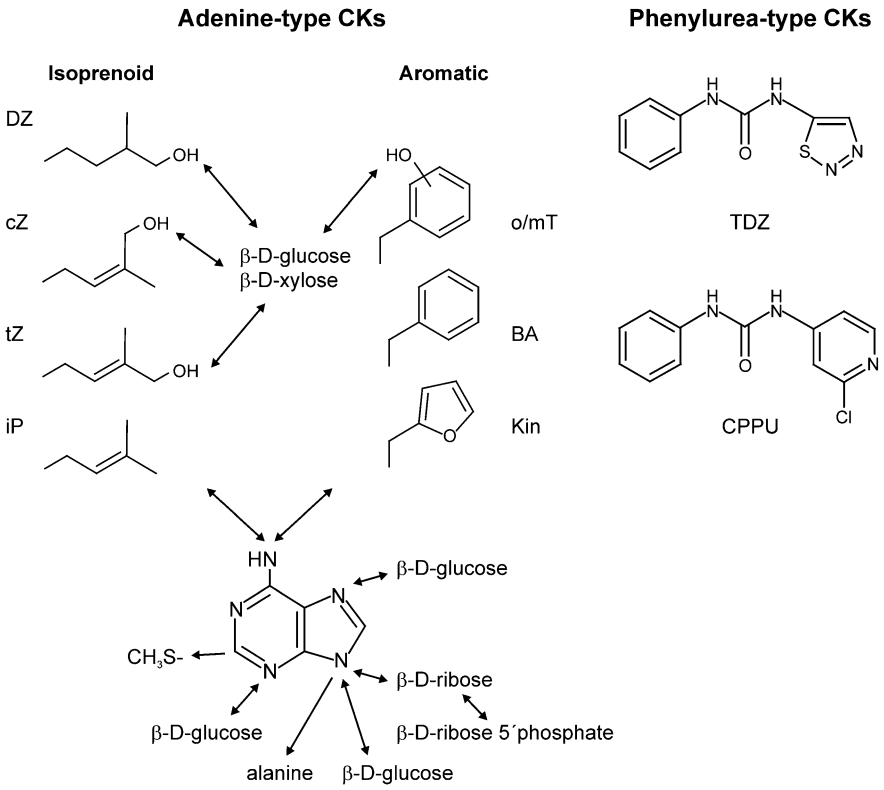

Fig. 1 Structure of adenine-type and phenylurea-type CKs. The isoprenoid or aromatic side chain is the main determinant of the biological activity of natural CKs, while substitution at other indicated positions corresponds to the formation of a specific type of CK conjugate via metabolic interconversion. *iP* N^6-isopentenyladenine, *DZ* dihydrozeatin, *tZ trans*-zeatin, *cZ cis*-zeatin, *Kin* kinetin, *BA* N^6-benzyladenine, *mT meta*-topolin, *oT ortho*-topolin, *TDZ* thidiazuron, *CPPU N*-(2-chloro-4-pyridyl)-*N*'-phenylurea

The endogenous levels of active CKs in plant tissues are regulated by enzymes that are involved in general purine metabolism as well as specific enzymes that catalyze CK biosynthesis (isopentenyltransferase, IPT), activation (LOG and CYP735As), deactivation (N- and O-glycosyltransferases), reactivation (β-glucosidase), and degradation (cytokinin oxidase/dehydrogenase, CKX). Activity of these enzymes along with that of proteins involved in CK perception (histidine kinases, HK), signal transduction (histidine-containing phosphotransmitters, HPt), and response regulators maintains a signaling homeostasis that in the following is referred to as "cytokinin status" (Fig. 2).

The cytokinin status of the plant depends on the localization and activity of these proteins and the expression of the corresponding genes. Most of these genes have been identified and assigned with respect to their roles in the regulation of plant growth and development. This has led to the development of new chemical

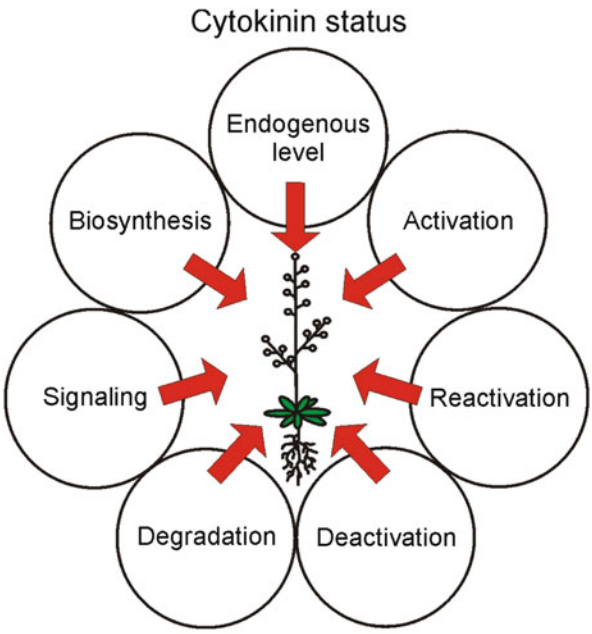

Fig. 2 The main determinants of plant cytokinin status. The endogenous levels of CKs that are active in signaling are synthesized and further regulated by the (re) activation, deactivation, and degradation by CK-specific enzymes and enzymes of general purine metabolism. The signaling machinery then receives the CK signal and translates it into specific biological responses

inhibitors that target key proteins in CK metabolism and signaling. The remainder of this chapter discusses strategies for modulating CK status of a plant based on transgenic approaches and treatment with exogenous chemical compounds and presents some of their potential applications in biotechnology.

2 Modulation of Cytokinin Status Through Control of Gene Expression

Based on the results of experiments with model plants (as described in this chapter and the reviews written by Werner and Schmülling 2009; Frébort et al. 2011; Peleg and Blumwald 2011; Zalabák et al. 2013; Wilkinson et al. 2012; and Spíchal et al. 2012), it has been suggested that the genes that control CK status are ideal targets for transgenic manipulation because they influence several agronomically important traits including tolerance of various stresses. During the past years, transformation procedures were the most limiting step in the generation of crops with modified CK metabolism, especially cereals (Zalabák et al. 2013). However, many of the major technical obstacles to this process have recently been surmounted, resulting in the creation of several new CK-modified transgenic crop lines. We therefore expect that in the near future, strategies based on the spatio-temporal modulation of endogenous CK levels will improve the transfer of the acquired knowledge into the preparation of transgenic crops.

2.1 Modulation of Cytokinin Levels Through Control of Biosynthesis

Isopentenyltransferase (IPT; EC 2.5.1.27) is a key enzyme in isoprenoid CK biosynthesis and was first used as a transgene over 20 years ago, when an Agrobacterial *IPT* gene was constitutively overexpressed in tobacco (Smart et al. 1991; Li et al. 1994). These fundamental experiments showed for the first time that the enhancement of endogenous CK production induces cell division and shoot formation from calli. Several subsequent studies have demonstrated similar positive effects due to the overexpression of various *IPT* isoforms under the control of different specific promotors. In general, the elevated CK levels observed in *IPT*-overexpressing plants cause what is known as "CK syndrome" (Hewelt et al. 1994) – a phenotypic change that is characterized by reduced root and stem growth, reduced apical dominance with a sequential release of axillary buds, reduced leaf expansion, and delayed leaf senescence (Beinsberger et al. 1991; Zhang et al. 1995; Gan and Amasino 1995; Faiss et al. 1997; Redig et al. 1996; Zubko et al. 2002). Stimulation of the CK status by IPT overexpression was utilized as a strategy to push yield or stress resilience. In addition, *IPT* overexpression under the control of tissue-specific and stimulus-responsive promotors has been explored. Most of these studies used an Agrobacterial *IPT* gene as the transgene. To date, the only plant *IPT* isoforms that have been used to modulate endogenous CK levels are those of *Arabidopsis*, *Petunia* (Zubko et al. 2005), and maize (Brugiere et al. 2008).

Shortly after IPT was confirmed to cause the overproduction of CK in transgenic tissues, the gene for this biosynthetic enzyme was used for transgenosis under the control of a range of different promotors, most of which are stimulus specific. In an early study (Gan and Amasino 1995), IPT was expressed in transgenic tobacco plants under the control of various senescence-specific promotors. IPT expression under the control of the senescence-associated promotor of the cysteine protease SAG12 from Arabidopsis yielded plants that exhibited delayed senescence (Gan and Amasino 1995). The P_{SAG12}:IPT construct described in that pioneering work has since been used many times in a range of different plant species. The general "CK syndrome" was observed in all cases, albeit to varying degrees and with some case-by-case variation. Notably, the introduction of the construct into lettuce caused prolonged retention of chlorophyll, increased resistance to pathogen attacks, and reduced nitrogen tolerance (McCabe et al. 2001). Ryegrass (Li et al. 2004) and maize (Robson et al. 2004) plants transformed with the construct exhibited an extended photosynthetically active lifespan. In wheat, the introduction of the construct delayed chlorophyll degradation under low nitrogen conditions (Sýkorová et al. 2008). In tomato, P_{SAG12}:IPT overexpression resulted in suppressed leaf senescence and advanced flowering while also causing a slight increase in fruit weight and fruit total soluble solids (Swartzberg et al. 2006). In contrast, when another *Arabidopsis* analogue of the cysteine protease SAG13 promotor was used to control the expression of IPT in tomato plants, it caused stem thickening, reduced internodal distances, and the loss of apical dominance

(Swartzberg et al. 2006). The two transgenic tomato lines were used to investigate the relationship between the effects of IPT and hexokinase on leaf senescence and seed germination. These studies revealed that IPT overexpression is not sufficient to rescue plants from the detrimental effects of hexokinase deficiency, and so the latter enzyme appears to have a stronger effect on the control of plant senescence (Swartzberg et al. 2011). In maize, IPT overexpression under the maize homologue of the SAG12 promotor (SEE1) perturbed the development of the reproductive organs: each spikelet only produced a single kernel, with each kernel containing a duplicated embryo (Young et al. 2004). In a similar study, the rice homologue of the cysteine protease (OsSAG39) promotor was cloned and used to achieve homologous overexpression of IPT in transgenic rice plants. This yielded an early flowering phenotype that matured rapidly without any loss of yield (Liu et al. 2010). In cotton, IPT overexpression under a cysteine protease promotor (specifically, the Ghcysp promotor from *Gossypium hirsutum*) also had positive effects on senescence, yield, and crop quality (Liu et al. 2012). Merewitz and coworkers recently used the P_{SAG12}:IPT construct to overexpress IPT in creeping bentgrass and characterized the stress responses of the resulting plants (Merewitz et al. 2010, 2011, 2012). An increased tolerance of water stress was observed, suggesting that IPT has positive effects on osmotic adjustment and promotes efficient water use. The plants also exhibited increased root viability and enhanced photosynthetic rates, particularly in mature leaves (Merewitz et al. 2010, 2011, 2012). Another promotor that has been used for the senescence-triggered expression of IPT is P_{SARK}, which derives from the senescence-associated receptor protein kinase gene. P_{SARK}:IPT was shown to increase drought tolerance in transgenic tobacco (Rivero et al. 2007, 2010), rice (Peleg et al. 2011), and peanut (Qin et al. 2011). A different study showed that IPT overexpression under the control of a promotor derived from the tomato ethylene oxidase gene *ACO1* generated plants that had elevated concentrations of cytokinin O- and N-glucosides in their generative shoots and consequently produced more flowers than wild-type plants. However, the individual flowers of the transgenic line had lower diameters than those of the wild-type (Khodakovskaya et al. 2009).

Several recently published studies have explored the consequences of using promotors from stress-responsive genes to control *IPT* expression. In tomato, the heat stress-inducible expression of IPT under the control of the heat shock protein 70 promotor (in the form of the P_{HSP70}:IPT construct) increased yields and promoted shoot growth under salinity stress (Ghanem et al. 2011). Similarly, a stress-inducible promotor from *Arabidopsis*, P_{RD29}, was used to control IPT overexpression in tobacco. The resulting transgenic line exhibited enhanced tolerance of salt stress together with prolonged chlorophyll retention (Qiu et al. 2012). Finally, IPT overexpression under the control of the cold-inducible promotor P_{COR15a} from *Arabidopsis* conferred increased low-temperature tolerance in sugarcane. Notably, the transgenic plants in this case had substantially higher total leaf chlorophyll contents than their wild-type counterparts under freezing conditions (Belintani et al. 2012).

Tissue-specific overexpression of IPT has been used in attempts to increase CK levels in the reproductive organs of plants in order to increase yields and crop quality. A range of tissue-specific promotors have been used to achieve this goal. The initial results in this area were not promising. For example, in tomato plants, IPT overexpression under a fruit-specific promotor produced islands of green pericarp tissue in otherwise ripe tomato fruits (Martineau et al. 1994). In canola, IPT expression under the promotor of the 2S albumin seed storage gene from *Arabidopsis* caused IPT expression in seeds. However, the only phenotypic consequence of this targeted overexpression was an increase in the number of siliques formed, due to an increased branching of inflorescences (Roeckel et al. 1997). IPT expression under the anther-specific TA29 promotor affected the morphology, floral organ systems, and reproductivity of transgenic tobacco plants (Sa et al. 2002). In tomato plants, IPT expression under the fruit-specific 2A12 promotor generated transgenic plants that exhibited hyperplasic placenta and produced few or no seeds (Mao et al. 2002). Conversely, tobacco plants that overexpressed IPT under the *vicilin* promotor had significantly greater dry seed weights than wild-type plants (Ma et al. 2002), suggesting a crucial role of CK for the regulation of tobacco embryo development. Similarly, lectin-driven IPT expression in tobacco seeds resulted in larger seeds, faster germination, and more rapid seedling development (Ma et al. 2008). In grapefruit plants, the only phenotypic consequence of IPT overexpression under the flower-specific promotor of the *APETALA3* gene from *Arabidopsis* was an increased chlorophyll content in the uppermost leaves. However, the expression of this promotor was found to be leaky (Pasqauli et al. 2009). In a similar experiment, the Arabidopsis *AtIPT4* gene was expressed under the flower-specific promotor of the *APETALA2* gene (also from *Arabidopsis*). This was shown to affect floral development, producing enlarged inflorescences and flower meristems (Li et al. 2010). An interesting result was obtained in a study where an Agrobacterial *IPT* gene was expressed under its native promotor in tobacco plants: strong IPT expression was observed in the leaves and stems of the transgenic plants but not in the roots. This ectopic expression delayed senescence without affecting root growth (Ma and Liu 2009). In lupin plants, IPT overexpression under the flower-specific tobacco promotor P_{TP12} enhanced yield with respect to seed number (Atkins et al. 2011).

An alternative approach to modulating CK levels in plants is based on silencing genes involved in their biosynthesis. The inactivation of up to three of the seven Arabidopsis *IPT* genes (Miyawaki et al. 2004) had negligible phenotypic effects compared to the pronounced consequences of IPT overexpression (Miyawaki et al. 2006), illustrating the high levels of functional redundancy within this gene family. However, the quadruple mutant *ipt1,3,5,7* had severely reduced CK levels and exhibited the typical phenotypic traits of cytokinin-deficient plants, including retarded shoot growth and enhanced root growth (Miyawaki et al. 2006), as well as increased tolerance of salt stress (Nishiyama et al. 2012). Analyses of the radial stem growth in *ipt* mutants revealed that CKs are the central regulators of cambial activity (Matsumoto-Kitano et al. 2008). Interestingly, chlorosis was observed in *Arabidopsis* lines with mutations in the genes encoding specific tRNA-dependent

IPT isoforms (AtIPT2 and 9; Miyawaki et al. 2004), including the single *tRNA:ipt9* mutant. In experiments using a single mutant, *Atipt3*, Igarashi et al. (2009) showed that CKs regulate the communication between reproductive and vegetative organs and thereby confirmed that CKs play a role in regulating reproductivity (Sa et al. 2002). These studies on the modulation of endogenous CK levels via the silencing of *IPT* genes have provided valuable insights into the mechanisms of CK biosynthesis. In addition, the loss-of-function mutants that were developed in these studies could potentially have applications in biotechnology if expressed under appropriate tissue-specific promotors.

Despite the diverse uses of *IPT*-containing constructs in the modulation of endogenous CK levels, the details of the CK biosynthetic pathways are still poorly understood. In particular, the regulatory mechanisms that link the biosynthesis of cytokinins and isoprenoids remain obscure, the substrate specificities of individual members of the IPT enzyme families have not yet been determined, and virtually nothing is known about the biosynthesis of aromatic cytokinins. In this context, it is interesting that a recent study demonstrated that changes in the farnesylation of IPT can have profound effects on its subcellular localization and catalytic activity (Galichet et al. 2008).

2.2 *Modulation of Cytokinin Levels Through Control of Metabolism*

2.2.1 Activation

The activation of cytokinin molecules represents the final step in cytokinin biosynthesis, and the first discovery of an enzyme that catalyzes this reaction was recently reported. Phosphoribohydrolase (named Lonely Guy, LOG, based on the rice *log* mutant flower phenotype that often contained only one stamen but no pistil) cleaves ribose 5′-monophosphates from CK nucleotides to release the corresponding active free CK bases (Kurakawa et al. 2007; Kuroha et al. 2009). After the first description in rice LOG function was investigated in *Arabidopsis*, which was found to contain nine LOG isoforms (Kuroha et al. 2009). The Arabidopsis studies focused on a line in which AtLOG was overexpressed under the control of a dexamethasone-inducible promotor. AtLOG overexpression in these plants caused significant changes in their CK distribution. However, the phenotypic consequences of AtLOG overexpression were generally less pronounced than those of IPT overexpression in the same species (see above). The overall phenotype of the AtLOG-overexpressing mutants was consistent with enhanced cell division: compared to wild-type plants, they exhibited increased cell counts in their vascular tissue, produced semidwarf shoots with increased numbers of axillary stems, had elevated chlorophyll in their leaves, exhibited retarded senescence when kept in darkness, and produced fewer lateral roots. Tokunaga and coworkers (2012)

recently prepared *Arabidopsis* lines with single and multiple *log* loss-of-function mutations. No dramatic change in phenotype occurred in single *log* mutants, but the septuple *log* mutants exhibited severely retarded root and shoot growth, with defects in the maintenance of the apical meristems (Tokunaga et al. 2012). The observed phenotypes were partially similar to those of AtCKX overexpressors (Werner et al. 2003) and multiple AtIPT loss-of-function mutants (Miyawaki et al. 2006; Kuroha et al. 2009).

The only *log* mutants that have been characterized in rice are loss-of-function mutants. These mutants exhibited similar phenotypic changes to those observed in their *Arabidopsis* counterparts. Specifically, the mutants exhibited premature termination of shoot meristem growth that resulted in the formation of a smaller panicle with a reduced number of branches compared to the wild-type, along with increased adventitious root formation (Kurakawa et al. 2007).

Overall, the results obtained in this area indicate that LOG plays a key role in CK biosynthesis and is also pivotal in the regulation of cytokinin activity during normal growth and development.

2.2.2 Inactivation

Cytokinins are inactivated through conjugation to mainly a sugar moiety catalyzed by cytokinin UDP-glycosyltransferase (UGT; EC 2.4.1). Inactivation can occur through either O- and N-conjugation. O-conjugated species can be reactivated by the action of β-glycosidase, and so CK O-glycosides have been tentatively identified as CK storage forms. The strategy to modulate the CK status of plants by changing the glycosylation of endogenous cytokinins was first achieved using the cytokinin UDP-glycosyltransferase gene *ZOG1* from *Phaseolus lunatus* under the control of the 35S promotor in tobacco plants (Mok et al. 2000). Several subsequent investigations demonstrated that changes in CK glycosylation can cause diverse phenotypic changes under different conditions (Martin et al. 2001a; Polanská et al. 2007; Havlová et al. 2008). In one notable case, Ubi:ZOG1 overexpressing tobacco plants were analyzed (Pineda-Rodo et al. 2008). In general, these plants had similar phenotypes to cytokinin-deficient plants, suggesting that cytokinin glycosides play important roles in plant development and especially in sex-specific floral development. Two maize analogues of the *ZOG1* gene have been isolated: *cisZOG1* (Martin et al. 2001b) and *cisZOG2* (Veach et al. 2003). Surprisingly, it was shown that the substrate for these maize enzymes is *cis*-zeatin, whereas the substrates of ZOG1 are *trans*- and *cis*-zeatin riboside. While the expression of both maize genes was analyzed in various tissues, no experiments involving the manipulation of their expression to modulate endogenous CK levels have yet been reported.

In *Arabidopsis*, five cytokinin glycosyltransferase genes were identified from 105 active UDP-glycosyltransferase (UGT) genes: isoforms 76C1, 76C2, 73C1, 73C5, and 85A1 (Hou et al. 2004). Mutant lines that overexpress these five cytokinin UGTs were prepared, and the 76C1 isoform was demonstrated to be

specific for cytokinins in vivo. However, 76C1 overexpression did not cause any discernible changes in phenotype or CK levels (relative to wild-type plants), and no increase in CK glycosyltransferase activity was detected in the 76C1-overexpressing mutant under normal conditions. The only evidence of any change in enzyme activity was an increase in the level of *trans*-zeatin 7-glucoside after treatment with high levels of exogenous *trans*-zeatin (Hou et al. 2004). In related studies, some *Arabidopsis* UGT76C2 gain- and loss-of-function mutants have recently been characterized (Wang et al. 2011). These mutants exhibited altered CK levels that were consistent with their genetic changes. However, there were no clear phenotypical changes associated with the mutations under normal conditions other than that the seeds of the *ugt76c2* mutant were somewhat smaller than those of wild-type plants. The UGT76C2oe and *ugt76c2* mutants were further analyzed to determine their phenotypes, CK contents, gene expression patterns, and chlorophyll contents following treatment with BA and when grown in darkness. The two mutant lines exhibited opposing behavior in these experiments, indicating that UGT76C2 affects CK activity by altering the extent of their *N*-glucosylation and thereby regulates plant responses in vivo (Wang et al. 2011).

In an analogous approach, Kudo and coworkers (2012) used a sequence homology approach to identify six putative UGTs in rice, three of which proved to be active. The three active UGTs were ectopically overexpressed in rice, revealing that overexpression of the *cZOGT1* and *cZOGT2* genes causes short-shoot phenotypes, delayed leaf senescence, and a decrease in the crown root number. Interestingly, *cZOGT3* overexpression did not cause any phenotypic changes (Kudo et al. 2012).

Based on their successful use of sequence similarity searches to identify UGT genes in maize, Meek et al. (2008) used this approach to identify five bean and soybean UGTs along with two UGT genes in rice and one in tomato plants. However, none of the corresponding proteins were found to be active on CKs. However, it should be noted that they were only tested for the capability to glycosylate *cis*- and *trans*-zeatin; in previous investigations into the activity of other potential cytokinin UGT enzymes, substrates such as dihydrozeatin N^6-isopentenyladenine, BA, kinetin, and *m*-topolin were also examined (Martin et al. 1999; Hou et al. 2004; Mok et al. 2005).

While much remains to be learned about the functions of CK glycosides in plants, the results discussed above strongly suggest that techniques for manipulating their abundance and distribution in plants are likely to provide important new insights into their role in CK homeostasis.

2.2.3 Reactivation

The reverse of CK glycosylation is the hydrolysis of CK glycosides to yield the corresponding free CKs. This reaction is catalyzed by CK β-glucosidases (EC 3.2.1.21; Brzobohatý et al. 1993). Most of the known cytokinin β-glucosidases possess broad substrate specificity. Only a few β-glucosidases with specificity for either O- or N3-cytokinin glucosides have been described

(Brzobohatý et al. 1993), and one β-glucosidase with a strict specificity for zeatin-O-glucoside was identified in *Brassica napus* (Falk and Rask 1995). It was quickly revealed that CK β-glucosidases play an essential role in early plant growth and development due to their ability to convert *trans*-zeatin-O-glucoside and dihydrozeatin-O-glucoside into their active free counterparts in developing embryos (Smith and van Staden 1978) and coleoptiles (Brzobohatý et al. 1993). Most of our current understanding of CK reactivation derives from experiments conducted on the maize β-glucosidase Zmp-60.1, which was first isolated from maize coleoptiles (Campos et al. 1992; Esen 1992). In maize, this enzyme is most active in the coleoptiles, followed by the roots and pistils (Esen 1993; Nikus and Jonsson 1999). It was subsequently shown to be localized in the plastids/chloroplast (Esen and Stetler 1993; Kristoffersen et al. 2000). To date, Zm-p60.1 is the only β-glycosidase that has been introduced into transgenic plants. Tobacco plants that ectopically overexpress Zm-p60.1 had CK profiles that differed from those of wild-type plants but did not exhibit any morphological changes. However, when these mutants were treated with exogenous tZ, they accumulated higher levels of active CK metabolites than wild-type plants (Kiran et al. 2006). This was accompanied by the inhibition of root elongation. In subsequent investigations, a vacuolar variant of Zm-p60.1 was generated and overexpressed in tobacco (Kiran et al. 2012). As was the case for native Zm-p60.1 overexpressors, plants from this mutant line did not exhibit any phenotypic deviation under normal conditions. However, seedlings supplied with exogenous tZ increased their fresh weight more rapidly than wild-type plants, suggesting that they were more responsive to exogenous tZ. The Zm-p60.1-overexpressing transgenic line was subsequently crossed with a tobacco line that overexpresses ZOG1 from *Phaseolus* (Martin et al. 2001a). The resulting plants had a CK status and phenotype that closely resembled those of the wild-type. This result indirectly suggests that the vacuole acts as a storage organelle for CK glycosides (Kiran et al. 2012). Zm-p60.1 is clearly a powerful tool for manipulating endogenous CK levels in plants. However, additional results in other plant species will be required in order to obtain a full understanding of CK reactivation and its role in CK metabolism.

2.2.4 Degradation

Another method of manipulating endogenous CK levels involves engineering their irreversible degradation. The enzyme that is responsible for cleaving cytokinin side chains is cytokinin dehydrogenase (CKX; EC 1.5.99.12). CKXs have been characterized in many plant species, including *Arabidopsis* (Bilyeu et al. 2001; Werner et al. 2001), rice (Ashikari et al. 2005), and maize (Houba-Hérin et al. 1999; Massonneau et al. 2004; Morris et al. 1999; Šmehilová et al. 2009). Moreover, it has been demonstrated that they are very potent modulators of CK status, prompting the creation of several transgenic plant lines that carry CKX genes. The first studies in this area involved lines that constitutively overexpress CKX, including a tobacco line that overexpresses *Arabidopsis* CKX isoforms (Werner

et al. 2001; Galuszka et al. 2007; Mýtinová et al. 2011), barley lines that overexpress a homologous CKX gene (Galuszka et al. 2004; Mrízová et al. 2013), or a heterologous CKX gene from maize (Mrízová et al. 2013), an *Arabidopsis* line that overexpresses an orchid CKX (Yang et al. 2003), and a tobacco line that overexpresses CKX from cotton (Zeng et al. 2012). All of these lines exhibit CK deficiencies that cause phenotypic changes which are consistent with the role of CKs as positive regulators of shoot growth and negative regulators of root growth. In keeping with the results obtained using the multiple *ipt* knockout lines discussed above, CKX-overexpressing *Arabidopsis* plants exhibited increased tolerance to drought and salinity stress (Nishiyama et al. 2011).

On the other hand, a reduction in OsCKX2 expression caused CK accumulation in rice inflorescence meristems. This caused the formation of an increased number of reproductive organs, leading to enhanced grain production in the rice cultivar Habataki (Ashikari et al. 2005). The effects of raising endogenous CK levels by silencing *CKXs* have also been investigated in barley (Zalewski et al. 2010), *Arabidopsis* (Bartrina et al. 2011), and tobacco (Zeng et al. 2012). In *Arabidopsis*, the simultaneous silencing of the genes *CKX3* and *CKX5* resulted in the formation of more flowers/siliques per plant and more seeds per silique, causing a 55 % increase in seed yield relative to wild-type plants (Bartrina et al. 2011). Similarly, the grain number was increased by around 30 % in a rice *Osckx2* loss-of-function mutant (Ashikari et al. 2005) and by around 20 % in a barley *Hvckx1* loss-of-function mutant (Mrízová et al. 2013). A positive correlation between TaCKX2.1 and TaCKX2.2 expression and grain number per spike has been demonstrated in wheat (Zhang et al. 2011). The silencing of *CKX* genes thus seems to be a promising method for increasing wheat production. However, it will be important to carefully select an appropriate *CKX* isoform (or combination of isoforms) to tailor this approach since there are more than ten currently uncharacterized *CKX* genes in the wheat genome.

Some groups have explored the scope for manipulating endogenous CK levels by means of tissue-specific CKX overexpression. ZmCKX1 overexpression under the control of pollen- and anther-specific promotors in maize led to male sterility (Huang et al. 2003). In another study, root-specific promotors were used to control the expression of *CKX* genes in order to create root-targeted CK deficiencies. This strategy was used to produce transgenic tobacco and *Arabidopsis* plants that overexpress AtCKX1 or AtCKX3 under the root-specific promotors PYK10 and WRKY6, respectively (Werner et al. 2010). The transgenic plants produced more extensive root systems than wild-type equivalents without any loss of shoot biomass, were more tolerant to drought and heat, and exhibited high levels of leaf mineral enrichment (Werner et al. 2010; Macková et al. 2013).

2.3 *Modulation of the Cytokinin Status Through Control of Signaling*

CK signaling is mediated by a two-component signal transduction system (TCS) that has two main stages: the perception of the hormone molecule by a sensor histidine kinase (HK) receptor and signal transduction from the receptor to the response regulators (RRs) via histidine phosphotransfer proteins (HPts). The response regulators are divided into two groups (type A and type B) based on their structures (Imamura et al. 1999; Mizuno 2004). RRs of both classes contain a receiver domain and an output domain. The output domains of the B-type RRs contain an important DNA-binding domain that is not found in the A-type proteins. This domain enables the type-B RRs to serve as transcription factors that promote the expression of genes involved in the regulation of CK signaling. Type-A RRs are involved in phosphorylation-dependent interactions (To et al. 2007) and may compete with type-B RRs for phosphorylation by the HPt proteins (Kieber and Schaller 2010) to suppress CK signal transduction. Together, the two RR types form a self-regulating negative feedback loop in CK signaling (Heyl and Schmülling 2003).

2.3.1 Perception

Three functional CK receptors have been characterized in *Arabidopsis*: CRE1/AHK4, AHK2, and AHK3 (Inoue et al. 2001; Suzuki et al. 2001; Yamada et al. 2001). Various single and multiple gain- and loss-of-function mutants were prepared to study the effects of modulating CK sensing on plant growth and development. The *ahk4 Arabidopsis* loss-of-function mutant has a CK-resistant phenotype in that CKs do not suppress its root growth, greening, or formation of shoots from calli (Ueguchi et al. 2001). The RNAi loss-of-function *ahk3* mutant was also found to be CK insensitive, exhibiting a dwarfed phenotype, accelerated germination, larger seeds, and increased root branching relative to wild-type plants, together with enhanced drought, salt, and cold stress tolerance (Kim et al. 2006). Interestingly, constitutive AHK3 overexpression in transgenic plants had no significant effects on leaf senescence (Kim et al. 2006). Studies on higher-order loss-of-function receptor mutants revealed that these genes contribute to the regulation of shoot growth, leaf senescence, seed size, germination, root development, and CK metabolism (Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006). Detailed phenotypic analyses revealed that the individual AHK receptors have partially redundant but differentiated functions and that the AHK2 and AHK3 receptors have particularly prominent roles, with opposing regulatory functions in roots and shoots (Riefler et al. 2006). Cytokinin-insensitive transgenic plants were shown to have enhanced tolerance to cold, drought, and salinity (Tran et al. 2007; Ren et al. 2009; Jeon et al. 2010). It was shown that the suppression of CK perception may be a useful tool to increase plant resistance to biotic stresses. CK

receptor mutants as well as plants with induced CK deficiency were found to be resistant to the development of clubroot disease caused by *Plasmodiophora brassicae* (Siemens et al. 2006; Galfe et al. 2009). Similarly, an *ahk3ahk4* mutant and a related triple knockout mutant were found to not respond to the gall-forming pathogen *Rhodococcus fascians*, demonstrating that AHK3 and AHK4 are necessary for symptom development (Pertry et al. 2010). In legumes (*Medicago*, *Lotus*), analyses of loss-of-function and gain-of-function mutations to CK receptors have revealed that CK perception plays a central role in nodule formation (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Tirichine et al. 2007).

2.3.2 Signal Transduction

Another way of manipulating CK sensitivity in plants would be to target the elements of the two-component signaling system that lie downstream of the receptors – the HPts and RRs. Transgenic plants with deficiencies in CK signal transduction and responsiveness due to higher-order mutations in the genes encoding His phosphotransfer proteins (*ahp1,2,3,4,5*; Hutchison et al. 2006) and type-B response regulators (*arr1,10,12*; Mason et al. 2005) have phenotypes that resemble those caused by genetic defects in CK perception. Similar phenotypes were also observed for plants that express 35S:ARR1-SRDX, a dominant repressor of response regulator ARR1 (Heyl et al. 2008). Experiments using the triple loss-of-function *arr1,10,12* mutant revealed that the three affected type-B RRs play a central role in regulating CK activity during vegetative development (Yokoyama et al. 2007; Ishida et al. 2008; Argyros et al. 2008). The type-B protein ARR2 was shown to promote plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis* (Choi et al. 2010). Other type-B RRs were identified as important regulators of plant growth, fertility, and the general development of the plant body (Taniguchi et al. 2007; Horák et al. 2008). Some type-A ARR genes were found to be expressed in response to cold, and *arr7* mutants showed increased freezing tolerance (Jeon et al. 2010). Studies on plants with multiple knockouts affecting both groups of RRs demonstrated that there is significant functional overlap between the individual members of each group and confirmed the roles of type-A and type-B RRs as negative and positive regulators of CK signaling, respectively (To et al. 2004; Mason et al. 2005).

Cytokinin response factors (CRFs) are members of the *Arabidopsis* AP2 gene family that are transcriptionally up-regulated by CKs and have been shown to work in tandem with the B-type ARRs (Rashotte et al. 2006). They rapidly accumulate in the nucleus in response to CK AHP phosphorylation. Experiments with *Arabidopsis crf* loss-of-function mutants revealed that this gene regulates the development of embryos, cotyledons, and leaves but does not affect root elongation (Rashotte et al. 2006). Plants with multiple *crf* mutations have small cotyledons like *ahk* multiple mutants. Furthermore, multiple mutations in CRF5 and CRF6 led to embryo abortion, resulting in a decreased number of seeds per silique (Rashotte et al. 2006). CRFs were subsequently found in other plant species (Rashotte and

Goertzen 2010) and have been further characterized in some species, such as tomato (Shi et al. 2012).

Genome-wide experiments confirmed that TCS proteins interact with various proteins that are not directly involved in CK signaling, which suggests that there is some crosstalk between the CK signaling system and other signaling pathways or tools for regulating protein function (Dortay et al. 2008). Numerous novel CK-regulated genes have recently been discovered, providing new insights into the translational consequences of CK signaling, which are also affected by crosstalk with other hormones and different environmental cues (Brenner and Schmülling 2012). These findings further emphasize the complexity and importance of TCS signaling and expose new approaches to studying the diverse functions of CKs in plants.

2.4 Modulation of Cytokinin Status Through Control of Transcription Factors

The most widely used methods of modulating endogenous CK levels in plants are based on modifying the activity of CK-metabolizing enzymes. One alternative to this strategy is to alter the CK status of the plant by targeting the various CK-regulated transcription factors.

Ectopic expression of different CK-regulated transcription factor genes in *Arabidopsis* caused phenotype changes similar to those observed in plants with elevated or depressed CK contents (Köllmer et al. 2011). Compared to wild-type plants, ectopic GATA22 overexpressors were found to have shorter roots with less branching, whereas HAT22 overexpressing seedlings had reduced chlorophyll levels and an earlier onset of leaf senescence. Plants overexpressing HAT4 suffered from defective inflorescence stem development that was accompanied by a decrease in root growth and branching. Conversely, *hat4* loss-of-function mutants had more extensive root systems than wild-type plants. Multiple phenotypic changes were observed in 35S:bHLH64-overexpressing plants, including enlarged larger rosettes with thicker and longer hypocotyls and reduced chlorophyll contents (Köllmer et al. 2011). Although these plants exhibited phenotypes that were consistent with changes in CK levels, their CK contents were not determined. The proposed role of this gene in the cross-regulation of CK signaling and other metabolic pathways is therefore not supported by the relevant experimental evidence at present. AtMYB2 is a transcription factor that has been shown to influence the onset of plant senescence (Guo and Gan 2011). It is expressed during the late stages of development in basal internode areas, where it acts as a suppressor of CK production in order to ensure proper plant development (Guo and Gan 2011). There is also evidence that AtMYB2 is probably a negative regulator of some IPTs because significant upregulation of these genes was observed in an *atmyb2* loss-of-function mutant, along with a bushy phenotype (Guo and Gan 2011).

2.5 Perspectives and the Identification of Additional Cytokinin Metabolic Enzymes and Their Genes

The preceding discussion clearly shows that there are many ways of manipulating endogenous CK levels in specific plant tissues. CKs work in concert with other plant hormones to control plant development and the responses to various stimuli. Another way in which this can be achieved is by manipulating the expression of genes that have indirect effects on CK homeostasis rather than directly affecting CK metabolism. It has been proposed that CKs are transported across the plasma membrane by at least two types of transporters (reviewed in Kudo et al. 2010; Muraro et al. 2011) – purine permeases (PUP; Bürkle et al. 2003) and equilibrative nucleoside transporters (ENT; Gillissen et al. 2000; Sun et al. 2005; Hirose et al. 2008). However, there is a lack of data on their roles in CK transport, and so further studies in this area are definitely needed.

Numerous enzymes that have been implicated in the initiation and progression of senescence appear to be sensitive to CK signaling. In plants, nutrients in senescing organs are remobilized to sustain other parts of the plant. This process is accomplished via a source-sink-type relationship that is mediated by enzymes belonging to an apoplastic phloem-unloading pathway. One of the enzymes that regulate hexose levels, an extracellular invertase, was found to be co-induced by CKs (Lara and others 2004). This invertase was shown to be essential for delaying CK-driven senescence, indicating that it plays a vital role in the molecular mechanism that underpins the initiation of senescence (Lara and others 2004). CKX activity was believed to play a role in this process because senescence is accompanied by significant reductions in CK levels. It is therefore possible to stimulate the phloem unloading of assimilates during storage organ maturation by manipulating endogenous CK levels in regulatory tissues (Galuszka et al. 2005).

While our current understanding of CK metabolism is relatively detailed, some issues remain to be resolved. The isoprenoid CKs can be synthesized via an iPMP-dependent pathway in which the first step is catalyzed by IPT and subsequent steps involve the cytochrome P450 monooxygenase enzymes CYP735A1 and CYP735A2 (EC 1.14.13; Takei et al. 2004), which hydroxylate the iP chain to form tZ. An iPMP-independent pathway has also been proposed to contribute to the active tZ pool in Arabidopsis (Astot et al. 2000), and it has been shown that the endogenous *cis*-zeatin in *Arabidopsis* is derived from tRNA (Miyawaki et al. 2006). However, this pathway does not seem to be capable of forming the high levels of cZ that are found in plant species where cZ-type cytokinins are prevalent (Gajdošová et al. 2011). Another potential route for cZ synthesis involves its formation from tZ, which could be catalyzed by a cis-trans isomerase (Bassil et al. 1993). However, the activity of this enzyme is low, and there is no experimental support for its proposed role. Another enzyme that uses zeatin as a substrate is zeatin reductase (ZR, EC 1.3.1.69), which converts tZ to dihydrozeatin (Martin et al. 1989). As with the zeatin cis-trans isomerase, the gene for zeatin reductase has yet to be cloned, and

further research will be needed to determine the potential of these enzymes as tools for adjusting the CK status of plants.

CKs are known to be inactivated by specific glycosyltransferases. However, only for few of these, the enzymatic activities have been characterized or the genes isolated. Efforts to identify potential CK glycosyltransferase genes in various plant species using approaches based on molecular biology have revealed that structure-based homology searching is not a particularly effective strategy for this purpose. Experiments with loss-of-function mutants have indicated that there may be other isoforms of these enzymes in plants that could have different specificities towards various CKs. In addition to the inactivation pathway, there is likely to be a complementary reactivation pathway involving enzymes that deglycosylate inactivated cytokinins. However, the only such enzyme that has been characterized and cloned to date is a β-glucosidase from maize. There is therefore a need for further investigations into these deactivation and reactivation pathways.

Finally, several as-yet unidentified natural CK-like compounds that may have similar functions to known CKs have recently been detected in various plant species (unpublished data). The physiological significance of these compounds remains to be determined, as does the nature of their associated metabolic pathways.

3 Chemical Tools to Modulate Cytokinin Status

Small molecule design programs and chemical library screening campaigns have revealed a number of bioactive small molecules that affect diverse processes in plants, including growth, development, reproduction, and immunity, among others (Kumari and van der Hoorn 2011). Synthetic compounds that affect plant responses to hormones such as auxin, gibberellin, and ethylene are known and widely used in research and practical agriculture. Despite the substantial progress that has been made with respect to the structures of the proteins that are involved in CK perception and metabolism over the last decade, only a few substances with applications in CK research and the potential for use in practical agriculture have been discovered.

Diverse adenylated and non-adenylated CK analogues that inhibit the physiological effects of CKs were synthesized in the 1970s in search for CK-antagonizing compounds (Iwamura 1978; Iwamura et al. 1979). However, the identification of CK receptors in *Arabidopsis* made it possible to investigate the mode of action for these compounds directly; investigations of this sort revealed that these compounds inhibit cell cycle progression (Spíchal et al. 2007) rather than functioning as competitive inhibitors of CK receptors (Spíchal et al. 2007; Arata et al. 2010). The first CK antagonists to be developed in a targeted way were derivatives of the aromatic CK BA: N^6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) and N^6-(2,5-dihydroxybenzylamino)purine (LGR-991). Both of these compounds are competitive inhibitors of CK perception that block the CK-binding sites of the CK receptors CRE1/AHK4 and AHK3 (Spíchal et al. 2009; Nisler et al. 2010). PI-55

suppresses the effects of CKs in various bioassays, accelerating the germination of Arabidopsis seeds and promoting root growth and the formation of lateral roots (Spíchal et al. 2009). Treatment with PI-55 had positive effects on seedling shoot and root growth as well as the fresh weight of treated seedlings of various plant species grown in the presence of Cd. This suggests that the modulation of cytokinin levels via treatment with exogenous inhibitors of cytokinin perception may be useful for protecting plants against the adverse effects of high Cd levels (Gemrotová et al. 2013).

A new specific antagonist of the Arabidopsis CK receptor CRE1/AHK4, N^6-(benzyloxymethyl)adenosine (BOMA), was recently identified in a study on synthetic derivatives of N^6-adenosine (Krivosheev et al. 2012). Another recently discovered noncompetitive CK antagonist is the phenylquinazoline derivative S-4893, which targets to the same receptor and strongly promotes root growth in *Arabidopsis* and rice (Arata et al. 2010). The crystal structures of various complexes formed between the CRE1/AHK4 sensor domain and different CK ligands have recently been published and used to rationalize the hormone-binding specificity of the receptor (Hothorn et al. 2011). In the future, it will be possible to use these structures as a basis for the design of new and potent CK agonists and antagonists.

Several synthetic urea-derived CKs that competitively inhibit CKX activity have been reported, including thidiazuron, CPPU, and CBPU (Hare and Van Staden 1994; Laloue and Fox 1989; Burch and Horgan 1989; Kopečný et al. 2010). In the most cases, their modes of binding to CKX have been clarified by structural analysis (Kopečný et al. 2010). The activity of CKX was shown to be strongly inhibited by unsaturated allenic (HA-1, HA-8) and alkyne substrate analogues (Houba-Hérin et al. 1999; Suttle and Mornet 2005; Kopečný et al. 2008). Kinetic and X-ray crystallographic studies proved that HA-1 and HA-8 behave as mechanism-based inhibitors, and it was therefore suggested that they might be useful tools for regulating CKX activity in plants (Suttle and Mornet 2005; Kopečný et al. 2008). A class of substituted 6-anilinopurines was recently identified as new and potent CKX inhibitors, and the molecular basis of their interaction with CKX was described (Zatloukal et al. 2008; Spíchal et al. unpublished results). In vivo treatment with the most effective inhibitors of this class, 2-chloro-6-(3-methoxyphenyl)aminopurine (INCYDE) and 2-fluoro-6-(3-methoxyphenyl) aminopurine (INCYDE-F), phenocopies the deregulation of CKX expression and promotes the development of yield-forming traits under field conditions (Spíchal et al., unpublished results). It was recently shown that INCYDE protects plants against the negative effects of Cd exposure in a similar way to PI-55, although most probably by a different mechanism (Gemrotová et al. 2013). Moreover, the stabilization of CK levels due to treatment with INCYDE confers enhanced resistance to the pathogen *Verticillium longisporum* in *Arabidopsis* (Reusche et al. 2013).

The deregulation of CK levels via the inhibition of CK biosynthesis might be another useful way of manipulating these important plant growth regulators. However, while the structure of the bacterial IPT protein has been determined and could be used as a template for the structure-based design of inhibitors that modulate plant

IPT activity (Sugawara et al. 2008), a chemical inhibitor of IPT with appropriate biological function has not been reported to date. An in silico screen of potential CK biosynthesis inhibitors was recently performed using genome-wide gene expression profiles and predictions of target sites based on global CK accumulation profile analyses (Sasaki et al. 2013). This investigation revealed that an inhibitor of cytochrome P450 monooxygenase, uniconazole (known already for a long time as inhibitor of gibberellin synthesis) targets CYP735As and thereby prevents tZ biosynthesis in *Arabidopsis* (Sasaki et al. 2013).

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The Life and Death Signalling Underlying Cell Fate Determination During Somatic Embryogenesis

Andrei Smertenko and Peter Bozhkov

Abstract Somatic embryogenesis (SE) is a sequence of stereotypical morphological transformations, which results in differentiation of cells into a plant body bypassing the fusion of gametes. As such, it represents a very powerful tool in biotechnology to propagate species with long reproductive cycles or low seed set and the production of genetically modified plants with improved traits. The initiation of SE can be divided into five major stages: (i) perception of extracellular signals or stress stimuli, (ii) transduction of the extracellular signal through the cytoplasm into the nucleus, (iii) induction of gene transcription required for embryogenesis, (iv) reorganisation of cytoplasm and (v) onset of embryonic development. The further embryonic development during SE resembles its zygotic counterpart and begins with the establishment of apical-basal asymmetry. The apical domain, the embryo proper, proliferates and eventually gives rise to the plantlet, while the basal part, the embryo suspensor, becomes a subject of terminal differentiation and gradually degrades via vacuolar programmed cell death (PCD). This PCD is essential for normal development of the apical domain. Some signalling events in the apical and basal domains share homologous components. Here, we describe our current knowledge on the control of life and death processes during SE.

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1 Introduction

SE was described by Williams and Maheswaran (1986) as "the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes". The lack of gamete fusion in SE implies high genetic homogeneity of all derived in this way plants. SE is a common process in nature (Raghavan 1976; Tisserat, et al. 1979; Vasil and Vasil 1980) and can occur as early in plant development as during embryogenesis. A group of cells in embryos of any developmental stage or even plantlets can give rise to new embryos leading to polyembryony or adventive embryony.

In mature plants of some species (e.g. *Kalanchoe*), dedifferentiation of somatic cells can also lead to embryo formation. Propagation through SE allows formation of multiple genetically identical embryos under favourable conditions avoiding the need to wait for the following reproductive season, and this reproductive acceleration bears vast evolutionary importance.

SE can commonly be initiated in vitro from non-differentiated cells which still posses "embryogenic potential" such as explants from microspores, ovules, embryos and seedlings (Williams and Maheswaran 1986). Cultivation of these explants on media containing an appropriate balance of plant growth regulators (PGRs) can induce proliferation of embryo-forming (embryogenic) callus cultures. The formation of embryos from "dedifferentiated" cells in vitro is called indirect SE (see chapter by Opatrný in this volume for the "dedifferentiation" of callus cells). Embryo development can also be initiated in vitro directly from explant cells without passage through callus formation phase, by a process called direct SE.

The production of physiologically normal and genetically homogenous plants through SE has multiple applications for biotechnology, from propagation of species, which take long time to reach the flowering stage or yield insufficient numbers of seeds, to production of genetically modified cultivars with engineered properties. The translational value drives research into characterisation of molecular pathways controlling SE and refined approaches to increase yield and quality. However, the conditions for SE are species specific and require optimisation throughout all its stages from the induction of SE until embryo maturation and germination. New genetic data from model species including thale cress (*Arabidopsis thaliana*) and Norway spruce (*Picea abies*) have significantly advanced our understanding of the molecular mechanisms regulating embryogenesis and have aided in the improvement of existing technologies and development of approaches for induction of SE in recalcitrant species.

Apart from its industrial importance, SE represents an important set of paradigms to study early signalling and morphogenetic processes in plant embryogenesis. As zygotic embryogenesis takes place deep within maternal tissues and the embryos are minute, the molecular, biochemical and cellular analyses of the developmental process are experimentally difficult. In contrast, SE yields macroscopic amounts of material at specific developmental stages for biochemical

studies. These exposed somatic embryos are also suitable for cell biology and live-cell imaging studies.

2 Biology of SE

While in nature SE often originates from cells still possessing "embryogenic potential" (e.g. polyembryony), the first important step of indirect SE is reprogramming of the cell fate which is accompanied by changes in the gene transcription (see below; see also chapter by Opatrný in the current volume). The first system of indirect SE was established in *Daucus carota* (Steward et al. 1958; Reinert 1958). The observations made in this system demonstrated that while callus cells were assumed to be totipotent and their ability to produce embryos should not be determined by the tissue of origin, in reality, embryogenic cultures from various species are composed of morphologically distinct cell types. One type, often referred to as proembryogenic masses, is composed of small, actively proliferating cells with dense cytoplasm, large nuclei, small vacuoles, smooth surface and high metabolic activity. These cells are accompanied by non-embryogenic cells, which are bigger, rougher, with larger vacuoles and therefore more translucent.

Somatic embryos can originate from a single cell (unicellular pathway; Haccius 1978) or from a group of two or more associated cells (multicellular pathway; Raghavan 1976). Both pathways appear to be redundant and can act in the same species in case of both direct (Maheswaran and Williams 1985; Trigiano et al. 1989) and indirect (Toonen et al. 1994; Fernandez et al. 2000; Somleva et al. 2000) SE.

2.1 SE Resembles Zygotic Pathway

The morphology of somatic embryo development is remarkably similar to zygotic embryogenesis (Fig. 1; von Arnold et al. 2002). Firstly, zygotic embryogenesis starts from a single cell followed by formation of globular embryo containing a defined number of cells. SE starts from a single cell or a group of cells and attains globular structure containing variable number of cells. How a group of cells initiates embryo formation is not clear, but considering our knowledge about zygotic embryogenesis, an asymmetric distribution of auxin is probably established (de Smet et al. 2010). In some instances, an early step in the initiation of SE communication with the surrounding cells is interrupted by increased cell wall thickness and blockage or dismantling of the plasmodesmata (Button et al. 1974). Inside the isolated single cell or cell cluster, the chain of signalling processes initiates cell divisions in parallel with the establishment of embryo polarity. In many cases, however, the embryo-forming group of cells retain plasmodesmata

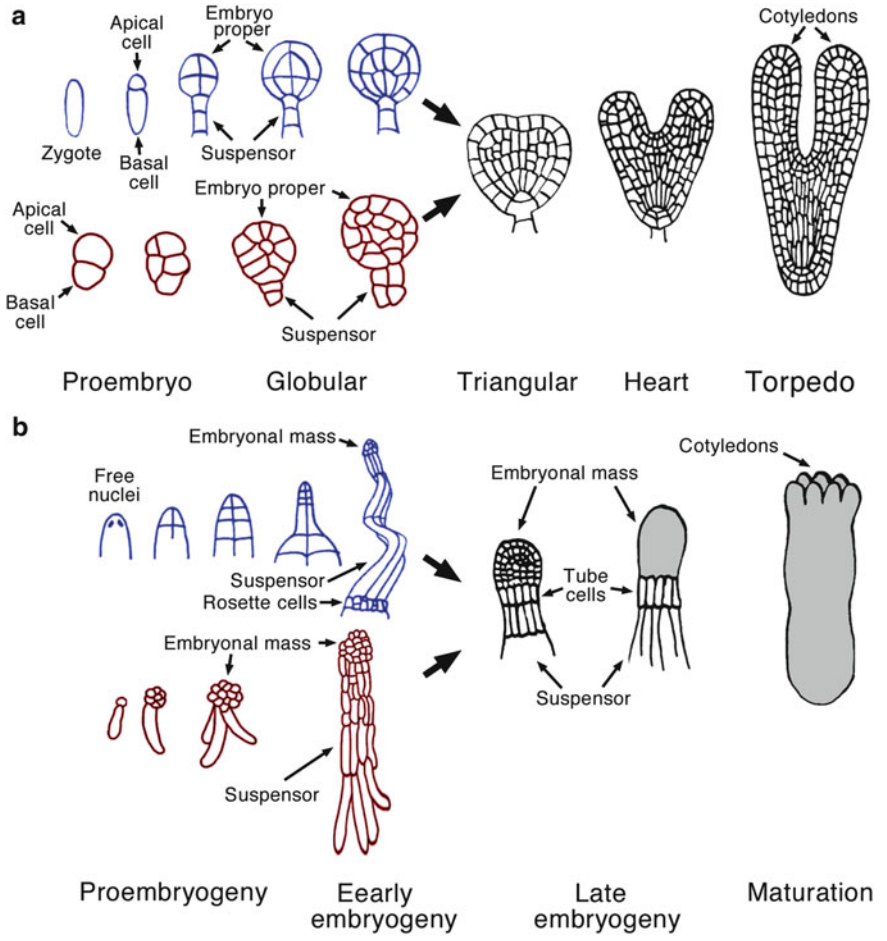

Fig. 1 Comparison of somatic and zygotic embryogenesis pathways in angiosperms (**a**) and gymnosperms (**b**)

Stereotypical morphological stages of zygotic and somatic embryogenesis are shown in *blue* and *red*, respectively. The stages of both pathways with common morphology are shown in *black*. The embryo structures are not drawn to scale

In angiosperms, both zygotic (shown for *Arabidopsis*) and somatic (shown for carrot; Street and Withers 1974) embryogenesis start with asymmetric divisions producing cells with different fates. The apical cell proliferates, eventually giving raise to the new plant, while the basal cell forms a terminally differentiated suspensor which disappears by the heart stage

The zygote in gymnosperms contains free nuclei, then undergoes cellularisation and divisions and gradually forms suspensors during the early embryogenic stage. During the somatic pathway, the polar structures composed of proliferating cells on one pole of the embryo and large vacuolated non-proliferating cells on the opposite pole appear already during proembryogeny (Fílonova et al. 2000b). The vacuolated cells give raise to the suspensor, which, similarly to the angiosperms, demises during maturation. Zygotic embryos in some genera contain small rosette cells at the base of suspensor, which can divide and form aberrant embryos (rosette polyembryony). Cleavage polyembryony, when the early embryo splits into four or more identical embryos while only a single embryo normally develops to maturity, is a common feature of multiple genera of

contacts with the neighbouring cells and do not display any apparent changes in the cell wall thickness (Williams and Maheswaran 1986).

The somatic embryo development encompasses key stages of zygotic embryogenesis characteristic of the respective taxon: heart and torpedo in case of dicotyledonous species; globular, scutellar and coleoptilar in case of monocotyledonous species; and early and late embryogeny in case of gymnosperm species (Fig. 1; Zimmerman 1993; Singh 1978). This requires establishment of two domains: apical and basal. The apical domain gives rise to shoot apical meristem, cotyledons and hypocotyl, while the basal domain originates root meristem and the root.

The embryo suspensor, a multicellular basally situated organ formed during the first divisions of zygote, plays a pivotal role in the establishment of apical-basal polarity (Zimmerman 1993; Kawashima and Goldberg 2010). The suspensor is a terminally differentiated organ subjected to gradual elimination by vacuolar PCD (Bozhkov et al. 2005a). Cell proliferation and tissue patterning events in the embryo proper must be balanced by terminal cell differentiation and death in the suspensor (see the Suspensor section below).

The mature somatic embryos resemble zygotic embryos, both morphologically and physiologically (Zimmerman 1993; von Arnold et al. 2002; Bozhkov et al. 2005a). Both exhibit apical-basal and radial polarity, possess primary shoot and root meristems and contain typical embryonic organs such as radicle, hypocotyl and cotyledons. The key genes controlling zygotic embryogenesis perform similar roles during SE (Mordhorst et al 2002). Somatic embryos accumulate similar nutrients required for subsequent germination of the seedling and target them to the same cellular compartments; however, the timing of accumulation and exact proportion between individual types of nutrients can vary (Merkle et al. 1995; Yeung 1995). In addition, somatic embryos may not require desiccation (desiccation is important for SE in *Medicago sativa* and *Picea* spp.) and skip the dormancy period. Instead, they initiate the shoot meristem and seedling growth, eventually producing morphologically normal and fertile plants.

2.2 Induction and Maintenance of SE by External Factors

Comparison of somatic and zygotic embryogenesis shows that the induction and maintenance of embryogenic potential requires exogenous factors, i.e. growth medium or maternal tissues, respectively. The subsequent progression of embryogenesis towards tissue patterning and organ establishment results from the execution of genetic programmes in the cells of globular embryos. This genetic programme results in a stereotypical sequence of morphological changes regulated

Fig. 1 (continued) gymnosperms (e.g. *Pinus*; Fílonova et al. 2002). In SE, cleavage polyembryony was reported even in species which lack this feature during zygotic embryogenesis (e.g. *Picea)*. In the latter case, the additional embryos form from the embryonal mass cells. Both zygotic and somatic pathways are shown for *P. abies*

intrinsically by conserved mechanisms with minimal or no impact from surrounding tissues or environment.

Initiation of embryogenic cell cultures is accompanied by the induction of specific sets of genes, primarily those responsible for control of the cell cycle (Zimmerman 1993; Feher et al. 2003; Yang and Zhang 2010). The core cell cycle machinery responsible for driving cell proliferation appears to be conserved between embryogenic cultures and plant meristems (Hirt et al. 1991; De Jong et al. 1993; Emons 1994).

2.2.1 The Role of Hormonal Factors

The gene expression as well as the pattern of cell divisions is regulated by plant growth regulators (PGRs) and composition of the growth medium. Synthetic auxins, in particular 2,4-dichlorophenoxyacetic acid (2,4-D), were originally used for induction and maintenance of embryogenic cell cultures. Other classes of PGRs including cytokinins (Sagare et al. 2000), abscisic acid (ABA; Senger et al. 2001), jasmonates and brassinosteroids (Jimenez 2005) were successfully used for initiation of embryogenic cell lines in different plant species. The variability in embryogenic induction can be explained by the dual role of PGRs. At supra-physiological concentrations, PGRs induce stress responses in addition to their role in signalling, and these stress responses play an essential role in reprogramming gene expression patterns (Feher et al. 2003; Karimi and Saidi 2010). In agreement with this hypothesis, abiotic stresses, such as heat stress, can induce SE even in the absence of added PGRs (Simmonds and Keller 1999; Dubas et al. 2011).

Maintenance of proliferating embryogenic cultures requires a continuous supply of exogenous PGRs auxin and cytokinin. In some instances, for example, in the alfalfa model, only a short (from several minutes and up to several hours) pulse of a relatively high concentration (100 μM) of auxin is sufficient to stimulate embryogenesis (Hirt et al. 1991).

Embryogenic cultures proliferating in the presence of auxin, with or without cytokinin, produce embryos resembling the globular stage of zygotic pathway but composed of variable number of cells. Transition to the next stages is induced by withdrawal of auxin from the medium. This leads to the onset of histogenesis and initiation of tissue and organ patterning. At the same time, the embryo starts to synthetise its own auxin (Michalczuk et al. 1992a, b). While the regulation of auxin distribution in somatic embryos remains unknown, the transition through successive stages of zygotic embryogenesis is accompanied by re-localisation of auxin efflux carrier (PIN proteins) and establishment of auxin gradients through the embryos (Fischer and Neuhaus 1996; Friml et al. 2002, 2004). The exogenously applied auxin interferes with the formation of these gradients and inhibits transition from globular to heart stage. Likewise, application of the polar auxin transport inhibitor 1-N-naphthylphthalamic acid to embryogenic cultures affects cell divisions and interferes with apical-basal patterning of somatic embryos (Fischer and Neuhaus 1996; Larsson et al. 2008).

The maturation of somatic embryos may require additional growth regulators, such as ABA. Although ABA can be produced endogenously by somatic embryos (Hatzopoulos et al 1990), the efficiency of embryo maturation is higher in the presence of exogenous ABA (von Arnold et al. 2002). In zygotic embryos, ABA induces dormancy and upregulates a set of specific genes, known as *Late Embryogenesis Abundant* (*LEA*; Dure et al. 1989). Although somatic embryos do not pass through a classical dormancy period, treatment with ABA induces several *LEA* genes including *DC8*, *DC59*, *ECP31* and *ECP40* (Zimmerman 1993), suggesting that ABA induces a semi-dormancy period in somatic embryos, which plays an important role in SE.

2.2.2 Nonhormonal Factors

Apart from hormones, pH and Ca^{2+} are important factors for the induction of SE. The optimal pH of the culture medium lies in the mild acidic range between 4.0 and 5.8 (Von Arnold et al. 2002), while alkaline media inhibit SE (Pasternak et al. 2002), suggesting that initiation of SE requires active ionic transport across membrane.

Calcium as a well-known secondary messenger during plant development and stress responses (Sanders et al. 1999), not surprisingly, also plays a role in SE, especially considering that stress plays an important role in the initiation of the whole process. For example, induction of embryogenesis in carrot cell cultures requires at least 0.2 mM calcium (Overvoorde and Grimes 1994), and increase of calcium concentration from 1 to 10 mM causes a twofold increase in the efficiency of embryogenesis (Jansen et al. 1990). Conversely, sequestering of calcium in the medium using ethylene glycol-bis(aminoethyl ether)-N,N′-tetraacetic acid (EGTA) inhibits embryogenesis (Anil and Rao 2000). Calcium could even counteract the inhibitory effect of 2,4-D on the initiation of embryogenesis from proembryogenic masses (Jansen et al. 1990).

3 Cell Fate During SE: "Pro-Life" Signalling

Considering the remarkable morphological resemblance of somatic and zygotic embryogenesis, it is assumed that all key regulators of the zygotic pathway discovered in *A. thaliana* and other systems using genetic approaches (De Smet et al. 2010) are equally active in SE. This assumption is supported by numerous experimental studies described below (Table 1). The research on SE is focused on the identification of key regulators that improve the induction and yield of the whole process. While the initiation of embryogenic cultures depends on the developmental stage of the starting plant material and factors in the growth medium (for details, see chapter by Opatrný in this volume) perceived by complementary cellular sensors, the maintenance of the embryogenic potential during subsequent

Table 1 Regulators known to improve the efficiency of somatic embryogenesis

Name	Functions	Species	Overexpression phenotype	References
Agamous-Like 15 (AGL15)	MADS-box TF	*A. thaliana*	Primary somatic embryogenesis from zygotic embryos and also in shoot apical meristem	Harding et al. 2003
		Glycine max	Enhanced indirect somatic embryogenesis	Thakare et al. 2008
BABY BOOM (BBM1)	AP2/ERF family TF	*Brassica napus*, *A. thaliana*	Direct somatic embryogenesis on seedlings	Boutilier et al. 2002; El Ouakfaoui et al. 2010
		Nicotiana tabacum	Sterility if constitutive; spontaneous organogenesis if inducible	Srinivasan et al. 2007
		Capsicum annuum	Indirect somatic embryogenesis in otherwise recalcitrant plants	Heidmann et al. 2011
		Populus tomentosa	Indirect somatic embryogenesis	Deng et al. 2009
EMBRYOMAKER (EMK)	AP2/ERF family TF	*A. thaliana*	Enhanced direct and indirect somatic embryogenesis	Tsuwamoto et al. 2010
LEAFY COTYLEDON1 (LEC1)	HAP3 domain TF	*A. thaliana*	Induction of direct somatic embryogenesis	Lotan et al. 1998
LEAFY COTYLEDON2 (LEC2)	B3 domain TF	*A. thaliana*	Induction of direct somatic embryogenesis without auxin	Stone et al. 2001; Ledwon and Gaj 2011
SHOOTMERISTEMLESS (STM)	Homeobox domain TF	*Brassica oleracea*	Enhanced efficiently of indirect somatic embryogenesis	Elhiti et al. 2010
WUSCHEL (WUS)	Homeobox domain TF	*A. thaliana*	Induction of direct somatic embryogenesis without auxin	Zuo et al. 2002
		Coffea canephora	Enhanced efficiently of indirect somatic embryogenesis	Arroyo-Herrera et al. 2008
HBK3	Homeobox domain TF	*P. abies*	Increased yield of somatic embryogenesis	Belmonte et al. 2007
RKD4	RWP-RK domain TF	*A. thaliana*	Short-term expression promotes somatic embryogenesis without auxin	Waki et al. 2011
Polycomb Repressive Complex 2 (PRC2)	Trimethylation of histone H3 on K27	*A. thaliana*	Inhibits embryogenesis, downregulation promotes embryogenesis	Chanvivattana et al. 2004

azadC	Inhibitor of DNA methylation	*A. thaliana*	Upregulation of STM and higher yield of somatic embryos	Elhiti et al. 2010
SERK1	Receptor kinase	*A. thaliana*	Enhanced efficiency of indirect somatic embryogenesis	Hecht et al. 2001
		Oryza sativa	Constitutive overexpression boosts the frequency of shoot regeneration in vitro and increased blast resistance in vivo	Hu et al. 2005
10 Gibberellin 2-oxidase 6 (GA2ox6)	Gibberellin metabolism	*A. thaliana*	Constitutive overexpression increases somatic embryo production	Wang et al. 2004

cultivation requires the activity of signalling and genetic pathways, which in turn lead to specific cellular responses such as cytoplasmic remodelling, modification of the division patterns and cell/tissue differentiation. The success of this reprogramming requires a developmental dichotomy: whereas some cells are assigned for survival ("pro-life"), others have to be eliminated by PCD ("pro-death"). In the following sections, the signalling "pro-life" will be considered.

3.1 Cell Wall Components

SE is accompanied by modifications in structure and molecular composition of the cell wall (Emmons 1994; Malinowski and Filipecki 2002). These changes are thought to be important to establish a balance of the mechanic forces required for the maintenance of specific cell shapes and cellular architecture and the determination of the division plane (Malinowski and Filipecki 2002). Apart of controlling the morphogenic events inside the cell, the cell walls are responsible for the communication with neighbouring cells via apoplastic or symplastic flow.

The cells produce a plethora of signalling factors, which upon export into the cell walls can spread through apoplast to surrounding cells and stimulate embryogenesis. In liquid cultures, these factors accumulate in the medium and promote embryogenesis in the whole culture (von Arnold et al. 2002).

Some of these signalling factors isolated from the conditioned medium of the embryogenic cell cultures were able not only to support embryogenesis in low-density embryogenic cultures, which otherwise fail to initiate embryo development (Smith and Sung 1985; de Vries et al. 1988), but could, in addition, induce embryogenic transformation of non-embryogenic cultures (Hari 1980). The outer cell wall surface of embryogenic cells contains specific arabinogalactan proteins (AGPs) and pectins, which are so specific that they can be used as markers of embryogenic cell cultures (Rumyantseva et al. 2003; Konieczny et al. 2005).

3.1.1 Extracellular Proteins

Several cell wall-modifying enzymes are differentially expressed during SE. For example, induction of SE in divergent species is accompanied by upregulation of xyloglucan endotransglycosylases (Malinowski and Filipecki 2002; Thibaud-Nissen et al. 2003; Rensing et al. 2005). This class of enzymes modifies the structure of xyloglucan chains and alters mechanical properties of the cell wall. SE of *Pinus radiata* is accompanied by upregulation of α-D-galactosidase (named SEPR1), which cleaves terminal α-galactosyl moieties of glycolipids and glycoproteins and can thus modify structure and properties of cell wall components (Aquea and Arce-Johnson 2008). Several members of the pectinesterase gene family (SEPR91, SEPR110 and SEPR114) are downregulated during early embryogenesis, but since pectinesterases can cause both stiffening and loosening of the cell

wall, it is difficult to understand the functional context of this downregulation, until the stiffness of the cell wall in embryogenic cells is measured.

The functions of many cell wall proteins in SE remain enigmatic. For example, a glycine-rich protein CEM6 was isolated from a screen for early embryogenesis-abundant genes in carrot cultures (Sato et al. 1995). It is upregulated from pre-globular to early heart stages and contains hydrophobic cell wall localisation signal. A gene encoding a similar glycine-rich protein Atgrp-5 was upregulated during SE in *A. thaliana* and eggplant (Magioli et al. 2001). CEM6 and Atgrp-5 were suggested to play a role in cell wall stiffness-related modifications required for early embryogenesis.

Extracellular glycosylated acidic class IV endochitinase or extracellular protein 3 (EP3) is involved in the transition from globular to heart stage in carrot embryogenic cultures (de Jong et al. 1992). The exact function of endochitinase is unknown; nonetheless, it appears to be a part of a phylogenetically conserved pathway, since endochitinase from sugar beet stimulates SE in the cell cultures of *P. abies* (Egertsdotter and von Arnold 1998). Interestingly, the endochitinase is expressed only in a subset of morphologically distinct cells in the embryogenic cultures located outside the proembryogenic masses and not in the developing somatic embryos themselves. In the seeds, endochitinase is not expressed in the embryos but in the inner integumentary cells of young fruits and in a specific subset of cells located in the middle of the endosperm of mature seeds (van Hengel et al. 1998). These findings suggest that endochitinase is required for the processing of signalling molecules which play a nurturing role during both somatic and zygotic embryogenesis.

The nonspecific lipid transfer proteins (LTP, Sterk et al. 1991) belong to an abundant class of proteins (extracellular protein 2, EP2) found in the conditioned medium of carrot embryogenic lines. The level of LTP expression in cotton cell lines is high before induction of embryogenesis and during the globular stage and then diminishes during post-globular stages (Zeng et al. 2006). The LTPs were implicated in the control of protoderm (the outermost layer of cells above a meristem) differentiation (Thoma et al. 1994; Dodeman et al. 1997). Correspondingly, ectopic overexpression of LTPs under control of a 35S promotor affects establishment of bilateral symmetry of the embryos and disturbs epidermal cell layer morphology (Francois et al. 2008). At the cellular level, LTPs were implicated in the transport of phospholipids or other nonpolar molecules from the endoplasmic reticulum to other cellular compartments (Sterk et al. 1991; Kader 1996; Toonen et al. 1997), so they might be responsible for the stabilisation and transport of signalling molecules through apoplast and symplast.

Germin-like proteins (GLP) belong to one of the most abundant groups of extracellular proteins found in embryogenic lines of *Pinus caribaea* (Domon et al. 1995). Several studies showed upregulation of transcription of GLP-encoding genes in embryogenic lines of other species (Neutelings et al. 1998; Wojtaszek et al. 1998; Çaliskan et 2004). In all cases, their expression was limited to embryogenic cells. GLPs are part of the cupin superfamily of ubiquitous plant proteins (Dunwell et al. 2008) with divergent primary sequences,

but conserved tertiary structure. They all are hydrogen peroxide-producing enzymes of two types: oxalate oxidase or superoxide dismutase. Embryogenesis-related GLPs are of the oxalate oxidase type, but their glycosylated form isolated from the conditioned medium possessed no apparent activity. Contrary, a high oxalate oxidase activity of GLPs was detected in the embryogenic lines of wheat (Çaliskan et al. 2004), leading the authors to conclude that active forms of GRPs reside in the cell wall and increase cell wall rigidity by producing hydrogen peroxide, which cross-links glucuronoarabinoxylan polymers. The involvement of GLPs in embryogenesis is further supported by the finding that transcription of an *A. thaliana* cupin At4g36700 is regulated by AGL1, a transcription factor, which controls both zygotic and somatic embryogenesis (Zheng et al. 2009).

3.1.2 Arabinogalactan Proteins

AGPs are a heterogeneous group of molecules composed of a polypeptide, a long chain of branched glycan and a lipid (Fig. 2a; Majewska-Sawka and Nothnagel 2000). Commonly, more than 90 % of the AGP molecule can be constituted by carbohydrates. Only specific types of AGPs can stimulate SE. For example, a fraction of AGPs recognised by the antibody ZUM18 promoted embryogenesis, while the fraction recognised by antibody ZUM15 were inhibitory (Kreuger and van Holst 1995). Removal of AGPs from the culture medium using anti-AGP antibody or AGP-binding synthetic phenyl glycoside (Yariv reagent) blocks SE (McCabe et al. 1997; Butowt et al. 1999; Thompson and Knox 1998; Chapman et al. 2000), while addition of AGPs to old cultures and cultures with low embryogenic potential can promote embryogenesis (Kreuger and van Holst 1995; Egertsdotter and von Arnold 1995). In contrast to the situation with endochitinases, cells producing active AGPs localise within embryos (McCabe et al. 1997).

Although numerous studies have demonstrated a signalling role of AGPs during embryogenesis (Egertsdotter and von Arnold 1995; Kreuger and van Holst 1995; Butowt et al. 1999; Thompson and Knox 1998; Chapman et al. 2000), the molecular mechanisms behind this activity remain poorly understood. One of the possible scenarios involves activity of chitinases. Chitinases can cleave off the glycosyl chains of AGPs (Domon et al. 2000; Passarinho et al. 2001), and the released oligosaccharins can serve as signalling molecules to promote SE (Darvill et al. 1992; Svetek et al. 1999). Chitinase EP3 co-localises with AGPs in developing seeds, providing further evidence that the EP3/AGP signalling module plays a key role in embryogenesis (van Hengel et al. 1998). In agreement with this hypothesis, inactivation of AGPs by Yariv reagent inhibits SE (Chapman et al. 2000). However, treatment of AGPs with endochitinase EP3 produces more active AGPs (Van Hengel et al. 2001). This questions the role of glycosyl residues of AGPs for the induction of SE. Furthermore, a phytocyanin-like domain of AGP from cotton that

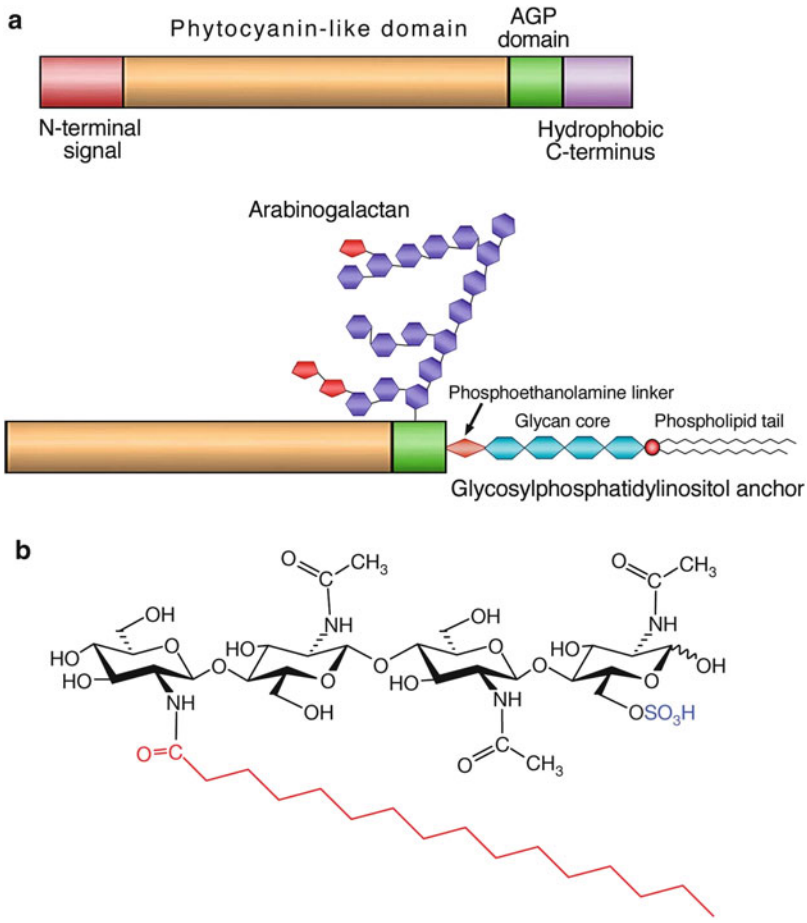

Fig. 2 Structure of the extracellular signalling molecules. (**a**) Hypothetical structure of an arabinogalactan protein (AGP; Poon et al. 2012). The N- and C-terminal domains of the precursor are cleaved, and then the resulting C-terminal region is modified by addition of an arabinogalactan and a glycosylphosphatidylinositol (GPI) anchor which targets AGP molecule to the cell surface. (**b**) Lipochitooligosaccharide (LCO) responsible for induction of arbuscular mycorrhiza produced by the fungus *Glomus intraradices* (Maillet et al. 2011). The structure of LCOs responsible for the induction of SE is unknown, but likely to resemble the structure given here. The sulphate group shown in *blue* is optional. The acyl chain can vary (shown in *red*) and in this particular case can be palmitic (C16:0) or oleic (C18:1 Δ9Z) fatty acids

lacks glycosylation motifs was alone sufficient to induce SE (Poon et al. 2012). It was so far not possible to assign regulation of SE to the protein or glycan components of AGPs owing to inconsistency of data obtained in different model systems. These inconsistencies may reflect species-specific features of AGP signaling.

3.1.3 Oligosaccharines

A class of oligosaccharines, called lipochitooligosaccharides (LCOs), was originally identified as nodulation (Nod) factors secreted by bacteria of the genus *Rhizobium* to promote plant cell division and formation of nodules for subsequent colonisation by *Rhizobium* (Spaink et al. 1991; Truchet et al. 1991). Nod factors have a uniform backbone made of 3 to 5 chitin (1,4-linked N-acetyl-D-glucosamine) residues with the N-acetyl groups on the terminal nonreducing sugar substituted for an acyl chain (Fig. 2b). The specific activity of LCOs is determined not only by the number of chitin residues and the structure of the acyl chain but also by addition of monosaccharides such as arabinose, mannose and fucose as well as the attachment of sulphate, acetate or carbamoyl groups (Downiw and Walker 1999). Rhizobial Nod factors can stimulate SE up to the globular stage by promoting cell divisions in *D. carota* (De Jong et al. 1993) and *P. abies* cell cultures (Egertsdotter and von Arnold 1998; Dyachok et al. 2000). Embryogenic cell lines produce their own type of LCOs with similar structure to Nod factors. The fraction of conditioned medium enriched with these LCOs can stimulate SE (Dyachok et al. 2002).

3.2 Perception and Transduction of Extracellular Signals

The soluble molecules present in the medium are perceived by the receptor kinases located in the plasma membrane. While the ligands of many receptor kinases are not known, it is plausible that they are produced in the cell walls. The signal can then affect cytoplasmic processes or be transduced into the nucleus where it modulates transcription.

3.2.1 Receptor Kinases

SE receptor-like kinases (SERKs) belong to an evolutionary conserved superfamily of leucine-rich repeat receptor-like kinases (LRR-RLK; Becraft 1998). The first member of this group was identified in maize using degenerate PCR primers to kinases (Walker and Zhang 1990). Later this group was extended by many key regulators of signal transduction during plant development and environmental adaptation, which, apart from SERK, include CLAVATA1 (CLV1), Erecta1, brassinosteroid-insensitive 1 (BRI1) and Crinkly4 (Becraft 1998). All members of this superfamily share an intracellular kinase domain, an extracellular leucine-rich repeats (LRR) domain and several juxtamembrane phosphorylation sites. LRR domains interact mutually resulting in homo- or heterodimerisation (see also chapter by Robatzek, this volume). LRR-RLK can autophosphorylate in

monomeric form and trans-phosphorylate in the oligomerised state modulating in this way interaction of kinases with other proteins and their effectors.

SERKs were originally identified from carrot embryogenic cultures (Schmidt et al. 1997). Their expression levels are elevated in proliferating embryogenic cultures and at the early stages of embryogenesis up to the heart stage (Schmidt et al. 1997; Somleva et al. 2000; Salaj et al. 2008). Like all LRR-RLK, SERKs localise to the plasma membrane (Shah et al. 2001). Similarly, during zygotic embryogenesis in *D. carota*, a SERK was expressed from the eight-celled stage through to the globular stage, while no transcript was detected in unfertilised flowers (Schmidt et al. 1997). The transcription of SERKs is upregulated by auxin (Nolan et al. 2003). The genomes of all angiosperm species examined so far contain several genes encoding SERKs, e.g. five in case of *A. thaliana* and six (excluding three SERK-like homologues) in case of *Medicago truncatula*. Four out of six *M. truncatula* isotypes are upregulated during induction of SE (Nolan et al. 2011), suggesting functional redundancy of individual members of the gene family. Consistently with this conclusion, only quadruple SERK knockout of *A. thaliana* exhibited embryo lethal phenotype (Gou et al. 2012). Ectopic expression of *Arabidopsis* SERK1 isotype promotes formation of somatic embryos (Hecht et al. 2001). Altogether, SERKs are potent signalling molecules for induction and regulation of SE.

There is an intriguing link between SERKs and brassinosteroid signalling. SERK1 was found in a complex with brassinosteroid-insensitive 1 (BRI1) kinase and can trans-phosphorylate BRI1 (Karlova et al. 2006). SERK1 phosphorylation is enhanced in the presence of brassinosteroid, and, in turn, BRI1 can trans-phosphorylate SERK1 (Karlova et al. 2009). So far, no substrates of SERK1 have been identified, but considering that the BRI1 pathway is dependent on SERK activity (Gou et al. 2012) and steroids are required for both somatic (Pullman et al. 2003) and zygotic (Schrick et al. 2004) embryogenesis, it is conceivable that SERKs, at least as one of their functions, can modulate brassinosteroid signalling during embryogenesis. Although they belong to the same phylogenetic group of LRR-RLK, SERK and CLV1 play opposite roles in SE. CLV1 reduces expression of transcription factors promoting embryogenesis and consequently inhibits SE (Elhiti et al. 2010).

3.2.2 Ca^{2+} Effectors

Induction of SE in carrot cell cultures requires at least 0.2 mM calcium (Overvoorde and Grimes 1994), and rising concentration from 1 to 10 mM causes twofold increase in the efficiency of embryogenesis (Jansen et al. 1990). Initiation of embryogenesis from proembryogenic masses of carrot and sandalwood on hormone-free medium is accompanied by increased calcium uptake from the medium, resulting in increased overall intracellular calcium content (Timmers et al. 1996; Anil and Rao 2000). In addition, a short spike of 10- to 16-fold increase of intracellular Ca^{2+} (up to 600–860 nM) was detected by Fura-2 ratiometric

analysis within 10 s after removal of 2,4-D from the medium (Anil and Rao 2000). Interference with the intracellular calcium homeostasis using calcium channel blockers or the ionophore A23187 resulted in complete suppression of embryogenesis (Overvoorde and Grimes 1994; Anil and Rao 2000), similarly to what have been observed when Ca^{2+} in the growth medium was omitted or chelated. This suggests that a specific pattern of intracellular calcium concentration is required to initiate embryogenesis. Consistently, inhibition of calcium signalling by W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide) decreased SE efficiency by 85 % (Anil and Rao 2000). Calcium influx can be translated into physiological responses via several classes of effectors (Galon et al. 2010). So far the role of two classes of calcium effectors during embryogenesis was addressed: calmodulins and calcium-dependent protein kinases (CDPK).

Transcription and protein accumulation of calmodulins can vary between different model systems. In several model systems, the transcription was fairly constant in the course of embryogenesis (Oh et al. 1992; Overvoorde and Grimes 1994), and no changes were detected in calmodulin methylation, which can alter specific activity of calmodulin (Overvoorde and Grimes 1994). In *P. abies*, however, the level of calmodulin was upregulated immediately after initiation of embryo development upon removal of PGRs (van Zyl et al. 2003). The expression of CDPKs and their activity was upregulated in calcium-dependent manner in both proembryogenic masses and somatic embryos (Anil and Rao 2000; Kiselev et al. 2008). Xu and colleagues (1999) used DM-Bodipy-PAA to label calcium changes at the early stages of zygotic embryo development. They reported that the probe preferentially labelled the basal part of the embryo. Therefore, it might be a calcium gradient, but not the overall concentration of calcium effectors, that could mediate the early stages of embryo polarisation and organ patterning.

3.3 Gene Expression

The exogenous signals can be transduced by protein kinase pathways into the nucleus resulting in the switch of gene expression. This switch can be attributed in part to the activity of transcription factors and in part to the epigenetic modifications of chromatin.

3.3.1 Transcription Factors

3.3.1.1 Heme Activator Protein 3 (HAP3) Related

HAP is a multimeric transcriptional activator complex, which recognises the CCAAT box and in plants consists of the three subunits HAP2, HAP3 and HAP5 (Edwards et al. 1998). While in yeast and animals each subunit is encoded by a single gene, in plants, each subunit is encoded by gene families. Two members of

the HAP3 family, LEAFY COTYLEDON1 (LEC1) and LEAFY COTYLEDON1 LIKE (L1L), are key regulators of zygotic embryogenesis (Lotan et al. 1998; Kwong et al. 2003). During the early stages of embryogenesis, LEC1 and L1L specify cotyledon cell identity and maintain the fate of suspensor cells (Meinke 1992; Meinke et al. 1994; West et al. 1994), while during the later stages, they control initiation and/or maintenance of the embryo maturation, but also suppress precocious germination (Parcy et al. 1997; Lotan et al. 1998).

Ectopic overexpression of *LEC1* leads to early growth arrest of the seedling and abnormal plant development, occasionally accompanied by the formation of somatic embryo-like structures (Lotan et al. 1998). Therefore, LEC1 seems to confer embryogenic potential to somatic cells. The gain-of-function mutant of *LEC1*, turnip (*tnp*), exhibits a milder phenotype with ectopic cell divisions and a loss of tissue identity (Casson and Lindsey 2006). Interestingly, the penetrance of the *tnp* phenotype is enhanced by auxin, inhibited by cytokinin, but not affected by ABA and GA. This attributes to LEC1 an important role in the switch of cell identity during auxin-induced dedifferentiation and the proliferation of embryogenic cells. The transcription of *LEC1* is negatively regulated by PICKLE, a chromodomain-helicase-DNA-binding protein 3 (CDH3) chromatin remodelling factor (Ogas et al. 1999). The contribution of LEC1 to SE is far from being clear, but elevated expression levels of *LEC1* are characteristic for embryogenic cell lines and not found in non-embryogenic cell lines of *A. thaliana* (Ledwon and Gaj 2011). Additionally, the conifer *LEC1* homologue (*HAP3A*) is upregulated during SE in *P. abies* and *Pinus sylvestris* (Uddenberg et al. 2011). Contrary, the ectopic expression of *CHAP3A* (*Picea mariana* homologue of LEC1) in embryogenic cell lines did not affect the efficiency of SE (Klimaszewska et al. 2010).

3.3.1.2 B3-Domain Transcription Factors

The B3-domain proteins belong to a plant-specific family of transcription factors with common tertiary structure of seven beta strands and two alpha helices, which binds to the major groove of the DNA double helix but recognises different motifs. This group includes several major regulators of embryogenesis: LEAFY COTYLEDON2 (LEC2), FUSCA3 (FUS3) and ABA INSENSITIVE3 (ABI3) of *A. thaliana* and Viviparous1 (Vp1) of *Zea mays* (McCarty et al. 1991; Luerssen et al. 1998; Stone et al. 2001). LEC2 and FUS3 are important for the early patterning and later embryo maturation stages, while ABI3 is important only for embryo maturation (Stone et al 2001). Overall, the roles of B3-domain and HAP3-related transcription factors during embryogenesis overlap (Lotan et al. 1998). Consistently, postembryonic ectopic expression of *LEC2* leads to formation of somatic embryos, calli and cotyledon/leaf-like structures (Stone et al. 2001), while downregulation of LEC2, FUS3 and ABI3 significantly inhibits both direct and indirect SE (Gaj et al. 2005).

LEC2 upregulates expression of two transcription factors Agamous-Like 15 (AGL15; see next section) and aux/IAA30 (Braybrook et al. 2006).

Transcription of *AGL15* is upregulated in the embryos, and consequently, overexpression of *AGL15* increases efficiency of induction of embryogenic cell lines in response to exogenous auxin (Harding et al. 2003). AGL15 in turn upregulates transcription of *aux/IAA30* and *LEC2* (Zheng et al. 2009). Aux/IAA30 regulates gene transcription in response to auxin (Sato and Yamamoto 2008; see also chapter by Skůpa et al., this volume). Therefore, these three transcription factors form a positive feedback loop essential for activation of an embryogenic programme in somatic cells. In addition, LEC2 increases transcription of two flavin monooxygenases, YUC2 and YUC4, responsible for the auxin synthesis. Consequently, the overall auxin level in the seedlings ectopically expressing *LEC2* was significantly higher than in the control plants (Stone et al. 2008).

Expression of FUS3 is regulated by auxin (Gazzarrini et al. 2004). Along with LEC1 and LEC2, FUS3 is essential for the induction of SE (Gaj et al. 2005). At the same time, FUS3 upregulates the synthesis of ABA and downregulates synthesis of GA during embryo maturation (Gazzarrini et al. 2004). This alteration of the hormonal balance inhibits cell divisions and delays senescence during leaf differentiation. In this way, FUS3 couples auxin signalling with the equilibrium of physiological responses to GA and ABA. However, the changes in the hormonal balance regulated by FUS3 do not apparently reduce embryogenic potential of cells in mature embryos.

Another member of the B3-domain proteins, VP1/ABI1-LIKE (VAL), represses transcription of *LEC1*, *L1L*, *FUS3* and *ABI3* genes, but not of *LEC2*, and knockout of *VAL* mimics ectopic expression phenotypes of *LEC1, L1L* and *LEC2* (Suzuki et al. 2007), resulting in embryo-like outgrowth in the region of apical meristem and callus-like formations on the roots.

3.3.1.3 Agamous-Like 15 (AGL15)

AGL15 belongs to a divergent family of eukaryotic transcription factors found in phylogenetically distant species from yeast to human and angiosperm plants. All members of this family contain a conserved MADS-box motif within their DNA-binding domain. The name MADS-box derives from the first letters of four originally identified proteins: *M*CM1 (minichromosome maintenance-defective1 from *Saccharomyces cerevisiae*), *A*G (AGAMOUS from *A. thaliana*), *D*EFA (DEFICIENS from *Antirrhinum majus*) and *S*RF (serum response factor from *Homo sapiens*). *AGL15* expression peaks during embryo development starting as early as the globular stage. In agreement with its role, AGL15 localises to the nuclei of both embryo proper and suspensor. After germination, *AGL15* is transiently expressed in the young shoot apical meristem and floral buds (Heck et al. 1995; Rounsley et al. 1995; Perry et al 1996; Fernandez et al 2000; Thakare et al. 2008). The expression of *AGL15* is under tight control of embryo development, and premature termination of embryogenesis in *lec1-2* abolishes expression of *AGL15* (Perry et al. 1996).

Embryogenic cultures exhibit high levels of *AGL15* expression (Thakare et al. 2008). Consistently, ectopic expression of *AGL15* increases the efficiency of both direct and indirect SE (Harding et al. 2003; Thakare et al. 2008), while knockout of *AGL15* reduces the efficiency of SE (Thakare et al. 2008). Importantly, AGL15 is integrated into different signalling processes during embryogenesis. Firstly, AGL15 is a component of the SERK complex (Karlova et al. 2006); secondly, its expression is controlled by LEC2 and is upregulated by auxin (Gazzarrini et al. 2004; Braybrook et al. 2006; Zhu and Perry 2005); thirdly, it promotes transcription of *LEC2, FUS3* and *ABI3* (Zheng et al. 2009); fourthly, AGL15 in cooperation with LEC2 and FUS3 reduces the content of active gibberellin and induces expression of gibberellin catabolising GA 2-oxidases GA2ox6 (Wang et al. 2004) and GA2ox2 (Zheng et al. 2009). Since it controls the level of active gibberellin, GA2ox6 is a potent regulator of SE: overexpression of *GA2ox6* increases efficiency of SE by almost sevenfold (Wang et al. 2004).

3.3.1.4 AP2/ERF Domain Proteins

APETALA2/ethylene-responsive factor (AP2/ERF) proteins are a family of plant-specific transcription factors involved in the regulation of a plethora of developmental processes, such as flower meristem identity, flower patterning, lateral root morphogenesis and response to environmental factors, in addition to causing pleiotropic effects on cell size, plant height and fertility (Dietz et al. 2010). The size of the AP2/ERF gene families in phylogenetically divergent species reflects this functional diversity and at the same time correlates with the complexity of morphology and life cycle. For example, the *A. thaliana* genome encodes 147 members, while *Selaginella moellendorffii* and *Chlamydomonas reinhardtii* genomes encode 57 and 14 members, respectively.

Several members of the AP2/ERF family regulate SE. The *M. truncatula* homologue of *A. thaliana* ERF, *MtSERF1*, is an ethylene-inducible gene expressed in zygotic embryos, proliferating embryogenic cell cultures and somatic embryos (Mantiri et al. 2008). The localisation of expression is restricted to the shoot apical meristem region of the heart-stage embryo. Knockdown of *MtSERF1* inhibits SE. Another member, *A. thaliana EMBRYOMAKER* (*EMK*), is expressed in early and mature embryos and has a redundant role in maintaining embryonic cell identity (Tsuwamoto et al. 2010). Ectopic expression of *EMK* promotes initiation of somatic embryos from cotyledons.

The best-studied member of the AP2/ERF domain family is BABY BOOM (BBM). Boutilier and co-authors (2002) reported that *BBM* is expressed during all stages of zygotic embryogenesis in *A. thaliana* starting from the globular stage and up to mature seeds. Furthermore, *BBM* was identified as a marker of SE in cell cultures of *Brassica napus* being expressed during all stages of the embryogenesis. Ectopic expression of *BBM* enhances SE and other morphogenic responses. Direct SE, formation of ectopic shoots and calli could be induced in *A. thaliana* and *B. napus* even without any PGRs (Boutilier et al. 2002; El Ouakfaoui et al. 2010).

Overexpression of *BBM* resulted in the induction of indirect SE in tobacco (Srinivasan et al. 2007), poplar *Populus tomentosa* (Deng et al. 2009) and the otherwise recalcitrant *Capsicum annuum* (green pepper; Heidmann et al. 2011) in the presence of PGRs.

In germinating seedlings, *BBM* is expressed in root meristems (Nawy et al. 2005), but constitutive expression of *BBM* does not lead to the formation of neither ectopic root meristems nor roots. This suggests that BBM works together with other regulatory elements in the tissue-specific environments. In agreement with this conclusion, BBM upregulates TUBBY-LIKE PROTEIN 8 (TLP8), a transcription factor of as yet unknown function (Passarinho et al. 2008). TLP8 in turn is a target of the transcription factor LEAFY (LFY), which is responsible for flower meristem identity and activation of pathways guiding specification of flower organs (William et al. 2004).

3.3.1.5 Homeodomain Transcription Factors

The homeodomain is a highly conserved 60-amino acid long region characteristic for transcription factors found in all eukaryotes. Homeodomain-containing transcription factors regulate different developmental processes including SE. One phylogenetic group of homeodomain containing transcription factors includes *A. thaliana* WUSCHEL (WUS) and its fourteen homologues (Mayer et al 1998; Palovaara and Hakman 2008; see also chapter by Opatrný in this volume). WUS plays an essential role in keeping cells in a state of proliferation and responsiveness to other developmental cues (Mayer et al 1998; Gallois et al 2002). This activity maintains shoot and flower apical meristems during embryonic and postembryonic development, but does not have any apparent role in the maintenance of the root meristem (Laux et al. 1996). *WUS* is expressed only in a group of cells (organising centres) underlying the shoot and floral apical meristems starting from the 16-celled embryo stage. The lack of *WUS* expression in the meristem cells suggests that it acts in a non-cell autonomous manner (Mayer et al. 1998). Genetic studies revealed that WUS is controlled by the LRR-RLK CLV1 pathway forming a self-regulatory loop in which CLV1 acts above WUS. While WUS promotes proliferation of the stem cells, which express members of the CLV1 pathway, the CLV1 pathway suppresses WUS on the transcriptional level and in this way controls the size of the stem cell niche (Haecker and Laux 2001). The discovery of a WUS-binding motif in the promoter of AP2/ERF transcription factor MtSERF1 suggests that CLV1 controls other transcriptional networks through WUS (Mantiri et al. 2008).

The efficiency of callus induction from *wus* and wild-type plants was the same (Mordhorst et al. 2002), indicating redundancy of WUS. The subsequent formation of somatic embryos was not assessed by Mordhorst et al. (2002), but in another work, genetic suppression of WUS by ectopic expression of *CLV1* significantly reduced the responsiveness to SE stimuli which could be rescued by higher concentrations of 2,4-D (Elhiti et al. 2010). Ectopic inducible expression of *WUS* inhibits seedling development but promotes direct SE from young embryos and

from differentiated tissues in the absence of 2,4-D. The embryos started to form from somatic cells after only several divisions (Zuo et al. 2002). Conversely, knockdown of *WUS* inhibits SE from *B. napus* microspores (Elhiti et al. 2010). The ability of WUS to reprogram vegetative tissues towards embryonic development makes it a very useful tool for overcoming difficulties of inducing SE in recalcitrant species. For example, ectopic expression of *A. thaliana WUS* in *Coffea canephora* promoted hormone-induced callus formation and increases the yield of somatic embryos by 400-fold (Arroyo-Herrera et al. 2008).

Angiosperm genomes contain close homologues of *A. thaliana* WUS and large families of WUSCHEL-related homeobox (WOX) proteins. Gymnosperms lack the WUS clade, but harbour WOX members falling into the conserved clades present in angiosperm species (Palovaara and Hakman 2008). Two members of the *P. abies WOX* gene family, *WOX2* and *WOX8/9*, from independent conserved phylogenetic clades exhibit higher level of transcription during all stages of zygotic embryogenesis, in embryogenic cell lines and during all stages of SE (Palovaara and Hakman 2008; Palovaara et al. 2010). However, the level of WOX2 was found to be similar in embryogenic and non-embryogenic cell lines (Palovaara and Hakman 2008).

The second group of homeodomain fold transcription factors important for SE are the Knotted1-like homeobox (KNOX) family proteins. This group regulates balance between cell proliferation and cell differentiation during tissue patterning and hence is important for plant development (Hay and Tsantis 2010). The founding member of this group, maize *Knotted1* (*kn1*; Vollbrecht et al. 1991), maintains stem cell niches in the shoot apical meristem by preventing premature differentiation of proliferating cells (Hay and Tsantis 2010), and correspondingly, *kn1* lacks a shoot apical meristem (Vollbrecht et al. 1991). The Arabidopsis KNOX family consists of two clades, KNOXI and KNOXII. KNOXI group consists of four members, *SHOOTMERISTEMLESS (STM)*, *BREVIPEDICELLUS (BP)*, *kn1-like in A. thaliana 2 (KNAT2)* and *KNAT6*. The role of KNOXII group in embryogenesis remains unknown. The phenotype of *stm* resembles *kn1* and *wus* in that it lacks shoot apical meristem (Long et al. 1996). This suggests that STM cooperates with WUS to maintain the stem cell niche and to regulate the balance between cell proliferation and differentiation, with the difference that *STM* is expressed in all cells of the shoot apical meristem, while *WUS* is expressed only in an organising centre subtending the meristem.

STM expression becomes detectable in the shoot apical meristem at the 32-celled zygotic embryo stage (Long et al. 1996) and persists in shoot apical and floral meristems during postembryonic development. *STM* is also upregulated during SE. The soybean *KNAT2* homologue *SBH* (soybean homeobox-containing gene) is expressed in early somatic embryos reaching maximum transcript level at the cotyledonary stage and decreasing thereafter (Ma et al. 1994). Ectopic expression of *B. napus STM* promotes SE (Elhiti et al. 2010). *P. abies* KNOXI homologues HBK (*h*omeo*b*ox of *K*NOX class) are markers and important regulators of SE. *HBK2* is expressed in somatic embryos, but not in a cell line lacking embryogenic potential (Hjortswang et al. 2002). *HBK1* and *HBK3* are upregulated immediately after initiation of embryogenesis on the medium without PGRs.

The expression of *HBK4* becomes apparent during later stages, after degradation of the suspensor, and together with *HBK2*, it plays a role in the establishment of shoot apical meristem (Larsson et al. 2012). Ectopic expression of another member of the *P. abies* KNOXI family, *HBK3*, enhanced the yield of somatic embryogenesis. The shoot apical meristem in these embryos was significantly larger than in wild-type embryos. Accordingly, the downregulation of *HBK3* inhibited embryogenesis (Belmonte et al. 2007).

The KNOXI and WUS pathways are intertwined in the regulation of SE. STM upregulates transcription of *WUS*, and conversely, higher level of WUS promotes SE. In addition, STM enhances transcription of genes involved in the hormonal perception (Elhiti et al. 2010), which implies that higher efficiency of SE can be achieved by simultaneous upregulation of both pathways.

3.3.1.6 RKD4

Arabidopsis RKD4 (RWP-RK domain 4) belongs to the RWP-RK group of plant-specific transcription factors conserved in all lineages starting from unicellular algae. The members of this group contain a highly conserved motif RWPxRK (Schauser et al. 1999) originally isolated as a key regulator of symbiotic root-nodule development. Mutation of RKD4 results in a smaller embryo suspensor, because of suspensor cell identity lost, and defective seed germination (Waki et al. 2011; Jeong et al. 2011). *RKD4* transcription was detected in all cells during early embryogenesis, but starting from the late globular stage, it was confined to the embryo-suspensor cells (Waki et al. 2011). Induction of ectopic expression of *RKD4* for 8 days activates the expression of embryogenesis-related genes, initiates embryogenesis in somatic cells and promotes SE, whereas constitutive ectopic expression of *RKD4* results in continuous proliferation without differentiation (Waki et al. 2011).

3.3.2 Epigenetic Mechanisms

The dedifferentiation of somatic cells and initiation of SE rely on the alteration of gene transcription profile and take up to several weeks. During this time, hormones and stress in addition to changing the gene transcription profile induce epigenetic modifications of the chromatin. This exemplifies notion that cell fate in plants depends on the “genomic memory” as well as positional information. So far, several epigenetic mechanisms have been implicated in the control of SE, including DNA methylation, histone post-translational modifications and microRNA (miRNA) pathways.

3.3.2.1 DNA Methylation

DNA methylation is one of the main heritable genomic modifications that modulate transcriptional activity of specific sequences. This involves covalent attachment of a methyl group to the cytosine base at the CpG, CpHpG and CpHpH sites (where p stands for phosphate and H denotes either A, T or C). The reaction is catalysed by three families of DNA cytosine methyltransferases: chromomethylase (CMT), domains rearranged methyltransferase (DRM) and methyltransferase (MET). In *Arabidopsis*, DRM2 acts as a de novo methylase, and DRM2, MET1 and CMT3 transfer methylation marks to the newly synthesised strand of DNA during replication (Cao and Jacobsen 2002).

Gene silencing regulated by DNA methylation plays a vital role in SE. For example, the promoter region of *LEC1* becomes hypomethylated prior to initiation of SE, whereas methylation levels increase during embryo maturation and subsequent vegetative growth. Hypermethylation of a region within the promoter of *LEC1* using RNA-directed DNA methylation (RdDM) downregulates its transcription. This indicates that transcription of *LEC1* is regulated by methylation of its promoter (Shibukawa et al. 2009). Experiments with modulation of global DNA methylation levels in vivo using inhibitors of methylation generated inconclusive results. Inhibition of methylation in carrot cultures with 5-azacitidine blocked embryogenesis (Yamamoto et al. 2005). In contrast, inhibition of methyltransferase 1 with the drug 5-aza-2′-deoxycytidine (azadC) promoted embryogenesis and increased transcription of the key embryonic regulator *STM* (Elhiti et al. 2010). 5-azacitidine is known to exert a general cytotoxic effect at higher concentrations (Čihák 1974), which could have been the reason for the inhibition of embryogenesis.

3.3.2.2 Histone Post-translational Modifications

Gene expression can also be regulated on the level of chromatin organisation. Chromatin is a complex of DNA and proteins inside eukaryotic nucleus. The basic repeat unit of chromatin is the nucleosome, a 146-base pair unit of DNA wrapped around an octamer protein core containing two copies of each histone protein subunits H2A, H2B, H3 and H4. Nucleosomes are linked together in a fibre of 30 nm thickness by the histone protein H1. This arrangement is important for packaging long DNA molecules into the small volume of the nucleus and at the same time serves as a mechanism of regulation of DNA replication and gene transcription. Transcriptionally inactive chromatin, called heterochromatin, considered to be more packed, while transcriptionally active euchromatin has more relaxed packing. A complex of proteins responsible for the modification of chromatin organisation facilitates the epigenetic regulation of genes (Jarillo et al. 2009). Post-translational modifications of histones, including methylation of lysine and arginine, acetylation or ubiquitination of lysine and phosphorylation of serine

residues, contribute to the establishment and maintenance of chromatin activity states (Costa and Shaw 2007).

Histone acetylation on ε-amino groups of lysine residues neutralises the overall positive charge of histones and weakens their interaction with the negatively charged DNA. This relaxes chromatin packaging and promotes the transition into the euchromatin state. Histone acetylation-dependent chromatin remodelling modifies transcription of factors regulating embryogenesis and in this way controls transition from proliferation to embryonic growth. Knockdown of *A. thaliana PICKLE,* which encodes subunit CHD3 of histone deacetylase complex, upregulates transcription of *LEC1* and delays transition from embryonic to vegetative growth (Ogas et al. 1999). Treatment of seedlings with a gibberellin synthesis inhibitor increased the penetrance of the *PICKLE* phenotype, indicating that its role in suppressing embryonic growth was partially complemented by a redundant gibberellin-dependent pathway. Furthermore, treatment of *P. abies* embryogenic cultures with the inhibitor of histone deacetylase Trichostatin A prior to induction of embryogenesis repressed embryo development and reduced transcription of a key regulator of embryogenesis, the B3-domain transcription factor *PaVP1* (Uddenberg et al. 2011).

Similar to acetylation, histone methylation modifies chromatin packing, but depending on the site, it may either inhibit or stimulate transcription. For example, S-adenosyl-methionine (SAM)-dependent transmethylation can modulate expression of cell cycle regulators (Jones and Wolffe 1999). Several members of the SAM metabolic pathway were upregulated during early stages of hormone-induced cellular dedifferentiation preceding the establishment of embryogenic cell cultures in cotton (Zhu et al. 2008) and during early embryogenesis in *P. abies* (van Zyl et al. 2003).

In addition to the SAM-dependent pathway, histone methylation-dependent silencing is controlled by the highly evolutionary conserved group of Polycomb (PcG) proteins. The Polycomb Repressive Complex 2 (PRC2) is a histone methyltransferase responsible for trimethylation of histone H3 on lysine 27 (Schubert et al. 2006). Plant PRC2 consists of four subunits: the first subunit is encoded by *CLF* (Curly Leaf), *Swn* (Swinger) and *MEA* (Medea); the second subunit by *FIE* (Fertilisation Independent Endosperm); the third subunit by *MSI1* (Multicopy Suppressor of Ira 1); and the forth subunit is encoded by *FIS2* (Fertilisation Independent Seed 2), *EMF2* (Embryonic Flower 2) and *VRN2* (Vernalisation 2). The PRC2 complex is responsible, in particular, for silencing of *STM* (Schubert et al. 2006). The knockout of *FIE* results in callus outgrowth on germinating seedlings (Bouyer et al. 2011). Reduced activity of PRC2 in double homozygous *clf* and *swn* plants results in callus formation and SE even in the absence of PGR (Chanvivattana et al. 2004).

3.3.2.3 MicroRNAs

MicroRNAs (miRNAs) are small, single-stranded, endogenous transcripts capable of forming a stem-loop hairpin structure that can mediate target gene silencing by RNA cleavage or translational inhibition. In this way, miRNAs can act cooperatively with transcription factors to fine-tune gene expression programmes during cell differentiation and proliferation (Willmann and Poething 2007; Willmann et al. 2011). Multiple examples demonstrate the key role of miRNA in the regulation of cell proliferation in vitro and SE. Analysis of the miRNA profile in embryogenic callus of rice identified several candidates (e.g. miR397 and miR398) with potential roles in maintaining cells in the meristematic state by mediating gene silencing (Luo et al. 2006). Using Japanese larch as a model system, Zhang and colleagues (2010) identified four distinct miRNA families with different expression in embryogenic and non-embryogenic calli. These miRNAs can target to multifunctional transcription factors involved in regulation of development, ABA signal transduction and response to abiotic stress. miRNAs regulate expression of the transcription factors LEC2 and FUS3 in early embryogenesis of *A. thaliana* (Willmann et al. 2011). A detailed analysis of miRNA profiles during successive stages of indirect SE in sweet orange (Wu et al. 2011) identified miRNA specific for early embryo development, globular embryos, cotyledon-shaped embryos and cell lines lacking embryogenic potential.

3.4 Cytoskeleton

The cytoskeleton is a complex network of cytoplasmic filaments and, in plants, is composed of two principal systems: microtubules and actin filaments. The cytoskeleton determines the composition and properties of the cell wall and interacts with the cell wall components in controlling the distribution of organelles and maintaining cytoplasmic architecture and cell shape. In addition, the cytoskeleton provides tracks for the intracellular transport of organelles and molecules during interphase and cell division. Microtubules are hollow tubes of 25 nm in diameter, composed of α- and β-tubulin heterodimers. During interphase, specific microtubules subtend the plasma membrane forming the "cortical array" controlling the direction of cell expansion, whereas a different set of microtubules tether and position the nucleus (for details, refer to the chapter by Nick in this volume). Actin filaments (F-actin) are 7 nm thick fibers composed of two twisted chains of protein actin. Like microtubules, F-actin can be cortical or endoplasmic.

The role of the cytoskeleton in SE was extensively studied during androgenesis (formation of embryos from cultured microspores) in rapeseed, *B. napus*. Direct androgenesis can be induced from immature pollen (microspores) of the responsive cultivar Topaz by heat stress (Simmonds and Keller 1999; Dubas et al. 2011; see also chapter by Opatrný in this volume). The process starts from a single easily accessible microspore cell containing, depending on the stage of development,

either one or two (vegetative and generative) nuclei (for review, see chapter by Lou et al. in this volume). In case of two nuclei, only the vegetative nucleus responds to the heat treatment. In both cases, the otherwise asymmetrically positioned nucleus moves to the centre of the cell, and subsequently, the microspore divides symmetrically. The central positioning of nucleus is important for the induction of androgenesis in rapeseed, but can vary among species. In case of osmosis-induced androgenesis in barley, the uninucleate microspore must divide asymmetrically to produce generative and vegetative nuclei. Contrary to *B. napus* system, the symmetric division of the microspore in barley does not lead to embryonic development (Maraschin et al. 2005).

The effect of heat stress on the induction of androgenesis in *B. napus* can be mimicked by destabilisation of microtubules using colchicine (25 μM for 42 h). This treatment caused relocation of the nucleus to the centre of the cell, followed by symmetric division and induction of androgenesis (Zhao et al. 1996). Similarly, depolymerisation of actin by 15-min treatment with high concentration (20 μM) of cytochalasin D followed by continuous incubation in a medium containing 2 μM cytochalasin D resulted in the relocation of the nucleus to the central position and induction of androgenesis (Gervais et al. 2000). Thus, induction of the embryogenesis might require a more flexible cytoskeleton. The basal cell (which is generally not surrounded by exine; for details, see the chapter by Lou et al. in this volume) undergoes a series of transverse divisions forming a suspensor-like file of 3–8 cells, while the apical cell (constrained by exine) passes through several rounds of transverse and longitudinal divisions leading to the formation of globular embryo proper (Dubas et al. 2011; Tang et al. 2013).

Induction of SE is accompanied by changes in the expression of the cytoskeletal structural proteins. For instance, an α-tubulin in wheat is upregulated (Singla et al. 2007), while α1-tubulin in *P. glauca* and β3-tubulin gene in *P. abies* are downregulated (Lippert et al. 2005; van Zyl et al. 2003). An *A. thaliana* β2/3-tubulin (At5g62700) is a target of the key transcription factor regulating embryogenesis, AGL15 (Zheng et al. 2009). An F-actin regulator actin-depolymerising protein 9 (ADF9) is a target of BABY BOOM during SE (Passarinho et al. 2008). Actin-depolymerising proteins sever microfilament bundles and facilitate plasticity of F-actin required for the morphogenesis.

4 Cell Fate Regulation During SE: "Pro-Death" Signalling

Plant development requires removal of specific organs and tissues (e.g. leaf and flower senescence) by PCD. In addition, PCD is indispensable for the differentiation of some tissues like xylem or bark. Consistently, numerous studies demonstrate the importance of PCD for both zygotic and somatic embryogenesis highlighting elimination of embryo suspensor as the key PCD process that defines the success of embryogenesis.

4.1 Functions of the Embryo Suspensor

The suspensor is a part of the embryo, which does not contribute to the postembryonic phase of plant life. Both the embryo suspensor and embryo proper originate from the zygote following the first division after fertilisation. The apical daughter cell gives rise to the embryo proper, while basal cell develops into suspensor. The embryo proper and suspensor remain interconnected during early stages of embryogenesis.

The suspensor is thought to have co-evolved with the colonisation of land by plants over 450 million years ago (Kawashima and Goldberg 2010) based on the observation of a suspensor-like structure (foot) in bryophytes. In higher plants, the suspensor can have different size and morphology, ranging from only one cell in orchids or a file of seven small cells in *A. thaliana* or *B. napus* to hundreds of cells exceeding the size of the embryo proper in *Phaseolus coccineus* (scarlet runner bean). Somatic embryos form suspensors in many systems (Zimmermann 1993), and like in the case of the zygotic pathway, the suspensor plays an essential role in embryogenesis (Suarez et al. 2004). Three main functions have been attributed to the suspensor: (i) positioning of the embryo in the sack close to endosperm in angiosperms or the megagametophyte in gymnosperms; (ii) conduction of nutrients to the embryo proper using specialised cellular structures responsible for the cell-to-cell transport, including plasmodesmata and cell wall outgrowths (Yeung and Meinke 1993); and (iii) synthesis of the hormones auxin, gibberellic acid, cytokinin and ABA, which are essential for setting up the embryo polarity and regulation of the early stages of embryogenesis (Kawashima and Goldberg 2010).

The suspensor is a terminally differentiated structure that is removed by PCD. The elimination of the suspensor starts at the late globular stage and terminates by the beginning of torpedo stage in angiosperms or during late embryogeny in gymnosperms (Bozhkov et al. 2005a). Therefore, the original cell proliferation programme of the suspensor has to be switched into the self-destruction programme, whereas embryo proper cells continue to express factors facilitating cell proliferation, histogenesis and organ patterning.

Genetic studies highlight the entwining and complex signalling networks, maintaining the balance between embryo formation and suspensor degradation. For example, in *Arabidopsis* mutants *raspberry* and *twin*, suspensor cells are reprogrammed from the cell death pathway to a meristematic fate, resulting in abnormal embryogenesis (Vernon and Meinke 1994; Yadegari et al. 1994; Zhang and Sommerville 1997). Perturbation of suspensor development in mutants of transcription factors (*wox8* and *wox9*), MAP (*yda, mpk3, mpk6*) and receptor (*ssp*) kinases or the vacuole formation gene *vcl1* leads to embryo lethality. In turn, defective development of the embryo proper in the mutants *rsy2*, *rsy3*, *sus1/dcl1*, *sus*, *twn*, *lec1* and *l1l* perturbs suspensor differentiation (Bozhkov et al. 2005a; Kawashima and Goldberg 2010). Inhibition of suspensor differentiation and death during SE has similar prohibitive effect on the progression of embryo development (Suarez et al. 2004; Helmersson et al. 2008).

The balance between survival and development of the embryo proper and death elimination of the suspensor plays a crucial role in zygotic embryo development and has a significant impact on the efficiency of SE. How this balance is established and then maintained is not clear, but intriguingly, the same groups of proteins can play a role in proliferation or cell death, depending on the molecular environment. For example, a member of evolutionary conserved family of the HAP3 transcription factors, LEAFY COTYLEDON1 (LEC1), is essential for specification of cotyledons during early embryogenesis and for the maintenance of suspensor cell fate (Meinke 1992; Meinke et al. 1994; West et al. 1994). Different members of homeodomain containing WUSCHEL transcription factors (WOX) that determine embryo polarity are expressed in the suspensor. WOX2 is specifically expressed in the apical cell, while other members of this family, WOX8 and WOX9, are expressed in the basal cell (Haecker et al. 2004). Understanding the molecular mechanisms of this balance can help to initiate embryogenic cultures and to enhance yield and quality of SE.

4.2 Vacuolar PCD and SE

Considering morphological and molecular markers available to date, two most common types of plant cell death have been categorised: vacuolar and necrotic (van Doorn et al. 2011). Vacuolar death is characterised by appearance of double membrane vesicles known as autophagic vacuoles (aka vacuoles, autophagosomes) that engulf portions of the cytoplasm. The cargo of autophagosomes is digested by fusion with lytic vacuoles (equivalent to the lysosomes of animal cells). Gradually, most of the cytoplasm becomes occupied by lytic vacuoles. The process culminates by rupture of the vacuole and apparently uncontrolled degradation of all remaining cellular content. Necrotic cell death is characterised by mitochondrial dysfunction, early rupture of the plasma membrane, protoplast shrinkage and incomplete processing of the cell corpse. This type of cell death typically occurs during plant responses to intracellular pathogens and acute abiotic stress (van Doorn et al. 2011).

Vacuolar PCD is essential for two main processes in plant embryogenesis: (i) establishment of embryo polarity and (ii) differentiation and subsequent elimination of the suspensor. The changes in the cellular organisation accompanying successive stages of vacuolar PCD during SE have been addressed in detail using the *P. abies* model (Fig. 3). This system has the advantage of developing very large multicellular suspensors of several millimetres in length. The suspensor cells undergo stereotypical stages of cell death similar to those reported in other angiosperm and gymnosperm species (Bozhkov et al. 2005a). Thus, there is a gradient of successive PCD stages along the apical-basal axis starting from the proliferating cells of the embryonal masses, initiation of terminal differentiation and commitment to PCD in the embryonal tube cells, execution of PCD in the proximal cells of the suspensor, and empty walled corpses at the distal end (Fig. 3; Smertenko et al. 2003; Bozhkov et al. 2005a).

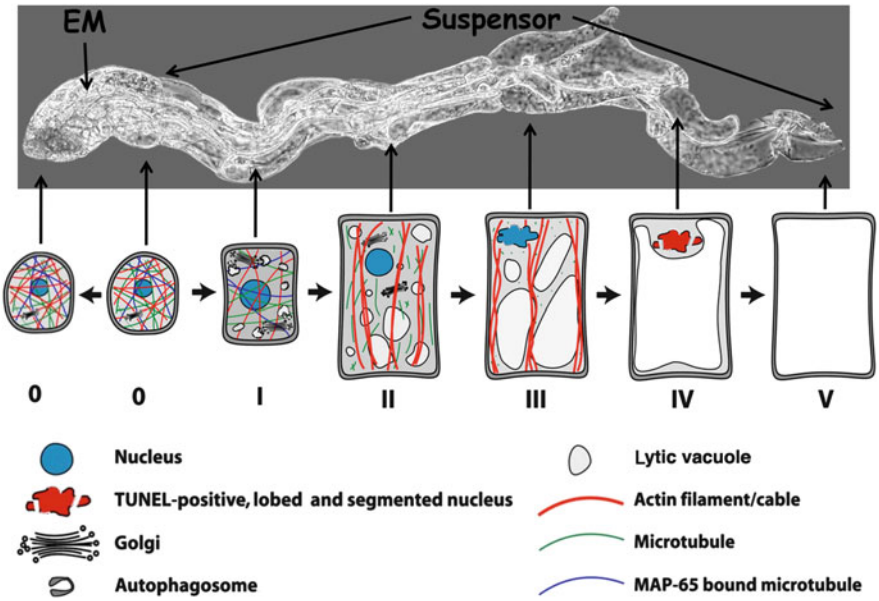

Fig. 3 Programmed cell death during somatic embryogenesis
A proliferating cell in the embryonal mass (stage 0) can either persist in this stage or switch to differentiation becoming an embryonal tube cell (stage I). The embryonal tube cell at some time point commits to PCD and undergoes a series of stereotypical morphological alterations (stages II to IV), which lead to stepwise clearance of all cellular content by stage V. Terminal differentiation and PCD can be observed simultaneously along the apical-basal axis of the early embryo, with each cell tier of the suspensor featuring one stage

Stage 0 represents meristematic cells of embryonal masses/embryo proper. Cell divisions within embryonal mass provide new cells for growth of both embryo and suspensor. How a meristematic cell initiates terminal differentiation on its path towards a suspensor cell is not known. One scenario proposes that cells in the basal part of the embryonal mass undergo asymmetric division generating daughter cells of unequal structure, molecular composition and fate. Here, the cell fate is determined by both cell composition and positional signalling. This scenario has been shown to occur in other plant systems. For example, during the first division of microspore in *B. napus*, the cell with larger contact surface with exine has a dense cytoplasm and lacks vacuoles, while the cell with the least contact surface possesses distinct vacuoles (Tang et al. 2013). Apart from the vacuoles, the rest of cytoplasmic features including microtubule organisation are indistinguishable in both types of cells (Dubas et al. 2011). The vacuolated basal cell divides to generate the suspensor, while the meristematic or apical cell gives rise to the embryo proper. In case of androgenesis in barley, the early embryo consists of two domains with structurally distinct cells (Maraschin et al. 2005). One domain contains small vacuolated cells descending from generative nuclei and destined for PCD. The larger cells with dense cytoplasm in the second domain originate from the

vegetative cells and form the embryo proper. Alternatively, the cell fate within embryonal mass could be determined by its position relatively to hormonal gradients. There is evidence indicating genetic control of the early cell specification events in the *Arabidopsis* proembryo. The basal cell expresses transcription factor WOX8, while apical cells express a homologue of WOX8, WOX2 (Haecker et al. 2004).

The presence of lytic compartments appears to be the earliest morphological marker of suspensor cell differentiation. While the embryo proper/embryonal mass cells in the embryogenic cultures of *P. abies* contain neither lytic nor protein storage vacuoles, embryonal tube cells can be distinguished by de novo formation of autophagosomes (Fílonova et al. 2000a; Fig. 3). In addition, the proliferation ceases, the cell shape changes from spherical to cylindrical, and the cytoskeleton undergoes dramatic reorganisation. The random network of microtubules seen in the meristematic cells becomes transformed into parallel array transversely to the apical-basal axis of the embryo (Fowke et al 1990; Smertenko et al. 2003). The proportion of microtubules bound to microtubule cross-bridging protein MAP65 is diminished in comparison to the embryonal mass cells indicating alterations of microtubule bundling.

All subsequent stages of vacuolar PCD take place in the suspensor, and each stage is typically confined to one tier of cells. Stage 2 is characterised by cell expansion, growing number of autophagosomes, formation of small lytic vacuoles, fragmentation of microtubule network and reduced MAP65 binding to microtubules. The cortical array of microfilaments is substituted by endoplasmic thick longitudinal actin cables (Binarová et al. 1996; Smertenko et al. 2003; Schwarzerová et al. 2010). The transformation of the F-actin network is accompanied by alteration of actin isotypes expression (Schwarzerova et al. 2010). F-actin cables contribute to the regulation of suspensor development by as yet unknown mechanism (Smertenko and Franklin-Tong 2011). Possibly, they regulate spatial distribution of autophagosomes, their fusion and growth of lytic vacuoles. In agreement with this hypothesis, treatment with the actin depolymerisation drugs latrunculin B and cytochalasin D impaired suspensor development and triggered cell death in the embryonal masses (Smertenko et al. 2003). A slight destabilisation of microfilaments by application of low concentrations of latrunculin B promoted cell death in the suspensors and increased the yield of mature embryos consistent with the role of actin in the control of PCD progression (Schwarzerová et al. 2010).

By stage 3, small lytic vacuoles gradually fill most of the cytoplasm and form several large vacuoles. At the same time, anisotropic cell expansion continues, F-actin cables persist, but the microtubule network disintegrates, and only microtubule fragments and tubulin aggregates remain in the cytoplasm. The main morphological feature of this stage is the onset of nuclear dismantling. The nuclear surface forms lobes, which subsequently become detached to produce nuclear segments that eventually are digested by the vacuoles. The nuclear pore complexes disassemble at the sites of nuclear segment dissociation, resulting in chromatin leakage to the cytoplasm.

During stage 4, the large lytic vacuole occupies the whole volume of the cell, F-actin cables disintegrate, the nucleus becomes fragmented, and chromatin aggregates and degrades. Nucleases cleave chromosomal DNA in the easily accessible inter-nucleosomal sites, resulting in accumulation of 50 kbp long chromatin loop-size fragments and 180 bp (and multiples of thereof) nucleosomal fragments (Fílonova et al. 2000a). The degradation of the nucleus and the biogenesis of lytic vacuoles are two autonomous processes. Some nuclei become digested before formation of central lytic vacuole is completed (Fílonova et al. 2000a). Mitochondria remain morphologically intact at this stage, but their biochemical activity is altered. The activity of alternative oxidase and external NADH dehydrogenase as well as fatty acid-mediated uncoupling increases, while the activity of mitochondrial K^+ channel decreases (Petrussa et al. 2009). The vacuolar PCD culminates in the rupture of the tonoplast and release of the lytic enzymes digesting all remaining cellular components and leaving a hollow walled cell corpse (stage 5; Fig. 3). The dead cells detach from the distal end of the suspensor.

Cell dismantling during vacuolar PCD in the suspensor takes about 5 days from the transition of a cell to the embryonal tube to complete clearance of the cell content. The transition from one stage of vacuolar PCD to the next takes approximately 24 h (a time required to form a new tier of the suspensor cells) with the exception of stage 5, which is apparently a swift process (Fílonova et al. 2000b).

4.3 Cell Death Proteases and SE

All metazoan species from worms to humans share three core families of proteins essential for the regulation of PCD: CED-3/caspases, CED4/Apaf-1 and CED-9/Bcl-2 (Aravind et al. 1999, 2001; Koonin and Aravind 2002). On the basis of classification of "domains of death" present in key metazoan proteins, a common ancestral cell death machinery was proposed for all eukaryotic species (Ameisen 2002; Koonin and Aravind 2002). A growing body of bioinformatics and experimental data show a lack of direct homologues to the key animal cell death regulators in plants. However, some of these regulators are replaced by structurally and functionally related proteins.

The caspases belong to a family of *c*ysteine-dependent *asp*artate-specific pro*tease*s (Cohen 1997; Thornberry and Lazebnik 1998). Members of this family are continuously expressed as inactive zymogens in almost all cell types. Following activation by an apoptotic signal, caspases cleave core components of the cytoskeleton, nucleus, signal transduction pathway, protein expression machinery, cell cycle regulators and both positive and negative regulators of cell death (Earnshaw et al. 1999). Combination of all proteolytic events compromises the survival pathways and promotes morphological changes characteristic for apoptosis. Since embryogenesis relies on cell death, caspase mutants exhibit abnormal embryo pattern formation and prenatal or perinatal lethality (Baehrecke 2002).

Plants do not have caspases but possess ancestral structurally related proteases called *metacaspases* (Uren et al. 2000; Tsiatsiani et al. 2011). Unlike caspases, metacaspases prefer substrates with arginine or lysine instead of aspartic acid in P1 position (Vercammen et al. 2004). On the basis of primary sequence and domain analysis, plant metacaspases are subdivided into two types. Type I metacaspases harbour N-terminal prodomain containing a proline-rich and a Zn-finger motifs. Type II metacaspases lack this N-terminal prodomain, but have a linker region between p20 and p10 catalytic subunits.

McII-Pa is the only member of type II metacaspases expressed in the embryo suspensor of *P. abies* (Suarez et al. 2004). McII-Pa is cytoplasmic during the first three stages of PCD, then associates with the nuclear membrane between stages 3 and 4 and finally localises inside the nucleus around condensed chromatin during later stages 4 and 5 (Bozhkov et al. 2005b). Enzymatically active mcII-Pa promotes nuclear lobing, chromatin condensation and DNA fragmentation in vitro and hence was suggested to be responsible for the disassembly of the nuclear envelope and the initiation of chromatin condensation. Knockdown of *mcII-Pa* results in the suppression of vacuolar cell death, inhibition of suspensor formation and failure of embryogenesis (Suarez et al. 2004).

The first natural substrate of mcII-Pa is a multifunctional regulator of gene expression, Tudor staphylococcal nuclease (TSN; Sundström et al. 2009), a member of multimeric complexes responsible for the transcription, mRNA splicing and RNA silencing (Tsiatsiani et al. 2011). TSN is a substrate of caspase-3, indicating that despite different substrate specificity of caspases and metacaspases, their substrates partially overlap. TSN and mcII-Pa are initially localised in the cytoplasm, where mcII-Pa-mediated cleavage of TSN may perturb post-transcriptional RNA processing and promote cell death.

Several other groups of proteases with no structural homology to caspases exhibit caspase-like substrate specificity in cell death-dependent manner (Bozhkov and Lam 2011). The first group is represented by *subtilisin-like serine proteases* (Vartapetian et al. 2011). These proteases recognise a wide range of peptidic substrates containing aspartic acid residue in P1 position, including VEID, VAD, YVAD, IETD, VDVAD and LEHD, but they do not cleave DEVD. On the basis of their substrate specificity, this group was initially named "saspases" for *s*erine *asp*aragine-specific prote*ases*. An alternative name for saspases is phytaspases (Vartapetian et al. 2011). Like caspases, saspases or phytaspases prefer particular residues in the positions P2, P3 and P4. Processing of phytaspases includes cleavage of a prodomain, and under normal conditions, the active enzyme is exported to the apoplast space. Induction of PCD triggers import of phytaspases back to the cytoplasm, where they play a key role in the execution of PCD (Chichkova et al. 2010).

Although the precise role of phytaspases/saspases in embryogenesis remains unknown, we have previously reported that VEIDase activity, typical of this group of proteases, can be detected in the embryonal tube and suspensor cells during early embryogeny of *P. abies* and that this activity plays an important role in the suspensor differentiation and somatic embryo development (Bozhkov

et al. 2004). Pharmacological inhibition of VEIDase activity promotes proliferation of meristematic cell clusters, suppresses suspensor differentiation, prevents cell death and consequently abrogates embryogenesis (Bozhkov et al. 2004). Intriguingly, VEIDase activity was shown to localise in autophagosome-like structures in barley endosperm cells during the early stages of developmental PCD (Boren et al. 2006).

The second group of proteases is constituted by *vacuolar processing enzymes (VPEs)* with an YVADase, caspase-1-like, substrate specificity (Hatsugai et al. 2004). VPE exhibits caspase-like catalytic dyad, the catalytic site and the substrate Asp pocket. In addition, it is produced as inactive precursor and undergoes proteolytic processing to become an active enzyme. The activity of VPEs is upregulated in response to tobacco mosaic virus (TMV) infection and the active enzymes localise to the vacuoles, where they regulate maturation of vacuolar proteins during senescence and stress-induced cell death (Hara-Nishimura and Hatsugai 2011). The role of VPEs in SE remains unknown.

The caspase-3-like activity with the cleavage specificity towards DEVD peptide was attributed to proteasome. This activity is principal for both pathogen-induced cell death and vacuolar cell death in xylem (Hatsugai et al. 2009; Han et al. 2012). A significant increase of DEVDase activity accompanied androgenesis in barley (Maraschin et al. 2005), suggesting proteasome could regulate PCD during embryogenesis.

Recently, a novel regulatory module consisting of cathepsin protease NtCP14 and its inhibitor cystatin NtCYS was shown to play a key role in the differentiation and elimination of suspensor in tobacco embryos (Zhao et al. 2013). High expression levels of NtCYS during early stages of embryogenesis suppress NtCP14 activity and allow suspensor to grow, while downregulation of NtCYS expression during later stages leads to higher NtCP14 activity and to the initiation of PCD. These findings demonstrate that several divergent proteolytic events act as part of the same pathway or in parallel pathways to control initiation and execution of PCD in the suspensor.

4.4 Redox Homeostasis

Many different types of stresses, such as heat, heavy metal, dehydration or osmotic stress, are efficient inducers of SE (Simmonds and Keller 1999; Ikeda-Iwai et al. 2003; Dubas et al. 2011). In agreement with this hypothesis, induction and progression of SE is accompanied by upregulation of genes responsible for production of reactive oxygen species (ROS) and for induction of oxidative stress (e.g. germin-like oxalate oxidase and peroxidase; Caliskan et al. 2004; Thibaud-Nissen et al. 2003; Marsoni et al. 2008). Exogenous application of hydrogen peroxide at low concentrations (0.25 mM) can increase the yield of SE (Luo et al. 2001). Therefore, ROS accumulation might tentatively induce transcription of genes required for the embryogenesis.

Accumulation of ROS is a hallmark of oxidative stress-related cell death (De Pinto et al. 2012), and treatment with hydrogen peroxide can trigger cell death responses in different types of cells including embryos (Sundström et al. 2009; De Pinto et al. 2012). It was suggested that production of ROS triggers expression of *glutathione S-transferase* (*GST*) genes (Nagata et al. 1994; Thibaud-Nissen et al. 2003; Marsoni et al. 2008), which catalyse reversible modification of the active groups of biological molecules and protect them against oxidation during intracellular transport (Cummins et al. 2011; see also chapter by Khan and Hell in the current volume). The spatial profile of *GST* expression in the embryos is not known, but upregulation of *GST* during SE (Joosen et al. 2007) indicates that GSTs protect specific cells in the embryo against premature death and reduction of GST expression allows cell death to proceed.

5 Concluding Remarks

SE starts from single cells or a group of cells with similar morphology and transcriptional profile in response to external stimuli either produced by the surrounding tissue *in plant* or added to the tissue culture medium. These stimuli cause genetic reprogramming that leads to the establishment of cell lineages with altered gene transcription pattern, different morphology and developmental fate. The first result of these multidimensional alterations is the formation of morphologically asymmetric (polar) structure. Following the initiation of SE, the process becomes autoregulatory and can sustain successive stages of the embryogenesis pathway without any or with minimal contribution from the external signals. In spite of how complex the subsequent stages may seem, the original establishment and persistence of this polarity from a physiologically homogenous group of cells, including subsequent terminal differentiation and demise of the embryo suspensor, represents a fundamental paradigm of plant developmental biology.

Transcription of specific sets of downstream genes in the divergent cell lineages during early stages of SE is responsible for the specific cellular physiology and morphology. These downstream genes encode mainly components of the cytoskeleton and membranes, proteases, cell cycle regulators and cell wall-modifying enzymes.

Molecules secreted by embryogenic cultures, such as AGPs and oligosaccharines, can efficiently induce embryogenesis (Fig. 4). The extracellular signals are perceived by receptor kinases at the membrane, primarily by SERKs. SERKs can activate divergent signalling pathways by forming complexes with other receptor kinases and, in addition to plant development, can regulate cell death and response to pathogen attack (Chinchilla et al. 2008). The interleukin-1 receptor kinase/Pelle-like kinase is required for division of the basal cell following the first division of zygote and establishment of the suspensor (Bayer et al. 2009). Therefore, distinct classes of receptor kinases are responsible for the induction of PCD in the suspensor and maintaining proliferation status in the embryo proper.

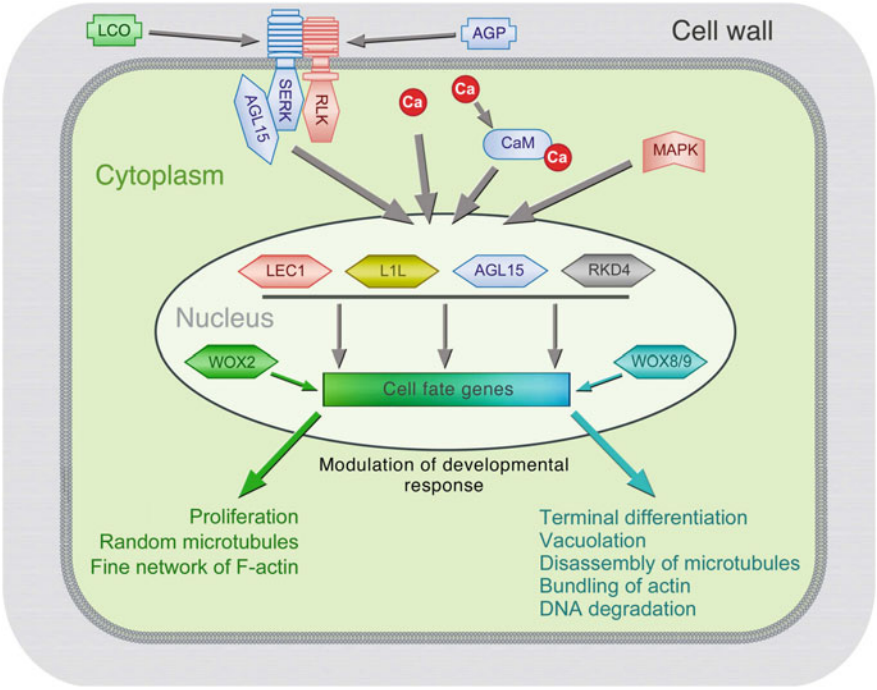

Fig. 4 The key components responsible for the balance of life-death processes during embryogenesis
Proliferation and death of cells are regulated by common key signalling pathways and gene transcription elements. The LCOs and AGPs directly or via interaction with components of the cell walls activate receptor kinases, consequently inducing the rise of intracellular Ca^{2+} concentration, calmodulin activity (CaM), MAP kinases activity and translocation of gene transcription regulatory elements to the nucleus. Some of these elements, like AGL15, are part of the receptor kinase complexes. In the nucleus, transcription factors LEC1, L1L, AGL15 and RKD4 control expression of core cell fate genes essential for both proliferation and terminal differentiation. The switch between the pathways is regulated by specific transcription factors exemplified by WOX2 expressed in the embryo proper and WOX8/9 which is expressed in the suspensor. In addition, cytoplasmic signalling pathways (e.g. depending on positional information) can direct the cell fate

The signal must then reach the nucleus in order to alter transcription patterns. Several pathways could be involved in the signal transduction. First is calcium-dependent pathway involving calmodulin and calmodulin domain kinases. Second is calcium-independent protein phosphorylation mediated by mitogen-activated kinases. Third, transcription factors can directly participate in the signal perception complex. For example, a MADS domain transcription factor, AGL15, is a component of SERK complex (Karlova et al. 2006) and relocates from cytoplasm into the nucleus of cells in embryo suspensor and embryo proper during early stages of embryogenesis (Perry et al. 1996). AGL15 upregulates expression of several transcription factors essential for embryogenesis including LEC2, FUS3 and

ABI3 (Zheng et al. 2009) and has a predicted phosphorylation motif RQVT20 within the conserved MADS domain (http://phosphat.mpimp-golm.mpg.de/; Heazlewood et al. 2008). Therefore, phosphorylation by SERK or other kinases could regulate AGL15 activity.

The establishment of apical-basal gradients during zygotic embryogenesis is controlled by WOX homeobox family of transcription factors. WOX8 and WOX9 are expressed in the basal cell, which will give rise to the terminally differentiated suspensor, while WOX2 is expressed in the apical cell destined to form the embryo proper. The RWP-RK transcription factor RKD4 regulates development of both embryo proper and suspensor (Waki et al. 2011; Jeong et al. 2011).

An extensive body of evidence accumulated during more than half a century of studies showcases the requirement of dynamic balance between pro-life and pro-death processes during SE. Understanding this balance is important to induce SE in recalcitrant species and to improve the yields of generated somatic seedlings by the existing biotechnologies. The original "symptomatic" analyses on the "macro-morphological" level have been recently complemented by cytological, biochemical and molecular studies. Owing to the genetically and physiologically diverse models used in this field to date, discrepancies have to be resolved. However, sequencing the genomes of many common SE models in combination with postgenomic technologies and more traditional techniques will help to sharpen the main concept, where the balance of "pro-life" versus "pro-death" signalling towards cell fate determines the induction and efficiency of SE.

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Molecular Cell Biology of Pollen Walls

Yue Lou, Jun Zhu, and Zhongnan Yang

Abstract The pollen wall comprises the outer exine and the inner intine layers. It plays important roles in protecting pollen from various environmental stresses including microbial attack and in cell-cell recognition during pollination. The exine is further divided into a sexine and a nexine layer. The material for the exine is provided directly by the tapetal cells. The pollen wall of each plant has its unique pattern. After meiosis, the four microspores are enwrapped by callose to form a tetrad. The pollen-wall pattern is determined at tetrad stage. In contrast, the intine is synthesized by the microspore itself. Many genes have been identified from male-sterile mutants in *Arabidopsis thaliana* and rice during recent years. The majority of these genes are involved in pollen-wall formation including tapetal development, sporopollenin biosynthesis and transport, callose wall and primexine deposition. This chapter introduces the recent advance of pollen-wall formation in genetic and molecular level.

The life cycle of angiosperms can be subdivided into vegetative development culminating in the mature sporophyte and reproductive development including the formation of the gametophyte generation, pollination and fertilization. The pollen grains corresponding to the male gametophytes are formed in the anther, where the reproductive microsporocytes are neighboured to nonreproductive cell layers. During development of the pollen grain, the pollen wall forms as a robust and viscous layer covering the pollen grain. The biological function of this pollen wall is to separate the microspore from the paternal tissue during its development in the anther, to provide physical and chemical resistance against environmental stresses in the mature pollen to ensure its survival and to provide a species-specific adhesion to the stigma surface. However, the structure of the pollen wall not only

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mediates important biological functions but provides insight into the dynamics of plant phylogeny and reports the genetic mechanism underlying pollen-wall ontogeny. Recent research shows that most phenotypes of male sterility are connected with the abnormal development of the pollen wall. In this review, we concentrate on the genesis of each layer in pollen wall and outline the events that are essential during pollen-wall development.

1 Overview of the Angiosperm Pollen Wall

1.1 Structure of the Pollen Wall

Pattern and structure of the pollen wall represent an important feature of plant taxonomic classifications and forensic identifications and therefore have been described for many species (Cutter 1971; Stanley and Linskens 1974; Blackmore and Barnes 1990; Scott 1994). Despite the morphological diversity among taxa, the principal structure of the pollen wall shares general principles (Fig. 1). The pollen wall consists of two main layers, the outer exine and inner intine. The exine can be further divided into the sexine (a reticulate layer) and the nexine (a flat layer). The sexine consists of a so-called baculum and a tectum, sculpted in a taxon-specific manner. The exine is subtended by the nexine that acts as skeleton for the exine. In contrast to the complex exine, the intine is a relatively simple layer, which is deposited between the plasma membrane and nexine. Finally, the pollen coat or tryphine fills the spaces between the baculum to surround the sculpted pollen wall.

1.2 Development of the Pollen Wall

Although the development of the pollen wall varies among species, the fundamental sequence of this process has been elucidated in detail (Scott 1994; Owen and Makaroff 1995; Blackmore et al. 2007; Ariizumi and Toriyama 2011). Pollen-wall formation initiates at the late stage of meiosis. The microsporocytes secrete callose onto the plasma membrane to form a callose wall. After meiosis, the four microspores are wrapped inside this callose wall to form a tetrad. In the tetrad, the primexine is deposited between the callose and the plasma membrane. It acts as a template for the sexine-sculpting pattern. When the plasma membrane becomes undulated, the sporopollenin precursors secreted by tapetum are deposited at the peaks of undulated membrane to form probaculae and protectum. Subsequently, callose and primexine are completely degraded, and the nexine layer appears surrounding the plasma membrane in the released microspore. Upon continuous addition of material derived from the tapetum, the sexine increases in size and associates with the nexine to accomplish the exine structure. Once the nexine layer

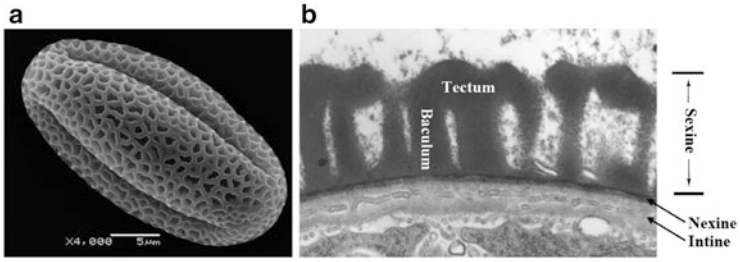

Fig. 1 Scanning (**a**) and transmission electron microscopy (**b**) of a mature pollen grain in Arabidopsis

has been formed, the intine is laid down between the plasma membrane and nexine layer and extends covering the entire microspore. Finally, the intine increases in thickness, and the pollen coat (tapetal fragments) is added to the exine cavities (Fig. 2).

1.3 Constituents of the Pollen Wall

The exine is mainly made up of sporopollenin, which is highly resistant to non-oxidative physical, chemical and biological degradation. Due to the small amounts of material, the insolubility of sporopollenin and technical limitations, the details of sporopollenin components and structure are far from understood. Moreover, there is evidence for differences in chemical pathways and modifications of sporopollenin between species, adding further complexities (Edlund et al. 2004). Compared with the complex exine, the components of intine are rather similar to the primary walls of plant cells, including cellulose, hemicellulose, pectin and proteins (Brett and Waldron 1990). As third component, the pollen coat accounts for 10–15 % of total pollen mass (Piffanelli et al. 1997) and is mainly composed of nonpolar esters and very long-chain wax esters (Scott and Strohl 1962; Bianchi et al. 1990).

2 Tapetum Plays an Essential Role in Pollen Wall Formation

2.1 Tapetum Development

The tapetum layer occurs universally for the land plants. It is of considerable physical significance because most nutrients for the pollen development are produced by, stored in and transported from the tapetum (Dickinson 1982). When

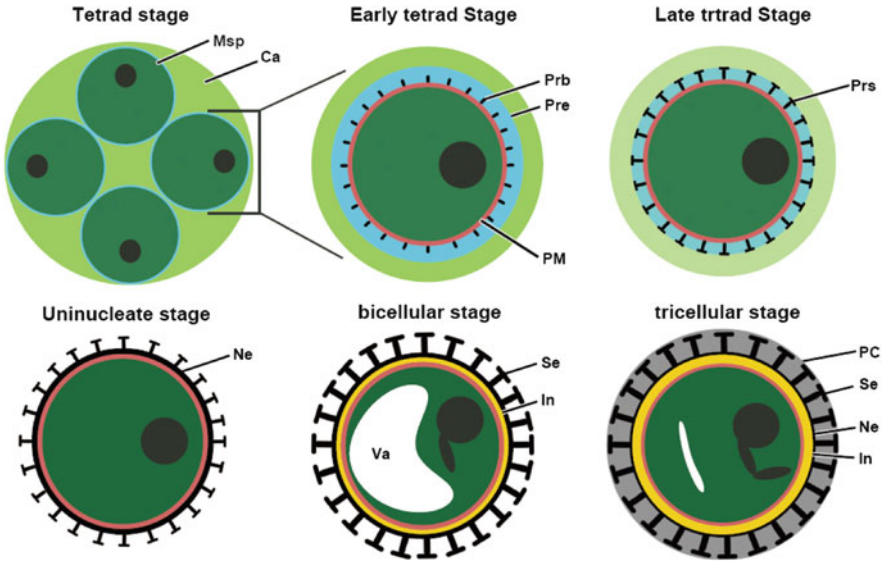

Fig. 2 Diagrammatic views of pollen-wall formation. *Msp* microspore, *Ca* callose, *Prb* probaculum, *Pre* primexine, *PM* plasma membrane, *Prs* prosexine, *Ne* nexine, *Se* sexine, *In* intine, *PC* pollen coat

RNase is expressed in the tapetum by means of a tapetum-specific promoter, this leads to male sterility, indicating an intimate association between tapetum and microspore development (Mariani et al. 1990). There exist two major types of tapetum in angiosperms. The secretory tapetum remains in its original position with the anther wall and finally autolyses (as in *Arabidopsis* and *Lilium longiflorum*). In contrast, the amoeboid tapetum forms a periplasmodium to intrude between the developing microspores (as in *rhoeo discolour* and *Tradescantia bracteata*) (Pacini et al. 1985). Tapetum development has been traced back in *Arabidopsis* to the L2 layer as one of three 'germ' layers (L1, L2 and L3) in the stamen primordia. The L2 layer gives rise to the primary parietal cells, secondary parietal cells and the L2-derived archesporial lineage. The tapetum is specified from this archesporial lineage as the innermost one of four somatic cell layers (Sanders et al. 1999). During meiosis, the tapetum undergoes dramatic morphological changes to prepare for its nutritive function for the microspores (Echlin 1971). The cytoplasm is condensed and packed with ribosomes, mitochondria, Golgi bodies, endoplasmic reticulum and vesicles. The tapetal cells pass mitosis without cytokinesis to form binuclear daughter cells. At a late stage of meiosis, the tapetum develops into polar secretory cells lacking a primary cell wall (Stevens and Murray 1981; Bedinger 1992). Meanwhile, the hallmarks of programmed cell death (PCD) become evident in the tapetum (Varnier et al. 2005). Following meiosis, the tapetum begins to provide the precursors of sporopollenin from the inner tangential face and the intercellular tapetal space to execute the pollen exine formation (Pacini and Juniper

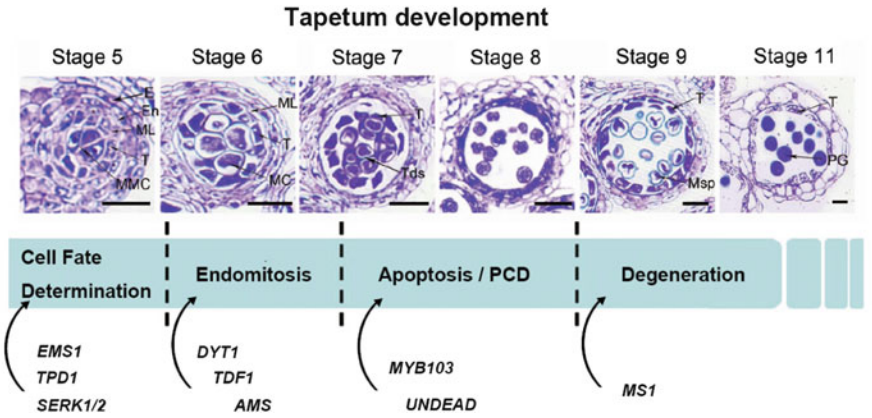

Fig. 3 Light microscope photograph of cross sections of *Arabidopsis* tapetum at different stages based on the 14 stages of anther ontogeny (Sanders et al. 1999). Tapetum development with main events at different anther stages includes cell fate determination, endomitosis, apoptosis/PCD and degeneration. Genes required for the respective processes are given for the different stages

1979). Later, the tapetum secretes the callase complex (also termed β-1,3-glucanase) to dissolve the callose wall, releasing microspores from the tetrad (Stieglitz 1977). After completion of the first microspore mitosis, the tapetum accumulates numerous elaioplasts and cytoplasmic lipid bodies for pollen coat formation. This material is discharged into the cavities of the exine surface after the tapetum degeneration (Mascarenhas 1975; Hesse and Hess 1993). The normal developmental process of tapetum in Arabidopsis is shown in Fig. 3.

The formation of binucleate cells and large stacks of extensive endoplasmic reticulum (ER) support a nourishing function of the tapetum for microspores/pollen-grain development including pollen-wall formation. At the meiotic stage, the tapetal nucleus undergoes divisions deviating from conventional mitosis and/or nuclear fusions. In *Zea mays*, the division takes place in the ordinary way, but without formation of a cell plate. The two daughter nuclei remain inside the tapetal cell. For other species, various types of division peculiarities have been described (Maheshwari 1950). However, the mechanism for these specific nuclear divisions is still unclear. The tapetum shows extensive ER stacks fused with the plasma membrane (Owen and Makaroff 1995). This might facilitate protein synthesis and secretory activity to release materials into the locule. In pollen development, the ER is the major site for glycerolipid biosynthesis (Benning 2008). AtGPAT1 and AtGPAT6 are members of the glycerol-3-phosphate acyltransferase (GPAT) family, which mediate the initial synthetic step of glycerolipid biosynthesis (Zheng et al. 2003; Li-Beisson et al. 2009; Li et al. 2012). Disruption of the *AtGPAT1* or *AtGPAT6* genes causes defective tapetum development with reduced ER profiles, and irregular exine deposition, leading to a partial degradation of pollen grains

(Zheng et al. 2003; Li et al. 2012). In the double mutant of *GPAT1* and *GPAT6*, the defective callose dissolution affects microspore release from tetrads (Li et al. 2012).

PCD is required for development and maintenance in many multicellular organisms (Vaux and Korsmeyer 1999, see also chapter by Smertenko and Bozhkov, this volume). It has been proposed that tapetum degeneration involves programmed cell death (PCD). The cytoplasmic or structural components of the degraded tapetum serve important functions during pollen maturation (Wu and Cheung 2000). Cytological features of PCD include cell shrinkage, condensation of chromatin, swelling of ER and persistence of mitochondria (Papini et al. 1999). A proper timing of tapetum degeneration is necessary for a normal microsporogenesis. Premature or delayed tapetal PCD causes male sterility. A plant aspartic protease is associated with tapetal degeneration. The *UNDEAD* gene encodes an A1 aspartic protease in Arabidopsis, and silencing of *UNDEAD* with siRNA leads to apoptosis-like PCD in premature tapetal cells (Phan et al. 2011). Two aspartic protease-encoding genes in rice, *OsAP25* and *OsAP37*, can promote cell death in both yeast and plant. The mutation of ETERNAL TAPETUM 1 (EAT1), an upstream regulator of OsAP25/37, delays tapetal PCD (Niu et al. 2013).

2.2 Genetic Pathway of Tapetum Development and Functions

Many genes involved in tapetum development and functions, especially those related to pollen-wall formation, have been identified in Arabidopsis (see Table 1). Among them, the *EXCESS MICROSPOROCYTES1* (*EMS1*)/*EXTRA SPOROGENOUS CELLS* (*EXS*) and *TAPETUM DETERMINANT1* (*TPD1*) genes trigger the signalling pathway for tapetal fate determination during early development (Ma 2005; Zhao et al. 2002; Canales et al. 2002; Yang et al. 2003). Later, several transcriptional factors regulate tapetum differentiation and pollen-wall formation. The genes *DYSFUNCTIONAL TAPETUM1* (*DYT1*) and *ABORTED MICROSPORES* (*AMS*) encode putative basic helix-loop-helix (bHLH) transcription factors (Zhang et al. 2006; Sørensen et al. 2003), whereas *DEFECTIVE in TAPETAL DEVELOPMENT and FUNCTION1* (*TDF1*) encodes a putative R2R3 MYB transcription factor (Zhu et al. 2008). These three genes are expressed in the tapetum and meiocytes/microspores during early development. Mutations in these genes cause tapetal hypertrophy extending into the locule and resulting in sporophytic male sterility. A further member of the R2R3 MYB family, *AtMYB103* (also named *MS188* or *MYB80*), apparently regulates the sexine formation, since in the respective mutant, the sexine layer is completely absent (Zhang et al. 2007; Zhu et al. 2010). AtMYB103 directly regulates a gene encoding an A1 aspartic protease named UNDEAD (Phan et al. 2011) leading to the model that the AtMYB103/UNDEAD system may regulate the timing of tapetal PCD, consistent with the observation that precocious PCD occurs in *atmyb103* (Zhu et al. 2010). Mutation of

Table 1 Related genes of pollen wall development

Development process	Gene	Locus	Function	Protein	Reference
Tapetum development	*EMS1/EXS*	AT5G07280	Tapetal differentiation	LRR receptor protein kinase	Zhao et al. (2002) Canales et al. (2002)
	TPD1	AT4G24972	Tapetal differentiation	A novel small protein	Yang et al. (2007)
	SERK1	AT1G71830	Tapetal differentiation	LRR receptor protein kinase	Albrecht et al. (2005)
	SERK2	AT4G21330	Tapetal differentiation	LRR receptor protein kinase	Colcombet et al. (2005)
	DYT1	AT4G21330	Early tapetal development	bHLH transcription factor	Zhang et al. (2006)
	TDF1	AT3G28470	Early tapetal development	MYB transcription factor	Zhu et al. (2008)
	AMS	AT2G16910	Early tapetal development	bHLH transcription factor	Sorensen et al. (2003) Xu et al. (2010)
	AtMYB103	AT5G56110	Tapetal PCD and exine formation	MYB transcription factor	Zhang et al. (2007)
	MS1	AT5G22260	Tapetal PCD and exine formation	PHD-finger transcription factor	Wilson et al. (2001) Yang et al. (2007) Ito et al. (2007)
	AtGPAT1	AT1G06520	Glycerolipid biosynthesis	Glycerol-3-phosphate acyltransferase	Zheng et al. (2003)
	AtGPAT6	AT2G38110	Glycerolipid biosynthesis	Glycerol-3-phosphate acyltransferase	Li et al. (2012)
Callose formation	*CalS5*	AT2G13680	Callose synthesis	Callose synthase	Dong et al. (2005) Nishikawa et al. (2005)
	CDKG1	AT5G63370	Callose synthesis regulation	Cyclin-dependent kinase	Huang et al. (2013)
	ARF17	AT1G77850	Callose synthesis regulation	Auxin response factor	Yang et al. (2013)

(continued)

Table 1 (continued)

Development process	Gene	Locus	Function	Protein	Reference
Primexine formation	*NPU1*	AT3G51610	Primexine deposition	Membrane protein	Chang et al. (2012)
	RPG1	AT5G40260	Primexine deposition	Sugar efflux transporter	Guan et al. (2008) Chen et al. (2010)
	DEX1	AT3G09090	Primexine deposition	Ca^{2+}-binding protein in secretory pathway	Paxson-Sowders et al. (1997) Paxson-Sowders et al. (2001)
	NEF1	AT5G13390	Primexine deposition	Putative plastid inner envelope membrane protein	Ariizumi et al. (2004)
Lipid metabolism and transporter	*ACOS5*	AT1G62940	Medium-chain fatty acids metabolism	Fatty acyl-CoA synthetase	de Azevedo Souza et al. (2009)
	LAP5	At4G34850	Fatty acids metabolism	Polyketide synthases	Kim et al. (2010)
	LAP6	At1G02050	Fatty acids metabolism	Polyketide synthases	Kim et al. (2010)
	CYP703A2	At1G01280	Medium-chain fatty acids metabolism	Hemethiolate monooxygenase (P450)	Morant et al. (2007)
	CYP704B1	AT1G69500	Long-chain fatty acids metabolism	Hemethiolate monooxygenase (P450)	Dobritsa et al. (2009)
	MS2	AT3G11980	Sporopollenin secretion	Putative tapetum microbody located fatty acid reductase	Aarts et al. (1997)
	TKPR1	AT4G35420	Fatty acids metabolism	Tetraketide alpha-pyrone reductase	Grienenberger et al. (2010)
	TKPR2	AT1G68540	Fatty acids metabolism	Tetraketide alpha-pyrone reductase	
	ABCG26	AT3G13220	Lipid transport	ATP binding cassette transporter	Quilichini et al. (2010) Dou et al. (2011) Choi et al. (2011)
Pollen aperture	*Inp1*	AT4G22600	Pollen aperture formation	Unknown protein	Dobritsa and Coerper (2012)
Callose dissolution	*A6*	AT4G14080	Callose dissolution	Glycoside hydrolase	Hird et al. (1993)
Intine formation	*AtUSP*	AT5G52560	Cell wall components biosynthesis	UDP-sugar pyrophosphorylase	Schnurr et al. (2006)

	CESA1	AT4G32410	Cellulose biosynthetic process	Cellulose synthase	Desprez et al. (2007)
	CESA3	AT5G05170	Cellulose biosynthetic process	Cellulose synthase	Desprez et al. (2007)
	RGP1	AT3G02230	Polysaccharide biosynthesis	Glycosylated polypeptides	Drakakaki et al. (2006)
	RGP2	AT5G15650	Polysaccharide biosynthesis	Glycosylated polypeptides	Dhugga et al. (1997)
	FLA3	AT2G24450	Cellulose deposition	Fasciclin-like arabinogalactan protein	Li et al. (2010)
Pollen coat	*FLP1*	AT5G57800	Pollen coat deposition	Lipid transfer protein	Ariizumi et al. (2003)
	CER1	AT1G02205	Wax biosynthesis pathway	Membrane decarbonylase	Jenks et al. (1995) Aarts et al. (1995)

the nuclear protein MS1, containing a leucine zipper-like and PHD-finger motives, causes tapetum vacuolation and defects in exine structure (Wilson et al. 2001; Vizcay-Barrena and Wilson 2006; Ito et al. 2007; Yang et al. 2007). Compared with the transcription factors *DYT1*, *AMS* and *TDF1*, the expression of the *AtMYB103* and *MS1* genes in tapetum and microspores occurs during a later developmental stage (Zhu et al. 2011).

Based on gene-expression profiling, several genetic networks have been proposed for tapetal development and pollen formation in Arabidopsis (Feng et al. 2012; Zhu et al. 2008; Xu et al. 2010; Phan et al. 2011; Ito et al. 2007; Yang et al. 2007; Wijeratne et al. 2007). Among these genetic networks, the transcriptional regulatory pathway involving DYT1-TDF1-AMS-MS188-MS1 could be confirmed by in situ hybridization analysis in the respective mutant background and by the phenotype of double mutants *dyt1–3 tdf1*, *tdf1 ams-2* and *ams-2 ms188–3* (Zhu et al. 2011). In this genetic pathway (Fig. 3), DYT1, TDF1 and AMS are sequentially activated to regulate early tapetum development, whereas AtMYB103 and MS1 are sequentially activated for late tapetum development and pollen-wall formation (Zhu et al. 2008, 2011).

3 Biosynthesis and Transport of Sporopollenin

Sporopollenin is assumed to consist of the heterogeneous materials derived from long-chain fatty acids, oxygenated aromatic rings and phenylpropionic acids (Guilford et al. 1988; Wehling et al. 1989; Wiermann and Gubatz 1992; Wilmesmeier et al. 1993; Piffanelli et al. 1998; Ahlers et al. 1999; Meuter-Gerhards et al. 1999). Further analyses including Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) and X-ray photoelectron spectrometry (XPS) have elucidated that the sporopollenin polymer has a uniform composition which may be linked via ether bridges (Bubert et al. 2002). By the hydrolysis and methylation py-GC/MS method, two UV-absorbing monomers of sporopollenin have been found in pollen (Blokker et al. 2005). Other studies on the chemical similarities between the walls of spores and pollen in early land plants suggest that the first land plants may have evolved the sporopollenin polymers to protect their spores, such that sporopollenin may have arisen as the first polyester-based extracellular matrix in plant (Bowman et al. 2007; Morant et al. 2007).

The complex biochemical pathways in the tapetum leading to the sporopollenin monomers required for pollen exine formation have been well documented (Fig. 4) (Ariizumi and Toriyama 2011). First, lauric acids (C_{12}) are esterified in the plastids of the tapetum to CoA by the fatty acyl-CoA synthetase ACOS5 (de Azevedo Souza et al. 2009). Subsequently, the resulting CoA esters enter the ER and are hydrolysed by a putative thioesterase for the regeneration of lauric acids. These lauric acids are then hydroxylated by specific members of the cytochrome P_{450} family in the ER, such as the *Arabidopsis* CYP703A2 that could be shown in vitro to efficiently catalyse the monohydroxylation at carbon atom 7 of lauric acids (Morant et al. 2007), whereas

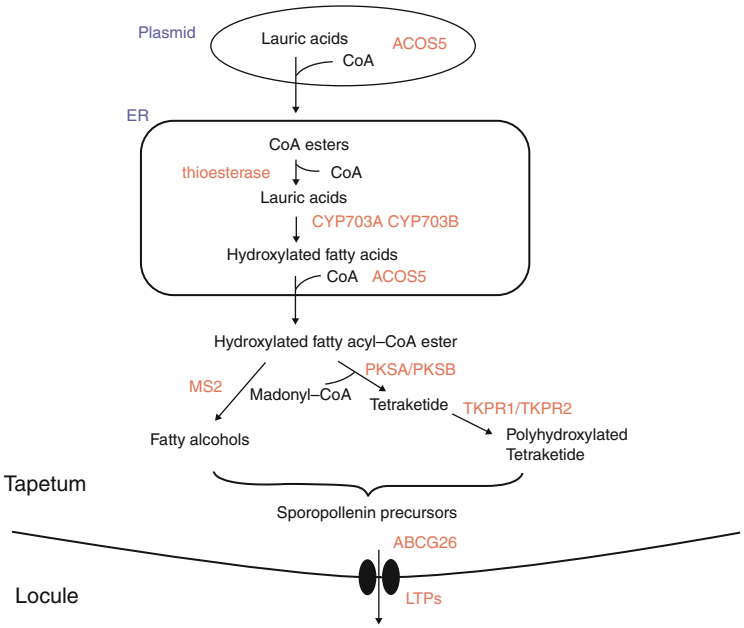

Fig. 4 Model for sporopollenin biosynthesis in the tapetum. Enzymes encoded by genes with known functions in sporopollenin biosynthesis are indicated in *red*. Fatty acids synthesized in plastids are esterified to CoA by ACOS5 and then hydrolyzed by a putative thioesterase in ER. The hydroxylated fatty acids produced by CYP_{450}s are regenerated to CoA esters by ACOS5. Sporopollenin monomers, including the fatty alcohols and tetraketide products which are catalysed by MS2 and PKSs/TKPRs, respectively, are exported by ABCG26 and lipid transfer proteins (LTPs) to the locule as sporopollenin building units for pollen-wall formation

CYP704B1 preferentially catalyses end-of-chain hydroxylation of longer fatty acids (C14 to C18) (Dobritsa et al. 2009). These findings suggest that at least two types of differentially hydroxylated fatty acids may serve as monomeric building blocks during the formation of esters from hydroxylated fatty acids to export them across the membrane, providing the substrates for reduction by the gene product of *Male Sterile 2* (*MS2*) (Schnurr et al. 2004; Morant et al. 2007). *MS2* encodes a fatty acyl ACP (Acyl Carrier Protein) reductase, which can convert palmitoyl-ACP to fatty alcohols providing the monomeric constituents of sporopollenin (Chen et al. 2011).

The second pathway required for sporopollenin biosynthesis is the formation of phenylpropanoids (Dobritsa et al. 2010). In *Arabidopsis*, *POLYKETIDE SYNTHASE A* (*PKSA*) and *PKSB* (also named *LAP6* and *LAP5*) encode chalcone synthases with anther-specific expression, involved in flavonoid biosynthesis (Dobritsa et al. 2010; Kim et al. 2010). Both proteins accept fatty acyl-CoA esters as reaction substrates and condense them to malonyl-CoA, yielding triketide and tetraketide α-pyrones as reaction products (Kim et al. 2010). Sequentially, TETRAKETIDE α-PYRONE REDUCTASE1 (TKPR1) and TKPR2 (previously

called DRL1 and CCRL6) reduce the carbonyl function of tetraketide α-pyrone compounds synthesized by PKSA/PKSB (Grienenberger et al. 2010). Genetic evidence suggests that the pairs of PKS and TKPR enzymes cooperating during phenylpropanoid biosynthesis also contribute to the sporopollenin precursors for exine formation (Grienenberger et al. 2010).

The sporopollenin originates from the tapetum as shown by ultrastructural evidence from different plant species (Heslop-Harrison 1968). For instance, the exine material lines the tapetal margin close to the surface of microspores in the *Gramineae* (Rowley et al. 1959), and the dark particles of tapetal cells observed in *Silene pendula* were found to be precursors presumably containing sporopollenin that eventually deliver the exine materials (Heslop-Harrison 1962). Therefore, the sporopollenin synthesized in the tapetum must be transported to the surface of the developing microspores. In Arabidopsis, ABCG26/WBC27, a member of the ATP-binding cassette (ABC) transporter superfamily, has been identified as potential transporter to export fatty alcohols and other derived monomers from tapetal cells to the surfaces of microspores during the formation of the exine layer. TEM analyses revealed a lack of sporopollenin deposition in the *abcg26-1*/*wbc27-1* mutant, resulting in absence of probacula, bacula and tectum (Choi et al. 2011; Quilichini et al. 2010; Dou et al. 2011). Furthermore, the *ABCG26*/*WBC27* is the direct target of transcript factor AMS, indicating that the sporopollenin translocation is under genetic control of the tapetal cells (Xu et al. 2010).

4 Pollen-Wall Pattern

The pollen-wall pattern is both under gametophytic and sporophytic control. It is determined by restriction of callose deposition, secretion of the primexine matrix, undulation of the plasma membrane and deposition of sporopollenin (Heslop-Harrison 1971; Sheldon and Dickinson 1983; Blackmore and Barnes 1987; Southworth and Jernstedt 1995). The initial pollen-wall pattern is laid down during the tetrad stage (Schmid et al. 1996). At this stage, each tetrad of microspores has already been casted by a callose wall, which is formed subsequently to meiosis. Then, the primexine is deposited between the callose wall and the plasma membrane of the microspore. Meanwhile, at the plasma membrane of the microspore, undulations appear, whose protrusions will be the sites for future probacular formation. Following the accumulation of sporopollenin, the probacula elongate, the protectum is formed adjacent to the callose, and the plasma membrane gradually returns to a smooth surface. Finally, the exine maturates by completion of the nexine and impregnation with sporopollenin, after the callose and primexine have disappeared (Paxson–Sowders et al. 1997).

4.1 Callose Wall Synthesis

Callose, a β-1,3-glucan polymer, is synthesized by callose synthase complexes which are presumed to be located in the microsporocyte membrane (Roberts 1990; Kauss 1996). After synthesis, callose surrounds the microsporocytes throughout entire meiosis. During meiosis and cytokinesis, a callosic septum grows centripetally to separate the individual microspores (Bhandari 1984; Cresti et al. 1992). Several biological functions of callosic walls have been proposed: (1) as mechanical barrier, callose fulfils an important role as a temporary wall that separates microsporocytes or the microspores themselves, such that they can disperse as single cells (Waterkeyn 1962); (2) as chemical barrier, it functions as a molecular filter isolating the developing microspores from the influence of the diploid tissue (Heslop-Harrison 1964); (3) as a source of glucose for the development of the cellulosic primexine, which provides the basic framework of the future exine (Larson and Lewis 1962); and (4) as a physical support for primexine assembly, which nucleates primexine subunits, increasing their local concentration and preventing them from diffusing into the anther locule (Nishikawa et al. 2005). However, it is uncertain whether the callose wall can directly act as a mould for the pollen-wall pattern. In *Ipomoea purpurea*, callose chambers mirror the imprint of the primexine matrix and were suggested to act as template for the primexine matrix and to define sculpturing patterns for the exine (Waterkeyn and Beinfait 1970). However, in some other species such as *Vigna* and *Caesalpinia*, the callose wall does not parallel the reticulate pattern of the exine (Takahashi 1989).

In *Arabidopsis*, 12 *CALLOSE SYNTHASE* (*CALS*) or *GLUCAN SYNTHASE-LIKE* (*GSL*) genes have been identified and classified into one gene family (Hong et al. 2001). In the tetrad, peripheral callose and interstitial callose are synthesized by different callose synthases. In the knockout mutant *cals5-2*, the peripheral callose of the tetrad is completely absent while the interstitial callose can still be observed. This shows that *CalS5* (*Gsl2*) is responsible for the synthesis of peripheral callose (Dong et al. 2005). Subcellular localization showed that both Gsl1 and Gsl5 are located on the cell plate, suggesting that they may be involved in interstitial callose synthesis (Hong et al. 2001; Enns et al. 2005). Recently, it has been reported that Auxin Response Factor 17 (ARF17) can directly bind the *Cals5* promoter region to regulate its expression for callose synthesis. In the *arf17* mutant, callose is significantly reduced and the primexine is absent, resulting in pollen-wall patterning defects (Yang et al. 2013). Auxin plays important roles during the entire lifespan of a plant, which affects cell division, elongation and differentiation (Ljung 2013, see also chapter by Skůpa et al., this volume). ARFs are the major component of auxin signalling. It is likely that auxin may regulate the pollen-wall pattern through ARF17. Similar to auxin, CDKs as further regulator of the cell cycle were found to participate in pollen-wall patterning. CDKs have been originally identified as key regulators of cell cycle transition by binding to their regulatory cyclin partners inducing their kinase activity (Morgan 1997). However, ablation of CYCLIN-DEPENDENT KINASE G1 (CDKG1), a member of this family of

cyclin-dependent protein kinases, resulted in aberrant callose deposition and defective pollen-wall formation during microspore development of Arabidopsis. The pre-mRNA splicing of the CalS5 gene was defective in *cdkg1* mutant. CDKG1 is proposed to be recruited to U1 snRNP through RSZ33 to facilitate the splicing of CalS5 for callose synthesis and pollen-wall pattern (Huang et al. 2013). Thus, CDKs might also act as splicing regulators for gene expression. The expression of CalS5 is regulated by both ARF17 and CDKG1, which shows that callose synthesis for pollen-wall formation is under complicated control.

4.2 Primexine Deposition and Plasma Membrane Undulation

The primexine is mainly composed of polysaccharides, proteins and cellulose (Heslop-Harrison 1963; Rowley and Southworth 1967; Dickinson and Heslop-Harrison 1977). It is initially delivered by Golgi-derived vesicles to the space between the microspore plasma membrane and the callose layer (Fig. 5a) (Dickinson and Sheldon 1984). After the primexine is formed, the microspore plasma membrane becomes undulated (Dahl 1986; Dickinson and Sheldon 1986; Skvarla and Rowley 1987; Takahashi 1993) (Fig. 5b). Subsequently, the undulating plasma membrane produces conspicuous peaks, and the probacula are extruded onto the peaks (Fig. 5c). Finally, the protectum is formed next to the callose to complete the exine pattern (Fig. 5d).

The primexine and the membrane undulation represent decisive factors to regulate the development of pollen-wall ornamentation. As the key organizers of pattern formation, the diverse properties of the primexine probably decide the pattern differences among taxa (Takahashi 1989; Gabarayeva and Rowley 1994; Anger and Weber 2006). The undulation of the plasma membrane contributes to the pattern in guiding probacula formation in the primexine. Different hypotheses have been proposed to explain the undulation of the membrane. In *Vigna unguiculata*, the primexine is secreted from microspores. The change of cytoskeletal tension in the microspore was suggested to cause the patterned undulation of the plasma membrane for the assembly of the exine layer (Southworth and Jernstedt 1995). In *Brassica*, fibrous materials are inserted into the invaginations in the plasma membrane. These materials will separate the peaks of the plasma membrane onto which the probacula are finally extruded (Fitzgerald and Knox 1995). In *Lilium*, the plasma membrane is proposed to be anchored at the callose wall at specific sites, and the areas between these sites are thought to retract from these anchor sites, finally forming the peaks and troughs of plasma membrane (Dickinson 1970; Dickinson and Sheldon 1986; Skvarla and Rowley 1987).

In *Arabidopsis*, several male-sterile mutants with defective primexine have been reported. Mutations of *DEFECTIVE in EXINE FORMATION1* (*DEX1*), *NO EXINE FORMATION1* (*NEF1*) and *RUPTURED POLLEN GRAIN 1* (*RPG1*) genes exhibit

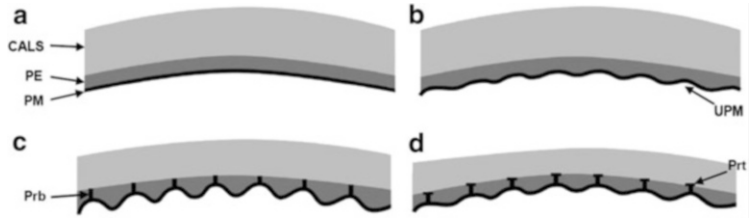

Fig. 5 The development of pollen-wall ornamentation. *CALS* callose, *PE* primexine, *PM* plasma membrane, *UPM* undulating plasma membrane, *Prb* probacula, *Prt* protectum

similar phenotypes: They all show irregular deposition of the primexine and reductions in plasma-membrane undulations, followed by random deposition of sporopollenin instead of a normal exine structure. As a result, no viable pollen comes to maturity (except for *rpg1* where a partial fertility is maintained). *DEX1* encodes a protein that is predicted to be membrane associated and contains several potential calcium-binding domains (Paxson-Sowders et al. 1997, 2001). The phenotype of the *dex1* mutant suggests that DEX1 may be a component of the primexine matrix and involved in the polymerization of the primexine. Alternatively, DEX1 could be part of the rough ER, processing and/or transporting primexine precursors to the membrane. *NEF1* encodes a plastidic integral membrane protein, which may indirectly change the composition of the primexine and/or sporopollenin or cause an imbalance between synthesis and transport of fatty acids (Ariizumi et al. 2004). *RPG1* encodes an MtN3/saliva family protein that is integral to the plasma membrane with seven putative transmembrane helices (Guan et al. 2008). Recently, *RPG1* has been renamed to *SWEET8*, because its gene product was found to act as a sugar efflux transporter (Chen et al. 2010). This is consistent with the idea that primexine formation is mainly dependent on polysaccharide polymerization delivered by RPG1. Also a second member of the MtN3/saliva family, RPG2, is reported to be involved in primexine deposition. It is proposed to play a redundant function of RPG1 during later stages of pollen development (Sun et al. 2013). In the *no primexine and plasma membrane undulation* (*npu*) mutant, the primexine is completely absent and the undulation of the plasma membrane cannot be observed (Chang et al. 2012). This suggests that the primexine determines plasma membrane undulation. *NPU* encodes a functionally unknown protein localized to the plasma membrane with two extracellular regions. Since NPU is a transmembrane protein just like RPG1, it may also act as a sugar transporter driving the transport of polysaccharide material essential for primexine formation. Although the primexine is supposed to be secreted by microspores itself (Southworth and Jernstedt 1995), these primexine mutants show dominant-recessive Mendelian inheritance which is evidence for a contribution of the maternal sporophyte tissue (Paxson-Sowders et al. 1997; Ariizumi et al. 2004; Guan et al. 2008; Chang et al. 2012; Ariizumi and Toriyama 2011).

4.3 Dissolution of the Callose Wall

The degradation of callose around the tetrad is one of the most dramatic cytological events in microsporogenesis. After the exine pattern has been established, the callose is broken down by a callase complex secreted from the tapetum (Stieglitz 1977). The glucanase activity expressed in the anther is mainly dedicated to callose-wall dissolution and the release of young microspores into the locules (Frankel et al. 1969). This activity peaks at the time of tetrad breakdown (Stieglitz and Stern 1973), suggesting that the appropriate timing of callose wall dissolution is specific and critical for normal microspore development. Several mutants and engineered plants with alterations in timing of β-1,3-glucanase expression provide evidence that both failure in callose degradation as well as its premature onset are the primary causes of male sterilities in several species (Izhar and Frankel 1971). In transgenic tobacco with premature callose degradation, the exine is sculptured in an irregular fashion, and sporopollenin is deposited randomly, leading to variable degrees of male sterility (Worrall et al. 1992). A molecular candidate for this enzyme is the gene product of Arabidopsis *A6* encoding a protein with similarity to β-1,3-glucanases. *A6* promoter::*GUS* and *RNase* fusions show that the *A6* gene is specifically expressed in the tapetum in a temporal pattern correlated with callase activity, suggesting that *A6* is probably part of the callase enzyme complex (Hird et al. 1993). However, the molecular mechanism regulating the callose dissolution has not been identified (Scott et al. 2004).

4.4 Formation of Pollen Apertures

Pollen apertures provide the exit points for the emerging pollen tube at the time of germination and also regulate water uptake during hydration (Heslop-Harrison 1979). Size, number, shape and position of pollen apertures are specific for a species and represent one of the taxonomy-defined elements of exine patterning (Furness and Rudall 2004). It has been proposed that the aperture position is controlled by microtubules (Heslop-Harrison 1971; Dickinson and Sheldon 1986), probably by microtubule-dependent modelling of the ER which subtends the plasma membrane underneath the prospective site of the aperture, such that vesicles carrying cellulosic or sporopollenin material are shielded from these sites, only leaving the intine layer (Schmid et al. 1996). Recently, a gene involved in aperture formation has been identified in *Arabidopsis thaliana*. In a mutant of this gene, *inaperturate pollen1* (*inp1*), all three apertures are lost although the pollen retains normal fertility. The INP1 gene product shows a tripartite subcellular localization in microspores when the maternal sporophyte still harbours a functional copy of this gene, demonstrating sporophytic control of aperture positioning. The aperture length is dependent on sporophytic gene dosage (Dobritsa and Coerper 2012).

5 Intine Development Is Controlled by the Gametophyte

The intine comprises cellulose, pectin and various proteins (Blackmore et al. 2007). It ensures the viability of the mature pollen grain as well as pollen-tube germination thus contributing to pollen survival and fertility (Brett and Waldron 1990; Edlund et al. 2004). Several genes involved in intine formation have been identified in *Arabidopsis*. *AtUSP* encodes a UDP-sugar pyrophosphorylase. It is the terminal enzyme in the *myo*inositol oxidation (MIO) pathway (Litterer et al. 2006) yielding precursors for the synthesis of glycolipids, glycoproteins and cell wall components including pectin and hemicellulose. Most pollen grains of the *usp* mutant lack intine layers and show a degraded cytoplasm, while there are no evident effect on the exine (Schnurr et al. 2006). *AtUSP* may involve in the synthesis of the matrix polysaccharides required for intine synthesis (Schnurr et al. 2006). The Arabidopsis genome contains five genes encoding reversibly glycosylated polypeptides (RGPs) (Dhugga et al. 1991,1997; Girke et al. 2004). *RGP1* and *RGP2* are specifically expressed in mature pollen. The *rgp1 rgp2* double mutant is lethal, and the malformed pollen grains are arrested due to the poorly defined intine (Drakakaki et al. 2006). RGPs may play a role on pectin and/or polysaccharide biosynthesis required for glycoprotein glycosylation during intine development (Drakakaki et al. 2006; Li et al. 2010). *CELLULOSE SYNTHASE* (*CESA)* genes encode catalytic subunits of the cellulose synthase complexes (CSCs), responsible for the deposition of cellulose (see also chapter by Nick, this volume). Mutations of *cesa1* and *cesa3* show the gametophytic lethality due to the uneven intine distribution, suggesting that the cellulose microfibrils provide a framework for deposition of intine polymers (Persson et al. 2007). The fasciclin-like arabinogalactan (FLA) proteins have been known for their role in the response to abiotic stress during plant development (Johnson et al. 2003; Shi et al. 2003; MacMillan et al. 2010). In a *FLA3* RNAi plant, the intine layer is absent leading to pollen abortion. Since FLA3 is distributed at the plasma membrane with a glycosylphosphatidylinositol anchor, it might modulate cellulose deposition during intine formation (Li et al. 2010). The gametophytic lethality of these mutants supports that the intine is controlled by male gametophyte.

6 Pollen Coat

The pollen coat is characterized by a complex lipid composition. Nonpolar esters form a semi-solid matrix, where proteins and other compounds are embedded. In the pollen coat, the long-chained lipids may function in cell-to-cell signalling, the lipid derivatives attract insects to facilitate pollen transmission and the carotenoids and flavonoids convey protection against UV radiation and microbial attack (Piffanelli et al. 1998; Doughty et al. 1993; Stephenson et al. 1997; Pacini and Franchi 1993; Paul et al. 1992).

Along with the sporopollenin, the tapetum also generates waxes regarded to be important for the pollen-pistil interaction (Preuss et al. 1993). As first step in wax biosynthesis, saturated C_{16} and C_{18} fatty acyl-CoAs produced in the plastid are further elongated to yield fatty acyl-CoA chains of 20–34 carbons in the ER. Then, these prolonged chains are converted to the various chemical classes of waxes by different biosynthetic pathways (Kunst and Samuels 2003). Genes acting at different steps of the wax biosynthetic pathway have been identified in *Arabidopsis* (Koornneef et al. 1989; McNevin et al. 1993). Mutations in *ECERIFERUM (CER)* affect the chemical composition of leaf and stem waxes (Jenks et al. 1995). The mutants *cer1*, *cer3*, *cer6*, *cer8* and *cer10* show male sterility. However, their fertility can be restored under conditions of high humidity (Koornneef et al. 1989; Hannoufa et al. 1996; Millar et al. 1999; Fiebig et al. 2000). *CER1* encodes a nove1 protein involved in the conversion of long-chain aldehydes to alkanes. Mutation of *cer1* not only alters the wax deposition on stem and fruits but also induces more numerous and smaller lipid droplets in the tryphine causing a defective exine structure (Aarts et al. 1995). The rice homologue of CER1, *Wax-deficient anther1* (*Wda1*), shows 56 % similarity on the amino acid level. In the *wda1* mutant, major components of waxes are reduced, and tapetal cells do not contain any orbicules or cytoplasmic lipid bodies causing a failure of exine formation. WDA1 is considered to mediate generation or secretion of very-long-chain aliphatic molecules, including the precursors for sporopollenin in anther walls (Jung et al. 2006).

FACELESS POLLEN-1 (FLP1) protein is also a member of the CER family (also named WAX2/CER3/YRE). It participates in the synthesis of wax in stems and siliques, components of the tryphine and the sporopollenin of exine (Ariizumi et al. 2003; Chen et al. 2003; Rowland et al. 2007; Kurata et al. 2003). In the *faceless pollen-1* (*flp-1*) mutant, stems and siliques are reduced in wax content, and excessive tryphine fills the interstices of the exine leading to a smooth surface. This is consistent with a role for the tapetum in the control of the tryphine. The exine structure of *flp-1* is sensitive to acetolysis, which may result from sporopollenin precursors in the tapetum being not properly secreted and/or transferred to the primexine (Ariizumi et al. 2003). The genetic evidence supports that sporopollenin and waxes may share partial biosynthesis pathway for pollen-wall development.

7 Summary

The pollen wall, as the most complex manifestations of plant cell walls, has the dual function to support plant gametogenesis and fertility. It consists of two main layers, the outer exine and the inner intine. The exine is controlled by the sporophyte and the intine is controlled by gametophyte. The formation of the exine comprises two developmental processes: pollen-wall pattern determination and deposition of sporopollenin precursors. Pollen-wall pattern determination is dependent on formation of a callosic wall, plasma membrane undulation and primexine deposition.

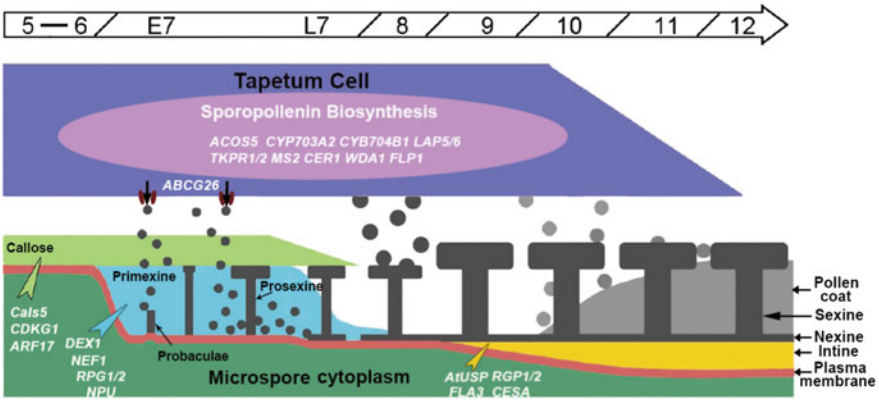

Fig. 6 Developmental model for pollen-wall formation with key genes involved in normal pollen-wall formation

The callose wall may provide a structural basis for the primexine deposition. In Arabidopsis, the gene products of CDKG1 and ARF17 regulate the transcript of *CalS5*, which is responsible for the synthesis of callose. Primexine formation and plasma membrane undulation successively provide the scaffold for sporopollenin deposition and polymerization. Genetic evidence shows that precise primexine formation requires the functions of the DEX1, NEF1, RPG1 and NPU proteins in Arabidopsis. Sporopollenin deposition and polymerization requires three important steps: synthesis, secretion and translocation of sporopollenin precursors. Genetic evidences have elucidated that all of these processes are controlled by tapetal cells. A transcriptional regulatory pathway involving DYT1-TDF1-AMS-MS188-MS1 has been proposed for tapetal development and pollen formation in Arabidopsis. In the metabolic pathway generating the material for pollen-wall formation, fatty acids are hydroxylated by specific members of the cytochromes P_{450} family; fatty acid modifications such as CoA-esterification and CoA-reduction are regulated by ACOS5 and MS2 respectively, whereas PKSA/PKSB and TKPR1/2 are required for phenylpropanoid biosynthesis. Subsequently, the sporopollenin precursors are transported from the tapetum to the surface of the microspores by the gene product of ABCG26, which is a direct target of AMS (Fig. 6).

Male sterility is an important trait in agriculture. Male-sterile varieties are valuable resources that greatly facilitate the production of hybrids via cross-pollination. In crops, heterosis can dramatically improve yield and quality, which is widely utilized in plant breeding. Because the exine is genetically controlled by the sporophyte tissue, defects in many genes essential for exine formation lead to male sterility. Therefore, manipulation of exine genes may provide novel strategies to generate male-sterile mutants which could be used for plant breeding in agriculture.

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Part II
Stress Tolerance

Plant Cell Responses to Cadmium and Zinc

Michal Martinka, Marek Vaculík, and Alexander Lux

Abstract Control of uptake, radial transport, translocation and accumulation of cadmium (Cd) or excessive amounts of zinc (Zn) from the polluted environment to vegetative and generative organs of plants is critical both for plants and in consequence also for human beings in relation with food safety. These processes are controlled by checkpoints at specific sites of the plant body. These checkpoints are represented by cells at the root surface, the root cortex, and the cells responsible for loading of the root xylem, the transition between the vascular systems of root and shoot, and the connecting tissues and cells at the nodes of the segmented stem. Control by these checkpoints is based on the structural and functional characteristics of specialized cells and tissues. The present contribution reviews the mechanisms of Cd and Zn uptake, transport and deposition, tissue and cellular localization as well as various proteomic and metabolomic responses. The knowledge on the responses of plant cells to Cd and excessive amounts of Zn might inspire further research focused on these topics and is essential to use plants for phytoremediation (restoration of contaminated sites) and phytofortification (improved quality of food and feed), thus improving human well-being.

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1 Cadmium and Zinc: Heavy Metals, Toxic Metals or Trace Elements?

Elements present in soil, water and air enter living organisms through food chains. Some of these elements, such as the intensively studied group of heavy metals, may be toxic. One of the most toxic heavy metals is cadmium (Cd) which, because of similarity to the essential element zinc (Zn), markedly influences many cell processes. Therefore, this chapter deals just with Cd and Zn. To understand the mechanisms of uptake, transport, toxicity and cellular detoxification may greatly help to eliminate potential toxic effects not only upon plants but also upon us as the final consumers.

Both Cd and Zn are located in the same column of the Periodic Table and in the environment often occur together. Cadmium is well known for its toxicity on all living cells. Various negative effects of this metal have been documented for the growth of both shoots and roots (Lux et al. 2011a; Gallego et al. 2012). Up to now no physiological function of Cd has been discovered for plant organisms, and Cd is therefore considered as non-essential metal for plants (Clemens et al. 2013).

In contrast, Zn plays an important role in plants, and an appropriate concentration of Zn is required for optimal plant growth and development. Unlike Cd, Zn is essential as micronutrient, and plants suffering from Zn deficiency show significant symptoms such as growth reduction or leaf chlorosis (Mengel and Kirkby 2001; Broadley et al. 2007; Marschner and Marschner 2012). Zinc is involved in various metabolic pathways and acts as cofactor of many enzymes and other proteins. However, excess of Zn is dangerous for plants, and various symptoms of Zn cytotoxicity, e.g. damage of chloroplast ultrastructure, vacuolation, increased number of peroxisomes and mitochondria with dilated cristae, have been described as well (Broadley et al. 2007; Azzarello et al. 2012).

Both, Cd and Zn, are frequently designated as "heavy metals". The use of this term is used for a group of elements with a relative atomic weight (density) exceeding 5 g cm^{-3} (Adriano 2001). This term is widely accepted and used in many biological, environmental or ecotoxicological studies and in the public usually provokes various negative emotions, stimulating feelings of toxicity and threat for mankind. Correctly, the use of this term would be appropriate only for a group of those elements, including Cd, which have been clearly shown to be non-essential, have no known function in plant metabolism and do not "naturally" occur in plants. However, this concept will be probably re-evaluated in the near future as recent findings indicate that roots of certain specific plants, especially belonging to the group of hyperaccumulators, might potentially actively forage for heavy-metal-polluted soils (e.g. Schwartz et al. 1999; Liu et al. 2010) contrasting with the usual situation, where species forage for unpolluted soils (e.g. Remans et al. 2012). A further problem of the term "heavy metal" is that it often comprises those elements, which are actively taken up by plants and accumulate present in the tissues in low concentrations, but in excess might be dangerous and negatively

influence metabolic and physiological processes. This is the case for the essential element Zn. Moreover, even elements that are not metals, but share some similarities, such as metalloids like arsenic (As) or antimony (Sb), are often incorrectly designated as "heavy metals". This also indicates that the use of the term "heavy metals" is inappropriate and a more precise terminology would be essential.

Those elements, which occur in plant tissues at low concentrations, typically less than 0.01 % (Adriano 2001), are designated as "trace elements", a term that is widely used in the field of plant nutrition, as many important elements play various roles in plant metabolism as cofactors of enzymes or components of different biomolecules. However, also unfavourable non-essential elements can be present in the tissues at the same concentration, taken up by plants either passively via the transpiration stream or actively by specific transporters, often primarily assigned for transport of other elements. Although the term "trace elements" has also been established in the literature (i.e. Kabata-Pendias and Pendias 2001), it is often not clear, whether a given element is essential or actually toxic for a plant, and this makes the use of the term difficult.

To overcome these terminological discrepancies, Ahrland et al. (1958) and Pearson (1968) suggested to use the so-called HSAB principle and distinguish three categories: class A metal ions ("hard"), class B metal ions ("soft") and "borderline" (acid) metal ions. Although this principle was later accepted by McNaught and Wilkinson (1997), also in the official chemical terminology (IUPAC – Compendium of Chemical Terminology), the number of publications that still use the terms heavy metals and trace elements prevail since then, indicating that the "HSAB principle" has not been really adopted in biology and environmental sciences. Both, trace elements and heavy metals, have been intensively studied because of their negative effects on all living cells. However, the use of both terms is confusing as toxicity often is observed only in excessive concentrations, such that the term "potentially toxic" would be more appropriate. Even important essential elements, including major macronutrients, can be toxic when administered in excess (Madrid 2010).

From this short survey it is evident that terms like "heavy metals", "toxic metals", "trace elements", "toxic elements" or "potentially toxic elements" should be used with care. For instance, Cd and Zn share some similarities, but are, in particular with respect to plant nutrition, also different. Whether especially Zn should be designated as toxic element or trace element remains a matter of debate on the background of the above mentioned disunion of terminology. However, for pragmatical reasons, and because there is no generally accepted terminology, we will use in the current chapter the term heavy metals for both Cd and Zn.

2 Control of Uptake and Translocation Within the Plant

Plants exposed to Cd or excessive amounts of Zn exhibit often (but not always) a gradient of Cd or Zn concentrations in their organs, usually descending from roots to shoots to lowest levels in fruits and seeds, but also in tubers (Chaoui et al. 1997). This indicates a set of barriers along the plant body and also limited translocation in both xylem and phloem (Lux et al. 2011a; Uraguchi and Fujiwara 2013). These barriers are based on the construction of plant organs, tissues, cells and their activities (Fig. 1).

The plant responses to Cd and Zn have been intensively studied and treated by several recent reviews (i.e. Sanita di Toppi and Gabbrielli 1999; Benavides et al. 2005; Broadley et al. 2007; Seregin and Kozhevnikova 2008; Hasan et al. 2009; Verbruggen et al. 2009; DalCorso et al. 2010; Lux et al. 2011a; Hassan and Aarts 2011; Lin and Aarts 2012; Sinclair and Kramer 2012; Clemens et al. 2013). In the current chapter, we will summarize the responses of individual tissues and cells to these two metals and focus on the checkpoints and main barriers for uptake and translocation of these elements.

The root is the organ where the majority of ions enter plant. Thus, the behaviour of the root, root architecture, structural characteristics of tissues and cells of individual roots and the distribution of transporters on the plasma membranes of individual tissues and cells should provide major checkpoints. Morphological, anatomical and cytological characteristics act in concert with metabolic responses to react to the presence of toxic elements or toxic concentrations of trace elements. Irrespective of the immense variability of plant species with respect to sensitivity for uptake and translocation of these ions, there are some general features of the tissue and cellular responses (Tamas et al. 2010; Kenderešová et al. 2012). As the key elements on the biochemical and physiological level, stress-modulated gradients of reactive oxygen species (ROS), antioxidants, auxin and ethylene have been proposed (Potters et al. 2009). The accumulation of ROS and water imbalance represent common symptoms of various stress factors. The elevated production of H_2O_2 as a signal molecule plays probably a key role in the induction of plant responses to abiotic stresses as for instance observed for “heavy-metal” stress in barley root tips (Tamas et al. 2010).

On the level of tissue and cellular structure, several mechanisms control transport along the axis of the plant body (Clemens et al. 2002), and a set of the general stress responses arrest cell division in the meristematic tissues and inhibit elongation (Potters et al. 2007, 2009; Vatehová et al. 2012). Cadmium interferes with the cell cycle and the effect differs markedly depending on the phase of cell cycle application as it was shown by Kuthanova et al. (2008). Internucleosomal DNA fragmentation occurs when Cd is applied in the S and G2 phase, whereas application of Cd in M and G1 phase is not accompanied by DNA cleavage. These findings were interpreted as induction of apoptosis-like programmed cell death (PCD) in the S and G2 phase being connected with preservation of genetic integrity of dividing meristematic cells. Suppression of autolysis and nonprogrammed character of cell

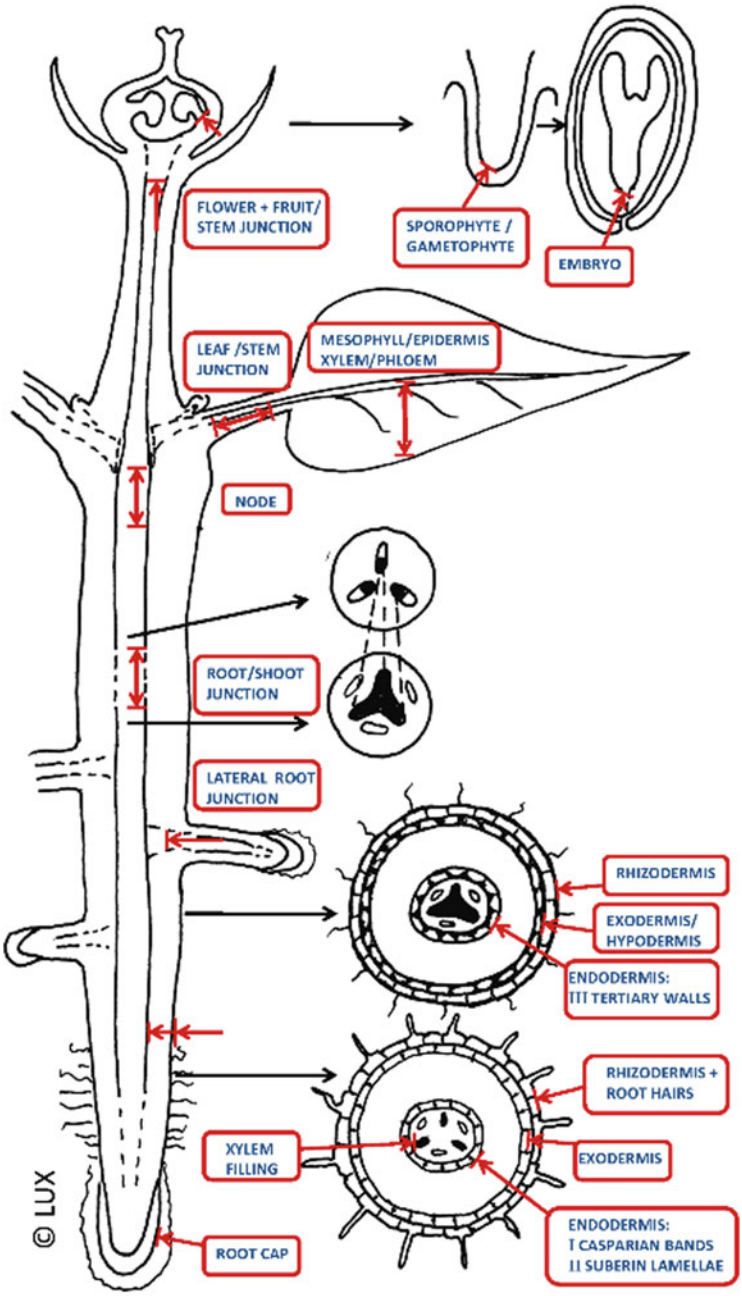

Fig. 1 Schematic illustration of putative control checkpoints of cadmium and zinc uptake and translocation along the plant body. The control occurs at the root surface (at the root cap, rhizodermis with root hairs). Radial transport is controlled by exodermis, endodermis and xylem loading. Translocation is controlled at the lateral root junctions, root-shoot junction, nodes, leaf-stem junction and distribution in leaf tissues. The terminal checkpoints are represented by stem-flower/-fruit junction, connection between sporophyte and gametophyte and the junction of embryo

death in differentiating cells (in G1/G0 phase) might protect the whole organism from the death and enable initiation of adaptation and recovery of the plant (Kuthanová et al. 2008).

Stress-induced morphogenic responses usually comprise three components: (a) inhibition of cell elongation, (b) localized stimulation of cell division and (c) alterations in cell differentiation status (Potters et al. 2007). Related with cell differentiation, ectopic deposition of some cell wall components reinforcing cell walls can provide additional barriers limiting the radial transport of ions into the vascular tissues of the plant root (Lux et al. 2011a, b).

2.1 The Root Surface

The most active parts of roots with respect to ion uptake are the apical and subapical regions (Frensch et al. 1996; Sun et al. 2013). In this region, the contact with solutes from the soil solution is made by cells of the root cap protecting the most sensitive part of the root tip – the root apical meristem, rhizodermal cells and root hairs.

The function of the root cap for the protection of root apex and the sensitive meristematic region against Cd was indicated in a study (Arduini et al. 1996), where Cd uptake and distribution were compared between stone pine (*Pinus pinea* L.), maritime pine (*Pinus pinaster* Ait.) and ash (*Fraxinus angustifolia* Vahl). The exceptionally long root caps in the two *Pinus* species play a protecting role against metal uptake at the root-tip zone. At higher concentrations of Cd, root cap cells perish as it was observed by Seregin et al. (2004) in maize, or the root cap border cells are sloughing off as observed in the shrub *Karwinskia* by Zelko and Lux (2004).

The root epidermis is the main tissue for the absorption of water and solutes from the liquid phase of the soil. For its specific structure and function in contrast with the epidermis of the aerial plant organs, it should be referred to more properly as rhizodermis (von Guttenberg 1968). Rhizodermal cells extend their absorption capacity by development of root hairs, usually short-lived, specialized absorption trichomes. These tip-growing extensions from root epidermal cells are important in nutrient uptake and in plant-soil interactions. They have been intensively studied both from the structural and functional point of view (for review see Datta et al. 2011; Jones and Dolan 2012; Marcon et al. 2013). Surprisingly, Cd treatment leads to an increased production of root hairs close to the root apex as found in various species, e.g. in maize (*Zea mays* L., Seregin and Ivanov 2001), radish (*Raphanus sativus* L., Vitória et al. 2003), barley (*Hordeum vulgare* L., Ďurčeková et al. 2007), sorghum (*Sorghum bicolor* L., Kuriakose and Prasad 2008) and Rhodes grass (*Chloris gayana* Kunth., Kopittke et al. 2010). This might be the consequence of accelerated maturation of rhizodermal cells and growth inhibition of the root caused by Cd. However, higher Cd concentrations can result in reduced root hair

production and disintegration of the rhizodermis (Seregin et al. 2004; Kuriakose and Prasad 2008; Gratão et al. 2009). Suberization of rhizodermal cell walls, occurring also under control conditions in older parts of roots more distant from the root apex, was found to be shifted closer towards the root tip in reaction to metal toxicity, a phenomenon that may be related with adaptation to osmotic stress induced by toxic metal concentrations (Seregin and Kozhevnikova 2008). Suberin is a major cell wall component forming a chemical barrier limiting the extracellular, or apoplasmic, transport of water and solutes in plant roots, which is supported by direct genetic evidence (Baxter et al. 2009). A thickening of rhizodermal cell walls was also observed in reaction to Zn in *Vicia faba* (Probst et al. 2009) and also in the hyperaccumulator *Sedum alfredii* (Jin et al. 2008).

Suberization is not the only activity of cells at the root surface cells that interferes with the uptake of ions from the soil. A second important mechanism is the production of mucilage and root exudates and interaction of these substances released from the root cells with the soil (Bertin et al. 2003). These processes have also attracted considerable attention during recent years with respect to toxic metals in polluted soils (Evangelou et al. 2012). Accumulating evidence shows that the composition of root exudates is relevant for the uptake of metals from the liquid phase of the soil, and this knowledge may be used also for phytoremediation purposes (Koo et al. 2010; Evangelou et al. 2012). The genotypic differences of barley cultivars with respect to Zn acquisition from the soil may be linked to differential carboxylate and amino acid composition of root exudates (Rasouli-Sadaghiani et al. 2011). However, root exudates of the hyperaccumulator *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) do not enhance metal mobilization, in contrast to root exudates of wheat (Zhao et al. 2001).

2.2 The Root Cortex

The next checkpoint following the rhizodermis or, more precisely, a set of checkpoints is present in the cells of the root cortex. This part of the root is represented by complex tissues whose main function is to control the radial transport of water and solutes realized by a single layer of endodermal cells present in all seed plants. The whole root cortex may be composed of only two cell layers as in the thin and most simple root organization as it is in *Arabidopsis* (Dolan et al. 1993), or it can be developed as multilayered broad zone with variable cell composition as in many cereals and other monocots (von Guttenberg 1968; Kutschera and Lichtenegger 1982). As already mentioned, for the uptake of water and solutes, only the relatively short subapical zone of actively growing roots is important, which also holds true for Cd (Piñeros et al. 1998) and Zn uptake (Broadley et al. 2007). In older parts of the roots, the peripheral tissues often degenerate or are sloughed off due to secondary thickening due to the activity of the vascular cambium or by the activity

of the cork cambium. The whole root cortex can be considered as a buffer zone (Lux et al. 2004a), and physical isolation of this buffer zone from the stele is critical for the control of solute transport to the vascular tissues and to the shoot.

The endodermis separates the cortex from the stele as an apoplasmic barrier (van Fleet 1961). Apoplasmic, extracellular movement of solutes to the xylem is restricted by suberin deposited in the endodermal cell walls (Baxter et al. 2009). The endodermis develops in three ontogenetic stages (von Guttenberg 1968; Schreiber et al. 1999; Geldner 2013). Stage I is characterized by impregnation of the Casparian bands developed in radial and transversal endodermal cell walls with suberin together with lignin (Schreiber et al. 1999; Grebe 2011). It is interesting that although this structure has been known for already almost 150 years (Caspary 1866), the factors regulating development of endodermal cells and the banded deposition of wall material have been elucidated only recently (Cui et al. 2007; Ropollo et al. 2011; Martinka et al. 2012; Geldner 2013). In stage II, the suberin lamellae are deposited over the whole inner surface of endodermal cells. In stage III, thick cellulosic secondary walls (sometimes classified as tertiary walls) are deposited over the inner surface of the cell. The whole cell wall is often lignified in this stage, and in some species the endodermal walls are also impregnated with silicon (Sangster and Parry 1976; Lux et al. 1999). As soon as stage III is completed, the radial transport of water and solutes is limited and the function of the endodermis is mostly confined to mechanical protection of the stele (Melchior and Steudle 1993; White 2001).

The three developmental stages of endodermis take place along the root axis and proceed in basipetal direction usually over a considerable distance from the root tip. In actively growing primary maize roots, the whole process of cell maturation may extend to almost 40 cm from the root tip (Schreiber et al. 1999). However, not in all species all three stages occur.

With respect to transport of water and solutes, as well as the regulatory function of the endodermis for uptake of Cd and excessive amounts of Zn, during stage I, the Casparian band only represents an important, but not completely tight and impenetrable barrier to solute movement through the apoplasm (Steudle et al. 1993; White 2001; Ranathunge et al. 2005). During stage II, the endodermis represents a more complete apoplasmic barrier to the radial flow of water and solutes to the xylem in the more mature parts of the root (Melchior and Steudle 1993; Peterson et al. 1993; Steudle and Peterson 1998; White 2001).

The importance of cell wall modifications for the control of toxic metal uptake (Cd) was shown by Schneider et al. (1999) and Schreiber et al. (1999). Comparison of root structural characteristics of willow clones differing in Cd uptake and translocation revealed a correlation between Cd movement and endodermal development. In clones characterized by low translocation characteristics, stages I and II of endodermal development were shifted closer to the root tip when compared with high Cd translocators (Lux et al. 2004b). Subsequent work has shown a close correlation between endodermal development and metal stress; both Zn- and Cd-treated plants developed suberin lamellae (stage II of endodermal development) closer to the root tips (Martinka and Lux 2004; Vaculík et al. 2009, 2012a, b;

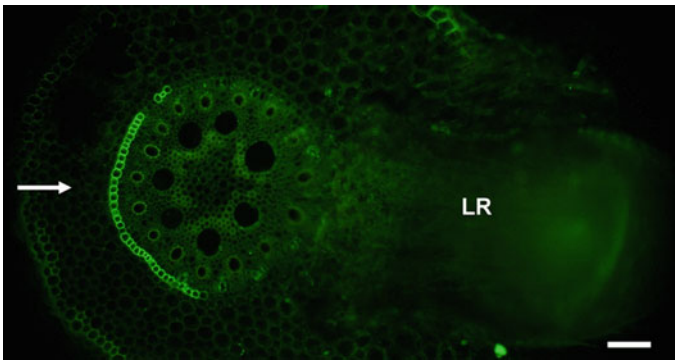

Fig. 2 Cross section of a maize root cultivated for 2 d between two sheets of agar and exposed unilaterally to medium containing 100 μM Cd (*arrow*). Section taken 10 mm from the root apex showing the development of suberin lamellae (*bright green fluorescence*) in the endodermis facing the exposed side. The lateral root (*LR*) emerged towards the control medium (without cadmium). In control conditions, the suberin lamellae were first deposited at a distance of about 80 mm from the root apex. Suberin was visualized with Fluorol yellow 088 under fluorescence (excitation filter TBP 400 + 495 + 570, beam splitter TFT 410 + 505 + 585, emission filter TBP 460 + 530 + 610, wavelengths are in nm). Scale bar: 100 μm

Stoláriková et al. 2012). Cultivation conditions influence Cd uptake and are related with endodermal development as shown by Redjala et al. (2011). Cultivation of maize in hydroponics results in more distant development of both endodermal and exodermal apoplasmic barriers from the root tip in comparison with cultivation in soil. This correlates with higher uptake of Cd in hydroponics, which may be explained as increased uptake by longer unprotected root tip in hydroponics. Endodermal cells respond to the presence of Cd by specific and rapid development of suberin lamellae as was shown by unilateral treatment of roots with Cd (Lux et al. 2011a; Fig. 2). The endodermal cells localized closer to the source of Cd (an agar plate with Cd) form the suberin lamellae earlier compared to the endodermal cells localized distant from the Cd source (facing the medium without Cd). The endodermis is a constitutive and ubiquitous barrier for apoplasmic transport. Additional barrier(s) might be developed in the hypodermal layers, subtending the rhizodermis. A so-called exodermis, a hypodermal layer of cells with Casparian bands, is found in roots of many Angiosperms (Perumalla et al. 1990; Peterson and Perumalla 1990). However, occurrence and development of this barrier depends on environmental conditions (Zimmermann and Steudle 1998). A relationship between the distance of exodermis initiation from the root tip and Cd uptake has been described for maize (Redjala et al. 2011).

The peripheral parts of the root cortex act as important barrier for the radial loss of oxygen and therefore have been well studied in rice and some other wetland plants (Armstrong and Armstrong 2005; Soukup et al. 2007). However, the deposition of cell wall material in the hypodermal/exodermal layers was also shown to be associated with reduced uptake of metal ions including Cd and Zn (Deng

et al. 2009). Mangrove species that harbour the "tightest barrier" with respect to radial oxygen loss took up the least amounts of Zn and also showed the highest Zn tolerance (Cheng et al. 2010). Additional evidence for the importance of suberin deposition in response to Cd stress was found in roots of the bulbous species *Merwilla plumbea*. These monocot plants do not form a regular periderm, due to the lack of secondary meristems. However, when these plants are exposed to high concentrations of Cd, they exhibited ectopic formation of suberized periderm in the peripheral zones of root cortex (Lux et al. 2011b).

2.3 The Loading into the Root Xylem

After having passed all barriers from the root surface over root cortex, including apoplasmic barriers, the metal ions enter the symplasm and are transported to the stele and xylem elements present there. It should be noted that the xylem elements represent part of apoplasmic space. A direct apoplasmic delivery of Cd and Zn to the stelar apoplasmic space is also possible, mainly in the root apical areas where the Casparian bands are not fully developed (White 2001; White et al. 2002; Broadley et al. 2007; Lux et al. 2004b; Redjala et al. 2009). Irrespective of the pathway, the loading into the root xylem seems to be one of the most critical steps for transport of both Cd and Zn (Papoyan et al. 2007; Ueno et al. 2008; Lu et al. 2009; Uraguchi et al. 2009; Verbruggen et al. 2009), and loading into xylem was found, for instance, responsible for the differential Cd accumulation of solanaceous species (Yamaguchi et al. 2011; Xu et al. 2012).

2.4 The Root-to-Shoot Junction, the Segmentation of the Stem and the Junction with Generative Organs

These steps are probably the least understood from both the structural and the physiological points of view, even though a strict selectivity and regulation of transport processes at these control points has been elucidated. Translocation of Cd from root to shoot via the xylem is the major process determining shoot and grain Cd accumulation in rice, as elegantly demonstrated by Uraguchi et al. (2009) using time-course analyses of xylem loading of Cd and shoot Cd accumulation comparing 69 rice accessions. The results showed strong correlation between Cd levels in xylem sap and those in shoots and grains.

The transport of ions from the root into the shoot base occurs after time lag. By tracing the translocation of radioactively labelled cadmium (^{107}Cd) from the culture solution into rice plants, the radial transport to the root xylem was deduced to be rapid and completed in less than 10 min. However, transfer to the shoot base occurred only after a time lag of 1.4 h. The structural base for this delay in transport may be found in the transition of vascular tissues between root and shoot, serving

Fig. 3 Distribution of ^{109}Cd in roots and shoots of maize seedlings grown hydroponically for 7 days in the presence of 34 nM ^{109}Cd monitors barrier of cadmium translocation at the root-shoot junction. Scale bar = 10 mm

also as a safety barrier (Fig. 3). The root-shoot barrier is considered as a major controller of Zn transport in plant (Sharma et al. 2013). This zone is structurally highly variable and comprises changes in the anatomical structure of conducting tissues that may relate with altered translocation of solutes. It is known that, even in cereals, there exists variability ranging from a very tight root vascular system, in which the vessels of the roots are separated from those of the shoots by tracheids on the one hand, up to a completely permissive system, where the vessels in the root are continuous with the vessels in the shoot (Aloni and Griffith 1991). The influence of vascular segmentation resulting in the formation of tight root vessels, including the junctions of lateral roots, which may also exhibit safety zone (Luxová 1986) has been a little studied topic in relation with uptake and translocation of toxic metals.

Vascular segmentation occurs also in the stem nodes (Esau 1965) and represents the next checkpoint for transfer of both nutrients and toxic elements along the shoot to the leaves and fruits. Accumulation of Cd in rice nodes was observed by

Fujimaki et al. (2010), and these authors concluded that nodes represent the most important regions for xylem to phloem transfer of Cd with a key role for the transport of Cd from the soil to the grains during the grain filling stage of rice. The final checkpoint for transport is located between stem and flowers and fruits. Results from studies using stable Zn isotopes indicate that two barriers of Zn transport into wheat grains may exist: between the stem tissue rachis and the grain, and the maternal and filial tissues in the grain (Wang et al. 2011c).

3 Membrane Transport

Heavy metals present in the apoplasmic space (following the terminology for transport processes proposed by Erickson 1986) are transported via plasma membrane transporters to the cytosol. While most of these transporters are able to transport both Zn and Cd (Morel et al. 2009), some of them are more specific for Cd (for instance, HMA3 from *N. caerulescens*, Ueno et al. 2011). Only a smaller fraction of metal ions is retained in the apoplasm by binding to cell walls, whereas the larger amount is available for plasma membrane transporters for symplasmic uptake (Redjala et al. 2009; Nocito et al. 2011). Cadmium and zinc are taken up by MTP1 (Yuan et al. 2012), by ZIP transporters (zinc-/iron-regulated transporter-like proteins), by orthologues of TaLCT1 transporters or via non-selective cation channels in the plasma membrane. Cadmium in chelated form might be also taken up by YSL transporters (yellow-stripe 1-like proteins), members of OPT superfamily oligopeptide transporters (e.g. Lux et al. 2011a; Gallego et al. 2012). After uptake, Cd ions are transported radially in the roots by the transporters NRAMP1 and NRAMP5 (natural resistance-associated macrophage protein) (Takahashi et al. 2011; Sasaki et al. 2012). As countermeasure against metal toxicity, cells can export Zn and Cd as free ions as well as in complexed forms from the cytosol through the plasma membrane via active transporters depending on ion homeostasis (Baekgaard et al. 2010; Migocka et al. 2011). The expression of metal transporters can be regulated by metal-regulated transcription factors (Farinati et al. 2010). The higher the activity of those transporters, the higher the ability of cells to accumulate and tolerate heavy metals (Pittman and Hirschi 2003; Wu et al. 2011). This export is energized either by ATP or by the electrochemical gradient generated by H^+-ATPase or by V-ATPase and pyrophosphatase (PPase), respectively (Migocka et al. 2011). In consequence of metal toxicity, the activities of ATPases and PPases can be inhibited. Since they provide the energy also for the sequestrating transporters, also the sequestration ability of cells can be impaired (Kabała et al. 2013). Sequestration of Zn and Cd into the vacuoles is mediated mainly by ABC (ATP-binding cassette) transporters, e.g. AtMRP7 (multidrug resistance-associated protein) (Wojas et al. 2009), CAX proteins (Pittman and Hirschi 2003; Wu et al. 2011) and P1B-ATPases (Wong and Cobbett 2009; Miyadate et al. 2011). Several of these transporters are activated by heavy metals and the activation energy decreases with the rising metal concentrations

(Leitenmaier et al. 2011; Migocka et al. 2011). In *Arabidopsis thaliana*, the activity of Zn-/Cd-transporting ATPases (HMA2 and HMA4) is the major mechanism for Cd root-to-shoot translocation, as their loss of function was shown to abolish root-to-shoot Cd translocation almost completely (to 2 % of the wild type) (Wong and Cobbett 2009). The C-terminal domain of AtHMA4 plays a dual role as Zn^{2+} and Cd^{2+} chelator (sensor with capacity to bind 10 Zn^{2+} ions per C terminus) and as a regulator for efficiency of Zn^{2+} and Cd^{2+} export (Baekgaard et al. 2010).

The ability of some plants to tolerate and hyperaccumulate heavy metals is not necessarily based on molecular properties of specialized transporters, but might be just consequence of expression pattern and level of transporter present in a wide range of species. For example, a higher expression (sevenfold) of the HMA3 gene (and thus increased sequestration of Cd to the vacuole) is at least partially responsible for the higher Cd accumulation in the ecotype Ganges of *N. caerulescens* as compared to the lower Cd-accumulating ecotype Pryon, as well as for the Cd hypertolerance of the Ganges ecotype (Ueno et al. 2011). Already sequestered toxic heavy metals can be released from the vacuoles by the tonoplast transporters NRAMP3 and NRAMP4 (Thomine et al. 2003; Lanquar et al. 2005; Oomen et al. 2009), which play a role in metal cation homeostasis in plants (Wei et al. 2009; Molins et al. 2013), and are important for the retrieval of seed Fe stores during germination as well as for the supply of essential metals to plastids to maintain photosynthetic function in leaves (Lanquar et al. 2005, 2010). Metals that have been released this way and metal ions taken up via the plasma membrane can be sequestered in the symplasm by partitioning into the vesicular portion of the endomembrane system (distinct from the vacuole or mitochondria) via NRAMP6, which is predominantly expressed in the dry seed embryo and to a lesser extent in aerial parts (Cailliatte et al. 2009).

A further important export destination of cytosolic Zn and Cd is the apoplasm. Proteins involved in metal efflux are the plasma membrane-localized transporters HMA2 (Mills et al. 2012; Satoh-Nagasawa et al. 2012), CET2 (Xu et al. 2009), CET3 and CET4 (Lang et al. 2011). All these proteins increase the concentrations of Zn and Cd in the apoplasm resulting in higher root-to-shoot translocation and increased metal accumulation in shoots (Xu et al. 2009; Lang et al. 2011). The long-distance translocation is enhanced even more, when the transporter (e.g. OsHMA2) releases the metal towards the xylem elements (Takahashi et al. 2012). The root-to-shoot Cd translocation via the xylem is the major and most common physiological process determining the Cd accumulation level in shoots (Uraguchi et al. 2009). Uptake into the cells of leaf and stem may be mediated by plasma membrane protein TcOPT3 expressed mostly in the aerial parts of plant (Hu et al. 2012b). In rice, the plasma membrane OsLCT1 efflux Cd transporter mediates the re-translocation of Cd from the leaves to the phloem after heading (Uraguchi et al. 2011), which is responsible for Cd accumulation in grain (Kashiwagi et al. 2009).

4 Subcellular Distribution

The literature report suggests that in root tissues, most of Cd is localized in the apoplasm, especially in cell walls, whereas only a small amount of Cd is found within the root cells themselves (e.g. Seregin et al. 2004; Liu et al. 2007b; Vázquez et al. 2007; Wierzbicka et al. 2007; Zhou et al. 2010; Ye et al. 2012; Lukačová et al. 2013). However, there are also findings indicating that the majority of Cd might be stored in the symplasm rather than in the apoplasm of roots and shoots in various plants that have been treated with Cd (Weigel and Jäger 1980; Lozano-Rodríguez et al. 1997; Shi et al. 2005; Fu et al. 2011). Redjala et al. (2009) found more ^{109}Cd in the symplasm of maize-root tissues treated with a low concentration of Cd (0.25 μM), whereas increasing the Cd concentration in the medium to 50 μM resulted in increase in total Cd content in the apoplasm. Similarly, Vaculík et al. (2012b) found a considerably higher amount of radioactively labelled ^{109}Cd in the symplasmic (90 %) as compared to the apoplasmic (10 %) fraction in both maize roots and shoots. Shi et al. (2010) found higher Cd concentrations in the symplasm compared to the apoplasm of the root of peanut, which differed from the situation in the shoot. These findings suggest that in plants treated with lower levels of Cd, most of this element is bound symplasmically and that the partitioning of Cd between apoplasm and symplasm might change when plants are exposed to higher level of this heavy metal (Vaculík et al. 2012b). Moreover, the patterns for the distribution of Cd/Zn between apoplasm and symplasm indicate that metal-tolerant plants can store more Cd and Zn in cell walls and apoplasm than metal-sensitive plants (Lux et al. 2011a).

The deposition and localization of heavy metals on the subcellular level has been investigated by several studies. The cell wall is one of the major storage sites for heavy metals in the cell. Liu et al. (2007a) found that most of Cd in the roots of two *Brassica* species was found in the cell walls. For *Elodea canadensis*, most of the Cd remained outside the cells and was bound there to the anionic fraction of cell walls (Dalla Vechia et al. 2005). For cotton roots, Daud et al. (2009) found that Cd was present in the form of electron dense granules and crystals attached to the cell walls. Similarly, Cd in the form of numerous black deposits was found in root-tip cell walls of epidermal, cortical and vascular cylinder tissues of various *Iris* species (Han et al. 2007; Zhou et al. 2010), or as deposits along the endodermal cell walls of maize roots (Wójcik and Tukiendorf 2005). Both Cd and Zn deposits were observed along the cell walls of xylem and phloem vessels in roots of Indian mustard (Maruthi Sridhar et al. 2005), and similarly, Zn was mostly detected in the cell walls of the xylem and of the parenchyma cells surrounding the vascular bundles of Zn-treated poplar leaves (Todeschini et al. 2011).

Cadmium and zinc ions are mainly transported across the plasma membrane by various influx and efflux transporters and cation channels (Broadley et al. 2007; Lux et al. 2011a; Gallego et al. 2012). Free ions of heavy metals, such as Cd and Zn, are dangerous for the plant cells when present in the cytoplasm, and therefore, they are mostly bound to specific thiol-containing ligands (glutathione or glutathione-based

phytochelatins) or small cysteine-rich peptides (metallothioneins). These Cd- and Zn-chelated complexes are later sequestered into the vacuoles as important step of metal detoxification in the cell. Therefore, vacuoles are, along with the cell walls, a further important cell compartment where heavy metals are localized. Deposits in the form of fibrous material were observed in vacuoles of bean root cells exposed to low concentrations of Cd (Vázquez et al. 1992). Gzyl et al. (2009) observed electron dense material in vacuoles of Cd-tolerant cells of cucumber suspension culture, and Daud et al. (2009) found Cd accumulated in the form of electron dense granules and crystals in vacuoles of cotton root cells. In addition to the vacuoles, Cd was also detected in the cytoplasm as granular deposits (Han et al. 2007; Van Belleghem et al. 2007). Similarly, Cd application increased the number of osmiophilic globules in the cytoplasm and nucleolus-associated bodies (Gzyl et al. 2009), and Cd ions were also localized in the cytoplasm of rhizodermal cells in the basal part of onion roots exposed to this heavy metal (Liu et al. 2007b). Van Belleghem et al. (2007) reported that in the endodermis of *A. thaliana* exposed to Cd, the metal was sequestered in the vacuoles in form of Cd/S fine granular deposits but present in the cytoplasm in form of large granular deposits. Concerning other organelles, Cd was localized in nuclei of Cd-treated root cells of bean (Vázquez et al. 1992) and also in nuclei of pericycle cells of maize roots (Wójcik and Tukiendorf 2005).

5 Accumulation in Various Tissues and Cells

Cadmium as well as Zn might enter the plant body through the cells, which are in direct contact with the environment. In the case of the root, the tissue responsible for safeguarding is the rhizodermis. As these types of cells are directly exposed to heavy metals in the liquid phase of the soil, the concentration of heavy metals in these cells is anticipated to be higher. There is evidence that Cd and Zn might accumulate in these cells and their derivates. For example, Küpper et al. (2000) found the accumulation of Cd and Zn in cell walls of the rhizodermis, and, similarly, Liu et al. (2007b) reported high concentration of Cd in the surface of onion rhizodermis cells. The highest concentration of Zn across the root was observed in the rhizodermis of hybrid poplar (*Populus* × *euramericana* clone I-214) exposed to elevated Zn, and the concentration of Zn decreased gradually from the surface to the inner part of the root (Stoláriková et al. 2012). The same pattern of Zn localization is characteristic also for secondarily thickened roots of grey mangrove (MacFarlane and Burchett 2000), spruce (Brunner et al. 2008), or species from the Salicaceae family (Di Baccio et al. 2009; Vaculík et al. 2012a). However, in these older roots, the bark peripheral tissue layers consist not only of rhizodermal cells but also other primary cortical tissues. Increased accumulation of Cd was found, for instance, in root hairs of *Biscutella laevigata*, a natural Cd hyperaccumulator (Pielichowska and Wierzbicka 2004), in maize (Seregin

et al. 2004), and Zn was found to be sequestered in root hairs of *Paulownia tomentosa* (Azzarello et al. 2012).

From the rhizodermis, Cd and Zn are transported across the root via the apoplasmic route through the intercellular spaces and cell walls, or need to pass through the plasma membrane of exo- and endodermal cells to reach the symplasmic space. It is well documented that ions cannot pass easily via the apoplasmic pathway in these cell layers, and, therefore, the concentration of Cd and Zn is higher in the root cells surrounding exo- and endodermis. This was clearly evident, for instance, in root cells of poplar, where Cd predominantly accumulated in the cells surrounding the central cylinder (Coccoza et al. 2008). Additional evidence that the endodermis serves as an effective barrier for Zn transport across the root comes from experiments on a hybrid poplar (*Populus* × *euramericana* clone I-214) exposed to elevated Zn, where the concentration of Zn was almost doubled in cells of innermost mesodermal cell layer adjacent to the endodermis with developed suberin lamellae, when compared to the cells of the pericycle (Stoláriková et al. 2012).

The accumulation and distribution of Cd and Zn particularly vary between various tissues of the root central cylinder. Only limited accumulation of Cd was observed in the pericycle, which probably relates with the development of lateral roots when plants are grown in the presence of Cd (Seregin et al. 2004). On the contrary, an increased accumulation of Cd and Zn is characteristic for vascular tissues. Vascular tissues are heterogeneous and consist of cells whose main function is transport (i.e. tracheal or sieve elements), but also from other accompanying and supporting cells with parenchymatous or sclerenchymatous character. As the main function of vascular cell types is to transport water and ions (transpiration stream), or assimilates (assimilation stream), they are directly exposed to heavy metals. For example, Isaure et al. (2006) localized most Cd in vascular bundles of *A. thaliana*, and, similarly, Hu et al. (2009) found increased concentration of Zn in xylem parenchyma cells of *Potentilla griffithii*, a Zn hyperaccumulator. Da Cunha and do Nascimento (2009) examined by light microscopy and histochemistry maize roots grown in soil contaminated by Cd and Zn and found that these metals accumulated, besides in exo- and endodermis, mainly in xylem, phloem and also in the pericycle. Wójcik and Tukiendorf (2005) found Cd in the cell walls of the metaxylem and inside some parenchymatic cells adjacent to the xylem of maize roots. Similarly, Han et al. (2007) found Cd deposited on the inner surface of xylem vessels in root tips of *Iris tectorum*. Vaculík et al. (2012a) observed that Cd was predominantly accumulated in the secondary xylem, while Zn was mostly localized in peripheral root tissues of willow. Large amounts of precipitated Cd in the phloem of *Arabidopsis* suggested that this metal might be re-translocated to the root from the shoot via these tissues (Van Belleghem et al. 2007).

From the root, heavy metals are translocated via the vascular system to the shoot. Therefore, cells of vascular tissues in stems, similarly as in the roots, might be the place with increased concentrations of heavy metals. This was documented by Maruthi Sridhar et al. (2005), as they observed Zn and Cd precipitates as black

deposits along the walls of xylem and phloem vessels in stems of Zn- and Cd-treated Indian mustard (*Brassica juncea*).

In leaves, accumulation of Cd was observed in petioles and central veins of *Arabidopsis halleri* leaves after hydroponic culture enriched for Cd for 3 weeks. However, the same plant showed a decrease of Cd accumulation in these cells and along the vascular bundles of leaves after 9 weeks of cultivation in the same hydroponic medium (Huguet et al. 2012). Higher concentrations of Cd were also detected in bundle sheath cells which accompany vascular tissues (Hu et al. 2009, 2012a). Da Cunha and do Nascimento (2009) found that Cd was mainly accumulated in cells which surrounded the main vein of maize leaf, and Van Belleghem et al. (2007) detected Cd in tracheids of *A. thaliana* leaves.

Cells of leaf mesophyll are characterized by a relatively low Cd accumulation when compared with the epidermis. This was attributed to a more efficient containment of the photosynthetically active cells of palisade or spongy parenchyma. Hu et al. (2012a) described a relatively low Cd accumulation in mesophyll cells of *Picris divaricata* and found high tolerance to Cd in individual protoplasts isolated from the mesophyll, a phenomenon that had been previously documented also in other studies performed on hyperaccumulators *T. caerulescens* (e.g. Ma et al. 2005; Wójcik and Tukiendorf 2005), *T. praecox* (Vogel-Mikuš et al. 2008), *P. griffithii* (Hu et al. 2009) and *Sedum alfredii* (Tian et al. 2009). However, higher concentrations of Cd and Zn in mesophyll over those in epidermal cells were observed in *A. halleri* (Küpper et al. 2000), and also for the Cd and Zn hyperaccumulator *N. caerulescens*, Cd content was heterogeneously distributed over individual cells (Küpper et al. 2007). Whether heavy metals are stored in these individual mesophyll cells with the function to protect the neighbouring parenchyma cells is still not clear. It should also be noted that although Cd and Zn concentrations per cell are usually lower in mesophyll than in other leaf cells, the major part of total heavy metals in the leaf are located in the mesophyll due to the high total volume of mesophyll tissue, and, therefore, this tissue is often a major storage site for Cd and Zn in the leaves (Ma et al. 2005).

The increased concentration of heavy metals in epidermal cells of aerial plant organs cannot be explained by uptake of these harmful elements from the air. The portion of heavy metals taken up by epidermis directly is relatively small. Moreover, the cells of shoot and leaf epidermis are mostly covered by lipid components like cutin, suberin and other waxy substances to prevent the cells from water leakage and the entrance of biotic and abiotic stressors (Schreiber 2010; Domínguez et al. 2011; Ranathunge et al. 2011). Although direct uptake through epidermis is negligible, relatively high concentrations of Cd and Zn have been found in epidermal cells of some plant species, especially those belonging to metal hyperaccumulators (e.g. Cosio et al. 2005; Vogel-Mikuš et al. 2008). The explanation for this apparent paradox is metal uptake by the roots, followed by the translocation via xylem vessels in the stem and final storage in leaf epidermal cells. It has also been reported that Cd is often stored in trichomes, unicellular or multicellular derivates of epidermal cells. Although they are similar to root hairs, only few specialized absorption trichomes of specific species actually participate in

uptake. Usually, their function is to cover leaves and stems and to protect them against abiotic stress factors, like intensive radiation or temperature changes, as well as against biotic stresses such as herbivores and insects (Fahn 1990).

Several studies documented that epidermal cells and leaf trichomes can accumulate heavy metals. Isaure et al. (2006) found that trichomes represent the main compartment of Cd accumulation in leaves of *A. thaliana*. Similarly, Pielichowska and Wierzbicka (2004) reported that the leaf epidermal cells and hairs of *B. laevigata* accumulated large proportion of Cd. Zinc was also detected in large amount in trichomes of the Zn hyperaccumulator *A. halleri* (Zhao et al. 2000), and in tobacco, both Zn and Cd were excreted into epidermal trichomes (Choi et al. 2001; Sarret et al. 2006). Broadhurst et al. (2013) found that Zn was concentrated in ovate spots around the tips of individual cells of multicellular larger trichomes and epidermal cells adjacent to the trichomes, but not in other epidermal cells of *P. divaricata*. In the same species, trichomes and cells of upper and lower epidermis are also characterized by Cd accumulation (Hu et al. 2012a). Heavy metals such as Cd and Zn can accumulate in other specialized epidermal cells – idioblasts – as it was observed in mulberry (Katayama et al. 2013). Therefore, it has been suggested that deposition of heavy metals in epidermal cells and their derivates is used as mechanism to cope with Cd and Zn in the environment, especially in species that hyperaccumulate heavy metals (Pielichowska and Wierzbicka 2004).

6 Influence on Gene Expression

Transcriptome changes after Cd and Zn treatment are dependent on ontogenetic stage, cell type, spatial localization and time period/term of treatment, metal concentration, species and genotype. Plant cells respond to Cd and high Zn concentrations by changes in expression of hundreds to thousands genes, among which some 40–60 % are upregulated and the rest are downregulated (Yamaguchi et al. 2010; Di Baccio et al. 2011; Zhang et al. 2012a; Lin et al. 2013). Most of those genes act in primary metabolism, protein structure and synthesis, signal transduction, nucleotide binding and transcription as well as transport and response to stress of abiotic and biotic stimuli, including sulphur metabolism (Herbette et al. 2006; Romero-Puertas et al. 2007; Cebeci et al. 2008; Harada et al. 2010; Di Baccio et al. 2011; Zhang et al., 2012). Already at mild metal concentrations, the changes in the transcriptome are pronounced (Yamaguchi et al. 2010; Lin et al. 2013). Cadmium increases the expression of genes for pathogenesis-related proteins and for the proteins involved in remobilizing carbon from other energy sources (Kieffer et al. 2008) and causes dehydration stress via repression of dehydration-related transcription factors and genes for aquaporin isoforms (Yamaguchi et al. 2010). The circadian clock genes are affected too and this could impact the plant responsiveness to photoperiod and temperature and hence developmental decisions such as flowering (Maistri et al. 2011). To cope with Cd, plants activate the sulphur assimilation pathway to provide an enhanced supply of

glutathione for phytochelatin biosynthesis (see also chapter by Khan and Hell, this volume), which is followed by gradual decrease of glutathione content and increase of phytochelatin content (Herbette et al. 2006; Romero-Puertas et al. 2007; Shanmugaraj et al. 2013). Additionally, several genes encoding enzymes involved in the biosynthesis of phenylpropanoids are induced rapidly (Herbette et al. 2006), which may contribute also to the synthesis of lignin and other compounds constituting apoplasmic barriers (Lux et al. 2011a). Cadmium induces genes encoding enzymes for the synthesis of trehalose and polyamine, as well as tryptophan, identified as critical factors for the resistance to various stresses (Zhao et al. 2010). Moreover, the cells increase the transcript levels for antioxidant enzymes like catalase and monodehydroascorbate reductase (Romero-Puertas et al. 2007), as well as other genes contributing to Cd resistance (like AtATM3, which very probably mediates transport of glutamine synthetase-conjugated Cd^{2+} across the mitochondrial membrane) (Kim et al. 2006). On the other hand, Cd downregulates genes for regulators of oxidative stress, such as CuZn-superoxide dismutase and Mn-superoxide dismutase, as well as of proteins involved in carbon metabolism (Kieffer et al. 2008). The cells of vascular tissues downregulate AtFRD3, a xylem-loading citrate transporter (Romero-Puertas et al. 2007; Yamaguchi et al. 2010), probably with the function to decrease the translocation of Cd bound to citrate, because the Cd loading into the root xylem is responsible for the differences in Cd accumulation in the aerial parts of plants (Xu et al. 2012).

Regulatory networks differentially modulate gene expression depending on time course and tissue in response to Cd and Zn. Gene expression is more responsive to chronic than to acute exposure (Herbette et al. 2006). Moreover, different parts of plants respond to the heavy metals differently, with some cells more adapted to cope with stress and deposition of metals than others. For example, trichomes are biologically active and stress-responsive cellular targets specifically or dominantly expressing genes of antipathogenic T-phylloplanin-like proteins, glutathione peroxidase and several classes of pathogenesis-related proteins and proteins linked with osmotic regulation (osmotin- and thaumatin-like proteins) (Harada et al. 2010). Even for closely related species, these expression patterns can show pronounced differences. The Zn/Cd hyperaccumulator *N. caerulescens* expresses many species-specific genes (Rigola et al. 2006), and the difference from its relative, the non-hyperaccumulator *Thlaspi arvense*, concerns approximately 5,000 differentially expressed genes, including genes involved in Zn transport and compartmentalization (Hammond et al. 2006). Comparison with other close relatives, *A. halleri* (Zn/Cd-tolerant, Zn hyperaccumulator) and *A. thaliana* (Cd-sensitive), reveals distinct signalling cascades in response to metals (Weber et al. 2006). Moreover, Zn tolerance in *A. halleri* involves strong constitutive expression of metal homeostasis genes (ZIP6, a putative cellular Zn uptake system and a member of the zinc-/iron-regulated transporter-like protein; AtHMA3, a putative P-type metal ATPase; ZAT/AtCDF1, a cation diffusion facilitator; and AtNAS3, a nicotinamine synthase) in the shoots to accommodate higher basal levels of Zn accumulation (Becher et al. 2004). In metallicolous genotypes of *Salix caprea*, cysteine biosynthesis genes are constitutively upregulated, while

some metallothioneins and cell wall-modifying genes were induced irrespective of genotype and metal uptake capacity (Konlechner et al. 2013).

This molecular information is useful for further development of gene-specific markers for screening and breeding of low Cd and high Zn lines of crops to cope with metal malnutrition (Chandel et al. 2010).

7 Protein and Metabolic Responses

Plant cells respond to the presence of Cd and Zn by changes of protein profiles significantly correlating with transcriptomic regulation (Fukao et al. 2009). The modulation in the levels of tens to hundreds of proteins depends on cell type, plant organ, type of metal and concentration, duration of exposure and the genotype (Fukao et al. 2009; Garcia et al. 2009; Durand et al. 2010). Upon exposure to Cd and high Zn concentrations, root cells up- and downregulate enzymes and their isoenzymes of specific metabolic pathways and cellular processes as manifestations of tolerance mechanisms (mainly of the heavy-metal detoxification and antioxidant processes), but also modulations of carbon, nitrogen and sulphur metabolic pathways (Sarry et al. 2006; Aina et al. 2007; Alvarez et al. 2009; Kim and Lee 2009a; Wang et al. 2011b).

Approximately half of the proteins upregulated by Cd in the root are involved in responses to oxidative stress and redox homeostasis (Alvarez et al. 2009; Lee et al. 2010). Cadmium increases the amount of the antioxidative enzymes and the transporters and proteins involved in the degradation of oxidatively modified proteins (Aina et al. 2007; Kim and Lee 2009b). These prompt antioxidative responses are necessary for the reduction of metal-induced oxidative stress in roots (Lee et al. 2010). The cells upregulate ferritin and glutamine synthetase (a key enzyme in glutathione biosynthesis) (Hradilova et al. 2010), glutathione S-transferases and glutathione peroxidase, resulting in an accumulation of ferritin, glutathione-derived metal-binding peptides and several different families of phytochelatins. These compounds bound to metals are exported from cytosol by glutathione-conjugate membrane transporters (see chapter by Khan and Hell, this volume) and thus help to maintain the low metal levels at sensitive sites (Roth et al. 2006; Sarry et al. 2006; Alvarez et al. 2009; Halušková et al. 2009; Kieffer et al. 2009a; Hradilova et al. 2010). Both metals, Zn and Cd, induce N- and O-glycosylation and thus cause pronounced changes in glycoprotein pattern profiles (Stefanic et al. 2012). Moreover, increased abundance of antioxidant enzymes, namely, superoxide dismutase, ascorbate peroxidase and catalase, ensures cellular protection from reactive oxygen species under Cd stress (wheat, Wang et al. 2011a; soybean, Hossain et al. 2012). However, higher concentrations of the metals can inhibit some enzymes of antioxidant system, such as glutathione reductase and ascorbate peroxidase, in the leaves (Kieffer et al. 2009b).

When the root system is not able to maintain Cd either in the rhizosphere or deposited in the roots, some amount of metal is translocated into the aerial parts of

the plant. After that, cells of shoot activate a reaction similar to a hypersensitive response during plant-pathogen interaction – enhanced abundance of stress-related proteins such as heat shock proteins and proteinases. This is accompanied by a massive secretion of pathogenesis-related proteins into the extracellular space (poplar, Kieffer et al. 2009a; barley, Pos et al. 2011). Additionally, proteins involved in phytohormone synthesis, energy metabolism and cytoskeleton remodelling are induced (*Triticum aestivum*, Wang et al. 2011a; *Solanum torvum*, Wu et al. 2013). Cadmium entering into leaf cells can impair a prime sensitive target – plastids (Molins et al. 2013) reducing photosynthesis – which results in reorganization of carbon and carbohydrate metabolism and subsequent inhibition of growth (poplar, Durand et al. 2010). The availability of photoassimilates for developmental processes is decreased, since instead they have to be utilized for energy generation (Hossain et al. 2012) or stored in the form of hexoses or complex sugars, thus acting as osmoprotectants (Kieffer et al. 2009a). Under such conditions, the non-photosynthetic cells do not only suffer stress from the direct exposure to metals but also from reduced supply with assimilates as consequence of leaf stress (Durand et al. 2010). This may be the reason why these cells upregulate enzymes of mitochondrial respiration (involved in glycolysis and TCA cycle) to provide more energy to meet the high energy demand to sustain tolerance to heavy-metal stress (poplar, Kieffer et al. 2009b; soybean, Hossain et al. 2012). Because of the lack of sufficient energy, the Cd-challenged cells downregulate several proteins involved in sucrose metabolism, protein synthesis and processing. However, the cells enhance the expression of molecular chaperones to help refold misfolded proteins and to stabilize protein structure and function, thus maintaining cellular homeostasis (Alvarez et al. 2009; Kim and Lee 2009a, b; Aloui et al. 2011; Hossain et al. 2012). Cadmium also increases expression of methionine synthase, fructose-bisphosphate aldolase, triosephosphate isomerase (playing roles in defence against Cd toxicity) (Kim and Lee 2009a), ATP sulphurylase, glycine hydroxymethyl-transferase, trehalose-6-phosphate phosphatase and latex allergen-like proteins (Roth et al. 2006). Moreover, the cells change the expression of proteins related to metabolism, protein destination and storage, signal transduction, energy and cell structure (Hradilova et al. 2010). To decrease the negative impact of Cd on the whole organism, the plants evolved a strategy of contrasting accumulation of Cd in the cells of different plant parts. In the aerial parts the metal is prevalently localized in the basal leaves. In those leaves, the nitrogen and sulphur metabolic pathways are activated to produce more glutathione as a source for phytochelatin production, whose main role is in chelating Cd. These chelate-polypeptide complexes accumulate in the vacuole, limiting the distribution of Cd to apical leaves. These metal-enriched leaves display reduced chlorophyll *a* synthesis and photosynthesis, followed by an over production of ROS, leading to senescence and cell death. On the other hand, in the apical leaves the total chlorophyll content increases as well as the photosynthetic complexes and enzymes involved in CO_2 fixation and carbohydrate metabolism. Thus, apical leaves seem to supply the energy required by the plant. Many proteins involved in carbon metabolism are less abundant, whereas proteins involved in remobilizing carbon from other energy sources are

upregulated. This contrasting accumulation of metals and detaching those parts with sequestered metals from the plant represents an active detoxification strategy in higher plants (Fagioni and Zolla 2009).

Plants hyperaccumulating heavy metals have to cope with even higher metal concentrations in their cells than the non-hyperaccumulating plants. *Phytolacca americana* L. is a Cd hyperaccumulator plant reacting to the presence of metals by alterations in protein expression pattern of leaves. While major changes are seen for photosynthetic pathways, and in the sulphur- and glutathione-related metabolisms, also proteins that attribute to the processes of transcription and translation and molecular chaperones are upregulated (Zhao et al. 2011). The hyperaccumulating plant *Arabis paniculata* shares these responses to Zn and Cd in similar pathways to a certain extent. The leaf cells react to Zn stress mainly by enhanced auxin biosynthesis as well as by enhanced energy and protein metabolism to maintain plant growth and correct misfolded proteins. In the case of Cd, plants activate antioxidative metabolism and processes involved in xenobiotic tolerance to sustain cellular redox homeostasis and metal transportation (Zeng et al. 2011). Cells of *N. caerulescens* possess a higher abundance of glutathione S-transferase proteins, ZIP family transporters and vacuolar Zn transporter MTP1, which contribute to relatively large amounts of Zn. The metal is stored in mesophyll cells in the form of Zn-nicotinamine complexes, while the epidermal cells use complexation with malate and citrate (Schneider et al. 2013).

Cadmium at higher concentrations initiates a gradual destruction of plant cells culminating in cell death. Cell death can occur in (at least) two forms (Van Doorn et al. 2011): The cell either dies through a relatively slow cascade of autolytic events proceeding in the cytoplasm, while the plasma membrane is still preserved (vacuolar programmed cell death), or, alternatively, can collapse rapidly, with the cell component remaining largely unprocessed (necrotic cell death). The type of ensuing cell death is dependent on the concentration of Cd and on the position in the cell cycle as observed in a study, where Cd was administered in a synchronized population of tobacco BY-2 cells. Exposure of these cells to high Cd concentrations (1 mM) induced necrotic death of all cells, whereas a 20 times lower concentration of Cd (50 μM) induced in some cells a vacuolar type of programmed cell death, and in others a necrotic type of cell death, depending on the position in the cell cycle (Kuthanová et al. 2008).

8 The Interactions in the Rhizosphere

The interactions in the rhizosphere between plants and microorganisms can significantly influence the responses of plants to heavy metals. However, our understanding of the complex interactions in the rhizosphere and the effect of other organisms on the plant-based mechanisms is still far from complete. Progress in the field can be expected from approaches, where controlled microbial inoculations of the

rhizosphere are investigated with respect to their effects on heavy-metal uptake by plants (Singh et al. 2011).

Bacterial and nonpathogenic fungal microorganisms modulate specifically gene expression and proteomic profiles of plants. For instance, in shoot cells of *A. halleri*, they induce proteins related to photosynthesis and abiotic stress (subunits of the photosynthetic complexes, Rubisco, superoxide dismutase and malate dehydrogenase) accompanied by reduction of plant defence-related proteins (e.g. endochitinases, vegetative storage proteins and beta-glucosidase) (Farinati et al. 2009, 2011). Roots of *Medicago truncatula* downregulate several of the Cd-induced proteins in the presence of the arbuscular mycorrhizal symbiont *Glomus intraradices*, and a part of proteins involved in the symbiotic program are recruited to counteract Cd toxicity through the mycorrhiza-dependent synthesis of proteins putatively involved in alleviating oxidative damages (Aloui et al. 2009). The mycorrhizal symbiosis between *Glomus irregulare* and *M. truncatula* alleviates the negative effects of Cd on photosynthesis through the increase in photosynthesis-related proteins coupled to a reduction in gluconeogenesis/glycolysis and antioxidant processes (Aloui et al. 2011).

9 Application in Phytotechnologies

The use of knowledge on plant cells and thus the use of plants in technologies – phytotechnologies – potentially offers solutions to cope with major environmental (metal contamination of soils and water), dietary (as Cd consummation and Zn malnutrition), energetic (enormous consumption of non-renewable energy sources) and aesthetical (landscape damage after mining activities) problems of today (Prasad et al. 2010).

Phytoextraction as one of principal phytoremediation technologies utilizes the remarkable ability of plants to concentrate elements in their bodies and thus to reduce the concentrations or toxic effects of contaminants (e.g. Cd or Zn in physiologically exceeding concentrations) in the environment. This relatively recent technology is perceived as cost-effective, efficient, eco-friendly, nonintrusive, solar-driven and aesthetically pleasing technology with good public acceptance (Salt et al. 1998; Cheraghi et al. 2011; Ali et al. 2013). It is possible to increase the efficiency of phytoextraction by using suitable amendments enhancing the mobility of metals and their uptake by plants and by using plants selected for reasonable efficiency in extracting heavy metals from soil and water (Nehnevajova et al. 2005; Marmiroli et al. 2011; Huang et al. 2013). Therefore, hyperaccumulating plants are also in the centre of interest. These plants possess a strongly enhanced rate of heavy-metal uptake, a faster root-to-shoot translocation and a greater ability to detoxify and sequester heavy metals in leaves (Baker and Whiting 2002; Rascio and Navari-Izzo 2011). The disadvantage of the majority of hyperaccumulator plants is a relatively low biomass production. Therefore, fast-growing woody species or crops, although they are accumulating less metals relative to biomass, are used more often in combination with advanced

agrotechnology, biotechnological improvement and plant selection based on cell biological knowledge. For example, the understanding of a relation between the development of apoplasmic barriers in root endodermal cells and the ability of root-to-shoot translocation of heavy metals in different willow clones helps to select more suitable plants for the phytoextraction of metals. The plants forming the apoplasmic barriers in a greater distance from the root apex are able to translocate larger amounts of metals to the aerial parts (Lux et al. 2004b). Higher concentration of metals in the aerial parts makes it easier to collect contaminants concentrated in plant body and thus enhances the efficiency of phytoremediation process. A problem arising from this increased translocation is the presence of larger amounts of metal in the shoots challenging the plants by increased metal toxicity. The increased root-to-shoot translocation of cadmium and zinc combined together with an enhanced cadmium tolerance was achieved biotechnologically by artificial expression of genes like metallothionein *MT2b* and heavy-metal-transporting ATPase *HMA4* in tobacco cells (Grispen et al. 2011). Likewise, biomass production and tolerance of poplar plants growing in metal-contaminated soils were enhanced by transformation of plants with the γ-glutamylcysteine synthetase as compared to wild type plants (Ivanova et al. 2011). The capacity of plants to extract the metals of interest from soils can be improved also by inoculation of soil/plants with bacteria (e.g. *Bacillus thuringiensis*, Babu et al. 2013; *Sphingomonas* sp., *Variovorax* sp., Zhang et al. 2013) or fungi (e.g. *Fusarium oxysporum*, Zhang et al. 2012b). Plant-microbial symbiosis generally enhances the metal extraction capacity of plants by changes in expression pattern and cellular metabolism, leading to increases of uptake, translocation and accumulation of metals. On the other hand, the ectomycorrhizal *Amanita muscaria* does not change significantly the heavy-metal concentration in the plants of *Salix dasyclados*, even though it is beneficial evident from the larger production of willow biomass (resulting from mycorrhiza) increasing the total amount of absorbed metals from soils (Hrynkiewicz and Baum 2013). Evaluation of genetic diversity associated with heavy-metal bioaccumulation potential, growth, physiological and biochemical responses of various plants to heavy metals helps to select the optimal genotypes for strategies using plants either to phytoremediate heavy-metal-contaminated areas (Mleczek et al. 2010; Marmiroli et al. 2011; Han et al. 2013; Konlechner et al. 2013) or to improve the quality of plant food.

A large portion of the world population suffers from malnutrition of trace elements, including zinc. Phytofortification can be used to meet the goal to produce sufficiently and sustainably safe and nutritious food, with enhanced amount of Zn. The result of phytofortification is a consumption of increased amounts of Zn in edible parts of plants. Successful phytofortification can be achieved through fertilization, crop breeding or biotechnology. Identification of plant interspecies and intraspecies variation in trace element accumulation and understanding the transport and homeostasis of trace elements will assist to approach this ultimate goal, but still many aspects have to be elucidated (Zhao and McGrath 2009).

On the one hand some plants, tolerant to heavy metals, are able to hyperaccumulate metals in shoots, which could be beneficial for phytoremediation

purposes in order to clean soil and water. On the other hand, tolerant food crops, exposed to heavy metals in their growth medium, may be dangerous as carriers of toxic metals in the food chain leading to food toxicity. There is an additional duality in plant tolerance to heavy metals: Food crops that are tolerant and/or hyperaccumulators could be used on one hand for phytoremediation but at the same time under controlled conditions could be used for food fortification providing essential metals (Verkleij et al. 2009). To solve the problem of Zn phytofortification without contamination of the plants and their consumer with Cd, it is possible to express in cells of otherwise heavy-metal low-accumulating plant a high-affinity and high-specificity Zn transporter (Dixit et al. 2010). Thus, the complex understanding of plant-metal interactions is essential to use phytotechnologies optimally. Extended knowledge of the uptake, translocation, storage and detoxification mechanisms in plants and of their interactions with microorganisms and the combined use of functional genomics, proteomics, metabolomics, genetic analysis and plant breeding are essential to understand and control the fate of metals in plants and food, nonfood and technical crops. The integration of knowledge about plant cells from different scientific views allows to optimize plant properties, making phytotechnologies more economically and socially attractive, decreasing the level and transfer of metals along the food chain and augmenting the content of essential minerals in food crops (Mench et al. 2009).

Plants can be also used for long-term biomonitoring of soils contaminated with heavy metals. The content of Zn and Cd in the leaves and fruiting catkins of *Populus alba* report the content of these metals in a soil after calibration based on bioaccumulation coefficients (Madejon et al. 2013). Further advantages of phytotechnologies are renaturation of contaminated areas (Han et al. 2013), the phytostabilization of soils against erosion and the stabilizations of essential elements availability (Ciadamidaro et al. 2013; Madejon et al. 2013). In addition, the use of phytotechnologies improves carbon sequestration and production of renewable energy sources, which contribute to sustainable land use management (Mench et al. 2009).

This review summarizes our growing recent knowledge about cadmium and zinc uptake, translocation, transport, distribution, accumulation and transcriptomic, proteomic and metabolomic responses of plant cells, including the induction of programmed cell death by these heavy metals. The chapter highlights the important checkpoints controlling the route of heavy metals through the plant body. These data are valuable for screening, breeding and cultivation of low Cd and high Zn lines of crops to maintain the food safety and thus the health of people.

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Applied Cell Biology of Sulphur and Selenium in Plants

M. Sayyar Khan and R. Hell

Abstract Sulphur (S) is required in considerable amounts by all organisms, while selenium (Se) is beneficial for plants and essential for animals albeit in only small amounts. Due to their chemical similarity that is in parts also shared with molybdenum and chromium, inorganic sulphate and selenate are taken up by plants and assimilated in reduced form into organic compounds, most notably cysteine and selenocysteine, respectively. Uptake, reduction, and storage of S and Se compounds underlie complex cellular processes that need to be understood before successful translation into improved plants for human and animal diets can be achieved. Genetic engineering, breeding, and plant production approaches use insights from cell biology and basic research to introduce tailor-made desirable traits related to S and Se metabolism. Several examples for this approach will be discussed. In terms of enhanced crop quality, the so-called push and pull approaches to improve seed S amino acid compositions draw heavily from cell biology research. In pull or sink approaches, the expression of S-rich seed storage proteins is put under the control of seed-specific promoters. Such proteins possibly carry a targeting signal for the endoplasmic reticulum to achieve deposition in protein bodies. The regulation of some health-promoting compounds of Se together with S compounds such as glucoraphanin in Brassicaceae has received considerable attention in the recent past. However, to achieve high contents of Se metabolites simultaneously with the health-promoting S-containing compounds is challenging due to the crosstalk between the two pathways. The concept of S-enhanced defence linking S nutrition of plants with enhanced synthesis of S-containing defence compounds has been supported by considerable experimental data from basic research. Some of the

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recent findings regarding the role and potential biotechnological applications of some of these S-containing defence compounds including glutathione, phytoalexins, and glucosinolates are discussed in this chapter. Much progress has been achieved in the enrichment of Se contents in Brassica plants, while more basic research is required to enhance seed S contents and to understand how S nutrition is linked to plant defence.

1 Sulphur and Selenium from a Plant Nutritional Perspective

Sulphur is one of the six essential macronutrients in plants and carries out a multitude of functions in its oxidised (sulphate) and reduced (sulphide) state (Hell et al. 2008; Takahashi et al. 2011). Major functions include iron-S clusters, several vitamins, methionine and S-adenosylmethionine, and glutathione (GSH). In addition, most redox processes operate via thiol groups. These functions range from structural and regulatory disulphide bridges in proteins via thioredoxins and glutaredoxins to modifications by sulfenylation, nitrosylation, and glutathionylation and are thus indispensable for signal transduction processes conferred by reactive oxygen species during biotic and abiotic stress (Suzuki et al. 2012). Selenium, on the other hand, is not required by land plants but considered as beneficial nutrient. However, some algae, e.g. diatoms, need Se to produce several seleno-proteins (Pilon-Smits et al. 2009). For the same reason, Se is essential in the diet of mammals where an increasing number of seleno-proteins are still being discovered. S and Se, but also molybdenum (Mo) and chromium (Cr), share strong chemical similarities according to their grouping in the chemical period system. Indeed, uptake and metabolisation of these four elements by plants show some similarities, although Mo (used as molybdate) is the quantitatively least required essential micronutrient in plants (Bittner and Mendel 2010) and Cr (as hexavalent chromate) is one of the most hazardous heavy metals not only for plants but also for life in general, due to its mutagenic, carcinogenic, and protein interaction potentials (Shanker et al. 2005).

Sulphate uptake in plants is an active process of sulphate/proton cotransport based on the proton motive force at the plasma membrane that is provided by the P-ATPase system. In all plants, sulphate transporters can be divided into high and low affinity types with respect to sulphate and grouped into four functional categories (Hawkesford 2008). The encoding genes underlie the S homeostasis network that controls expression changes in response to supply and demand (Takahashi et al. 2011). They carry a STAS (sulphate transporter and anti-sigma factor antagonist) domain that has been shown to be required for trafficking to the plasma membrane (Shibagaki and Grossman 2005) and needs to be phosphorylated for activity (Rouached et al. 2005). So far, no successful engineering approaches have been reported that enhance sulphate uptake to improve crop quality. Group 1 sulphate transporters are of the high-affinity type, localised in the plasma membrane, and mostly, but not exclusively, expressed in roots where they are responsible for

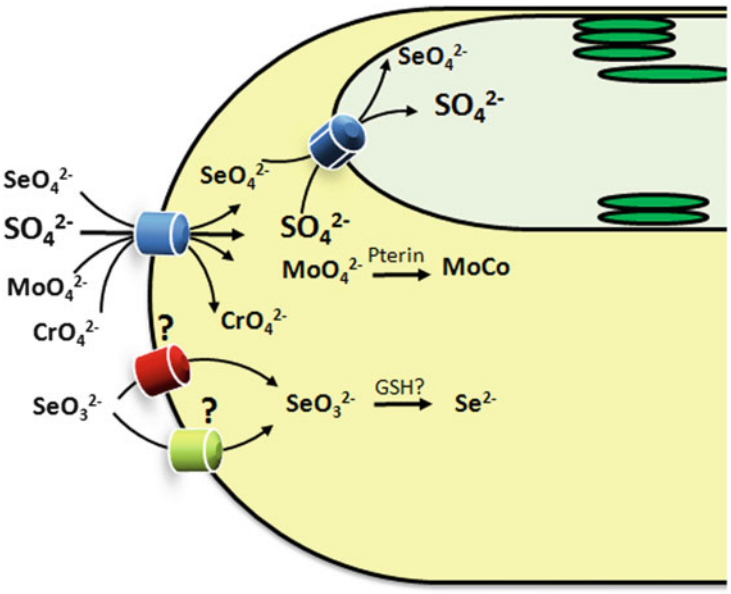

Fig. 1 Uptake of sulphur and chemically related inorganic compounds. *Blue barrel* represents sulphate transporters, *red barrel* represent phosphate transporters, and *light green barrel* represents silicon influx transporters. GSH, glutathione

the major uptake of sulphate from the soil (Fig. 1; Yoshimoto et al. 2002). Group 2 sulphate transporters reside also in the plasma membrane and are mostly expressed in the vasculature where they mediate long-distance-related loading processes. Group 3 members were shown to be localised in the inner plastid envelope and are responsible for import of sulphate from the cytosol into plastids (Cao et al. 2013), and group 4 transporters are localised to the tonoplast, where they export sulphate from the vacuole to the cytoplasm (Kataoka et al. 2004). The selenate, molybdate, and chromate anions are believed to enter plant cells in an unspecific way by the activity of these sulphate transporters (Fig. 1; Shibagaki et al. 2002; Schiavon et al. 2008; Bittner and Mendel 2010; Schiavon et al. 2012; Valdez-Barilla et al. 2011). Once arrived in the cytoplasm, molybdate is inserted into the pterin precursor to form the molybdenum cofactor that carries one or two S atoms depending on the target enzyme type (Teschner et al. 2010). Chromate can exert its toxic functions as soon as it arrives in the cytoplasm (but also in the nucleus) including interaction with SH groups of proteins and interference with GSH metabolism (Shanker et al. 2005). Thus, Mo and Cr are directly and indirectly related to S metabolism. In addition to selenate ($SeO4^{2-}$), also selenite ($SeO3^{2-}$) can be taken up by plants. Little is known about the mechanism, but it seems to be an active process according to metabolic inhibitor studies. Since phosphorous deficiency enhanced selenite uptake in wheat, it was suggested that selenite might enter the plant as a side activity of phosphate transporters (Li et al. 2008), but also

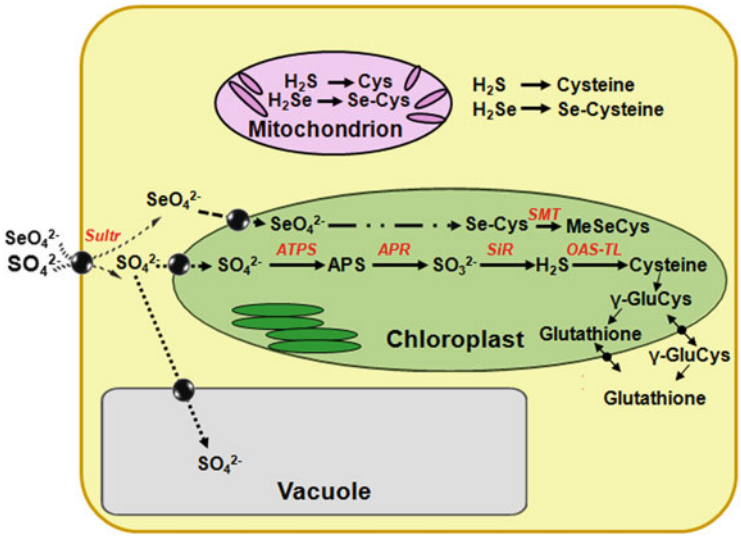

Fig. 2 Compartmentalisation of primary sulphur metabolism in *Arabidopsis*. The *dotted lines* in the chloroplast represent the steps catalysed by the enzymes shared by sulphur and selenium metabolism. Sulphur sulphate transporters, ATPS ATP sulphurylase; APR, adenosine phosphosulphate reductase; SiR, sulphite reductase; OAS-TL, *O*-acetyl serine (thiol) lyase; SMT, selenocysteine methyltransferase

silicon influx transporters have been suspected to be responsible for selenite uptake (Zhao et al. 2010).

Sulphate and selenite undergo further metabolisation in several cellular compartments (Fig. 2). Sulphate can be activated by ATP and ATP sulphurylase in the cytosol and the plastids. Reduction to sulphide takes place exclusively in the plastids via adenosine phosphosulphate reductase and sulphite reductase (Khan et al. 2010; Takahashi et al. 2011). Sulphide is inserted into cysteine in a two-step process that is catalysed and regulated by the cysteine synthase complex that exists not only in plastids but also in mitochondria and the cytosol (Hell and Wirtz 2011). Selenate, but not molybdate and chromate, is also reduced by the same assimilatory sulphate reduction pathway, yielding selenide and finally selenocysteine (Se-cysteine). In Se-resistant plants, Se-cysteine is specifically methylated to produce the nonprotein amino acid methyl-selenocysteine (MetSeCys; Fig. 2) by selenocysteine methyltransferase (SMT). The SMT enzyme has been shown for *Astragalus* to be predominantly localised within the chloroplast, which is the major site of S and Se assimilation in plants (Sors et al. 2009). Consequently, the toxic misincorporation of the Se amino acids into proteins is lowered (LeDuc et al. 2004). It should be noted that selenite can also be reduced nonenzymatically by GSH (Anderson 1993). There is evidence for this process only from *Escherichia coli* and yeast, but since the intracellular chemical conditions are comparable, there is good reason to assume that selenite can also be reduced in the cytoplasm and plastids of plants (Figs. 1 and 2; NG and Anderson 1978; Müller et al. 1997).

Since cysteine forms the central hub for distribution of reduced S into methionine and all other downstream metabolic processes, Se can be expected to be discriminated only in some cases, but to enter the usual metabolic pathways in others, for instance, to form Se-methionine. However, reduced Se cannot replace sulphide in most functions including Fe-S clusters, disulphide bridges, and catalytic processes. As a consequence, such proteins are dysfunctional and high Se concentrations are toxic for the plant. The fate of such proteins in plants is unclear, but recent evidence indicates that they are recognised by cellular surveillance mechanisms and degraded by the ubiquitin/proteasome pathway (Sabbagh and Van Hoewyk 2012). In contrast, Se-requiring organisms like bacteria and mammals have evolved special mechanism including a specific tRNA codons to allow targeted synthesis of Se-cysteine for protein translation (Pilon-Smits et al. 2009)

2 Sulphur from an Animal and Human Nutritional Perspective

In the human body, sulphur is the seventh most abundant element and consequently cysteine and methionine are required in the diet. Methionine is one of the nine essential amino acids for humans, and cysteine is regarded as conditionally essential, since methionine can be converted into cysteine via a trans-sulphurylation pathway. Thus, both amino acids are considered as equivalent from a nutritional perspective (Jez and Fukagawa 2008). In addition, the documented links between S and human health have triggered efforts to enhance the contents of nutritionally valuable S compounds in crop plants. But also high-yield animal production, e.g. of chicken and pigs, relies on balanced diets and even supplementation of amino acids. However, the most important food and feed crops differ considerably in their amino acid composition. Members of the legume family (e.g. soybean) are low in S amino acid contents, whereas cereals (e.g. maize) are low in lysine content and comparatively mediocre in cysteine and methionine contents (Wang et al. 2003). To this end, cell biological research using transgenic plants contributed significantly to the development of the so-called push and pull approaches.

Push approaches aim at the fortification of food and feed by enhancing biosynthetic pathways that lead to cysteine and methionine. The accumulation of these free amino acids in the target organs, usually seeds, is supposed to be integrated into seed storage proteins. Indeed, on average, seeds contain 20–40 times more S amino acids bound in proteins as compared to free amino acids (Chiaiese et al. 2004). Using model species such as tobacco, three main targets were analysed: uptake of sulphate, reduction of sulphate by adenosine phosphosulphate reductase, and synthesis of cysteine and of methionine. As mentioned before, primary sulphate uptake at the roots is under control of a network of signals and difficult to enhance without severe interference with other uptake pathways (Takahashi et al. 2000). However,

analysis of seeds of mutants of the plastid-localised sulphate transporter group 3, and of the tonoplast-localised sulphate transporter group 4 in *Arabidopsis*, suggests that manipulation of these uptake mechanisms may have a greater effect on seed protein composition (Zuber et al. 2010a, b). Overexpression of the central regulatory step of sulphate reduction catalysed by adenosine phosphosulphate reductase in maize pushed the pathway towards higher contents of cysteine and methionine, but apparently caused negative side effects on plant growth and has remained the only experiment in this direction. Overexpression of the two enzymes of cysteine synthesis, serine acetyltransferase or *O*-acetylserine (thiol) lyase, in either the cytosol or plastids, yielded several-fold increases of free cysteine in leaves but only minor increases of methionine (Sirko et al. 2004). This suggests that the trans-sulphurylation from cysteine to methionine in plants is regulated independently from the cysteine pathway. Overexpression of serine acetyltransferase in plastids under the control of a seed-specific promoter in *Lupinus angustifolius*, a grain legume, achieved high accumulation of free cysteine levels, but was not limiting for methionine or storage protein synthesis (Tabe et al. 2010). Much attention was paid to the overexpression of the central enzyme of trans-sulphurylation in plants and γ-cystathionine synthase (CGS) in plastids, leading towards enhanced synthesis of methionine. While belonging to the aspartate family of amino acids, plant methionine in several species exerts additional feedback control of its synthesis by an intricate mechanism of mRNA stalling at the ribosome when the nascent polypeptide chain of CGS is produced. A domain in the first exon of the mRNA determines translation and mRNA stability via binding of S-adenosylmethionine (Onouchi et al. 2004). Expression of a methionine-insensitive form of CGS in soybean seeds yielded increased free and protein-bound methionine contents if the overall amino acid content also increased, pointing to a connection between free amino acids and translation of seed storage proteins (Song et al. 2013).

This link becomes also relevant in the second major approach to enhance S amino acid contents. The pull approach uses naturally occurring or engineered proteins with high cysteine and methionine content. Examples are members of the soluble 2S albumin fraction and the 12S storage protein fraction where 8–15 % of residues belong to S-containing amino acids (Khan and Hell 2008). Expression of such protein-encoding genes under the control of seed-specific promoters creates sinks for free amino acids. If possible, such proteins carry a targeting signal for the endoplasmic reticulum to achieve deposition in protein bodies (Vitale and Hinz 2005). Several approaches aiming at improvement of legume seed amino acid composition have been reported. At best they just manage to reach the recommended limits of the Food and Agriculture Organization of the United Nations (Tabe and Higgins 1998; Boothe et al. 2010). Further work will include the combination of push and pull approaches in double-transformed plants. However, even stronger elevations apparently require parallel increases of contents of the other amino acids to meet the demands for S protein composition. Seed development including filling with storage compounds follows strict genetic programmes. Flexibility has evolved towards nutrient limitation as shown for

nitrogen and sulphur deficiency, when, for example, S-rich storage proteins are exchanged against proteins poor in S (Tabe et al. 2002; Higashi et al. 2006). Several lines of evidence point to similar compensatory mechanisms when storage proteins are overexpressed or downregulated or when storage proteins are replaced by proteins with improved properties. In one case, rice was transformed to express an S-rich 2S sunflower storage protein at high levels, but the amino acid composition and the total protein content remained largely the same, due to concomitant downregulation of other, endogenous, storage proteins (Hagan et al. 2003). In a different case, the overexpression of seed glutenin caused a decrease in the contents of endogenous prolamins in wheat (Scossa et al. 2008). These experiments suggest that a seed filling sensor may exist that monitors seed metabolite and/or volume and adjusts seed reserve contents (Lin et al. 2013). In summary, the combination of basic and applied research revealed numerous novel biological processes, and it will be up to cell biology to enable successful manipulation of seed protein composition.

3 Selenium and Human Health

Phytoremediation of Se-contaminated soils and human nutrition are the primary areas of interest in Se metabolism (Sors et al. 2005; Khan and Hell 2008; Rayman 2008). This essential micronutrient has important benefits for human and animal nutrition (Beck 2001; Beckett et al. 2004; Finley 2005; Ip 1998; Rayman 2002; Whanger 2004) and is needed in trace amounts with a recommended daily intake of 50–70 $\mu g \cdot day^{-1}$ for a person (U.S. department of Agriculture 2003). Examples for essential human seleno-proteins are GSH peroxidases, thioredoxin reductases, and iodothyronine deiodinases (Lu and Holmgren 2009). Numerous people (about 500–1,000 million) have been estimated to suffer from Se deficiency worldwide (Combs 2001). Deficiency of Se on one hand is associated with several health disorders (Rayman 2000; Whanger 2004); excess of Se on the other hand is toxic to animals (Lemly 1997; Wilber 1980) and humans (Van Vleet and Ferrans 1992) with a quite narrow gap between deficiency and toxicity (Zhu et al. 2009; Terry et al. 2000). Since the metabolism of Se leads to the production of a wide array of products (Ganther and Lawrence 1997; Ganther 1986), the biological activity of Se as an essential nutrient, cancer-preventive agent, or toxic compounds is not only dependent on the amount but also on the chemical form of Se that is produced during the course of metabolism (Ganther 1999).

Recently, the function of Se as health-promoting compound together with S in secondary compounds from Brassicaceae has received considerable attention. One of the first reports showing the effectiveness of Se in reducing the incidence of cancer in humans came in 1977 when mortality from cancer was examined as a function of Se intake/levels in whole blood in 27 countries, and an inverse association was found for several organs (Schrauzer et al. 1977). Interest in the use of Se as a chemopreventive agent peaked in 1996 when the results from a Se

supplementation study indicated that low-level, nontoxic supplementation with 200 μg Se per day could reduce the incidence of several types of common cancers when Se was supplied in the form of Se-treated yeast (Clark et al. 1996). This eventually led to the investment of the well-designed and large-scale Se supplementation trial known as 'Se and vitamin E cancer prevention trials (SELECT)'. The main aim of SELECT was to determine, among other issues, whether the provision of Se to men could reduce their risk of prostate cancer (Lippman et al. 2009). These results published initially in 2009 and then updated by additional follow-up studies (Klein et al. 2011) were not very promising since they indicated that, under their experimental conditions, Se was ineffective in reducing the incidence of prostate cancer. Although in contrast to animal data and epidemiological trends in humans with regard to the inverse association between Se intake and cancer incidence, at least for some organs sites, the supplementation data from clinical intervention trials may appear conflicting at this point. It has been argued that the difference in the form of Se and/or differences in the background Se levels of the study population may account for the apparent discrepancy. Efforts to understand the basic biology of seleno-proteins and Se compounds and their impact at physiological conditions may lead to optimised strategies based on better insight into Se supplementation (Bera et al. 2013).

4 Approaches to Modulate Selenium Contents in Plants

Improved agricultural practice, identification and utilisation of suitable donor genotypes, and genetic engineering approaches are some of the promising strategies to achieve plants with high contents of Se metabolites. However, to ensure high contents of Se metabolites while simultaneously safe guarding sufficient amounts of health-promoting S-containing compounds is a challenging task due to the fact that Se shares the initial steps for uptake and assimilation with S owing to their similar chemical properties which subsequently causes metabolic interference. The crosstalk between Se and the equally important S compounds in broccoli plants provides a nice example of the unintended interaction of manipulating a single desirable compound. The use of a Se-enriched commercially available broccoli cultivar in animal cancer trials (Finley 2003; Finley et al. 2000) revealed that as a result of Se fertilisation, the production of a potentially anticarcinogenic compound of S (sulforaphane) was inhibited by about 75 % compared with unfertilised controls (Charron et al. 2001). In view of such considerations, the production of the so-called Se-enriched functional foods at low/no compromise on other equally important bioactive compounds, specifically that containing S, is a major challenge for plant scientists that requires a comprehensive knowledge of the genes affecting S and Se uptake, assimilation, as well as metabolism coming from model and other organisms (Khan and Hell 2008).

It is important to mention here that, under field conditions, the formation of Se compounds in plants is limited by the low availability of selenate in most soils

which is the case in most of the Western European countries, where subsequently human Se intake is below the dietary recommendations (Combs 2001). Improved agricultural practices in Finland have shown that the addition of Se to agricultural fertilisers had increased the human dietary intake of Se by Finish people from 20 to 30 μg day^{-1} (in 1986) to 80 to 90 μg day^{-1} (in 1989), with the primary food source being wheat (Makela et al. 1993). Potatoes (Poggi et al. 2000), tomatoes, strawberries, radish, lettuce (Carvalho et al. 2003), and soybeans (Yang et al. 2003) provide further good examples for the effective production of Se-enriched foods through Se fertilisation.

5 Selenium Phytoremediation

Contrasting with attempts to increase Se uptake, many regions of the world face the problem of Se toxicity, leading an increasing demand for the cleanup of Se-contaminated soils. The observation that excessive Se bioaccumulation is toxic to wildlife has further increased interest in the phytoremediation of Se (Bañuelos 2001; Berken et al. 2002; Wu 2004). In this context, phytoextraction and phytovolatilisation are potentially promising strategies for remediation of Se-contaminated environments. Phytovolatilisation is particularly attractive, since in this strategy the plants metabolise inorganic Se to relatively nontoxic volatile forms such as dimethylselenide (DMSe) and dimethyl diselenide (DMDSe), which are then diluted and degraded after their escape to the atmosphere (Lewis et al. 1966; Terry et al. 2000), such that Se is completely removed from the local food chain (Atkinson et al. 1990). Phytoremediation of Se has been achieved under field conditions by planting fast-growing plant species such as Indian mustard (Bañuelos et al. 1997), which can accumulate Se up to several hundred micrograms per gram (Bañuelos and Schrale 1989). Overexpression of the *selenocysteine methyltransferase (SMT)* gene from the Se hyperaccumulator *Astragalus bisulcatus* (Hook.) Gray in *Arabidopsis* and Indian mustard has already been demonstrated to result in increased Se tolerance, accumulation, and volatilisation (LeDuc et al. 2004). However, the advantage conferred by SMT was less pronounced when Se was supplied as selenate instead of selenite. Double transgenic lines overexpressing both *ATP sulphurylase (ATPS)* and *SMT* resulted in substantial accumulation of Se in Indian mustard when Se was supplied as selenate (LeDuc et al. 2006), underpinning the importance of this step in Se metabolism.

There are still certain open questions that need to be thoroughly investigated. For example, it is still not well understood how plants take up the different forms of Se. Also, the subsequent xylem transport to the shoots needs to be elucidated. Selenate and selenite are the two major inorganic forms of Se available for plant uptake in the soil, and their abundance is dependent on redox potential and pH (Zhao et al. 2010). From the perspective of S metabolism, Se shares many chemical properties with S, both with respect to the forms in which it exists and its molecular interactions; like S, Se can adopt several valence states as selenide (Se^{2-}),

elemental Se (Se^0), selenite (Se^{4+}), and selenate (Se^{6+}) (Läuchli 1993; White et al. 2004). Based on the chemical similarity between selenate and sulphate, selenate is believed to be taken up via sulphate transporters supported by considerable experimental evidence (Terry et al. 2000; Sors et al. 2005). For example, the competitive inhibition of selenate by sulphate in barley (Leggett and Epstein 1956) suggests that both ions are imported by a common transporter. The high-affinity sulphate transporter Sultr1;2 has been identified in *Arabidopsis* using selenate as analogue of sulphate (Shibagaki et al. 2002) and has been shown to be a major contributor in the acquisition of selenate (El Kassis et al. 2007). A selenate-induced expression of sulphate transporters has been reported in several studies (Hsu et al. 2011; Takahashi et al. 2000; Van Hoewyk et al. 2008; Yoshimoto et al. 2002).

By contrast, little is known about the uptake mechanism of selenite in plants, and it was suggested in the past that selenite absorption may be an entirely passive process (Arvy 1993; Shrift and Ulrich 1969), since there was no evidence that selenite transport is mediated by membranes transporters (Arvy 1993; Asher et al. 1977). However, it has been demonstrated that, in wheat, selenite uptake is an active process since the uptake was significantly inhibited by the metabolic uncoupler CCCP (Li et al. 2008). It was argued that the uptake of selenite is at least mediated by phosphate transporters based on the evidence that phosphorus deficiency enhanced selenite uptake in wheat. Conversely, some of the earlier studies reported that increased phosphate concentration is correlated with decreased selenite uptake in different plant species (Broyer et al. 1972; Hopper and Parker 1999). However, the mechanism by which selenite is taken up by the roots is not understood. Recent findings suggest the involvement of the silicon influx transporter OsNIP2;1 in selenite uptake in rice (*Oryza sativa*). Defects of OsNIP2;1 led to a significant decrease in the Se concentration of the shoots and xylem sap in selenite-supplied plants which was not the case when Se was administered as selenate (Zhao et al. 2010).

Also the long-distance transport of Se from root to shoot is not well understood. Selenate is transported more easily than selenite as reported in several studies (Asher et al. 1977; Arvy 1993; Zayed et al. 1998). In fact, it has been demonstrated for Indian mustard that only 10 % of the absorbed selenite was transported from root to shoots, whereas selenate whose uptake was only twofold higher was rapidly transported into shoots (de Souza et al. 1998). These findings suggest that plants transport substantial amounts of selenate into the leaves compared to selenite. The reason for the poor translocation of selenite to the shoots has not been thoroughly investigated, but it has been suggested that this could be due to the rapid conversion of selenite into organic forms of Se such as selenomethionine (SeMet) (Zayed et al. 1998), which are retained in the roots.

The development of crop genotypes with improved Se accumulation and tolerance traits from both a nutritional and a phytoremediation perspective are the future challenges which could be achieved either via screening of existing germplasm or through conventional breeding. However, conventional breeding often is subjected to certain limitations like species barriers and lack of suitable donor parents available for breeding. As an alternative, genetic engineering approaches to

introduce tailored desirable traits offer potential as biotechnological tools. Strategies using genetic engineering rely on the knowledge of genes and their expression across different species that play a key role in Se metabolism. The results from fundamental plant biology research are providing valuable insight as a prerequisite for future biotechnological applications.

6 Sulphur in Relation to Plant Stress: The Role of Glutathione

Sulphur-containing defence compounds (SDCs) play a significant role for the survival of plants under biotic and abiotic stress. SDCs include elemental S (S^0), H_2S, GSH, various secondary metabolites including some phytoalexins, and S-rich proteins (Rausch et al. 2007). Experimental data in the recent past have already supported the basic concept of S-enhanced defence (Bloem et al. 2005; Haneklaus et al. 2005; Kruse et al. 2007, 2012).

The tripeptide thiol, glutathione (GSH) plays a crucial role for S metabolism and stress resistance. In plants, GSH has been implicated in protection against various forms of stresses by scavenging reactive oxygen species (Foyer and Noctor 2005; Mullineaux and Rausch 2005), sequestration of heavy metals (Cobbett and Goldsbrough 2002; Freeman et al. 2004; see also chapter by Martinka et al. in this volume), and detoxification of xenobiotics (Dixon et al. 1998). During detoxification, GSH S conjugates are formed and sequestered in the vacuole for degradation. The degradation is initiated by cleavage of the γ-peptide bond between cysteine and glutamate (Meyer and Rausch 2008). Apart from that, GSH is also believed to act as a systemic messenger in the hypersensitive response due to its rapid accumulation after fungal attack (Edwards et al. 1991; Foyer and Rennenberg 2000; Gullner and Komives 2001). The GSH-related antioxidative system plays a regulatory function during plant defence responses, because GSH mainly accumulates during incompatible plant pathogen interactions that often lead to oxidative burst in plant tissues (Gullner and Komives 2001). GSH participates primarily in buffering oxidative burst in plant tissues, either directly or via the ascorbate-GSH cycle (Foyer and Rennenberg 2000; Winterbourn and Metodiewa 1999). However, GSH seems to play also a direct role for disease resistance as suggested in the context of the S-containing phytoalexin camalexin. Resistance to *P. brassicae* was restored when the excised leaves of the camalexin-deficient *Arabidopsis* mutant *pad2-1* characterised by reduced levels of GSH were fed with GSH (Parisy et al. 2006). Indeed, GSH has been shown to be required for the synthesis of camalexin and glucosinolates in *Arabidopsis* (Geu-Flores et al. 2011). The accumulation of GSH in response to various pathogens (Fodor et al. 1997; Mou et al. 2003; Vanacker et al. 2000) is indicative of an increased demand for GSH by the host, presumably to adjust disturbed redox homeostasis. It has therefore been hypothesised that GSH deficiency affects cellular homeostasis which eventually

results in compromised plant fitness. Although this possibility cannot be ruled out, there is increasing evidence for a direct role of GSH in plant defence beyond that of a mere redox buffer (Foyer and Noctor 2005; Mou et al. 2003; Noctor 2006).

The chemical reactivity of the thiol group of GSH makes it a very versatile biomolecule to serve a broad range of functions in all organisms. High cellular GSH is correlated with enhanced tolerance against cadmium (Cd) and nickel (Ni) in various plants (Zhu et al. 1999; Freeman et al. 2004; see also chapter by Martinka et al. in this volume), while heavy-metal exposure has been shown to stimulate GSH synthesis in roots and cultured cells (Rüegsegger and Brunold 1992; Schneider and Bergmann 1995). In contrast, reduced levels of GSH in the *cad2-1*, or lower biosynthetic rates of GSH in the *sir1-1* mutants of *Arabidopsis*, were linked with hypersensitivity to Cd (Cobbett et al. 1998; Howden et al. 1995; Khan et al. 2010), indicating the importance of GSH in heavy-metal stress.

The synthesis of phytochelatins (PC) is a main factor for basal As and Cd tolerance (Clemens 2006; Cobbett and Goldsbrough 2002; see also chapter by Martinka et al. in this volume), and PC deficiency causes Cd and As hypersensitivity in *Arabidopsis thaliana* (Ha et al. 1999). Phytochelatins are a family of cysteine-rich peptides and regarded as a principal class of heavy-metal chelators in plants. Upon exposure to heavy metals, for example, Cd, the phytochelatin (expressed as GSH equivalents) may accumulate tenfold relative to free GSH (Heiss et al. 1999, 2003 and references cited therein), thereby generating a strong metabolic sink for GSH. A threefold decrease of reduced GSH within 3 h of cadmium exposure has been shown in maize seedlings (Nocito et al. 2006). The availability of GSH, therefore, is essential for PC synthesis, particularly during heavy-metal exposure.

Genetic manipulation of GSH-related and PC synthesis genes in plants is considered a promising strategy to enhance stress tolerance to various heavy metals. Genes encoding enzymes such as γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), glutathione reductase (GR), ATP sulphurylase (APS), serine acetyltransferase (SAT), phytochelatin synthase (PCS), and glyoxalases (glyoxalase I and II) are potential candidates to engineer tolerance to heavy-metal stress through modulation of GSH and PC levels. In fact, overexpression of some of these genes in various plants has already been demonstrated to confer higher tolerance and accumulation of heavy metals (Yadav 2010).

6.1 Biosynthesis of GSH

GSH is a major nonproteinaceous thiol compound with low molecular weight present in all plant cells except for some plant species where GSH is functionally replaced by homologous tripeptides produced at comparable levels. The biosynthesis of GSH from its constituent amino acids (glutamate, cysteine, and glycine) requires two ATP-dependent sequential steps catalysed by γ-glutamylcysteine synthetase (GSH1 in plants; GSHA in bacteria) and glutathione synthetase

(GHS2 in plants; GSHB in bacteria). The first enzyme catalyses the formation of the atypical peptide bond between the γ-carboxylic group of glutamate and the amino group of cysteine. Subsequently, the peptide bond between the carboxylic residue of cysteine and the amino group of glycine is catalysed by GSH2. Glycine can be replaced by other amino acid residues in some plant species. For example, many members of the Fabaceae contain β-Ala instead of glycine to form homoglutathione (hGSH) (γ-Glu-Cys-β-Ala) in addition to GSH (Klapheck 1988; Price 1957). Molecular analysis of the hGSH biosynthesis in *Medicago truncatula* showed that homoglutathione synthetase (hGSH2) is closely related to GSH2 and has been suggested to be derived from GSH2 via gene duplication (Frendo et al. 2001). In plants, cDNAs for both GSH1 and GSH2 have been cloned and functionally expressed (May and Leaver 1994; Ullmann et al. 1996; Wang and Oliver 1996), and numerous studies have characterised the expression patterns at the transcript level. Many of these studies have revealed that in response to the different developmental or environmental cues that induce GSH biosynthesis, the expression of *GSH1* is more responsive in accordance with its proposed role as catalysator of the rate-limiting step (Xiang and Oliver 1998; Xiang et al. 2001). However, in some cases concomitant increases in the transcript levels of *GSH1* and *GSH2* have been reported; for instance, in *Brassica juncea*, transcript levels of both genes were increased (Schäfer et al. 1998). In *Arabidopsis*, sequence analysis of the genome has confirmed that *GSH1* and *GSH2* are present as single genes (May and Leaver 1994; Ullmann et al. 1996). Both GSH1 and GSH2 of *A. thaliana* harbour canonical transit peptides for plastid localisation; and their localisation in the plastid has later been experimentally verified (Wachter et al. 2005). Transcript analysis of *GSH1* has uncovered two mRNA populations with either a short or a long 5′-UTR, but both spanning the entire coding sequences. Transient transformation experiments with fusion constructs of these two transcript versions, bearing short or long 5′-UTRs with a GFP and a RFP reporter, respectively have provided evidence for an exclusive plastidic localisation of GSH1 irrespective of the length of the 5′-UTRs of GSH1 transcripts. The same was found for *B. juncea* as well (Wachter et al. 2005). Likewise, multiple transcript populations were found for GSH2; however, in this case, the larger, but less abundant, transcript encoded a plastidic GSH2 protein, whereas the prevalent shorter transcript that lacked a complete transit peptide sequence encoded a cytosolic GSH2 protein (Wachter et al. 2005).

7 The Biosynthesis and Role of Sulphur-Based Phytoalexins

Phytoalexins are functionally defined antimicrobial secondary plant compounds of low molecular mass that are synthesised de novo in response to abiotic and biotic stress and constitute an important part of the plant defence repertory against pests and pathogens (Hammerschmidt 1999; Pedras et al. 2011a). This chemically

heterogeneous group of compounds is also produced by a wide range of crop plants including Brassicaceae, Solanaceae, Vitaceae, Fabaceae, and Poaceae and is generally regarded as molecular markers for disease resistance (Ahuja et al. 2012). Since the introduction of the concept of phytoalexins in 1940, they have been extensively studied, not only with respect to their roles in defence against pests and pathogens but also with respect to their health-promoting effects (Boue et al. 2009; Holland and O'Keefe 2010; NG et al. 2011; Pedras et al. 2011a; Smoliga et al. 2011).

In the Brassicaceae, 44 phytoalexins have been reported so far from both cultivated and wild species which are basically indole alkaloids derived from (*S*)-tryptophan and mostly contain a S moiety. On the basis of their structural features, they have been assigned to six groups (reviewed in Pedras et al. 2011a). Since they are produced in very small quantities, it is not feasible to isolate them in substantial amounts from stressed plant tissues. However, chemical synthesis is a good alternative to provide the quantities of phytoalexins required to study their biological activities, ecological functions, and other biological properties (Pedras et al. 2011a). The structure, biology, detoxification mechanisms, and anticancer activity of cruciferous phytoalexins have been thoroughly reviewed (Kutschy and Mezencev 2008; Pedras et al. 2011a, b).

The biosynthesis of phytoalexin requires an induction step which is generally known as 'elicitation', as healthy plants do not produce these compounds under normal (unstressed) conditions. The elicitation of phytoalexins can occur under a great variety of conditions for most plant species, including abiotic factors, such as abiotic stress through heavy metals or UV radiation, as well as biotic stress inflicted by different microbes or their metabolites and biopolymers, including polysaccharides, oligosaccharides, peptides, and proteins (Smith 1996). These microbial cell components are known as elicitors, out of which the general elicitors are termed either MAMPS (microbial associated molecular patterns) or PAMPS (pathogen associated molecular patterns) (Jones and Takemoto 2004; Montesano et al. 2003; Nürnberger et al. 2004). The use of elicitors for the induction of phytoalexins and other defence responses in Brassicaceae has potential for crop protection (Walters et al. 2005). The amino acid (*S*)-tryptophan (Trp) which is biosynthesised from anthranilic acid via the shikimate pathway is the primary precursor for the majority of the cruciferous phytoalexins (Pedras et al. 2011a). Oxidative decarboxylation of (*S*)-Trp to indolyl-3-acetaldoxime (Naur et al. 2003) is reportedly a common step for the biosynthesis of most Brassicaceae phytoalexins and indolyl glucosinolates (Hull et al. 2000; Mikkelsen et al. 2000; Zhao et al. 2002). The antimicrobial activity of phytoalexins is generally discussed to be associated with an activity as plasma membrane disruptors of microbial pathogens or as inhibitors of respiration (Smith 1996). Compared to other Brassicaceae phytoalexins, the physiological role of camalexin, the phytoalexin produced by the model plant *Arabidopsis*, is better understood due to the availability of large number of mutants. These include phytoalexin-deficient mutants like *pad1, pad2, pad3, pad4, and pad5,* but also some other mutants affected in camalexin induction like *bos* and *ald1*. The biosynthesis of camalexins is not restricted to leaves, since they are also synthesised in the

roots in response to specific elicitors (Bednarek et al. 2005). Their biosynthesis is typically localised in proximity to the site of pathogen infection as shown by gradients of camalexin after infection with *Botrytis cinerea* (Kliebenstein et al. 2005) or *Alternaria alternata* (Schuhegger et al. 2007). The localised accumulation of camalexin was found to be correlated with a localised induction of tryptophan and camalexin biosynthetic genes (Schuhegger et al. 2006, 2007). Since camalexins show considerable structural diversity, they probably have multiple modes of action and may react with several cellular targets (Pedras et al. 2011a).

Recent advances in the studies of phytoalexins in *Arabidopsis* (similar to other crops from different families including Brassicaceae, Solanaceae, Vitaceae, Fabaceae, and Poaceae) have allowed insight into fundamental aspects of plant defence stimulating novel strategies of disease control. The majority of the biosynthetic steps leading to camalexins have been identified, but some of the intermediate steps and their conversion remain to be elucidated. It is important to mention that despite these complex and multiple defence responses, plants, for example, many Brassicaceae, remain susceptible to various microbial diseases which appears to be in some cases related to the detoxification of their phytoalexins by pathogens. This has been shown for many plant pathogenic fungi which are able to detoxify cruciferous phytoalexins by significant levels using a variety of reactions (reviewed in Pedras et al. 2011a). Research on phytoalexins provides useful information to engineer pathogen resistance into cultivated species including monocots that have also been intensively studied with respect to phytoalexins. A better understanding of their mode of action and of the detoxification mechanisms used by pathogens to bypass this line of defence will promote biotechnological strategies based on the directed control of phytoalexin production in specific tissues and at specific developmental stages (Ahuja et al. 2012). An example for such advanced plant-protection strategies is the application of phytoalexin detoxification inhibitors, for example, paldoxins, to cure specific fungal diseases (Pedras et al. 2007; Pedras 2008; Pedras and Yaya 2010). Interestingly, there exist phytoalexins from Brassicaceae that in turn inhibit fungal detoxifying enzymes, demonstrating that phytoalexins can convey various and different ecological functions. These functional diversity of phytoalexins could also explain why plants under stress usually biosynthesise not only one phytoalexin but also complex blends (Pedras et al. 2011a). Although promising, it is clear that considerable work is required to advance current knowledge to a level where immediate applications are foreseeable.

8 Glucosinolates and Their Potential for Future Biotechnological Application

Glucosinolates are among the well-known S-containing glucosides involved in the so-called activated plant defence systems. Of the approximately 200 known glucosinolate structures identified so far (Clarke 2010), the majority is found in

the order Brassicales which include the agriculturally important crop species in the families Brassicaceae, Capparaceae, and Caricaceae. Together with the enzyme myrosinase, glucosinolates constitute the so-called mustard oil bomb which is regarded a potent binary defence system against generalist insects (Hopkins et al. 2009) and nonadapted pathogens (Bednarek et al. 2009; Clay et al. 2009; Geu-Flores et al. 2011). The current interest in metabolic engineering and heterologous production of glucosinolates is mainly derived from the different bioactivities of glucosinolates and their hydrolytic products, some of which are goitrogenic (i.e. inhibit the function of the thyroid gland) in certain animals, whereas others are cancer preventive (Halkier and Gershenzon 2006; Hopkins et al. 2009; Juge et al. 2007; Traka and Mithen 2009; Gasper et al. 2005; Geu-Flores et al. 2009, 2011 see also chapter by Sadot in the current volume). This has sparked great interest in controlling the levels of specific glucosinolates or their degradation products.

8.1 Biosynthesis of Glucosinolates

After more than a decade of glucosinolate research during the postgenomic era of *Arabidopsis*, the biosynthetic pathway is well known, and most of the genes involved in the biosynthesis are already identified (Sønderby et al. 2010). Glucosinolates are derived from amino acids and are usually divided into three groups based on their precursor amino acids: (i) aliphatic glucosinolates derived from methionine, alanine, leucine, isoleucine, and valine; (ii) benzenic glucosinolates derived from phenylalanine or tyrosine; and (iii) indolic glucosinolates derived from tryptophan (Fahey et al. 2001). Recent progress in glucosinolate research include the identification of genes involved in the biosynthesis of glucosinolates which proceeds through three independent stages (Sønderby et al. 2010), i.e. side-chain elongation of precursor amino acids, side-chain decoration, and formation of the core glucosinolate structure. Elongation and decoration of the side chain are actually responsible for most of the known glucosinolates structures (Fahey et al. 2001). Some 40 structures found in *Arabidopsis* are primarily derived from methionine and tryptophan (Kliebenstein et al. 2001). Details and the current state of knowledge on the construction of glucosinolate core, side-chain elongation, and secondary modifications have been reviewed (Sønderby et al. 2010). It is, however, worth mentioning that despite an advanced understanding of the glucosinolate pathway at the biochemical level, the source of reduced sulphur in the glucosinolate structure has remained elusive and was thought to be cysteine for decades (Geu-Flores et al. 2011; Sønderby et al. 2010). This assumption was mainly based on feeding experiments using ^{35}S-labelled cysteine in which the radiolabelled S from cysteine was found to be more efficiently incorporated into glucosinolates compared to radiolabelled S from methionine or thioglucose (Wetter and Chisholm 1968). Such experiments, however, were inconclusive due to the incorporation of the label into GSH even in the

presence of the γ-Glu-Cys synthetase inhibitor buthionine sulfoximine (Bednarek et al. 2009; Schlaeppi et al. 2008). The same was true for the incorporation of ^{35}S-labelled GSH into glucosinolates since the label also incorporated into cysteine, which was the other S donor candidate. The first indication for an involvement of GSH, and not cysteine as a direct S donor, came from the analysis of the allelic mutants *pad2* and *cad2* impaired in GSH biosynthesis due to a mutation in the first committed enzyme (GSH1) for the biosynthesis of GSH. In contrast to the expectation that these plants would accumulate more cysteine than wild-type plants, both the mutants showed impaired levels of indolic glucosinolates upon induction (Schlaeppi et al. 2008). Given the fact that both these mutants contain less GSH and that feeding with GSH restored the induction, these results pointed to GSH as donor of reduced S. Additional indications for role of GSH as a direct S donor came from de novo engineering of glucosinolates in plants (Geu-Flores et al. 2009): Transient coexpression of the five known genes in benzylglucosinolate (BGLS) biosynthesis in the leaves of *Nicotiana benthamiana* resulted in the production of low amounts of BGLS, but with a concomitant increase of a related GSH conjugate, S-[(Z)-phenyl-acetohydroximoyl]-L-glutathione (GS-B) to ~ 80-fold higher levels. In contrast to the original hypothesis that cysteine is an S donor, GS-B was found to be a true intermediate, and its accumulation was correlated with the absence of GS-B-processing enzymes. Analysis of coexpression databases led to the identification of γ-glutamyl peptidase 1 (GGP1), whose cotransformation in *N. benthamiana* minimised the accumulation of GS-B and boosted the production of BGLS by ~ 5-fold. Furthermore, recombinant GGP1 was shown to metabolise the γ-glutamyl residue of GS-B in vitro (Geu-Flores et al. 2009). Although these studies in *N. benthamiana* suggested that GSH and not cysteine is the S donor and that GGP1 is the GSH conjugate-processing enzyme, experimental evidence to demonstrate this in the naturally occurring glucosinolate-producing plant was still missing. Such experimental evidence was recently provided when the role of GSH as an S donor in the biosynthesis of glucosinolates and GGP1 and GGP3 as cytosolic enzymes metabolising GSH conjugates was demonstrated in *A. thaliana* (Geu-Flores et al. 2011). These newly gained insights make the shift of a long-lived paradigm in glucosinolates biosynthesis.

9 Outlook

Sulphur and selenium are uneven twins. They share considerable chemical and metabolic similarities but differ strongly with respect to abundance, requirement, and toxicity. Further development of phytoremediation of excess selenium by selenium accumulator plants as well as enrichment of food plants will depend on the understanding of mechanisms of selenate uptake at the plasma membrane, role of glutathione for reduction of selenite in the plastids, and surveillance and degradation of dysfunctional proteins containing Se amino acids. In addition, the connection between selenium and redox homeostasis needs to be investigated to find

the switches that have to be activated to achieve improved plant genotypes for phytoremediation and human consumption.

Enhanced cysteine and methionine contents, especially in legume species, are a desirable and long-sought trait for food and feed. Here, recent advances of mechanisms at the biosynthetic side ('push') and accumulation side ('pull') in seeds need to be combined to achieve sulphur amino acid contents that reach the levels recommended by nutritionists. Such mechanisms refer not only to biochemical pathways but involve cell biological processes leading to formation of protein bodies.

The concept of using sulphur nutrition of plants with enhanced synthesis of sulphur-containing defence compounds to achieve the so-called sulphur-enhanced defence has now been largely confirmed in laboratory size experiments. However, the functional links between nutrition and contribution to innate immunity need to be explored to allow introduction of this kind of complex trait into crop plant breeding. Thus, the uneven twins of sulphur and selenium provide ample opportunity to improve plant properties provided that biotechnological approaches are continued.

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Endocytosis: At the Crossroads of Pattern Recognition Immune Receptors and Pathogen Effectors

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Abstract Understanding the receptors and signalling pathways that underpin recognition and immune responses to potentially pathogenic microbes is an important area in basic research and agriculture. Pattern recognition receptors at the cell surface are the primary sensors of potentially pathogenic microbes. Membrane recycling and late endosomal pathways are engaged in the interaction between plants and pathogens at the level of receptor endocytosis, but also as targets of effectors secreted by the pathogen to reprogram these trafficking pathways to their benefit. This illustrates the complexity and dynamic changes associated with plant immunity and is reviewed in this chapter focusing on PRRs as endosomal cargoes and regulators of endosomal pathways as effector targets.

1 Plant Immune Pathways: PTI and ETI

Plants are hosts to a large microbial community, forming interactions with mutualistic, commensal and pathogenic microbes. The outcome of such interactions depends on an intimate molecular dialogue between the host's immune system and microbial molecules (Boller and He 2009; Win et al. 2012). In recent years, a class of plasma membrane located plant immune receptors has come to attention as the primary sensors of potentially pathogenic microbes. These cell surface immune receptors are called pattern recognition receptors (PRRs) and typically detect pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), microbe-derived molecules conserved across whole classes of microbes important. Upon recognition of their specific ligand, PRRs mount a series of immune responses that can be defined according to their cellular location and timing (Table 1): (i) early responses at the plasma membrane (changes in ion fluxes, generation of reactive

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Table 1 Overview of PAMP-triggered responses over time and at location (According to Boller and Felix 2009; Melotto et al. 2006; Faulkner et al. 2013)

	Plasma membrane	Cytoplasm	Nucleus	Cell	Leaf	Plant
<1 min	Receptor complex formation					
<5 min	H^+ efflux Ca^{2+} influx					
<15 min	ROS burst	Kinase activation				
<1 h	Receptor endocytosis		Early gene expression	Stomatal closure		
<1 day				Actin bundling	Ethylene accumulation	
			Late gene expression	Plasmodesmata closure	SA accumulation Callose deposition	
>1 day						Growth arrest Immunity

oxygen species (ROS)); (ii) late responses at the plasma membrane (deposition of callose); (iii) early responses in the cytoplasm (activation of receptor-like cytoplasmic kinases (RLCKs), mitogen-associated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs)); (iv) late responses in the cytoplasm (production of hormones such as ethylene and salicylic acid (SA)); (v) responses in the nucleus (changes in defence gene expression); and (vi) responses at the cellular level (closure of stomata and plasmodesmata) (Boller and Felix 2009; Melotto et al. 2006; Zeng and He 2010; Faulkner et al. 2013). It is considered that these responses collectively result in plant immunity, referred to as PAMP-triggered immunity (PTI).

Pathogenic microbes are able to overcome this level of immunity and reprogram host cells for their own benefit. This is achieved through different strategies: (i) mutation of PAMPs to avoid recognition; (ii) secretion of so-called apoplastic effectors that can sequester PAMPs and (iii) secretion of so-called cytoplasmic effectors, which target, e.g. PRRs and downstream signalling components to suppress PTI (Boller and Felix 2009; de Jonge et al. 2010; Win et al. 2012). In addition, pathogens reprogram cytoskeletal dynamics (see below; Takemoto and Hardham 2004). To counter these molecular attacks, plants have receptors at both the plasma membrane, and in the cytoplasm. Cytoplasmic receptors are frequently nucleotide-binding (NB) leucine-rich repeat (LRR) immune receptors that detect these effectors and trigger defence responses (Win et al. 2012). Apoplastic effectors are detected by receptors at the cell surface, which in this case are structurally related to the PRRs, and in fact the distinction between PAMPs and apoplastic effectors may be less clear in some cases (Thomma et al. 2011). The immune responses mounted by NB-LRRs and cell surface receptors recognising apoplastic effectors are referred to as effector-triggered immunity (ETI). In contrast to PTI, ETI is typically strong and often associated with a rapid localised cell death known as the hypersensitive response (HR). Historically, immune receptors of the ETI system were termed resistance (*R*) genes (Jones and Dangl 2006). This type of resistance is currently most sought after in agricultural strategies to establish pathogen resistance. It occurs in a plant cultivar-pathogen strain-specific manner, but its dependence on highly specific molecular interactions means it is susceptible to effector mutation and evolution, leading to the breakdown of resistance (Jones and Dangl 2006; Boller and He 2009; Wulff et al. 2011). By contrast, PTI generally confers broad-spectrum immunity, an important aspect for agricultural application (Lacombe et al. 2010). Taken together, the molecular interplay between plants and pathogens is complex but can be in principle separated into three steps: (i) PRRs detect PAMPs at the cell surface to initiate PTI, (ii) pathogens secrete apoplastic and cytoplasmic effectors to overcome PTI, and (iii) host-specific encoded cell surface receptors and NB-LRRs can detect apoplastic and cytoplasmic effectors, respectively, that in turn mediate ETI (Fig. 1).

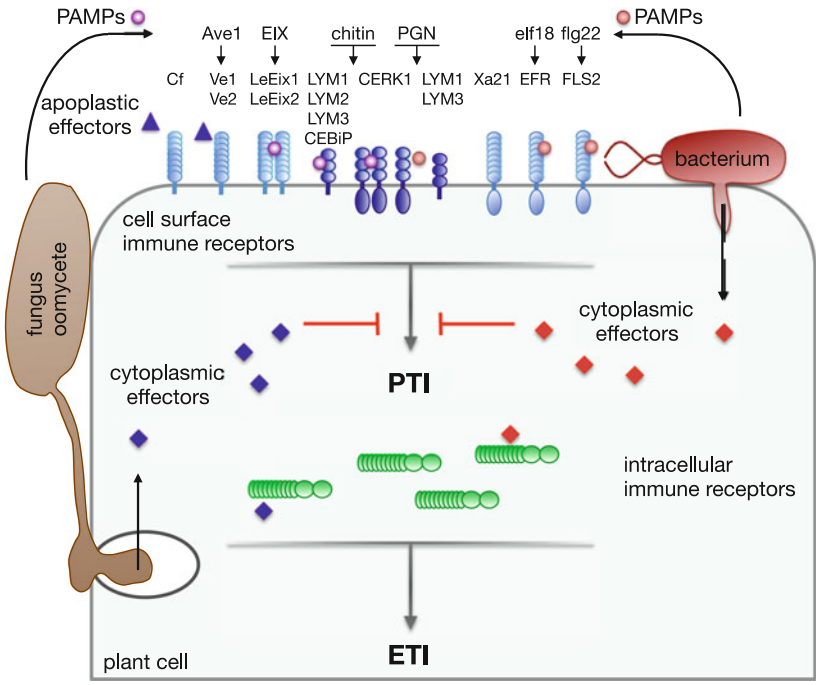

Fig. 1 Overview of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) at the cell surface, suppression of PTI by effectors and effector-triggered immunity (ETI) from intracellular immune receptors. Flagellin (flg22), EF-Tu (elf18) and peptidoglycan (PGN) have been identified as bacterial PAMPs (*red circles*), and chitin and xylanase (EIX) are known as fungal PAMPs (*pink circles*). PAMPs are perceived at the cell surface by pattern recognition receptors (PRRs), which can carry LRR or LysM domain in their extracellular parts (indicated as *light blue and purple*, respectively). Effectors secreted into the apoplast (*triangles*) can also be perceived by cell surface receptors, e.g. the Cf and Ve receptors. The primary mode of action of cytoplasmic effectors (*red and blue diamonds*) is the suppression of PTI, but in some cases these effectors are detected by specific intracellular immune receptors (*green*) that activate ETI (Modified from Win et al. 2012)

2 Pattern Recognition Receptors: RLKs and RLPs

The immune receptors encoded by PRRs are mostly type-I transmembrane proteins and fall into two classes, receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The latter lacks an intracellular signalling kinase domain, in contrast to the RLKs (Boller and Felix 2009). Both RLKs and RLPs can carry large LRRs in their ectodomains or lysine motifs (LysM), by which the interaction with their cognate ligands is mediated (Mueller et al. 2012; Liu et al. 2012). To date, a number of PRRs have been identified in plants (Fig. 1). This includes the LRR-RLKs FLAGELLIN SENSING 2 (FLS2), EF-Tu RECEPTOR (EFR), resistance to *Xanthomonas oryzae pv. oryzae* (Xa21) all conferring bacterial resistance (Zipfel et al. 2004, 2006; Song et al. 1995). The LRR-RLPs ETHYLENE-INDUCING

XYLANASE receptors (LeEix1/2) are PRRs mediating immunity to the fungus *Trichoderma*, and, although not classically considered but structurally related to PRRs, the cell surface receptors Ve1/2 confer specific resistances to races of *Verticillium* fungi (Ron and Avni 2004; de Jonge et al. 2012). A second class of important LRR-RLPs comprises the Cf receptors that trigger specific resistances to races of the *Cladosporium fulvum* fungi (Rivas and Thomas 2005).

LRR-containing PRRs sense both proteinaceous and carbohydrate-based ligands. FLS2 and EFR bind bacterial PAMPs; FLS2 recognises a 22-amino-acid conserved domain in the N-terminus of flagellin (flg22), while EFR binds the 18 amino acids of the EF-Tu N-terminus (elf18). LeEix2 recognises the fungal PAMP Xylanase (EIX) (Mueller et al. 2012; Zipfel et al. 2006; Ron and Avni 2004). In addition, Ve1 and the Cf receptors detect apoplastic effectors. Ve1 recognises Ave1 of *Verticillium*, an effector, which is also present in the genomes of other fungi (de Jonge et al. 2012), and the Cf receptors recognise *C. fulvum* Avr effectors (Rivas and Thomas 2005).

Another important class of PRRs is represented by the LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) and the LysM-RLPs LYM1/3 (Fig. 1). These receptors detect bacterial peptidoglycan (PGN) as well as fungal chitin triggering immunity to both bacteria and fungi (Willmann et al. 2011; Liu et al. 2012; Gust et al. 2012). By contrast, LYM2 and the LysM-RLP CHITIN ELICITOR BINDING PROTEIN (CEBiP) are so far only described as receptors for chitin (Kaku et al. 2006; Faulkner et al. 2013). Current data indicate that the cognate PAMPs of these LysM-type PRRs are glucan-composite molecules from bacteria and fungi. Bacterial lipopolysaccharides (LPS) are similarly glucan-composite molecules that act as PAMPs, but yet no receptor has been identified in plants (Livaja et al. 2008). Cryptogein is a protein secreted by the oomycete *Phytophthora cryptogea* and the INF1 elicitin is produced by *Phytophthora infestans*, both of which are recognised by host cells at the plasma membrane and trigger immune responses (Dahan et al. 2009; Chaparro-Garcia et al. 2011). While some binding proteins have been identified, their cognate PRRs remain elusive.

Most PRRs require complex formation with a second receptor for active signalling. FLS2, EFR, Ve1 and LeEix2 all require BRI1-ASSOCIATED KINASE 1/SOMATIC EMBRYO RECEPTOR KINASE 3 (BAK1/SERK3) for defence signalling. BAK1 is an LRR-RLK that was initially identified as co-receptor of the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and also functions as co-receptor of PRRs (Chinchilla et al. 2007; Schwessinger et al. 2011; Fradin et al. 2009; Bar et al. 2010). In the case of FLS2, FLS2 alone is sufficient to confer flg22 binding, and this triggers rapid formation of a stable complex with BAK1 necessary for signal transduction (Chinchilla et al. 2007b). Although FLS2 homodimer formation has been described, its role in regulating immunity is much less clear (Sun et al. 2012). By contrast, CERK1 differentially forms homodimers or heterodimers with CEBiP for chitin-triggered immunity in a species-dependent manner (Shimizu et al. 2010; Liu et al. 2012; Shinya et al. 2012). In addition, LeEix2 acts together with LeEix1 to mediate EIX perception (Ron and Avni 2004; Bar et al. 2010), and both the Cf receptors and Ve1 interact with

SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR), an LRR-RLK (Liebrand et al. 2013). This illustrates the dynamic and complex interactions of PRRs initiating PTI and ETI signalling and opens questions of how cell surface receptors engage in molecular interaction across the plasma membrane to convey intracellular processes.

3 Plant Endocytic Pathways: Recycling and Degradation

The internalisation of plasma membrane, plasma membrane-localised proteins and molecules from the apoplast is termed endocytosis and is central to modulating membrane composition, molecular communication across the plasma membrane and signal transduction (Scita and Di Fiore 2010; Reyes et al. 2011). Endocytosis is involved in a number of plant processes, including the establishment of cellular polarity by the endocytosis-mediated discrete localization of the PIN auxin carriers (see also chapter by Skůpa et al., this volume), cell plate formation during cytokinesis and abiotic and biotic stress responses (Chen et al. 2011; Dhonukshe et al. 2008; Beck et al. 2012a; Sutter et al. 2007; Takano et al. 2005). Different endocytic trafficking routes serve these tasks and specificity is determined by the composition of the endocytic machinery and the destination of the cargo (Fig. 2a). Although there is evidence for clathrin-independent endocytosis, the major endocytic pathway in plants is mediated by clathrin (Chen et al. 2011; Li et al. 2012) and has been implicated in the internalisation of the PIN auxin carriers and BRI1 (Dhonukshe et al. 2008; Irani et al. 2012). Interestingly, clathrin-mediated endocytic trafficking of some proteins is inhibited by high levels of auxin and SA, suggesting hormonal control of this trafficking pathway (Robert et al. 2010; Du et al. 2013).

Clathrin-mediated endocytosis begins with the recruitment of adaptor proteins and clathrin to the plasma membrane cargo. Clathrin-coated pits are formed and then released from the plasma membrane by scission factors (Chen et al. 2011). The recognition of plasma membrane cargo for the internalisation by adaptor proteins can be mediated by the tyrosine-based endocytic motif Yxxϕ (whereby ϕ represents an amino acid with a hydrophobic side chain), which is classically associated with clathrin-mediated endocytosis and first implicated in plant endocytosis by heterologous expression of the human TRANSFERRIN RECEPTOR (hTfR) in Arabidopsis protoplasts (Boll et al. 1996; Ortiz-Zapater et al. 2006). Tyrphostin A23 can block the interaction between the motif Yxxϕ and adaptor proteins and thereby interferes with the endocytosis of the plasma membrane cargo (Ortiz-Zapater et al. 2006). This inhibitor is therefore often used to dissect endocytic trafficking but should be taken with care, as its primary effect is to inhibit tyrosine kinase activities. Another important recognition signal of plasma membrane cargo for endocytosis is posttranslational modification with ubiquitin, whereby ubiquitination can regulate both internalisation from the plasma membrane and targeting of cargo to vacuoles (Tanno and Komada 2013; Herberth et al. 2012).

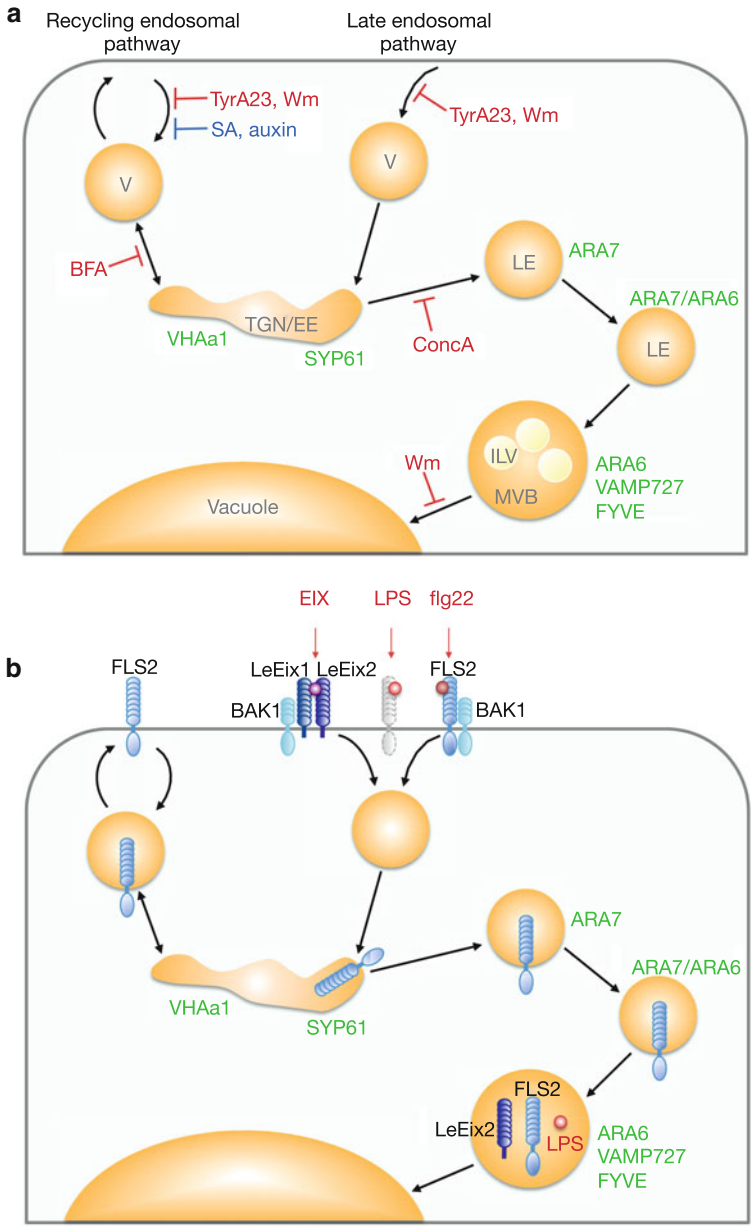

Fig. 2 (**a**) Overview of the endosomal trafficking pathways, recycling and late endosomal trafficking, indicating commonly used markers for the different membrane compartments (*highlighted in green*) and commonly used inhibitors to block trafficking at specific stages (*highlighted in red*); interference of endosomal trafficking by the hormones auxin and salicylic acid (SA) is indicated (*blue*). Compartments are labelled as follows: *V* vesicles, *EE* early endosome, *TGN trans*-Golgi network, *LE* late endosome, *MVB* multivesicular body, *ILV* intraluminal vesicles. (**b**) Overview of the endosomal trafficking by the pattern recognition receptors FLS2 and LeEix2 triggered by their

Ubiquitination at the so-called PEST motif, an amino acid motif enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), has been associated with sorting endocytosed cargo for the late endosomal pathway (Seaman 2008).

Endosomes, the membrane vesicles formed after pinching off from the plasma membrane, are highly mobile compartments and their trafficking is connected to the actin cytoskeleton (Grebe et al. 2003; Newpher et al. 2005). Upon internalisation into endosomes, the cargo is trafficked to the *trans*-Golgi network (TGN), a plant-specific endomembrane compartment, which functions as intracellular sorting organelle for cargo perceived from the Golgi-derived biosynthetic pathway as well as from the endocytic pathway (Viotti et al. 2010). Interestingly, the TGN appears to resemble the early endosomal compartment in plants. At the TGN, cargoes can be recycled back to the plasma membrane (Fig. 2a), and a key component controlling this recycling pathway is the ADP-ribosylation factor (ARF)-guanine nucleotide exchange factor (GEF) GNOM (Geldner et al. 2003). GNOM is the target of the chemical compound Brefeldin A (BFA), which is widely used in plant cell biology to disrupt endomembrane trafficking that in plant meristematic cells causes the accumulation of TGN/early endosomes into so-called BFA bodies due to the inhibition of the recycling pathway (Geldner et al. 2003).

From the TGN, internalised cargo can be alternately trafficked to the late endosomal pathway (Fig. 2a). Here, the cargo is sorted into late endosomal compartments or multivesicular bodies (MVBs) that in turn can fuse with the tonoplast and release the intraluminal vesicles for vacuolar degradation (Luzio et al. 2010; Reyes et al. 2011). This process can be inhibited by Wortmannin, a chemical compound affecting the function of phosphoinositide 3-kinases (PI3Ks), triggering the fusion and thereby the enlargement of MVBs (Tse et al. 2004). In addition, Wortmannin affects the internalisation from the plasma membrane, highlighting a role for PI3Ks at this step of the endocytosis process. Although not extensively studied in plants, the ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT (ESCRT) machinery is present at endosomal compartments and has been implicated to regulate endosomal trafficking of ubiquitinated cargo (Raiborg and Stenmark 2009; Scheuring et al. 2011; Reyes et al. 2011).

The various compartments along the endocytic trafficking route can be visualised using endocytic tracers such as FM4-64, a lipophilic dye that stains the plasma membrane, early and late endosomes and the tonoplast in a time-dependent manner (Bolte et al. 2004). In addition, a number of genetically encoded fluorescent fusion protein markers can be used to label distinct and overlapping endosomal compartments (Fig. 2a; Geldner et al. 2009; Lu et al. 2012). A widely used marker for the TGN/early endosome is the V-ATPase VHAa1, a proton pump required for the acidification of the early endosome (Dettmer et al. 2006). VHAa1 is targeted by concanamycin A (ConcA), an inhibitor of V-ATPases, and therefore ConcA

Fig. 2 (continued) cognate PAMPs (*highlighted in red*) flagellin (flg22) and Xylanase (EIX) and the internalisation of the bacterial PAMP lipopolysaccharide (LPS), for which no receptor is known to date (*grey dashed*). Co-localisation with known membrane markers is indicated (*green*)

treatment abolishes the maturation from early to late endosomes, preventing sorting of cargo for vacuolar degradation (Dettmer et al. 2006). Alternatively, the syntaxin SYP61 is used as a marker of the TGN (Drakakaki et al. 2012). Prevalent markers of endosomal compartments are also members of the Rab GTPase family, of which the Rab5 GTPases ARA7/RabF2b and ARA6/RabF1 are commonly used to visualise early and late endosomes, respectively, whereof ARA6/RabF1 is more specific for late endosomal labelling (Ueda et al. 2001, 2004; Ebine et al. 2012). Late endosomes can also be visualised using the fluorescent fusions of VAMP727, a v-SNARE, as well as the FYVE biosensor (referring to the Cys-rich proteins Fab1, YOTB, Vac1 and EEA1), which binds to phosphatidylinositol-3-phosphates enriched in these endosomal compartments (Ebine et al. 2012; Vermeer et al. 2006).

Endocytosis of cell surface receptors has been discovered in plants relatively recently and so far described for only a few LRR-RLKs. This paragraph will briefly summarise aspects of endocytic trafficking of non-PRR receptors. BRI1, BAK1/SERK3 and another member of the SERK family, SERK1, are cargoes of the endosomal recycling pathway (Shah et al. 2002; Russinova et al. 2004; Aker and de Vries 2008). They localise to FM4-64-labelled endosomes that accumulate into BFA bodies. BRI1 and BAK1 can interact at endosomes and endosomal localisation of either one or both receptors is enhanced upon their co-expression (Russinova et al. 2004). Moreover, the bioactive, fluorescent-labelled brassinosteroid analogue castasterone is internalised into endosomes and BRI1-positive compartments (Irani et al. 2012). Thus, there is significant evidence for BRI1 endosomal signalling: (i) BRI1 and BAK1 interact at endosomes; (ii) BRI1-positive endosomes are labelled with castasterone, a BRI1 ligand; (iii) the negative regulatory kinase BKI is not present at endosomes; and (iv) cells treated with BFA exhibit enhanced brassinosteroid signalling (Russinova et al. 2004; Geldner et al. 2007; Irani et al. 2012). However, this notion is open for discussion because a recent study demonstrated that BRI1 endocytosis is mediated by clathrin, and that upon inhibition of clathrin-mediated endocytosis by Tyrphostin A23 and overexpression of the C-terminal domain of the clathrin heavy chain (referred to as clathrin HUB), brassinosteroid signalling was not inhibited (Irani et al. 2012). This data therefore point to BRI1 signalling from the plasma membrane.

Alternatively, endocytosis can attenuate signalling of cell surface receptors. Endosomal trafficking of SERK1 is under control of the KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP), a negative regulator of many RLKs, indicating endocytosis of plasma membrane receptors as a mechanism for signal downregulation (Shah et al. 2002). Attenuation of signalling has also been suggested to play a role in CLAVATA 3 (CLV3) responses in the maintenance of stem cells. CLV3 triggers vacuolar trafficking of its cognate receptor CLAVATA 1 (CLV1), thereby reducing the plasma membrane levels of the LRR-RLK (Nimchuk et al. 2011). In addition to these LRR-RLKs, trafficking to the recycling and late endosomal pathway was also reported for PRRs as discussed below as well as Arabidopsis CRINKLY 4 (ACR4) and the S-locus RECEPTOR KINASE 3 (SRK3), respectively, which are both non-LRR-type RLKs (Tian et al. 2007; Ivanov and Gaude 2009).

4 Endocytosed Cargo in Immunity: PRRs and Microbial Molecules

The possibility of a role for endocytosis in the recognition of microbial molecules can be assessed at three levels: (i) the subcellular localization of the cognate receptor, (ii) the subcellular localization of the microbial molecule directly and (iii) the effect of the microbial molecule on endocytic trafficking. To date, our knowledge is most advanced for PRR endocytosis, i.e. subcellular localisation of the cognate receptor, which is discussed in detail below. Using functional fluorescent fusions, FLS2 was detected at ER-derived vesicles of the biosynthetic secretory pathway, at the plasma membrane and at endosomes (Robatzek et al. 2006; Lee et al. 2011; Beck et al. 2012b; Choi et al. 2013). In the absence of ligand, FLS2 constitutively recycles between the plasma membrane and BFA-sensitive endosomes (Fig. 2b; Beck et al. 2012b). Although BAK1, the co-receptor of FLS2, also undergoes endosomal recycling, this route of endocytic trafficking of FLS2 is independent of BAK1 (Aker and de Vries 2008; Beck et al. 2012b). This is in contrast to the endocytic trafficking of FLS2 activated by its ligand flg22 (Fig. 2b; Chinchilla et al. 2007a; Beck et al. 2012b). Here, BAK1 is necessary for FLS2 endocytosis, possibly reflecting the induced complex formation between FLS2 and BAK1 to reconstitute an active signalling receptor complex (Chinchilla et al. 2007b). Following flg22 treatment, activated FLS2 is enriched in plasma membrane microdomains and traffics to FM4-64 stained early and late endosomal compartments in a time-dependent manner (Fig. 2b; Keinath et al. 2010; Beck et al. 2012b; Choi et al. 2013). These FLS2-positive endosomes are not part of the BFA body but rather associate around it, suggesting insensitivity to BFA treatment (Beck et al. 2012b). It is striking that FLS2 is a cargo for two different trafficking routes, dependent on the activation status of the receptor; in the absence of its ligand, FLS2 enters the recycling endosomal pathway while upon flg22 elicitation, the activated FLS2 receptors traffic to the late endosomal pathway. This observation is further supported by the notion that SA does not inhibit flg22-induced FLS2 endocytosis, whereas it impairs the clathrin-dependent formation of BFA bodies (Du et al. 2013). A further hallmark of activated FLS2 endocytosis is its high ligand specificity, as no FLS2 endosomes are detected when eliciting with an flg22 antagonist, the PAMP elf18 or the unrelated ligand CLV3 (Robatzek et al. 2006; Segonzac et al. 2012; Choi et al. 2013).

Late endosomal trafficking of activated FLS2 was also confirmed by quantitative co-localisation studies combined with time course analysis in stable Arabidopsis transgenic lines and using transient expression in *Nicotiana benthamiana* (Fig. 2b; Beck et al. 2012b; Choi et al. 2013). At early time points after flg22 treatment, FLS2 was present at TGN compartments labelled with SYP61. Interestingly, FLS2 showed no clear co-localisation with VHAa1 at this compartment (Choi et al. 2013). FLS2-positive endosomes showed sequential co-localisation at compartments labelled with SYP61, both SYP61 and ARA7/RabF2b, and ARA7/RabF2b following flg22 elicitation (Choi et al. 2013). During flg22 treatment,

FLS2-positive endosomes also exhibited co-localisation with ARA7/RabF2b and the later endosomal/MVB markers ARA6/RabF1 and VAMP727 (Beck et al. 2012b; Choi et al. 2013). Quantitative analysis showed that while high levels of FLS2 co-localisation were found at ARA7/RabF2b-positive endosomes during intermediate stages of flg22 treatment, the highest incidence of co-localisation between FLS2- and ARA6/RabF1-positive endosomes occurred at later time points (Beck et al. 2012b). Taken together, this suggests that activated FLS2 is transported through a distinct early endosomal/TGN-type compartment and matures into an intermediate endosomal compartment with TGN/late endosome characteristics and then into a late endosome/MVB, demonstrating the rapid sorting of FLS2 into the late endosomal pathway (Fig. 2b; Beck et al. 2012b; Choi et al. 2013). These findings are supported by inhibitor studies, where FLS2 endosomal numbers increased upon ConcA treatment that prevented its sorting into late endosomes. This suggests that FLS2 traffics through an early endosomal/TGN compartment carrying VHAa1. Further, Wortmannin treatment increased the size of FLS2-positive endosomes by promoting the fusion of MVBs (Beck et al. 2012b) and reduced the number of FLS2 endosomes, suggesting a role for PI3Ks for internalisation from the plasma membrane (Robatzek et al. 2006; Beck et al. 2012b).

Internalised FLS2 did not only show co-localisation with Rab GTPases, but required the function of some members of the Rab GTPase family (Beck et al. 2012b; Choi et al. 2013). In Arabidopsis, transient overexpression of a dominant negative version of ARA7/RabF2b, which can no longer bind GTP, inhibited flg22-induced endocytosis of FLS2 to such extent that no FLS2-positive endosomes were detected in these cells (Beck et al. 2012b). This suggests that ARA7/RabF2b possibly functions at early stages of FLS2 endocytosis, in agreement with FLS2 co-localisation to early endosome/TGN-type compartments (Choi et al. 2013). In addition, two members of the Rab11 GTPase family, referred to as RabA in Arabidopsis, have been implicated in regulating FLS2 endosomal trafficking (Choi et al. 2013) as transient expression of a dominant negative version of RabA6a in *N. benthamiana* revealed extended co-localisation between internalised FLS2- and SYP61-positive compartments without impact on its co-localisation with ARA7/RabF2b. This points to a requirement of functional RabA6a for FLS2 trafficking from the TGN to the TGN/MVB intermediate compartment (Choi et al. 2013). By contrast, co-localisation of internalised FLS2 and SYP61 was reduced upon transient expression of a dominant negative version of RabA4c in *N. benthamiana* (Choi et al. 2013). As dominant negative RabA4c did not affect the co-localisation between FLS2-positive endosomes and ARA7/RabF2b, this indicates that internalised FLS2 from the plasma membrane could be transported directly to ARA7/RabF2b-labelled endosomes (Choi et al. 2013), supporting the finding that expression of dominant negative ARA7/RabF2b inhibits FLS2 endocytosis (Beck et al. 2012b).

Curiously, Tyrphostin A23 treatment did not fully inhibit FLS2 endocytosis, but caused a reduction in FLS2 endosomal numbers (Beck et al. 2012b). Because Tyrphostin A23 is known to target the interaction between the μ2 adaptin of the

adapter protein 2 (AP2) complex for the recruitment of cargo to clathrin-coated vesicles, other adaptin-AP2 complexes maybe involved in the process of FLS2 endocytosis. This may also underlie the insensitivity of FLS2 endocytosis to SA, which appears to interfere with clathrin-mediated processes of recycling endocytosis (Du et al. 2013). Furthermore, FLS2 carries non-canonical Yxxxϕ motifs instead of the classical Yxxϕ endocytic motif in its cytosolic domain (Beck et al. 2012a), which has been associated with clathrin-mediated endocytosis and is present in many RLKs (Geldner and Robatzek 2008). The involvement of the clathrin and the Yxxxϕ motifs in FLS2 endocytosis needs further clarification.

FLS2 also carries a PEST-like sequence in its cytoplasmic domain (Beck et al. 2012a; Salomon and Robatzek 2006). This suggests a role for ubiquitination in FLS2 endosomal trafficking, and indeed, flg22-induced endocytosis is inhibited upon MG132-mediated depletion of free ubiquitin (Robatzek et al. 2006). Moreover, mutation in the PEST-like sequence affected FLS2 internalisation (Salomon and Robatzek 2006). This provides indirect evidence that ubiquitination is a signal for FLS2 internalisation, compatible with the suggestion that FLS2 traffics in the late endosomal pathway (Beck et al. 2012b). Ubiquitination of FLS2 furthermore suggests its degradation, which is in agreement with FLS2 late endosomal trafficking. FLS2 ubiquitination has been demonstrated in vitro and in vivo and can be conferred by the plant U-box E3 ubiquitin ligases PUB12 and PUB13 as well as the bacterial effector AvrPtoB, an E3 ubiquitin ligase mimic secreted by *Pseudomonas syringae* pv. *tomato* DC3000 (PtoDC3000) bacteria (Göhre et al. 2008; Lu et al. 2011). PUB12 and PUB13 were found to interact constitutively with BAK1 and upon flg22-induced FLS2/BAK1complex formation, suggesting these E3 ligases mediate the ubiquitination of FLS2 (Lu et al. 2011). Consequently, the flg22-triggered degradation of FLS2 is inhibited in *pub12*/*pub13* mutants, which exhibit enhanced flg22-induced ROS production and disease resistance against PtoDC3000 infection (Lu et al. 2011). Yet, it is unclear whether the PUB12/PUB13-mediated ubiquitination involves an endocytic process. It also remains to be demonstrated at which level and to what extent flg22-induced FLS2 endocytosis intersects with FLS2-mediated immunity.

The current evidence suggests that FLS2 endocytosis is necessary for proper flg22-induced responses. Wortmannin treatment not only reduces FLS2 internalisation and enlarged FLS2-positive endosomes but also reduces flg22-induced activation of MAPKs while not affecting ROS production (Chinchilla et al. 2007a). Likewise, the PEST mutant variant of FLS2, $FLS2^{P1076A}$, conferred flg22-induced inhibition of seedling growth in complemented *fls2* loss-of-function mutants but not flg22-induced ROS (Salomon and Robatzek 2006). A further mutant variant of FLS2 impaired in flg22-induced endocytosis, $FLS2^{T867V}$, carries a mutation of a potentially phosphorylated threonine in the FLS2 cytoplasmic *juxta* membrane region and did not complement the enhanced disease susceptibility to PtoDC3000 infection when expressed in *fls2* mutants (Robatzek et al. 2006). Despite these data pointing at a role for FLS2 endocytosis in positively regulating at least some FLS2-mediated responses, they are not fully conclusive as chemical inhibitors such as Wortmannin can have broad and therefore rather unspecific

effects. FLS2 endocytosis is impaired upon treatment with the kinase inhibitor K252a and does not occur in *bak1* null mutants (Robatzek et al. 2006; Chinchilla et al. 2007b), indicating that it is essential to determine if FLS2 endocytic mutant variants have altered kinase activity or are altered in their ability to form a complex with BAK1.

Flg22-induced FLS2 endocytosis is not only observed for the Arabidopsis receptor in its homologous system and heterologously in *N. benthamiana* but is also seen for LeFLS2 from tomato when expressed in *N. benthamiana* (Beck et al. 2012a). This suggests that endocytosis of PRRs is a conserved process across plant species. Rice Xa21 is present in vesicles that showed similarity to SCAMP1-labelled endosomes, but no direct evidence for Xa21 endocytosis has been provided thus far. Interestingly, accumulation of Xa21 depends on an E3 ligase (Chen et al. 2010; Wang et al. 2006), but a role of this E3 ligase in Xa21 trafficking has not been established. In a possibly homologous scenario, the E3 ligase SINA4 interacts with *Medicago* SYMBIOSIS RECEPTOR KINASE (SYMRK), an LRR-RLK critical for the interaction between plants and symbiotic microbes. SINA4 mediates the re-localisation and removal from the plasma membrane of SYMRK, but as yet this has not been linked with endocytic processes (Den Herder et al. 2012).

In addition to the above-described LRR-RLKs, LeEix2, which codes for an LRR-RLP from tomato, has been found to localise at endosomes (Bar and Avni 2009). In contrast to the RLKs, where the fluorescent tag was always fused to the C-terminus of the receptors, here the fluorescent tag was fused to the N-terminus of LeEix2, between the signal peptide and the LRR domain (Bar and Avni 2009). Transiently expressed LeEix2 in *N. benthamiana* was present at FYVE-positive vesicles, suggesting trafficking to late endosomes under EIX elicitation, which could be affected by Wortmannin treatment and LY294002, a PI3K inhibitor described for animals (Fig. 2b; Bar and Avni 2009). In addition, treatments with 1-butanol and dynasore, inhibitors of phospholipase D-mediated vesicle formation and dynamin activity, respectively, inhibited LeEix2 endocytosis, while BFA treatment exhibited only a minor effect (Sharfman et al. 2011). Inhibitors of LeEix2 endocytosis also impaired EIX-induced HR but did not affect EIX binding to LeEix2, significantly linking LeEix2 endocytosis with defence signalling (Bar and Avni 2009; Sharfman et al. 2011). LeEix2 carries a classical Yxxϕ motif in its cytoplasmic tail that, when mutated, similarly impairs endocytic trafficking of this receptor and abolishes EIX-triggered, LeEix2-mediated HR (Ron and Avni 2004; Bar and Avni 2009). Furthermore, overexpression of EHD2, an EH (Eps15 homolog) domain protein that is known to regulate endosomal trafficking in animals, inhibited LeEix2 endocytosis, EIX-triggered HR and production of ethylene (Bar and Avni 2009). Knock-down of EHD2 specifically enhanced EIX-induced defence responses with respect to those triggered by flg22 (Bar and Avni 2009). Curiously, both endocytosis of LeEix2 and EIX-induced responses are inhibited upon LeEix2 complex formation with LeEix1 (Bar et al. 2010). Complex formation requires the presence of BAK1/SERK3, which interacts with LeEix1 but not LeEix2 (Fig. 2b; Bar et al. 2010). Altogether, these findings show that LeEix2 is transported to the

late endosomal pathway and, when either chemically or genetically perturbed, impacts the EIX-elicited defences. This indicates close intersection between the endocytosis process of PRRs and regulation of their immune responses but also implies differences between EIX- and flg22-triggered pathways. Beyond LeEix2, there is only indirect evidence for a role of endocytosis for RLP-type PRRs; the Yxxϕ motif is present in the cytoplasmic tails of the Cf and Ve receptors (Geldner and Robatzek 2008). Moreover, a recent report identified tomato and *N. benthamiana* SOBIR1/EVR as interactor and positive regulator of Cf-2-, Cf-4- and Ve1-mediated immunity in plants of the Solanaceae and in Arabidopsis, and was found to localise at vesicles (Liebrand et al. 2013). Its Arabidopsis homologue functions in floral organ abscission and is located in FM4-64-labelled endosomes (Leslie et al. 2010).

Subcellular localisation of several PAMPs has been investigated and can be correlated to PRR location to identify trafficking pathways. FITC and gold labelling of LPS derived from *Xanthomonas campestris* pv. *campestris* showed internalisation of this PAMP into ARA6/RabF1-labelled endosomes in *Nicotiana tabacum* suspension cells, indicating trafficking to the late endosomal pathway for vacuolar degradation (Fig. 2b; Gross et al. 2005). LPS internalisation was impaired by amantadine, which can inhibit clathrin-mediated endocytosis in animals and therefore points at LPS internalisation in a clathrin-mediated process. In contrast to its receptor LeEix2, FITC-labelled EIX localised at plasma membranes but localisation at vesicles was not mentioned (Ron and Avni 2004; Sharfman et al. 2011). No localisation data are yet available for bacterial flagellin or flg22. Biochemically, flg22 binds to membranes and binding is irreversible in intact cells (Bauer et al. 2001), which could suggest uptake together with FLS2. Likewise, the oomycete PAMP cryptogein binds to a protein at plasma membranes with characteristics of a receptor (Bourque et al. 1999). Other evidence further supports the hypothesis of a role for endocytosis in cryptogein-triggered immunity. Cryptogein enhances the internalisation of FM4-64 labelled endosomes and the number of clathrin-coated vesicles in BY2 suspension cells (Leborgne-Castel et al. 2008). Overexpression of the clathrin HUB domain inhibited not only the stimulatory effect of cryptogein on clathrin-mediated endocytosis but also reduced the expression of defence genes (Adam et al. 2012), again pointing at an intersection between the regulation of endocytic processes and immunity. Curiously, this elevation of endocytic processes is affected in cells silenced for NtRbohD, the NADPH oxidase responsible for the PAMP-triggered ROS production (Leborgne-Castel et al. 2008). This may suggest that the observed increase in endocytosis is caused by the cryptogein-induced ROS rather than being an indirect measure of cryptogein uptake. In the case of PRR endocytosis, this seems unlikely because ROS produced upon elf18 elicitation does not induce FLS2 endocytosis (Robatzek et al. 2006). However, it is possible that elf18 and flg22 stimulate and/or regulate endocytic processes in addition to the internalisation of their cognate PRR, e.g. through SA production as a late PAMP response, and subsequently affecting endosomal recycling (Du et al. 2013).

5 Pathogen Effectors: Cytoskeletal Dynamics and Targets

The relevance of a pathway in immunity often becomes clear when this pathway or its components are targets of pathogen effectors (Win et al. 2012). PtoDC3000 secreted HopM1 targets the ARF-GEF AtMIN7/BEN1 (Nomura et al. 2006). HopM1 localises to FM4-64-labelled and VHAa1-positive endosomes and co-localises with AtMIN7/BEN1 (Nomura et al. 2011). These studies suggest that HopM1 interferes with AtMIN7/BEN1 function at the early endosome/TGN. HopM1 mediates the proteasomal degradation of AtMIN7/BEN1 and this loss of AtMIN7/BEN1 function results in reduced callose deposition elicited by both flg22 and PtoDC3000. Further, it enhances susceptibility to bacterial infection, even following flg22 pretreatment (Nomura et al. 2006, 2011). In addition to its role in PTI, AtMIN7/BEN1 is also a positive regulator of ETI, as *Atmin7*/*ben1* mutant plants were decreased in resistance induced by the SA analogue BTH as well as upon infection with avirulent strains of PtoDC3000 (Nomura et al. 2011). Also, AtMIN7/BEN1 was found to regulate endosomal recycling, thereby interfering with the formation of BFA bodies (Tanaka et al. 2009). This opens the possibility that the effect of loss of AtMIN7/BEN1 function on ETI is associated with the SA regulation of trafficking underlying the recycling endosomal pathway (Du et al. 2013). However, it remains to be addressed whether HopM1 targeting of AtMIN7/BEN1 at the TGN primarily affects the biosynthetic secretory or endosomal pathway or both. AvrE is another effector critical for bacterial virulence (Badel et al. 2006). Expressing the AvrE homologue from *Erwinia amylovora* in yeast revealed that this effector strongly delayed endocytic trafficking of FM4-64 to the vacuole (Siamer et al. 2011). AvrE also affected polarisation of the actin cytoskeleton which might explain the delay in trafficking of the late endosomal pathway (Siamer et al. 2011).

The actin cytoskeleton is critical for endocytic processes, in particular for endosome mobility. FLS2 endosomes are closely associated with the actin cytoskeleton, and their mobility is affected in the presence of inhibitors that disrupt the actin cytoskeleton, e.g. Latrunculin B (Beck et al. 2012b). However, even under Latrunculin B treatment, the formation of flg22-induced FLS2 endosomes was still apparent and only abolished in the presence of 2,3-butanedione monoxime (BDM), an inhibitor of myosins (Beck et al. 2012b). These data suggest cooperative function of the actomyosin cytoskeleton in the internalisation of FLS2 from the plasma membrane and subsequent trafficking to the vacuole. A recent study reported that both flg22 and PtoDC3000 trigger an increase in actin filament density and disruption of the actin cytoskeleton, resulting in enhanced susceptibility to bacterial infection (Henty-Ridilla et al. 2013). Moreover, the ACTIN-DEPOLYMERISING FACTOR 4 (ADF4), which functions in severing actin filaments, is important in ETI as well as for some PTI responses (Porter et al. 2012). Taken together, these findings highlight the involvement of the actin cytoskeleton in plant immunity. Whether the change in actin bundling affects the trafficking of secretory vesicles and/or endosomes or their dynamics needs to be

addressed in order to dissect the specifics of the role of the actin cytoskeleton plays in immunity.

Reorganisation of the actin cytoskeleton has long been described in immunity against filamentous pathogens. For example, actin filaments form large bundles and are focussed at attempted penetration sites in Arabidopsis epidermal cells infected with non-host pathogens of *Colletotrichum*, powdery mildew fungi and the oomycete *Phytophthora sojae* as well as virulent and avirulent strains of the oomycete *Hyaloperonospora arabidopsidis* (Takemoto and Hardham 2004; Takemoto et al. 2006, 2003; Shimada et al. 2006). A similar observation was made in parsley cells infected with the non-host oomycete strain *P. infestans* (Schütz et al. 2006). These cytoskeletal rearrangements also suggest reprogrammed vesicle trafficking including the endocytic pathways. Barley ARFA1b/1c, an ARF GTPase, localises to MVBs, which like ARA6/RabF1-positive endosomes focally accumulate beneath attempted penetration sites of powdery mildew, and is necessary for callose deposition at this location (Böhlenius et al. 2010; Nielsen et al. 2012). An important component of penetration resistance against powdery mildew in Arabidopsis is the plant syntaxin PEN1/SYP121, which focally accumulates at attempted penetration sites and is required for papillae formation (Meyer et al. 2009). Although there is evidence for PEN1-mediated delivery of material at attempted penetration sites via exocytosis of MVBs, a recent study suggests that this process also depends on the BFA-sensitive pathway regulated by GNOM (Nielsen et al. 2012). This opens the possibility that the recycling endosomal pathway is also involved in plant immunity.

Enhanced resistance to powdery mildew infection in Arabidopsis is caused by loss of function of ENHANCED DISEASE RESISTANCE 1 (EDR1), which can be suppressed by a specific mutation of KEEP ON GOING (KEG), a modular protein carrying, e.g. a RING E3 ligase domain (Gu and Innes 2011). Both EDR1 and KEG localise at SYP61-positive early endosomal/TGN compartments and interact with each other (Gu and Innes 2011). KEG regulates transport of internalised plasma membrane proteins such as BRI1 via the late endosomal pathway to the vacuole at the level of ARA6/RabF1-positive MVBs (Gu and Innes 2012). In addition, KEG appears to act on the biosynthetic secretory pathway, affecting the secretion of apoplastic localised proteins such as the C14 protease and PR1 (Gu and Innes 2012). This illustrates that KEG is involved in the regulation of several distinct vesicle trafficking routes. Importantly, virulent powdery mildew strains trigger degradation of KEG, thereby overcoming plant immunity (Gu and Innes 2012).

A role for the late endosomal pathway in plant immunity is further supported by the notion that ARA6/RabF1- and FYVE-positive endosomes localise at the haustoria of *H. arabidopsidis* and *P. infestans* in successful infections of Arabidopsis and *N. benthamiana* epidermal cells. Additionally, Arabidopsis mutants in genes coding for ESCRT-I components confer enhanced susceptibility to infection with virulent *H. arabidopsidis* (Lu et al. 2012). Expression of dominant negative versions of some ESCRT components affects the replication of tomato bushy stunt virus in *N. benthamiana* linking endosomal trafficking to mechanisms of viral infection (Barajas et al. 2009). Taken together, current knowledge suggests

that both the recycling and the late endosomal pathways are engaged in processes of plant immunity and that successful pathogens target components of these endosomal pathways to the benefit of microbial proliferation. This also becomes apparent when considering that BFA and Wortmannin, inhibitors widely used as chemical probes in cell biology, are fungal toxins.

6 Cell Biology in Immunity: Conclusions and Future Perspectives

Perception of flg22 by FLS2 has been found across the plant kingdom (Boller and Felix 2009). However, many PRRs are restricted to a plant family or genus, such as EFR only known from *Brassicaceae* and Ve1 from tomato (Zipfel et al. 2006; Fradin et al. 2009). It is conceivable that these family-specific PRRs have been evolved to counteract particular pathogens infecting these plant families. This opens the possibility to transfer these PRRs between plant families and thereby engineer novel resistances in a plant family that otherwise would be susceptible. Indeed, EFR was successfully transferred from Arabidopsis to tomato (Lacombe et al. 2010). EFR expressing *N. benthamiana* and tomato plants gained responsiveness to elf18, to which these plants are normally insensitive. As a result, these EFR transgenic plants conferred resistance to pathogens that normally infect *N. benthamiana* and tomato. Likewise, it is possible to transfer Ve1 from tomato to Arabidopsis (Fradin et al. 2011). These examples illustrate that components identified from fundamental research in model organisms can be applied in crops to engineer resistance and vice versa, e.g. to study the molecular pathways. The need for new resistant traits could be at least partly addressed by identifying many novel PRRs from a variety of plant families.

Links between endosomal trafficking and plant immunity have been demonstrated at a number of different levels: (i) internalisation of PAMPs and PAMP-induced endocytosis of PRRs; (ii) PAMP-induced changes in overall endocytosis and dynamic re-localisation of endosomes upon infection; (iii) altered immune responses upon chemical and genetic inhibition of endocytosis; (iv) targeting of endosomal pathways by pathogen effectors; and (v) mutants in endosomal trafficking regulators exhibiting altered defence responses and immunity upon pathogen infection. This includes the pioneering work on FLS2 endocytosis, the effects of HopM1 and the pathways of focal accumulation triggered upon attempted penetration of powdery mildew. However, it is still not clear how endocytosis of PRRs regulates the responses mediated by these receptors and what are the molecular components involved in PRR endocytosis. Given the fundamental role of endosomal trafficking in processes such as molecular communication between microbes and host cells across the plasma membrane, it is highly likely that many more effectors will target the endosomal pathways and their components.

Therefore, it will be crucial to identify to what extent pathogens reprogram endosomal trafficking, and the respective roles of effectors and effector targets.

Over the last decade, plant cell biology has advanced with regard to microscopy techniques that improve sensitivity, resolution and tissue penetrance as well as with the development of a suite of markers labelling the different endomembrane compartments. However, we are still limited by dependency on genetically encoded fluorescent-tagged fusion proteins, either of endosomal markers or the cargoes. This requires that the fluorescent fusion proteins maintain functionality, which is not always the case and can hamper analysis of trafficking (Ntoukakis et al. 2011). Also, the markers used to label the endosomal compartments are often overexpressed, which can cause alterations to the compartment and/or the trafficking pathway, e.g. when using Rab GTPases (Jia et al. 2013). The use of genetically encoded fluorescent fusion proteins currently limits plant cell biology to a couple of transient expression systems (suspension cell cultures, biolistically transfected epidermal cells, *Agrobacterium*-mediated expression in *N. benthamiana*) and stable transgenic lines mostly in Arabidopsis. Consequently, cell biology research in plants has to date coalesced around Arabidopsis root meristem cells. However, there are evidently cell type-specific differences (Robinson et al. 2008; Craddock and Yang 2012), and notably many agriculturally important pathogens infect leaf tissue and/or cannot be studied in Arabidopsis. These challenges need to be addressed together with the development of microscopy and image analysis tools that enable the monitoring of the dynamic and quantitative subcellular changes triggered by microbes (Salomon et al. 2010; see also the chapter by Barton et al., this volume). The knowledge obtained so far provides novel insights into the plant immune system, and future research will bring further understanding of the molecular mechanisms that will be also relevant for improving crops in agriculture.

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Part III
Plant Metabolism

Plant Compounds Acting on the Cytoskeleton

Einat Sadot

Abstract The cytoskeleton is a network of filamentous protein polymers that regulate various aspects of the lives of eukaryotic cells, among them shape, adhesion, motility and division. Plant-derived compounds have made a major contribution to human medicine for thousands of years and continue to serve as an indispensable source of therapeutic substances. Drug discovery from medicinal plants has yielded compounds that act on the cytoskeleton; some of these are in clinical use, including colchicines, the vinca alkaloids, taxanes and podophyllotoxins. Compounds that are produced in living organisms usually have a complex chemical structure that is naturally designed to interact with cellular components. Therefore, although high-throughput screens based on mixtures of natural products are more difficult to handle and less favoured by the pharmaceutical companies, they still have recognized, highly valuable advantages. This review summarizes the history and recent developments in the field of plant compounds that act on the cytoskeleton.

1 Introduction

1.1 Natural Compounds as a Source for Drug Discovery

Plant-derived drugs make up a significant segment of natural product-based pharmaceuticals (Schmidt et al. 2007). Approaches such as bioprospecting, in which samples are collected from natural resources based on indigenous and traditional medical knowledge, have been central to the discovery of botanical therapeutics and continue to be important (Schmidt et al. 2007, see also chapter by Sangwan and Sangwan, this volume). Natural products are thought to present preferred structures

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for drug discovery (Li and Vederas 2009). This is because they are produced in live cells, and their chemical structure is designed by the cell machinery for interactions with biological molecules such as proteins, lipids and carbohydrates. In cells of different organisms they can efficiently bind their biological targets and either enhance or inhibit their activities. Indeed, natural products have been a source for many approved drugs (Balunas and Kinghorn 2005; Butler 2004; Schmidt et al. 2007) and serve as scaffolds for combinatorial library design (Grabowski et al. 2008). Although various methods of drug discovery, such as combinatorial chemistry (Dolle et al. 2008), drug design (Zhang et al. 2009), functional genomics (Kramer and Cohen 2004), data mining (Yang et al. 2009), peptide libraries (Marasco et al. 2008) and epitope mimetics (Robinson et al. 2008) have been introduced, and pharmaceutical discovery efforts currently favour high-throughput screens, natural products have been a most productive source, leading to the development of drugs such as anticancer and anti-infection agents (Harvey 2008; Butler 2008; Lucas et al. 2010). Thus it is agreed that natural products provide an infinite resource for drug discovery (Li and Vederas 2009; Newman and Cragg 2007, 2012; Harvey and Cree 2010; Koehn and Carter 2005; Pan et al. 2012).

1.2 Microtubules

Microtubules (MTs) are dynamic polymers of α-/β-tubulin heterodimers arranged head to tail to form hollow tubes of 25 nm in diameter and up to several micrometres in length. MT dynamics is tightly regulated both spatially and temporally (Sammak and Borisy 1988; Schulze and Kirschner 1988). This regulation involves nucleotides, MT-associated proteins, kinases and phosphatases, as well as coordinated interactions with other cytoskeletal components, such as actin filaments and integrin-containing adhesion sites (Desai and Mitchison 1997; Kodama et al. 2004; Small et al. 2002). This intrinsic dynamic behaviour and its regulation are crucial for MT-specific functions such as cell-shape maintenance, cell division, cell signalling (see also chapter by Nick in the current volume), intracellular vesicle and organelle transport and cell polarity and locomotion. The critical role of MTs in cell division makes them very suitable targets for the development of chemotherapeutic drugs (Jordan and Wilson 2004; Mollinedo and Gajate 2003; Pasquier et al. 2006; Zhou and Giannakakou 2005; Dumontet and Jordan 2010; Lu et al. 2012; Stanton et al. 2011). The effectiveness of drugs that originated in plants and are targeted to MTs was validated by the successful use of the first approved drugs – vinca alkaloids (from *Vinca rosea*) and taxanes (from *Taxus brevifolia*) – for the treatment of a wide variety of human cancers (Honore et al. 2005). Their clinical success prompted a worldwide search for new derivatives and compounds with improved characteristics, such as solubility, pharmacokinetics and oral route administration (Balunas and Kinghorn 2005; Honore et al. 2005).

1.3 The Actin Cytoskeleton

Actin is a globular, nucleotide-binding protein of ~42 kDa; its polymerization yields filaments of 7–9 nm in diameter. Within cells, actin structure and dynamics are controlled directly or indirectly by many classes of actin-binding and regulating proteins (Pollard et al. 2000; Rottner and Stradal 2011; Zaidel-Bar and Geiger 2010). Alterations in contractile tension generated by the actin–myosin cortex are of central importance in the development of the phenotype of morphologically transformed neoplastic cells with invasive behaviour (Vasiliev 2004). Among the morphological changes that provide the basis for invasive migration are (1) decreased area occupied by the cell on the substrate, (2) decreased number of actin stress fibres and (3) decreased number of mature focal cell-substrate contacts (Vasiliev 2004). Indeed, some transformed cells have been found to express reduced levels of cytoskeletal proteins, such as vinculin, α-actinin, tropomyosin and EPLIN, which promote either cell adhesion or actin stress-fibre stabilization. Restoring their expression can reverse some transformed phenotypes (Bharadwaj et al. 2005; Gluck et al. 1993; Maul et al. 2003; Rodriguez Fernandez et al. 1992).

2 The Search for New Plant Compounds Affecting the Cytoskeleton

2.1 Difficulties in Using Natural Compounds in High-Throughput Screens

As described further on, the nature of natural plant extracts makes them more difficult to use in high-throughput screens compared to synthetic chemical libraries (Koehn and Carter 2005; Li and Vederas 2009). Difficulties include the following: (1) problems of reliable access and supply. Limited amounts of plant samples which have been collected in remote regions by special expeditions might be difficult to replenish. In addition, plants are protected by local governments' intellectual property issues, raising some uncertainty with regard to collection of biomaterials and especially species that are considered endangered as a result of the 1992 Rio Convention and the 2010 Nagoya Convention on Biological Diversity (Oliva 2011). (2) Seasonal or environmental variations in plant metabolomics can complicate the initial detection of active compounds and subsequent repetition of assays or purification. For example, different temporary plant organs, such as flowers, fruits or leaves, as well as permanent organs, may produce different compounds and be affected differently by changes in environmental conditions such as weather, day length, water availability and other factors associated with the specific growth niche. (3) The crude extract might hamper high-throughput screening methods due to coloured and/or toxic ingredients. (4) The initial extract of natural materials

usually contains multiple compounds and may only contain very small quantities of the desired bioactive substance, often in a mixture with structurally related molecules. (5) The compound of interest might have poor solubility. (6) Activity might depend on synergy between several compounds which might be lost during the purification steps. (7) The limited amount of material might be insufficient for nuclear magnetic resonance (NMR)-based structure verification, because milligrams of purified compound are usually necessary. (8) Laborious and time-consuming effort must be invested to complete the structural characterization via repetitive purification steps along with bioassays and NMR analysis; in an increasing number of cases, this process ultimately leads to the revelation that the active molecule is already known.

One of the solutions to overcoming these difficulties would be the creation of libraries of purified natural products (Cremin and Zeng 2002). Bugni et al. (2008) took this idea one step further by developing an automated high-pressure liquid chromatography mass spectrometry (HPLC-MS) fractionation protocol to generate natural product libraries that are sufficiently pure for high-throughput screens. The products were characterized by accurate mass during production to eliminate duplicates of known compounds and to accelerate the identification of novel compounds (Bugni et al. 2008).

2.2 Difficulties to Detect Subtle Changes in the Cytoskeleton During Cell-Based High-Throughput Screens

The cell-based high-throughput screen for changes in cytoskeleton organization adds even more difficulties. This is due to the addition of high-resolution fluorescence microscopy. These systems typically require extensive human involvement for (1) cell growth in multicell plates; (2) preparation, dilution and addition of the different extracts; (3) maintenance of optimal illumination for microscopy; (4) scanning and focusing; (5) image acquisition; (6) archiving; (7) displaying; (8) processing; and (9) analysing (Abraham et al. 2004).

In view of these needs, automatic microscope systems were developed. In addition, computerized analyses of microscope images were described, including the development of algorithms that could be used for high-throughput experiments (Mitchison 2005; Perlman et al. 2004). However, the capacity to perform rapid automated data acquisition at high resolution, combined with an automated analysis pipeline – which is required for the quantitative determination of subtle changes in cytoskeletal organization – was developed only later. Kam and colleagues have established a rapid high-resolution automatic image-acquisition technology based on a laser-assisted autofocusing device with 0.2-s focusing times (Liron et al. 2006). Image-acquisition software was written to organize images in six dimensions; X, Y, Z, time, wavelength and position in the multi-well plate (Paran et al. 2006). The developed computerized image analysis allowed image segmentation and the

collection of quantitative data on morphology and fluorescence intensity of objects, as well as on cell area, co-localization of several colours and more (Paran et al. 2007). Using a 60 × 0.9 air objective and this automated system, Paran et al. (2007) were able to screen a natural chemical library for subtle perturbations in focal contacts, labelled with fluorescent paxillin, and to isolate an active compound. Another software, FiberScore, was developed to automatically identify fluorescence associated with fibres, which allowed the calculation of total length of fibres, intensity per unit length, direction of fibres and more (Lichtenstein et al. 2003). Coupling FiberScore to the above pipeline allowed the screening of chemical compounds affecting the cytoskeleton (Paran et al. 2006). Screening of cDNA libraries for genes affecting the cytoskeleton was also facilitated. For example, the screening of a YFP-labelled cDNA library from Arabidopsis for plant proteins associated with actin stress fibres of fibroblasts (Abu-Abied et al. 2006) yielded new actin-associated proteins as well as a new protein targeted to three organelles (Abu-Abied et al. 2009, 2006).

2.3 Screening for Compounds Affecting the Cytoskeleton

Since the cytoskeleton, and specifically MTs, have long been recognized as an advantageous target for drugs that could potentially serve for cancer therapy (Dumontet and Jordan 2010; Kingston 2009), screens were designed to search for more compounds that affect their integrity. For example, a colorimetric screen was created in which MTs were polymerized in the presence of the compounds and then filtered and stained. This screen was designed to find stabilizing compounds and proved to be more sensitive than standard turbidity or sedimentation assays (Bollag et al. 1995). Using this screen, epothilones derived from an extract of the myxobacterium *Sorangium cellulosum* were found to stabilize microtubules (Bollag et al. 1995).

Other screens were based on cells in tissue culture. For example, the mutant CHO cell line Tax 2–4 is dependent on paclitaxel for survival and growth. The Tax 2–4 cells die in the absence of paclitaxel because they have an unstable MT network due to a mutation in β-tubulin (Abraham et al. 1994). This cell line was used to screen natural compounds for those that would directly or indirectly stabilize MTs. The indolocarbazole K252a, which was isolated from *Nocardiopsis* sp. bacteria, was found to indirectly stabilize MTs by inhibiting a serine/threonine protein kinase (Abraham et al. 1994). Another two-step screen was aimed at the isolation of compounds that perturb mitosis (Haggarty et al. 2000): first the cells were treated with the compound library and tested in a cytoblot assay for reactivity with TG-3, an antibody that recognizes the phosphorylated form of the protein nucleolin. This protein is phosphorylated during mitosis and hence increased signal indicates a compound that arrests cells in mitosis. In the next step, the compounds that were able to arrest cells at mitosis were tested in an in vitro MT polymerization assay (Haggarty et al. 2000). This yielded 52 compounds that destabilized and

1 compound that stabilized MTs, as well as a drug, monastrol, which targeted the mitotic Eg5 kinesin. Later, a high-throughput fluorescent cell-imaging method was developed for phenotypic screening (Yarrow et al. 2003). In this screen, cells were treated with the compounds and stained with DAPI (a DNA stain) and phalloidin (an actin stain), which determined cell shape. A 10× objective was used to score for round cells with chromosome condensation. Typically, compounds that target tubulin cause a characteristic disruption of chromosome distribution, ranging from clustering of chromosomes at the spindle, to completely random organization (Yarrow et al. 2003). This screen yielded several compounds including inhibitors of the mitotic kinesin Eg5. Other cell-based screens in which automatic fluorescence microscopy was used were then described, for example, a screen in which cells were stained for 11 different cell components, among them actin and tubulin, and different multidimensional drug profiles were determined (Perlman et al. 2004). In another screen, cells were stained by the Hoechst dye for DNA and by anti γ-tubulin antibodies for centrosomes. This screen was aimed at drugs that lead to centrosome duplication (Perlman et al. 2005). Compounds that block MT polymerization or alter centrosome composition were found in a screen in which ciliogenesis was followed (Wu et al. 2012). An additional cell-based screen took advantage of the flagellum-driven motility of *Chlamydomonas* (Engel et al. 2011). Here a compound ciliabrevin was found which inhibited MT movement and retrograde actin flow in Drosophila S2 cells (Engel et al. 2011). Another cell-based screen was carried out in plants. Plant MTs established distinct cortical arrays in interphase cells, which govern the direction of cellulose microfibril deposition (Paredez et al. 2006a, b) and consequently dictate cell shape (Wasteneys 2004; Wasteneys and Collings 2004; Wasteneys and Fujita 2006; see also chapter by Nick in the current volume). Morlin (7-ethoxy-4-methyl chromen-2-one) was found in a screen of 20,000 compounds that could lead to a swollen root phenotype in Arabidopsis. Morlin was further found to compromise rates of both MT elongation and shrinkage (DeBolt et al. 2007). A collection of 4,000 bioactive compounds was screened in a cell-based assay using tobacco BY2 cells expressing GFP-tubulin. In the presence of 19 compounds, cells became spherically swollen. Among these compounds cobtorin was found to disrupt the parallel alignment of cortical microtubules and cellulose microfibrils (Yoneda et al. 2007, 2010).

2.4 A Cell-Based Screen of Natural Plant Compounds Affecting the Cytoskeleton

A specific screen for natural plant-derived compounds affecting the cytoskeleton of animal cells was carried out in Israel. The Israeli flora is rich in ethnobotanically characterized medicinal plants with traditional uses. More than 2,600 plant species are known, of which more than 700 are noted for their use as medicinal herbs or botanical pesticides (Ali-Shtayeh and Abu Ghdeib 1999; Ali-Shtayeh et al. 1997,

2000; Ali-Shtayeh et al. 1998; Azaizeh et al. 2003; Dafni et al. 1984; Friedman et al. 1986; Silva and Abraham 1981; Yaniv et al. 1987). Plants were collected from botanical gardens and in the wild in Israel and also taken from different collections stored and maintained by the Israel Gene Bank (http://igb.agri.gov.il/collections_page.pl). Some of the plant samples were divided, and different organs (fruits, seeds, roots, leaves, etc.) were stored separately. A total of 2,000 alcoholic extracts were screened for their ability to affect three cytoskeletal components (Fig. 1): actin stress fibres (Halpert et al. 2011) and MTs (Fig. 2) in Ref52 fibroblasts and HeLa cells and nuclear localization of β-catenin in SW480 colorectal cancer cells (Gregorieff and Clevers 2005; Kinzler et al. 1991). Cells were grown in 96-well plates, treated with the compounds for 1.5 h and then fixed and stained with fluorescent antitubulin or anti-β-catenin antibodies, or with phalloidin to stain actin fibres. The plates were either screened by the automated microscopy device described above (Liron et al. 2006; Paran et al. 2007, 2006) or manually. About 10 % of the extracts led to disruption of the actin cytoskeleton and only one compound iripalidal from *Iris germanica* was found to increase cell adhesion and stress-fibre formation (Halpert et al. 2011). One anti-MT compound, a glucosinolate (Fig. 2a–f), was isolated from *Arabis alpina*, a plant from the Brassicaceae that grows on Hermon Mountain in the north of Israel (Fig. 2g). The anti-MT activity was followed by cell staining in a series of fractionation assays using several thin layer chromatography (TLC) separations and NMR verification. This eventually led to the identification of the active compound as L-9-(methylsulfonyl)propyl isothiocyanate (Fig. 2h), a member of the glucosinolates that are typical to crucifers (Brassicaceae) (Al-Gendy et al. 2010; Jackson and Singletary 2004; Kjaer and Schuster 1972). Interestingly, sulforaphane – a glucosinolate from broccoli – was found to inhibit tubulin polymerization in vitro (Jackson and Singletary 2004; see also chapter by Khan and Hell in the current volume).

3 Aromatic Volatile Compounds Acting on MTs

Aromatic plants emit volatile growth inhibitors (Muller et al. 1964; Weir et al. 2004), termed allelochemicals by the plant physiologist Hans Molisch in 1937 (Inderjit and Duke 2003; Molisch 1937; Rice 1984). Allelopathy is the influence of a chemical released from one living plant on the development and growth of another plant (Inderjit and Duke 2003; Molisch 1937; Rice 1984). The word allelopathy is composed of the Greek words “allelon”, meaning mutual, and “pathos”, meaning suffering (Inderjit and Duke 2003; Molisch 1937). The presence of allelochemicals in aromatic shrubs was first established in the early 1960s (Muller et al. 1964) and these became a topic for research (Angelini et al. 2003; Baldwin et al. 2006; Barney et al. 2005; Dicke et al. 2003; Dudai et al. 2009; Yaguchi et al. 2009). Their antibacterial and antifungal activity was recognized, as well as their ability to inhibit seed germination and plant growth (Angelini

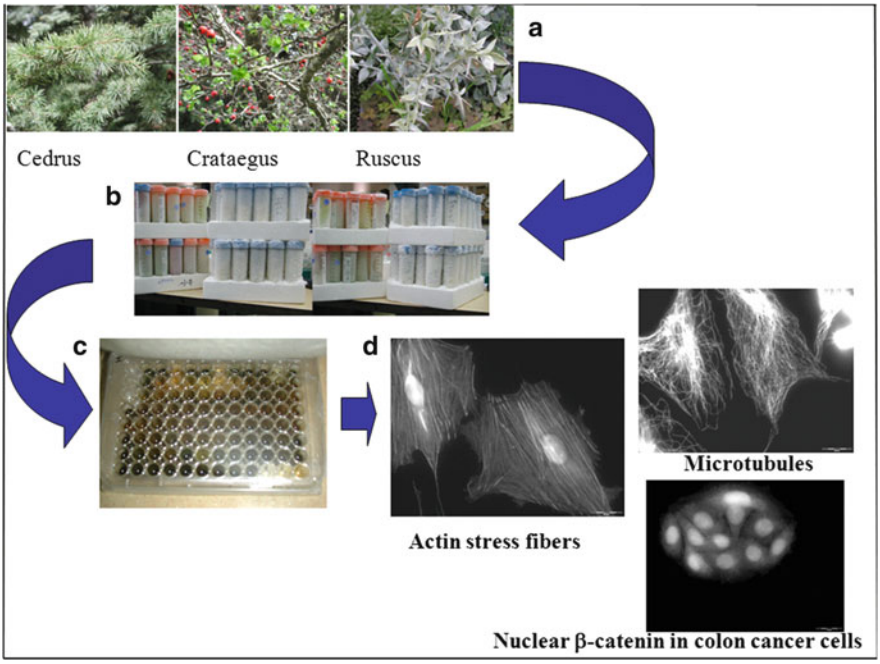

Fig. 1 A screen of natural plant products that affect the cytoskeleton. (**a**, **b**) Plant samples were collected in 50-ml tubes, immediately cooled and later frozen in a −80 °C freezer. The samples were ground in liquid nitrogen and freeze-dried in a lyophilizer. (**c**) Alcoholic extraction was performed and the extracts were kept in 96-well plates. (**d**) Cells were grown in 96-well plates and the culture was brought to 50 % confluence. The cells were treated with the extracts for 1.5 h and then fixed and immunostained (M. Halpert, O. Altshuler, Y. Moskovitz, M. Abu-Abied, M. Ron, A. Cohen, and E. Sadot)

et al. 2003; Baldwin et al. 2006; Barney et al. 2005; Dicke et al. 2003; Dudai et al. 2009; Yaguchi et al. 2009). Their mode of action against bacteria and other pathogens was widely studied; however, not much is known about their mode of action against plants or mammalian cells. Aromatic oils have been found to act as typical lipophiles which interfere with the prokaryotic cell wall and membranes, leading to leakage of macromolecules and lysis (Bakkali et al. 2008). In eukaryotic cells, aromatic oils have been shown to cause permeabilization of the plasma and mitochondrial membranes as well as apoptosis and necrosis (Bakkali et al. 2008). In plants, aromatic oils have been found to inhibit cell division and to lead to membrane disruption and oxidative stress (Maffei et al. 2001; Nishida et al. 2005; Romagni et al. 2000; Singh et al. 2006, 2009).

The mode of action of some aromatic oils is just beginning to be understood. Low doses of citral, in the micromolar range, were shown to disrupt MTs, but not actin fibres, in Arabidopsis seedlings as well as in rat fibroblasts. The effect was dose and time dependent and reversible (Chaimovitsh et al. 2010). Citral could also inhibit MT polymerization in vitro (Chaimovitsh et al. 2010), and mitotic plant

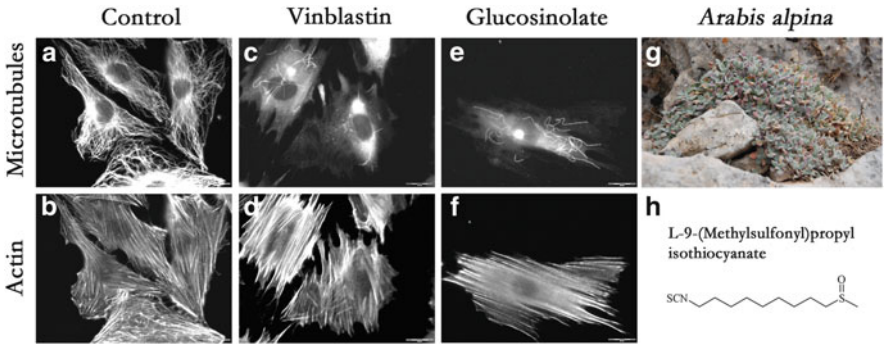

Fig. 2 Identification of an anti-MT compound from the plant *Arabis alpina*. Ref52 cells were either untreated (**a**, **b**) or treated with vinblastine as a positive control (**c**, **d**) or with a purified fraction containing the active compound from *A. alpina* (**e**, **f**). (**a**, **c**, **e**) Cells immunostained for MTs; (**b**, **d**, **f**) cells stained with phalloidin for actin as a control for compound specificity. The MTs, but not actin, are seen to be disrupted in the presence of vinblastine or the glucosinolate isolated from *A. alpina*. Scale bar = 20 μm. (**g**) *A. alpina* on Hermon Mountain, Israel, where the plant was collected. (**h**) The chemical structure of the purified active compound (a glucosinolate) (Y. Moskovitz, J. Riov, H. E. Gottlieb, M. Abu-Abied, A. Cohen, and E. Sadot unpublished data)

MTs were found to be more sensitive to citral than interphase MTs (Chaimovitsh et al. 2011). Citral is a volatile monoterpene, and an essential oil component of various aromatic plants, such as lemongrass (*Cymbopogon citratus*). It is actually a mixture of two acyclic monoterpenes: geranial (*trans*-citral or citral A) and neral (cis citral or citral B) (Lewinsohn et al. 1998). Interestingly, citral has been shown to induce apoptosis in cancerous cells (Chaouki et al. 2009; Dudai et al. 2005); another monoterpene, menthol, has been reported to stabilize MTs (Faridi et al. 2011).

A further screen for potential anti-MT and/or anti-actin activities in other volatile terpenes was performed (Table 1). In addition to citral, anethol and geraniol were found to disrupt MTs but not actin, myrcene and γ-terpinene partially disrupted MTs with no detectable effect on actin, caryophyllene disrupted actin, but not MTs, and limonene and methyl chavicol disrupted both MTs and actin, suggesting a non-specific effect. Carvacrol, eugenol, geranic acid, piperitone and thymol did disrupt neither MTs nor actin.

4 Anti-MT Plant Compounds and Their Derivatives: Advances in Their Development

MT-targeting agents interact with tubulin through mainly four characterized binding sites: colchicine, vinca alkaloid, taxane and epothilone-binding sites (Lu et al. 2012). While epothilone is a natural product isolated from the myxobacterium *S. cellulosum* (Bollag et al. 1995), the other three compounds are

Table 1 A screen of volatile aromatic terpenes affecting MT or actin fibres in HeLa cells. HeLa cells expressing RFP-tubulin were grown in multi-well plates. Standards of gas chromatography mass spectrometry (GCMS) of different monoterpenes were used for the screen. A 1-μl aliquot of each standard was diluted in 100 μl DMSO and then further diluted 1:2000 in culture media. After 30 min, the cells were fixed and stained for actin using fluorescent phalloidin. + disruption, +/− partial disruption, − no effect (O. Altshuler, N. Dudai and E. Sadot unpublished data)

Terpenes	Microtubule disruption	Actin disruption
Anethol	+	−
Carvacrol	−	−
Caryophyllene	−	+
Citral	+	−
Eugenol	−	−
Geranic acid	−	−
Geraniol	+	−
Limonene	+	+/−
Menthone	−	+
Methyl chavicol	+	+/−
Myrcene	+/−	−
Piperitone	−	−
Thymol	−	−
α-Pinene	−	+/−
β-Pinene	−	+
γ-Terpinene	+/−	−

derived from plants. In this section we elaborate upon known plant-derived anti-MT compounds and their derivatives. It is worth noting that there are many more natural products and derivatives of marine, fungal and bacterial origin, as well as synthetic compounds, that are known to interact with tubulin but are not listed here (Jordan and Wilson 2004; Mollinedo and Gajate 2003; Pasquier et al. 2006; Zhou and Giannakakou 2005; Dumontet and Jordan 2010; Lu et al. 2012; Stanton et al. 2011).

4.1 Colchicine

Colchicine is found in *Colchicum autumnale* and has been used medically for hundreds of years (Peterson and Mitchison 2002). Studies carried out during the late 1960s to identify the cellular target of colchicine led to the discovery of tubulin and established the field of MT research (Weisenberg et al. 1968; Shelanski and Taylor 1967; Borisy and Taylor 1967a, b). Later, the site of colchicine binding to the tubulin heterodimer was characterized (Ravelli et al. 2004). It was shown that colchicine binds to β-tubulin at its interface with α-tubulin. Upon binding, colchicine prevents that the typical curved structure of tubulin straightens, a conformational change that is necessary for dimer assembly into the protofilament (Ravelli et al. 2004). This leads to MT depolymerization. In 2009, colchicine was approved as a drug for the treatment of familial Mediterranean fever and gout diseases

(Lu et al. 2012). Chemical derivatives of colchicine were synthesized with the aim of reducing its cytotoxicity, and their ability to disrupt MTs was verified (Goto et al. 2002; Tang-Wai et al. 1993; Fournier-Dit-Chabert et al. 2012). Other colchiciodes have been found in seeds of the plants *Colchicum crocifolium* Boiss (Alali et al. 2010) and *Gloriosa superba* L. (Liliaceae) (Joshi et al. 2010). Although their cytotoxicity has been verified, no anti-MT activity has been reported so far.

4.2 Vinca Alkaloids

The first natural products to enter into clinical use were the vinca alkaloids vinblastine and vincristine (Kingston 2009). These compounds were isolated from the plant *Catharanthus roseus* (L.) G. Don (previously known as *V. rosea* L.) (Noble et al. 1958; Svoboda et al. 1962) and were found to promote MT depolymerization. Vinblastine binds at the interface between two dimers of α- and β-tubulin and inhibits longitudinal head-to-tail contact in the protofilament (Gigant et al. 2005). Both compounds are used in combination chemotherapy for the treatment of various types of cancer (Kingston 2009). Chemical derivatives of vinblastine have been synthesized for clinical use. These include vindesine (Dancey and Steward 1995), vinorelbine (Mano 2006), vinflunine (Kruczynski et al. 1998) and anhydrovinblastine (Ramnath et al. 2003).

4.3 Taxanes

The taxanes were originally isolated from coniferous trees of the genus *Taxus*, in a screen for natural products with antitumor activity. Taxol, later named paclitaxel, was isolated from the bark of *T. brevifolia* (Wani et al. 1971), and docetaxel is a semisynthetic paclitaxel analogue produced from its precursor found in the needles of *Taxus baccata* (Fumoleau et al. 1997). Several years after its discovery, it was found that Taxol interferes with mitosis (Fuchs and Johnson 1978) by MT stabilization (Schiff et al. 1979; Schiff and Horwitz 1980). The exact taxol-binding site was found to be on the β-tubulin subunit, at the inner face of the MT, adjacent to the binding site between two protofilaments (Nogales et al. 1999, 1998, 1995). Today, paclitaxel and docetaxel are widely prescribed antineoplastic agents for a broad range of malignancies (Yared and Tkaczuk 2012). Chemical derivatives of paclitaxel and docetaxel with increased solubility and reduced side effects in patients have been designed and tested clinically (Yared and Tkaczuk 2012).

4.4 Podophyllotoxin

Podophyllotoxin was isolated from the roots and rhizomes of *Podophyllum* spp. and has been known as a medicinal compound since the nineteenth century (Lu et al. 2012). It was found to disrupt MTs (Loike et al. 1978), but some of its chemical derivatives have lost this activity. Podophyllotoxin competes with colchicine for the same binding site on tubulin (David-Pfeuty et al. (1979) and references therein). Today, podophyllotoxin derivatives are in clinical use in combination chemotherapeutics (Stanton et al. 2011).

4.5 Combretastatins

Combretastatins are antimitotic agents isolated from the bark of the South African tree *Combretum caffrum*. Combretastatins A1 and B1 (Pettit et al. 1987) and A4, A5 and A6 (Pettit et al. 1995) have been shown to inhibit MT assembly and compete with colchicine for the tubulin binding site. Analogues with enhanced water solubility preserve MT-depolymerization activity (Babu et al. 2011). The use of combretastatin-A4-phosphate as a vascular-disrupting drug to impair tumour blood vessel networks has been validated in animal and human tests (Siemann et al. 2009).

4.6 Chalcone Derivatives

In screens for cytotoxicity and antitubulin activity, specific chalcone derivatives were isolated from *Calythropsis aurea* (Myrtaceae) (Beutler et al. 1993), the fern *Pityrogramma calomelanos* (Sukumaran and Kuttan 1991), *Piper aduncum* (Orjala et al. 1994) and the Chinese mint *Scutellaria barbata* (Ducki et al. 1996). Specific characterization revealed that α-Methyl chalcone inhibits MT assembly by binding to the colchicine-binding site of tubulin (Ducki et al. 1998, 2009).

4.7 Maytansinoids

Maytansinoids are derived from several plants, such as *Maytenus ovatus, M. serrata, Colubrina texensis* and *Putterlickia verrucosa* (Kupchan et al. 1972). The anti-MT activity of these compounds has been verified (Ikeyama and Takeuchi 1981).

4.8 Noscapinoids

Noscapinoid is an opium alkaloid found in *Papaver somniferum*, which arrests a variety of cell types in mitosis and inhibits the growth of several human and murine neoplasms, including lymphoma, thymoma and breast cancer (Ye et al. 1998). Noscapine has been found to alter MT dynamics, apparently without altering interphase MT arrays (Landen et al. 2002), which conferring its rather selective effect on rapidly dividing cells (Zhou et al. 2002).

4.9 Steganacin

Steganacin is a lignan lactone isolated from *Steganotaenia araliacea* Hochst. It has the capacity to compete with colchicine for the tubulin binding site and to inhibit MT polymerization (Schiff et al. 1978). Other related compounds isolated from the same tree, such as steganangin, steganolide A, episteganangin and steganoate A, had anti-MT activity (Wickramaratne et al. 1993).

4.10 Allicin

Allicin is one of the active compounds in garlic, *Allium sativum* (Cavallito et al. 1944). Allicin is a potent MT-disrupting reagent that interferes with tubulin polymerization by reacting with tubulin SH groups (Prager-Khoutorsky et al. 2007).

5 Plant Compounds Acting on the Actin Cytoskeleton

The number of natural products that affect the actin cytoskeleton and the number of reported screens for new actin effector compounds are lagging behind those related to MTs. The most common actin-disrupting or stabilizing natural compounds are used mainly for basic science research; they are not in clinical use and are not derived from plants (Jordan and Wilson 1998). These include the depolymerization agents cytochalasins, extracted from the mould *Chaetomium globosum* (Low et al. 1979; Spudich and Lin 1972), and latrunculins, isolated from the marine sponge *Latrunculia magnifica* (Spector et al. 1983) and other sponges. The actin-stabilizing compound phalloidin originates from the toxic fungus *Amanita phalloides* (Lengsfeld et al. 1974), and the actin-polymerization-inducing compound jasplakinolide from the marine sponge *Jaspis johnstoni* (Bubb et al. 1994). Other anti-actin compounds have been reported (Spector et al. 1999; Espina and Rubiolo 2008), mostly isolated from marine organisms, although several

plant-derived natural compounds that directly or indirectly affect the actin cytoskeleton have also been published. For example, a fraction containing iripallidal from *Iris germanica* was shown to increase cell adhesion and stress-fibre formation in a Rac1-dependent manner in HeLa cells (Halpert et al. 2011). Narciclasine isolated from bulbs of *Narcissus tazetta* also increases actin stress-fibre formation in glioblastoma cells in a Rho-dependent manner (Lefranc et al. 2009). In a screen for small molecules affecting cell adhesion, dehydro-α-lapachone, a compound from the tree *Tabebuia avellanedae*, was found to promote downregulation of actin stress-fibre formation and ubiquitination of Rac1 (Garkavtsev et al. 2011). Baicalein, a flavonoid from *Scutellaria baicalensis*, inhibits Rho kinase and actin stress-fibre formation in H9c2 cells (Oh et al. 2012). Capsianoside F from *Capsicum annuum* L. var. *grossum* promotes F-actin in Caco2 cells (Hashimoto et al. 1997). Pectenotoxin 2 produced by the marine alga *Dinophysis* promotes actin depolymerization in vitro (Butler et al. 2012), and lobophorolide isolated from the brown alga *Lobophora variegata* (Kubanek et al. 2003) stabilizes actin dimers and inhibits F-actin polymerization (Blain et al. 2010).

6 Conclusions

Plant-derived compounds form a long list of potent chemicals; some of their derivatives constitute successful chemotherapeutic compounds that are currently being used for anticancer treatment. These are represented by the tubulin-binding drugs that inhibit the function of the mitotic spindle, thereby arresting the cell cycle in mitosis. However, these compounds not only affect proliferating tumour cells, they also have significant side effects on non-proliferating cells. Therefore, there is a growing interest in developing novel antimitotic drugs that target MT-related or non-related components. Screens designed to target the spindle checkpoint regulators have yielded small molecules that inhibit specific kinesins or Aurora, polo-like, and MPs1 kinases (Dorer et al. 2005; Harrison et al. 2009; Kaestner and Bastians 2010; Peterson and Mitchison 2002).

Additional target which might be more effective in mitotic cells than in interphase cells is the tubulin carboxypeptidase (TCP) that remove COOH-terminal tyrosine residue from α-tubulin. Since tubulin tyrosine ligase is suppressed during tumour progression (Lafanechere et al. 1998), Glu-tubulin is accumulated in these cells which promote defects in spindle positioning and promotes tumour aggressiveness (Vasiliev et al. 2004). A screen of natural products for specific inhibitors of TCP that do not disrupt microtubules yielded two compounds from the plant *Vernonia perrottetii*, which were found to be sesquiterpene lactones (Fonrose et al. 2007).

The classical natural products targeting the actin cytoskeleton are toxic and not in clinical use. Other screens have been designed to target small GTPases (Berndt et al. 2011), some of which tightly regulate the actin cytoskeleton (Hall 2005), or other compounds that regulate actin polymerization (Peterson et al. 2001).

Taken together, future screens of natural plant-derived compounds might be carefully designed with the above considerations in mind to reveal the next generation of cytoskeleton-affecting compounds.

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Secondary Metabolites of Traditional Medical Plants: A Case Study of Ashwagandha (*Withania somnifera*)

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Abstract Ashwagandha is an important traditional medical plant and has been used for more than 3,000 years in Ayurveda and traditional medicine. Ashwagandha is used extensively for pharmacological and medical purposes. Therefore, the plant has attracted scientific attention worldwide. The medicinal properties of Ashwagandha are attributed to specific secondary metabolites such as alkaloids and withsteroids–withanolides. Withanolides are C_{28} steroidal structures built on an ergostane framework with oxidation at C_{22} and C_{26} to form a lactone ring. Withanolides are biosynthesised through the triterpenoid source pathway, and during recent years, tremendous progress in understanding withanolide biosynthesis and genomics has been achieved. This chapter provides a glimpse on major secondary metabolites from Ashwagandha, their distribution, occurrence, biosynthesis, genomics, and biotechnology.

1 Introduction

Ayurveda is an Indian system of medicine that has evolved for over more than 3,000 years by advancing knowledge on properties of different plants to cure various diseases. A large number of drug development programmes around the world are based on plants described for their pharmacological properties in traditional system of medicine such as Ayurveda, Siddha, Unani, and traditional Chinese medicine. These traditional medical systems serve as key resources of knowledge to derive phytomolecule-based modern pharmacological data and lead structures for drug development. The Solanaceae not only harbour important food plants but also are rich in several members used as medicinal plants, as described in ancient Ayurvedic literature and other traditional pharmaceutical traditions. In addition to

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the written record, the importance of this family is also supported by local healing practices that rely on oral tradition. The genus *Withania* is a member of this family and comprises of about 20 species. Among them, *W. somnifera* and *Withania coagulans* are those most important for medical application (Tuli and Sangwan 2010). Recently, the potential of a third species, *W. ashwagandha*, has been identified from Indian germplasm collections using multidisciplinary approaches (Kumar et al. 2011). Particularly, different parts of *W. somnifera* (Ashwagandha or winter cherry) constitute essential ingredients of hundreds of Ayurvedic formulations. The pharmacological activities of *W. somnifera* include promotion of physiological and metabolic recovery, antiarthritic and anti-ageing effects, and improvement of cognitive functions in a geriatric context as well as the recovery from neurodegenerative disorders (Lal et al. 2006; Misra et al. 2005; Sangwan et al. 2004a). Since the nature of these pharmacological effects closely resembles those of ginseng (*Panax* species), *Withania* is often designated as 'Indian ginseng'. In comparison to *W. somnifera*, *W. coagulans* has been far less explored for its phytochemical activity, despite its traditional use as earliest vegetable rennet suitable to coagulate milk. Recent pharmacological investigations demonstrate variable biological activities that differ from those of *W. somnifera*. These include antimicrobial, anti-inflammatory, antitumor, hepatoprotective, antihyperglycemic, cardiovascular, immunosuppressive, free radical scavenging, and antidepressant activities (Maurya and Akanksha 2010).

Phytochemically, several tropane alkaloids were the molecular candidates identified in Ashwagandha reported to mediate some biological activity. However, subsequent phytochemical analyses discovered a new group of abundant and highly diversified compounds subsequently termed withanolides (after the genus, where they had been discovered). Pharmacological investigations with extracts enriched in some of these withanolides matched significantly with the properties described for the plant or its part as described in Ayurveda. Thus, the curative properties of this plant could be attributed to the large and structurally diversified withanolides. Several studies could even link the therapeutic activity to individual specific withanolide moieties isolated from the herb (Kinghorn et al. 2004; Bargagna et al. 2006; Ichikawa et al. 2006; Kaileh et al. 2007). Although tropane alkaloids specific for the Solanaceae like tropine or pseudotropine had been reported also for *W. somnifera* long before the isolation of withanolides (Khanna et al. 1961), a link of these alkaloids with the therapeutic efficacy of Ashwagandha had not been followed or reported ever since. Nevertheless, the herb is still traded under the claim of its 'alkaloid content'.

Withanolides constitute a novel group of compounds descending from the triterpenoids. They are diversely functionalised molecules based on an ergostane skeleton. Biosynthetically, these specialised metabolites may diverge from the ubiquitous sterol pathway at the level of 24-methylene cholesterol (Sangwan et al. 2008). Withanolides are far from widespread in the plant kingdom but are synthesised abundantly only in a few genera of the Solanaceae family, with *W. somnifera* yielding the most prominent amounts and diversified forms. Leaves and roots of the plant are most preferred for their therapeutic properties in

traditional systems of medicine, and these are the plant parts sequestering the most significant amount of these compounds. The highly prolific sets of pharmacological activities have been linked with specific withanolides from *W. somnifera* and ranging from anti-inflammatory, antitumour, and antioxidative activities. Moreover, these withanolides inhibit cyclooxygenases and lipid peroxidation, modulate immunity, and restore neural functions by activating nitric oxide synthase (Rasool et al. 2000; Luvone et al. 2003).

Despite the growing body of evidence for pharmacological activities of the secondary metabolites (withanolides, withanamides, and tropane alkaloids) of Ashwagandha, information on the underlying biosynthetic pathways and the genes encoding for the enzymes of the pathways is still limited. Most of this information emerged from the New Millennium Indian Technology Leadership Initiative (NMITLI) programme on Ashwagandha funded by the Council of Scientific and Industrial Research (CSIR) in India since 2001. In addition to its own focussed research outcomes in terms of knowledge, products, and technology, the contributions and success of this cooperative research programme have tremendously catalysed the interests of the researchers worldwide for Ashwagandha.

Parallel to the understanding of the withanolide biosynthetic pathway (Sangwan et al. 2007, 2008), chemical synthesis of active withanolides A has also been pursued (Jana et al. 2011). However, given the complexities of the structures of these molecules, the chemical synthesis may remain of academic interest. At least in the near future, the plant will continue to be the sole economic source of these compounds. Therefore, programmes of plant improvement through conventional breeding and biotechnological manipulation based on knowledge on the pathway represent the most promising strategies.

2 Pharmacological Activities of Ashwagandha Extracts and Its Active Compounds

Ashwagandha has been regarded as an excellent source for anti-stress and rejuvenating activity comparable to ginseng (*Panax ginseng*), although the two herbs belong to different plant families (Solanaceae versus Araliaceae) that are also quite far apart phylogenetically. Traditionally, Ashwagandha has been used as a liver tonic, antiarthritic, and rejuvenating herb particularly in geriatric treatments (Jain 1991). As cellular mechanisms, suppression of free radical generation, anti-inflammatory activity; suppression of cancer cell proliferation accompanied by apoptosis through inhibition of NFκB; significant induction of axons, dendrites, presynapses, and postsynapses in the brains of mice; and amelioration of neuronal dysfunction in mice suffering from Alzheimer's disease have been reported (Ghosal et al. 1989; Luvone et al. 2003). Various activities were found to be associated with mainly root extracts and individual pure withanolides, such as withaferin A, withanolide A, and withanolide D (Table 1; Bhattacharya et al. 2002; Oh et al. 2008;

Table 1 Biological and pharmacological activities of *Withania somnifera* L. extracts and compounds

Extract/ phytomolecules	Activity	References
W. somnifera extract	Nootropic effect, prevention of increase in lipid peroxidation	Dhuley 2001
W. somnifera extract	Cytotoxic effect on macrophages	Iuvone et al. 2003
Root extract	Antioxidant activity	
Root extract	Aphrodisiac activity	Ilayperuma et al. 2002
Root powder	Enhanced immune system	Davies and Kuttan 2002
Root extract	Inhibition of angiogenesis	Mathur et al. 2006
Leaf extract	Increased tumour apoptosis	Kaur et al. 2004
Sitoindoside VII–VIII	Antistress activity	Bhattacharya et al. 2002
Withanone	Prevention against industrial metabolites	Priyandoko et al. 2011
Withanolide A	Neurite regeneration and synaptic reconstruction	Kuboyama et al. 2005
Withanolide D	Antileukemic agent, apoptosis inducer	Mondal et al. 2010, 2012
Withanolides	Neurite growth activity	Zhao et al. 2002
Withanolides	Anxiolytic antidepressant	Bhattacharya et al. 2002
Withanolides	Breast cancer cell death	Wang et al. 2012
Withaferin A	Annexin-mediated actin filament aggregation	Falsey et al. 2006
Withaferin A	Induction of apoptosis in human leukaemia cells	Oh et al. 2008
Withaferin A	Tumour proteasome as primary target	Yang et al. 2007
Withaferin A	Tumour inhibitor and antiangiogenic agent targeting vimentin intermediate filament	Bargagna et al. 2006
Withaferin A	ROS generation and mitochondrial dysfunction in apoptotic cells	Malik et al. 2007
Withaferin A	Apoptosis and inhibition of growth of human breast cancer cells	Stan et al. 2008
Withaferin A and sitoindoside	Brain cholinergic, glutamatergic, and GABergic agent	Schliebs et al. 1997
Withaferin A	Anticancer activity	Mandal et al. 2010
Withaferin A	Radiation-induced apoptosis	Yang et al. 2011
Ethanolic extract, withaferin A	Immunomodulatory, immunoprophylactic	Kushwaha et al. 2012
Withaferin A	Inhibition of Herpes simplex virus DNA polymerase	Grover et al. 2011
Withaferin A	Apoptosis in human melanoma cells through generation of reactive oxygen species	Mayola et al. 2011
Withaferin A	Improve islet transplant	Sorelle et al. 2013
Withaferin A	Proteasomal inhibitor	Khedgikar et al. 2013

Yang et al. 2007; Bargagna et al. 2006; Malik et al. 2007; Stan et al. 2008; Schliebs et al. 1997; Mandal et al. 2008; Kushwaha et al. 2012). In addition, immunostimulatory properties have been attributed to extracts from the root of *W. somnifera* (Kushwaha et al. 2012). This study has revealed that selected Indian chemotypes of Ashwagandha (NMITLI-101, NMITLI-118, NMITLI-128), as well as pure withanolide–withaferin A, possessed immunomodulatory activity (Kushwaha et al. 2012). Oral administration of aqueous ethanolic extract of chemotype 101R (10 mg/kg) as well as withaferin A (0.3 mg/kg) 7 days before and after challenge with the human filarial parasite *Brugia malayi* offered protection in *Mastomys coucha*. This protection was correlated with impaired development of *B. malayi* larvae by pretreatment with withaferin A leading up to almost two thirds reduction in the incidence of adult worms. Moreover, among the female worms that had managed to develop, a large percentage (more than 60 %) also showed defective embryogenesis. Withanone, a close structural analogue of withaferin A and predominating abundance, has not attracted much medical interest until recently when withanone was proposed to be effective as health adjuvant. Priyandoko et al. (2011) have shown that withanone protects human cells from methoxyacetic acid (MAA)-induced toxicity (Table 1). Withanone protected normal human cells from MAA toxicity by suppressing ROS levels, DNA and mitochondrial damage, and induction of cell defence signalling pathway (Priyandoko et al. 2011). These findings warrant further basic and clinical studies that may promote the use of withanone as a health adjuvant in a variety of consumer products, where toxicity has been a concern because of the use of ester phthalates (Priyandoko et al. 2011).

Recently, withaferin A has been shown to cause the redistribution of vimentin intermediate filaments in fibroblasts from their normal arrays extending throughout the cytoplasm into perinuclear aggregates (Grin et al. 2012). Microtubules become wavier and sparser, and the number of stress fibres has been shown to increase. Very recently, withaferin A was shown to act as a strong inhibitor of the inflammatory response in islets protecting against cytokine-induced cell damage while improving survival of transplanted islets (Sorelle et al. 2013). These studies suggest that withaferin A could be incorporated as an adjunctive treatment to improve the performance of islets after transplantation (Sorelle et al. 2013). So far, withaferin A has been apparently the most studied molecule out of all withanolides and has been associated with various pharmacological activities till date (Table 1). Recently withaferin A has also been found to harbour antiviral properties, and a mechanism of action has been proposed (Grover et al. 2011) based on docking and molecular dynamics simulation studies. Withaferin A might bind with the DNA polymerase of the *Herpes simplex* virus. Binding of withaferin A is also shown for the aberrant tumour proteasome beta5 subunit leading to an inhibition of the tumour-related chymotrypsin-like activity of this aberrant proteasome suggested to be responsible for the antitumor effect of withaferin A (Yang et al. 2007). For a further important but relatively far less abundant withasteroid, withanolide D, a distinct set of pharmacological activities has been elucidated (Mondal et al. 2010, 2012). Withanolide D ($C_4\beta$-$C_5\beta$,$C_6\beta$-epoxy-1-oxo-, 20b, dihydroxy-20S, 22 R-witha-2,

24-dienolide) has been shown to effectively induce apoptosis in leukaemia cell lines (MOLT-4 and K562), as well as in primary cells from patients irrespective of their lineages. This withanolide D-induced apoptosis correlated with an early accumulation of ceramide by the activation of neutral sphingomyelinase (Mondal et al. 2012). Further studies reported the withanolide D-induced cellular apoptosis in which mitochondria and p53 were intricately involved both in p53 wild-type and null cells (Mondal et al. 2012). Thus, these recent findings highlight new possibilities of recruiting withanolide D as alternative anticancer agent along with the existing chemotherapeutic agents potentially targeted towards mitochondria-mediated apoptosis (Mondal et al. 2012).

3 Diversity of Secondary Metabolites in Ashwagandha

The therapeutic potential of Ashwagandha is owing to the presence of wide variety of phytochemicals which are present in small amounts in all plant parts (Table 2). The earliest phytochemical investigations of Ashwagandha focussed on alkaloids. In fact, the therapeutic activities have been ascribed to alkaloids for a long time. Only later, withanolides were identified as the most prolific and predominant group of compounds isolated from Ashwagandha. More recently, additional new types of secondary metabolic compounds of minor abundance, such as withanamides and calystegines, have been shown to harbour pharmacological activity. This immense potential of compounds renders Ashwagandha a promising model for medicinal plants in general.

3.1 Alkaloids

Due to novel drugs derived from natural plant alkaloids (Cordell et al. 2001; Newman et al. 2003; Ortholand and Ganesan 2004) and in particular based on the presence of a tropane ring (Gross 2004), the interest for tropane alkaloids has enlarged in recent times. Tropane alkaloids are widely produced in the Solanaceae, and their potential for medical treatment has been recognised by modern medicine long back and is the base for the use of these plants in traditional systems of medicine. These drugs can be applied in different forms like pure molecules and tinctures. This group of alkaloids comprises *N*-methylpyrrolinium-derived nicotine alkaloids, tropine-derived true tropane alkaloids, and pseudotropine-derived nortropane alkaloids, also called calystegines (De Luca and St Pierre 2000; Drager et al. 1994; Drager 2004). The tropane alkaloids hyoscyamine (its racemic form being atropine) and scopolamine are used as anticholinergic agents acting on the parasympathetic nervous system used for the treatment of spasms as sedative agents and for dilation of the pupil (mydriasis) by ophthalmologists (Zayed and Wink 2004).

Table 2 Occurrence of secondary metabolites from Ashwagandha (*W. somnifera*)

Secondary metabolite	Plant part	Reference
Withaferin A	Leaves	Tursunova et al. 1977
Withanone	Roots, leaves	Chaurasiya et al. 2007, 2009
Ashwangandholide	Roots	Subbaraju et al. 2006
Withanamides A–I	Fruits	Jayaprakasam et al. 2004
2,3 Dihydrowithaferin A (viscosalactone B), withaferin A, withanolide A, withanolide D, withanolide B, 27-hydroxywithanolide A	Roots	Mishra et al. 2008
Withanolide dimer sulphide	Roots	Subbaraju et al. 2006
Withasomnillide, withasomniferanolide, somnifer-withanolide, somniwithanolide	Stem	Deb 1980; Lal et al. 2006
Withaferin A, withanone, 27-deoxy-17 hydroxy withaferin A, withanolide D, 27-deoxywithaferin A, 2,3-dihydrowithaferin A, withanolide P, withanolide F, dihydrowithanolide D	Leaves, fruits, stem, roots	Mishra et al. 2005
27-Hydroxy withanolide A, withanone, isowithanone, trihydroxywitha-24-enolide	Berries and seeds	Lal et al. 2006
Tropane alkaloids – tropine, pseudotropine, nicotine, withasomine, anaferine	Roots and leaves	Tuli and Sangwan 2010
Withanamides – withanamide A, withanamide B, withanamide C, withanamide D, withanamides E–I	Berries	Jayaprakasam et al. 2004
Flavonoids and phenolics – dihydroxykaempferol, quercetin, querceti-3-rutinoside, quinic acid, scopoletin, and aesculentin	Roots	Nur-e-Alam et al. 2003; Tuli and Sangwan 2010
Terpenoids and sterols – stigmasterol, stigmasterol glucoside, sitosterol, beta-amyrin	Fruits	Mishra et al. 2005, 2008

The relatively newly discovered group of (pseudo) tropane alkaloids, the calystegines (Fig. 1), resemble monosaccharides in structure and have been shown to be strong glycosidase inhibitors (Asano et al. 2000), suggesting that the calystegines have potential as antidiabetic compounds. *W. somnifera* roots contain alkaloids in varying levels up to 0.3 %, and the leaves are reported to contain some unidentified alkaloids as well. Though alkaloids were the earliest secondary metabolites reported from Ashwagandha, no information on their biosynthetic pathway, genes, and enzymes has been available until recently.

One of the key steps of the pathway catalysed by tropinone reductases (TRs) has been analysed very recently (Kushwaha et al. 2013). Two discrete tropinone reductases (TRs) bifurcate the tropane alkaloid pathway at the intermediary stage of tropinone into tropine and pseudotropine streams similar to the situation known from some other genera of Solanaceae (Leete 1990). The catalytic reaction products of TR-1 and TR-2, respectively, lead to the generation of hyoscyamine/scopolamine and calystegines. TR-I (EC 1.1.1.206) catalyses the NADPH-dependent

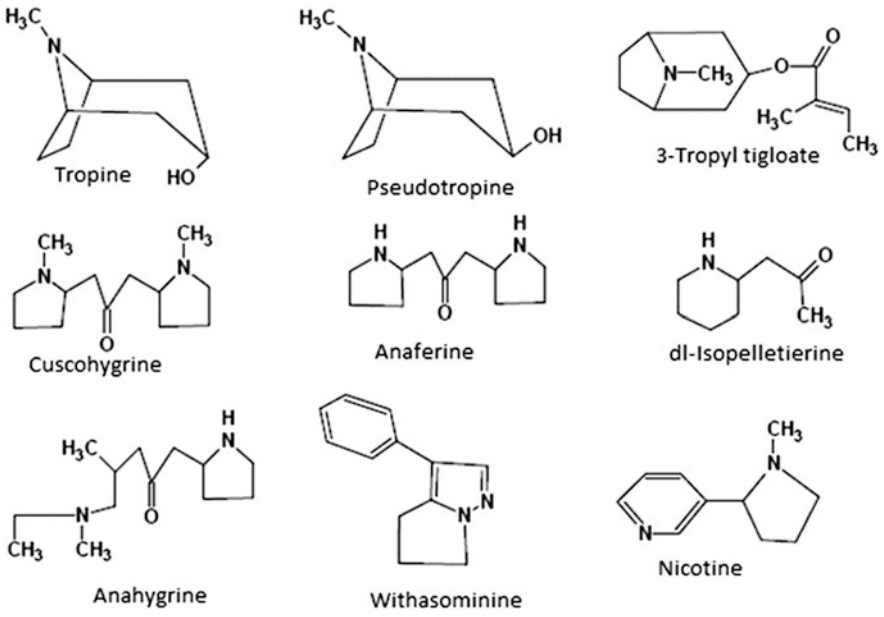

Fig. 1 Structure of alkaloids from Ashwagandha

reduction of the 3-keto group of tropinone to the 3α-hydroxy group, whereas TR-II (EC 1.1.1.236) converts the same keto group to the 3β-hydroxy form. Sequence analysis of TRs indicates that these are the members of the short-chain dehydrogenase/reductase family. When both tropane and calystegines are produced by a given species, the relative expression levels of the two TRs in the tissue (Hashimoto et al. 1992) will determine the partitioning of metabolite flux towards each form as there seems to be no interconversion between tropine and ψ-tropine in vivo (Yamada et al. 1990). Genes of one or both TRs have been isolated from several Solanaceae species, such as *Hyoscyamus niger*, *Datura stramonium*, *Solanum tuberosum*, and *Anisodus acutangulus*, which are known to accumulate high levels of hyoscyamine, scopolamine, and calystegines (Nakajima et al. 1993; Richter et al. 2006; Kai et al. 2009). Overexpression of *TR-I* has been reported to considerably enhance the production of tropane alkaloids in root cultures of *Atropa belladonna* and *A. acutangulus* (Richter et al. 2005; Kai et al. 2011). Presence of tropinone reductase homologues in plant species that are not known to produce any of the tropane alkaloids (e.g. *Arabidopsis thaliana*, *Cochlearia officinalis*) suggests that these enzymes might play additional roles in other metabolic pathways (Keiner et al. 2002; Brock et al. 2006).

Until recently, biosynthesis of tropane alkaloids has been considered to occur exclusively in roots and is subsequently transported to the aerial organs. Accordingly, the expressions of the relevant genes of the metabolic pathway have been shown to be active in root tissue. However, in a recent report, a tropine-forming

Table 3 Secondary metabolism-related genes isolated and characterized from Ashwagandha

Gene	Reference
1-Deoxy-D-xylulose-5-phosphate synthase (DXS)	Gupta et al. 2013
1-Deoxy-Dxylulose-5-phosphate reductase (DXR)	Gupta et al. 2013
3-Hydroxy-3-methylglutaryl coenzyme A reductase gene	Akhtar et al. 2013
Farnesyl diphosphate synthase (FDPS)	Gupta et al. 2011, 2012
Squalene synthase gene	Bhat et al. 2012
Squalene epoxidase	Razdan et al. 2013
3b-Hydroxy sterol glucosyltransferase (3SGT)	Sharma et al. 2007
3β-Hydroxy-specific sterol glucosyltransferase	Madina et al. 2007a
27β-Hydroxy glucosyltransferase	Madina et al. 2007b
Sterol glucosyltransferase	Chaturvedi et al. 2012
Tropinoe reductase 1	Kushwaha et al. 2013a; 2013b
Cyp 450 reductase	Rana et al. 2013

tropinone reductase (TR-I) cDNA was isolated from the leaf tissues of *W. coagulans*. The ORF was deduced to encode a polypeptide of 29.34 kDa. The recombinant His-tagged protein was functionally active implying parallel operation of the de novo biosynthetic pathway in aerial tissues. This not only represents the first report on a gene and enzyme of secondary metabolism for this commercially and medicinally important vegetable rennet species (Kushwaha et al. 2013a; 2013b) but also shows for the first time that there exists an independent tropane alkaloid synthesis in aerial tissues at least in Ashwagandha (Tables 2 and 3).

3.1.1 Multi-tissue Biosynthesis of Tropane Alkaloids

Among the secondary compounds of plants, alkaloids have been the group best studied with respect to their sites of synthesis, transport, and storage. The observations recorded in these studies suggest that there is no general site(s) of synthesis. For instance, nicotine and tropane/nortropane alkaloids of the Solanaceae plants are synthesised in the roots and transported to the aerial parts for storage, whereas the monoterpene-indole alkaloids of *Rauwolfia* and *Catharanthus* are synthesised in both the root and the leaf. The benzylisoquinoline alkaloids of *Papaver* are synthesised in the metaphloem (of both root and shoot) and stored in the laticifers and capsules, but the quinolizidine alkaloids in *Lupinus* are synthesised in the leaves and transported to roots (De Luca and St Pierre 2000; Drager 2004). In addition to tropane and its relative nicotine and calystegines for which the Solanaceae are renowned, a few genera of the family also accumulate a novel class of secondary metabolites called withanolides, the ergostane skeleton-based phytosteroids named after *W. somnifera*.

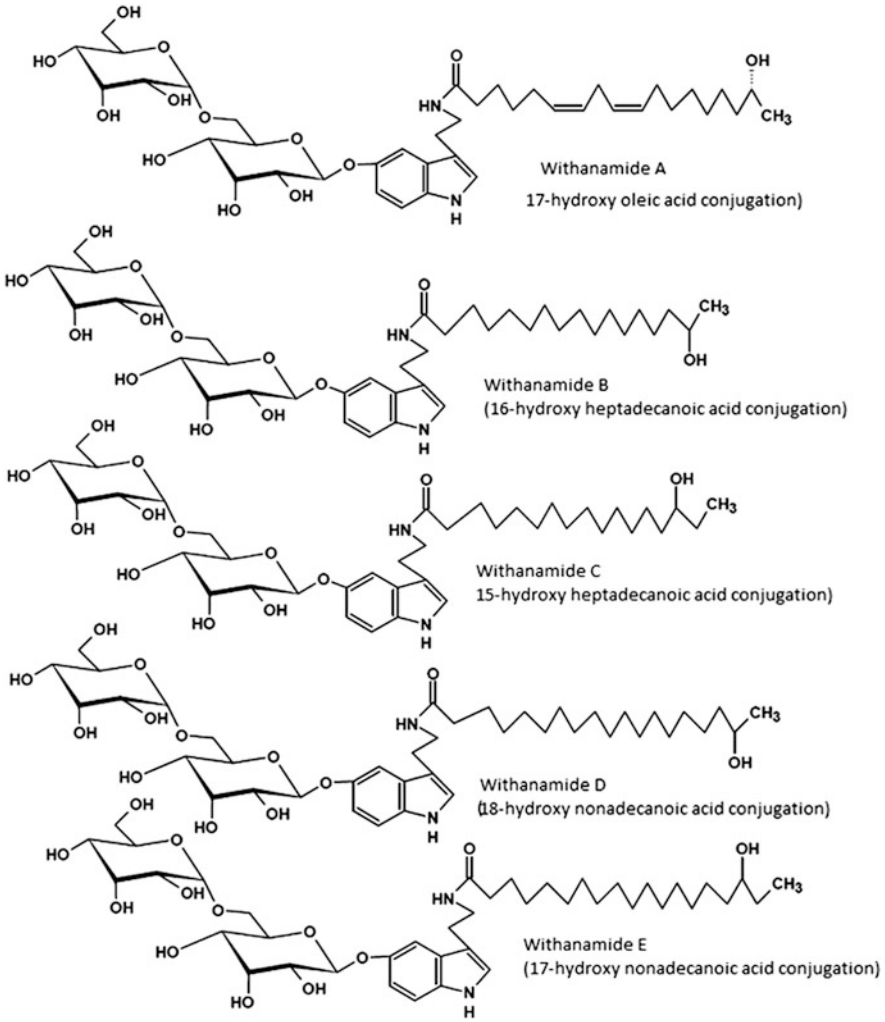

Fig. 2 Structures of withanamides A to E from Ashwagandha

3.2 Withanamides and Other Secondary Metabolites

A new group of novel amido compounds has been isolated from berries of Ashwagandha (Jayaprakasam et al. 2004). The withanamides are amido-conjugated compounds of serotonin diglucoside and long-chain hydroxyl fatty acids such as withanamides A to E (Fig. 2; Table 2). Chemical characterisation using established chemical and spectral methods showed that withanamides consist of long-chain hydroxyl fatty acid moieties, glucose and serotonin. Withanamides have also been shown to be pharmacologically active as potential inhibitors of lipid peroxidation.

Calystegines, as third novel group of compounds, are highly diversified in Ashwagandha. Calystegines are nonesterified polyhydroxylated alkaloids that had been discovered in roots of *Calystegia sepium* (Convolvulaceae) and then in *A. belladonna* (Solanaceae). So far, 25 calystegines that are structurally distinct have been reported for Ashwagandha (Drager et al. 1994; Drager 2004). In addition to these two novel and less abundant group of compounds, several commonly occurring flavonoids, phenolics, triterpenoids, and sterols (Table 2) have also been found in Ashwagandha (Nur-e-Alam et al. 2003; Mishra et al. 2005, 2008; Tuli and Sangwan 2010).

3.3 Withanolides from Ashwagandha

A growing body of evidence suggests that most of the therapeutic properties of Ashwagandha can be attributed to the withanolides. Withanolides are structurally similar to steroids that are ubiquitous in all organisms from microbes to man. Therefore, the withanolides are also known as withasteroids. In plants, major steroids, which are ubiquitously encountered, are sterols such as sitosterol, stigmasterol, or campestanol and brassinosteroids. Withanolides are structurally different from other steroids and not found outside the plant kingdom and are restricted mostly to the Solanaceae. The term withanolide originated from *Withania*, where these compounds had been discovered. Withanolides are most prolifically encountered in Ashwagandha among all Solanaceae and are characterised by a C_{28} basic steroidal skeleton with a nine-carbon side chain in which C_{22} and C_{26} are appropriately oxidised to form a six-membered δ-lactone ring (Fig. 3).

A 1-keto or hydroxy function in ring A is also a general feature of the withanolides. From the biogenetic point of view, the withanolides can be considered to harbour a cholestane-type structure with an extra methyl group at C-24 and various oxygenated groups or double bonds placed at different sites of the skeleton (Glotter 1991; Budhiraja et al. 2000). The withanolides were isolated during standard procedures of extraction, isolation, and structure elucidation of major as well as minor phytochemicals in the search for their potential pharmacological activities. In contrast to targeted isolation, such unbiased screens sometimes yield unexpected biologically active phytochemicals and provide a better chance to encounter novel results. Withanolides can be isolated from fresh as well as from dry material of Ashwagandha, which allows to understand metabolic intermediates accumulated under total arrest of metabolism (Sangwan et. al. 2004a, b). Conventional extraction of withanolides in pure methanol is suboptimal, but extraction of the fresh herb in 25 % methanol results efficiently extracts withanolides from the tissue (Sangwan et al. 2005; Chaurasiya et al. 2008; Lal et al. 2006). Almost all parts of Ashwagandha contain withanolides, but in specific composition. Although the withanolide profile is characteristic for each organ, some withanolide species occur in two or more plant parts. Leaves, stems, and berries (including seeds) are

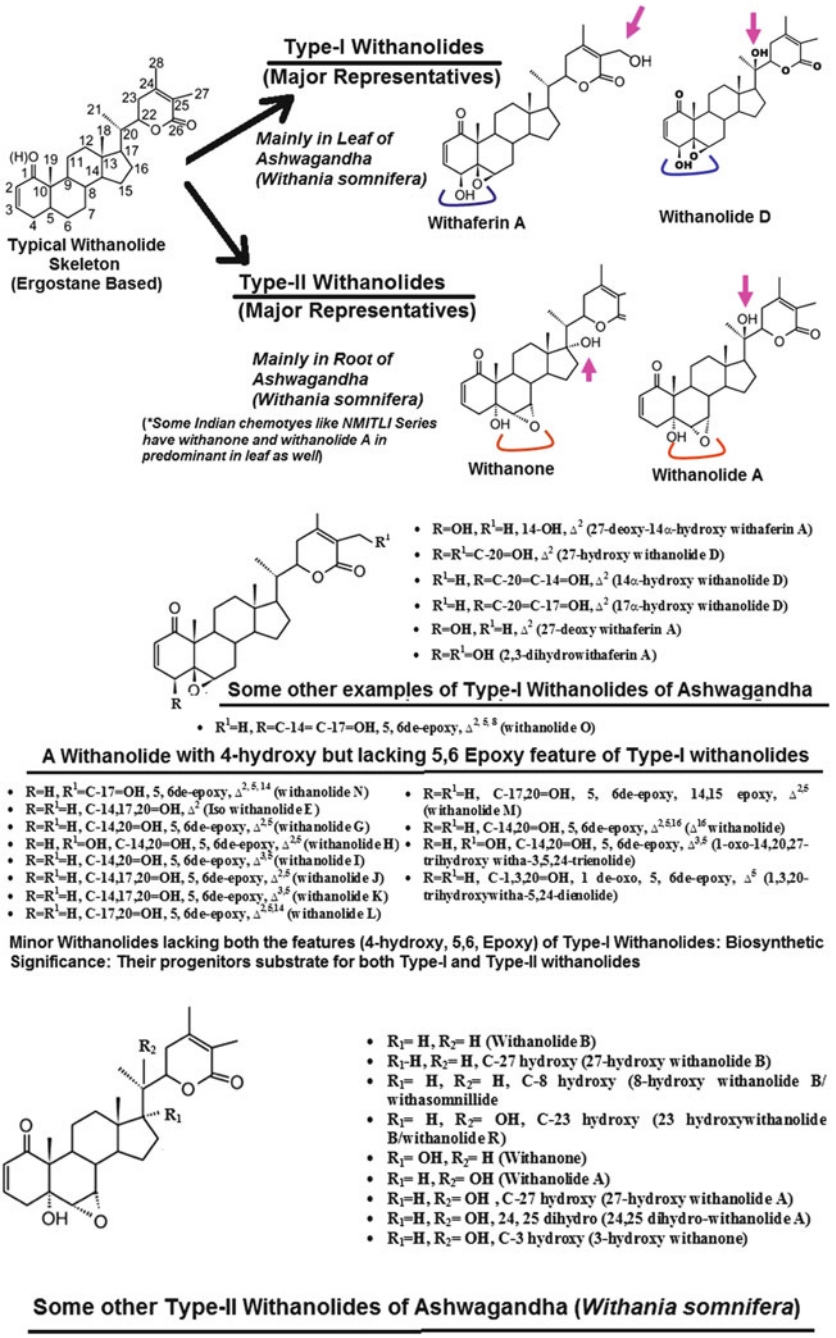

Fig. 3 Structures and organ distribution of major withanolides from Ashwagandha

the most productive source of withanolides (Chaurasiya et al. 2008). Although the roots are the major object of prescriptions in Ayurvedic and folklore systems of medicines, only a few steroids and some alkaloids have been identified from root tissue (Davies and Kuttan 2002; Ray and Gupta 1994). However, recently several withanolide-type compounds have been characterised in roots (Misra et al. 2008, 2012). Among withanolide-type compounds, several had been known earlier, such as 2, 3-dihydro-3-hydroxywithaferin A, viscosalactone B, withaferin A, withanolide D, withanolide B, withanolide A, 27-hydroxy withanolide B, and 27-hydroxy withanolide A (Misra et al. 2008). The structures of these compounds were elucidated by spectroscopic methods including VIS, UV, IR, ^{1}H NMR, ^{13}C NMR, 2D NMR, and mass spectroscopy (Misra et al. 2008). Also, a peculiar withanolide dimer sulphide has been isolated from the roots of Ashwagandha and found to inhibit the growth of cancer cell lines (Subbaraju et al. 2006).

Phytochemical studies on the shoot of Ashwagandha yielded five new withanolides from the stem bark, namely, withasomnilide, withasomniferanolide, somniferanolide, somniferawithanolide, and somniwithanolide (Table 2).

Ashwagandha leaves contain many important withanolides, as two major compounds, withanone with a total yield of 0.222 % dry weight and withaferin A with a yield of 0.166 % dry weight, had been identified (Glotter et al. 1977; Rahman et al. 1993). Ashwagandha is peculiar, because it simultaneously produces both lead structures: the 5α-hydroxy-67α-epoxide and the 4β-hydroxy-56β-epoxide. Among the minor withanolides isolated and detected in leaves, 27-deoxy-17-hydroxywithaferin A is the most abundant along with withaferin A (0.825 % dry weight) accompanied by withanolide D with 0.047 % (Chaurasiya et al. 2007). Furthermore, other minor withanolides were 27-deoxywithaferin A, 2, 3-dihydrowithaferin A, withanolide P, withanolide F, dihydro-27-deoxywithaferin A, and dihydro-withanolide D (Table 2; Fig. 3). In addition to several known withanolides, a new dienone withanolide was reported for leaves (Rahman et al. 1993). The other new withanolide, 3α-methoxy-2,3-dihydro-27-deoxywithaferin A, was isolated from leaves of *W. somnifera* along with withaferin A, 27-deoxywithaferin A, 2,3-dihydrowithaferin A, and withaferin 3-β-methoxy-2,3-dihydrowithaferin A (Anjuneyulu et al. 1997). The finding of withaferin A with immunosuppressive activity from shoot tips (Furmanowa et al. 2001) and the identification of very interesting withanolides of unusual chemical features from Ashwagandha leaves (Misra et al. 2005) show that the potential of leaf tissue has not yet been fully exploited. Also the Ashwagandha fruits have been shown to contain major withanolides in addition to substantial amount (10 %) of fatty acid oil rich in linoleic acid (60 % of the oil fraction). Specifically, the seeds also contain withanolides (Table 2). A specific withanolide, 7α, 17α-dihydroxy-1-oxo-5β, 6β-epoxy-22R-witha-2,24-dienolide, has been characterised through chemical identification and spectral data (Kundu et al. 1976). Two withanolides along with some minor amounts of coumarins and triterpenoids were isolated from Ashwagandha fruits (Ahmad and Douh 2002). A chloroform extract from fresh Ashwagandha berries yielded stigmasterol, its glucoside, withanone, 27-hydroxy withanolide A, along with two new withanolides,

namely, iso-withanone and 6α,7α-epoxy-1α,3β,5α-trihydroxy-witha-24-enolide (Lal et al. 2006).

3.4 Chemotypic and Metabolic Diversity of Withanolides

W. somnifera occurs in several chemotypes different with respect to their withanolide composition. A withaferin A-rich chemotype has been identified from germplasm of Indian origin, though earlier reports had reported withaferin A only for two non-Indian chemotypes (one from South Africa, the other from Israel). However, Ashwagandha chemotypes from both wild and cultivated Indian populations with withaferin A in all plant parts have been identified (Kaul et al. 2009). The quantitative dynamics of withaferin A production in Indian populations, the inter-chemotypic hybrids, five previously reported chemotypes from Israel, South Africa, and India, and the inheritance of traits were analysed in a detailed chemogenetic study of this complex species. Also withanolide chemotypes have been studied extensively (Chaurasiya et al. 2009). These studies suggest two major types. In type 1, withaferin A and withanolide D with lactonisation at 5, 6 positions dominate (Fig. 3), whereas in type II, the major withanolides are withanone and withanolides with lactonisation at carbon positions 6, 7 mainly occurring in roots (Fig. 3). A wide collection of genotypes categorised as elite, wild, and cultivated accessions with respect to metabolic diversity and protein patterns were assessed by RAPD to link phytochemical with genetic diversity. This study revealed correlation of genetic clustering with chemotype with respect to the predominant presence or absence of specific withanolides. A clade rich in withaferin A as well as in withanone could be separated from a clade rich only in withaferin A, a clade rich only in withanone, and clusters rich in either withanolide D or withanolide A. This study demonstrated for the first time existence of the discrete chemotypes in the Indian population of the plant (Chaurasiya et al. 2009), and except one similar work (Dhar et al. 2006) represents the only systematic study on withanolidal diversity in relation to genetic diversity. Such information is the key to selection, genetic improvement, conservation, and management of useful accessions in gene banks as well as the development and preservation of novel chemotypes or chemotype hybrids and recombinant inbred lines for effective utilisation. Previous studies suffered from the limitation that mainly secondary sources (from botanical gardens) rather than primary samples from wild habitats had been used (Fig. 3). A countrywide collection of wild Ashwagandha accessions by a research group at the Central Institute of Medicinal and Aromatic Plants (Council of Scientific and Industrial Research) under the New Millennium Indian Technology Leadership Initiative (NMITLI) of the Indian Government has revealed that India possesses the widest chemotypic diversity of *W. somnifera* in the world. The huge phytochemical variability of commercially available herbal preparations with respect to even a single withanolide possibly

reflects this chemotypic diversity of plants harvested from wild habitats by the drug manufacturers (Sangwan et al. 2004a).

The significance of wild collections cannot be overestimated, particularly in view of the high degree of self-pollination in this species. As a part of systematic studies on qualitative as well as quantitative phytochemical variability, several distinct lines of *W. somnifera* have been developed as an Indian core collection of diversity and characterised molecularly and phylogenetically (Sangwan et al. 2005, 2007; Sabir et al. 2007; Chaurasiya et al. 2009). The chemical diversity has been corroborated by high-throughput NMR metabolomics of the original chemotypes like NMITLI-101, NMITLI-108, and NMITLI-118 (Fig. 5a) developed at the Central Institute of Medicinal and Aromatic Plants (Council of Scientific and Industrial Research).

4 Tissue Specificity for the Biosynthesis of Secondary Metabolites

The secondary metabolites of *W. somnifera* are produced and stored in a tissue-specific manner. Even for a specific chemical subset, differential tissue distribution of different members is significant. The qualitative and quantitative profile of withanolides, as most prodigally produced metabolites in Ashwagandha, are substantially tissue specific (Chaurasiya et al. 2009). As medicinal properties of the herb are largely attributed to specific withanolide moieties, extracts prepared from different parts of the plant differ in the nature and/or efficacy of their pharmacological activities. Thus, traditional utilisation of specific tissues for specific therapeutic purposes appears to bear a scientific basis. In terms of comprehensive metabolomics, a study by Chatterjee et al. (2010) has revealed tissue-specific presence and concentration of primary and secondary metabolites in leaf versus root tissues of *W. somnifera*. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots could be profiled unambiguously. These included 11 bioactive sterol–lactone molecules. The study also revealed substantial qualitative and quantitative differences between the leaf and root tissues, particularly with respect to the secondary metabolites (Chatterjee et al. 2010). Biosynthetic or metabolism-related research on withanolides has been launched only a few years ago, but there are already evidences of withanogenesis inherent to both leaf and root, contrary to the traditional view that withanolides are synthesised in the leaves and transported to roots (Sangwan et al. 2008).

As per traditional practice, both roots and leaves are prescribed for medicinal purposes in an ailment-specific manner (Kaileh et al. 2007; Sangwan et al. 2005). This practice matches with the overall withanolide richness of the two organs as well as discrete predominance of some specific withanolide moieties in each organ. Extensive phytochemical investigations of the two organs have also revealed a considerable qualitative overlap of several minor or pharmacologically non-active

withanolides (Sangwan et al. 2004a, 2008). The biological function of withanolide tissue specificity is not known at this time. In fact, the functional aspects of withanolides for the plants are currently unknown beyond the general speculation that withanolides similar to other specialised metabolites might act in defence (Madina et al. 2007a, b; Sharma et al. 2007; Sangwan et al. 2008). Our preliminary observations that some *W. somnifera* accessions, which lack a specific major withanolide, show clearly altered flowering and fruiting behaviour and growth patterns (including a dwarf phenotype) indicate that withanolides might act also as growth regulators per se or may cross talk with brassinosteroids by competing for shared metabolic precursors. However, this hypothesis still warrants experimental verification. More insights into these questions are expected from our ongoing work to identify the enzymes and genes involved in the putative position-specific hydroxylation (by cytochrome P450) based on a metabolic model drawn from Ashwagandha root chemoinformatics, wherein withanolide B occupies an anaplerotic position for diverse metabolic transformations into withanolide A, withanolide R, withanone, and 27- hydroxy withanolide B.

Withanolide A is one of the most promising withanolides of *W. somnifera* in view of strong molecular evidences of its ability to induce nerve development and nerve growth promotion effect on synaptic reconstruction. Because of the root-specific production of withanolide A, root cultures particularly hairy roots that can grow rapidly in simple media and can be easily upscaled in a bioreactor form the first choice for such productions. However, despite several reports of *Agrobacterium rhizogenes*-transformed hairy roots of *W. somnifera*, the presence of withanolide A has not been detected. However, multiple shoot cultures of Ashwagandha raised under different combinations of benzyl adenine and kinetin influenced not only their morphogenetic response but also the level of withanolide A in the in vitro shoots. Interestingly, withanolide A, which is usually hardly detectable in the aerial parts of Ashwagandha, was detected in substantial amounts in multiple shoots (Sabir et al. 2008). The productivity of withanolide A in such cultures varied considerably (ca. tenfold, from 0.014 to 0.14 mg per gram fresh weight) with the change in the hormone composition as well as genotype of the explants. The enhanced de novo biogenesis of withanolide A in shoot cultures has been corroborated with radiolabeled incorporation-based biosynthetic studies using [2-(14) C] acetate as a precursor (Chaurasuya et al.2007). Production of withaferin A, the usual predominant withanolide of native shoots, has also been detected in the in vitro cultures (Sangwan et al. 2007). Although withanolides have been isolated from several tissues of the plant including roots, stem, leaf, and berries, whether these tissues are biosynthetically competent to synthesise withanolides wholly de novo or partially has remained a source of speculation. Further biogenetic analysis of withanolide A in native roots and in vitro-cultured normal roots has revealed that *in planta* biosynthesis of withanolide A by the roots takes place ab initio from isoprenogenic primary metabolites such as acetate and glucose.

5 Secondary Metabolites Withanolides

5.1 Biosynthesis of Withanolides

Very limited information has been available until recently on the biosynthetic aspects of withanolides. However, there is a very strong international surge in interests of researchers on biochemical, molecular, biological, and biotechnological aspects of withanolide biosynthetic pathways. A focussed and systematic series of investigations at the interface of chemistry, biochemistry, biology, and genetic and chemotypical resources in the frame of the Indian NMITLI programme has attracted global attention. Now, fundamental information about the genes and proteins involved in withanolide pathway has become available, in addition to the biochemical pathways and processes related to withanogenesis. Recent literature exhibits an increasing number of genes encoding the enzymes of the pathways involved in or related to the biosynthesis of withanolides and other secondary metabolites including functional and biochemical characterisation (Gupta et al. 2012; Akhtar et al. 2013; Sabir et al. 2013; Kushwaha et al. 2013a). Also several attempts have been made to analyse pathways from the perspectives of integration of early processes, ontogenetic regulation, and terminal transformation steps (Chaurasiya et al. 2007; Sangwan et al. 2008).

Biosynthesis of withanolides emanates from the central triterpenoid–sterol pathway with metabolic divergence even as early as the generation of 24-methylene cholesterol (Sangwan et al. 2008). The pathway comprises five segments: (a) isoprenogenic routes, (b) stem route of triterpenic isoprenoid hydrocarbons, (c) early events of cyclisation and oxidative modification common with phytosterols and brassinoids, (d) synthesis of withanolide progenitors and derived diversified withanolides, and (e) terminal conjugative transformations.

5.1.1 Isoprenogenic Routes

Unlike animal systems, plants possess two independent pathways for the synthesis of isopentenyl diphosphate (the monomeric building block for the homologous series of isoprenoids) – a mevalonate (MVA) pathway and a mevalonate-independent pathway (also called DOXP pathway). There is a huge variability in the specificity as well as in the relative participation of the two pathways that allow to accommodate the biosynthetic needs of different terpenoid subclasses (hemi-, mono-, sesqui-, tri-, tetra-terpenoids), even between the individual members within a subclass. Also other factors such as plant species, plant organ, or tissue type contribute to this variability. Recent studies, where the fate of precursors detectable by NMR through the stable ^{13}C isotope was followed in *W. somnifera* (so-called retrobiosynthetic strategies), have revealed that withanolide biosynthesis recruits both MVA (mevalonate) and DOXP (deoxy xylulose pathway) pathways of isopentenyl pyrophosphate (IPP) synthesis (Chaurasiya et al. 2012). A pictorial

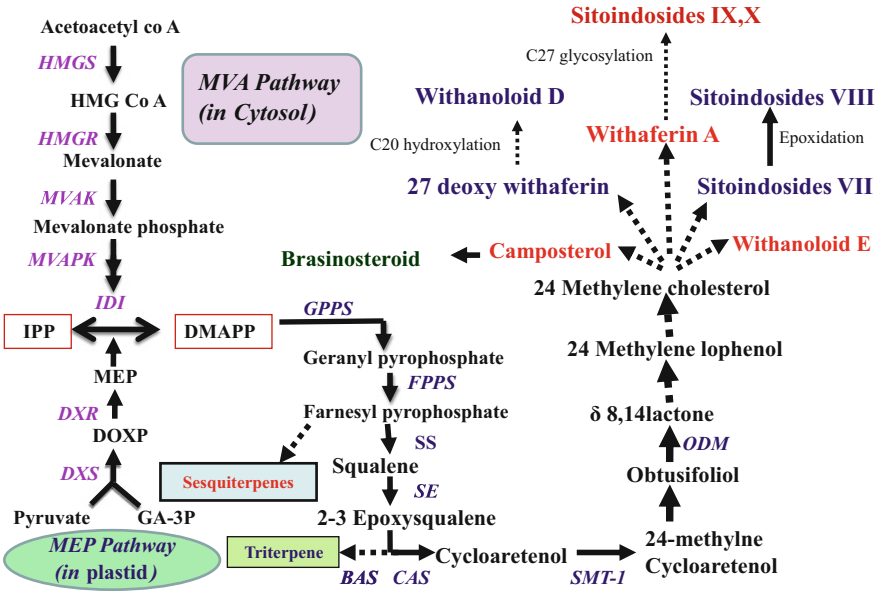

Fig. 4 Enzymes and genes of the withanolide biosynthetic pathway. *ACT* acetyl-CoA thiolase, *HMGS* 3-hydroxy3-methylglutarylco-A synthase, *HMGR* 3-hydroxy3-methylglutaryl CoA reductase, *MVAK* mevalonate kinase, *MVAPK* mevalonate phosphate kinase, *MVAPPD* mevalonate pyrophosphate decarboxylase, *GPPS* geranyl pyrophosphate synthase, *FPP* farnesyl pyrophosphate synthase, *SS* squalene synthase, *SE* squalene epoxidase, *CAS* cycloartenol synthase, *BAS* β-amyrin synthase, *SMT-1* sterol methyl transferase 1, *ODM* obtusifoliol demethylase, *dashed lines* indicate multiple steps

representation of the biosynthetic systems is presented in Fig. 4. The DOXP pathway serves as a plastid-derived alternative route for isoprenoid biosynthesis. The first couple of committed and regulatory steps for terpenoid biosynthesis through the DOXP pathway is represented by the reactions catalysed by DXS and DXR, whereas the mevalonate pathway begins from acetyl coenzyme A and is regulated at the level of a step catalysed by the hydroxymethyl glutaryl coenzyme A reductase (HMGR).

5.1.2 Stem Route of Triterpenic Isoprenoid Hydrocarbons

The trunk route of isoprenoid biosynthesis comprises a sequence of condensation steps of different degrees of polymerisation of the monomer units to generate the series of C_5, C_{10}, C_{15}, C_{20}, C_{30}, and higher prenyl pyrophosphates that provide the progenitors of mono-, sesqui-, di-, tri-, and higher terpenes. Squalene pyrophosphate serves as prenylated precursor and dephosphorylates through a carbocation mechanism under the catalysis of squalene synthase to generate squalene. Thus, squalene is a spin-off metabolite of the isoprenoid trunk route that is metabolised

into diverse triterpenoids (C_{30}) as well as their descendant specialised metabolites ($C_{30\pm n}$) including sterols (typically $C_{28–29}$), withanolides (C_{28}), and other phytosteroids. The five-carbon IPP monomer and its isomer dimethylallyl diphosphate (DMAPP) combine in a head-to-tail manner to form geranyl diphosphate (GPP, C_{10} prenyl pyrophosphate) under the catalysis of GPP synthase. GPP is then released and processed by monoterpene synthases to diverse spin-off parental monoterpenes such as geraniol, linalool, myrcene, or limonene, whilst GPP produced by farnesyl diphosphate synthase (FPP synthase) remains enzyme bound for a second sub-reaction, where additional IPP in *trans*-configuration is coupled to yield farnesyl diphosphate (FPP, C_{15} prenyl pyrophosphate). FPP may again be spinned off to generate sesquiterpenes under the catalysis of sesquiterpene synthases or processed further in the trunk pathway to serve as substrate to produce higher prenyl pyrophosphates and their spin-off higher terpenoids. Thus, for triterpenoids, two molecules of FPP condense in a head-to-head manner to produce squalene (C_{30}) under the catalytic action of squalene synthase (Fig. 4).

5.1.3 Early Events of Cyclisation and Oxidative Modification

Epoxidation of squalene to 2,3-squalene oxide involves atmospheric oxygen and the catalytic action of squalene epoxidase, a cytochrome P_{450} enzyme. Squalene-2, 3 epoxide occupies a highly anaplerotic position in steroid and triterpenoid biosynthesis and serves to deliver diverse progenitor molecules that are further diversified by progressive functionalisation. A battery of triterpene synthases, like cycloartenol synthase, lanosterol synthase, thalianol synthase, amyrin synthases, and lupeol synthase, catalyse these progenitors. Withanolide biosynthesis appears to follow the same early steps of squalene 2, 3-epoxide cyclisation and subsequent metabolic transformation steps as common for membrane phytosterols (like stigmasterol and sitosterol) and brassinolides. Based on a chemoinformatic analysis, it has been hypothesised that the pathway conveying withanolide biosynthesis could be a prolongation of the brassinolide pathway (Sangwan et al. 2008). Accordingly, it has been hypothesised that withanogenesis may metabolically originate from 24-methylene lophenol and/or 24-methylene cholesterol and/or campesterol (Sangwan et al. 2008). Whilst radiotracer studies carried out with 24-methylene cholesterol as precursor indicate that an intermediate from the sterol pathway is linked to withanogenesis (Lockley et al. 1976; Glotter 1991), alternative plausible metabolites have still to be tested as yet in this regard. Since the functionalisation reactions of secondary metabolism are quite versatile and can use multiple substrates, it is not very odd to examine the possibility that withanolide biosynthesis might be committed from multiple points of the sterol–brassinolide pathway (Sangwan et al. 2008). Physiologically, this hypothesis is consistent with our present knowledge on the functional aspects of sterols and brassinolides. Sterols are membrane constituents and their levels determine several critical cellular functions and processes, while brassinolides are hormones. Intracellular levels of membrane sterols and brassinolides are very low and have to be tightly controlled to

maintain a balance of cellular activities, plant growth, and development. In contrast, the levels of withanolides are several orders of magnitude higher (Sangwan et al. 2008). Therefore, withanolide biosynthesis in Ashwagandha may have role in regulating sterol and brassinolide homeostasis by serving as major carbon shunt from the shared pathway segment (Sangwan et al. 2008). Although lanosterol synthesis from squalene 2,3 oxide under the enzymatic action of lanosterol synthase has been considered to be restricted to fungi, some recent studies have revealed the presence of lanosterol in a few higher plants. As function, it has been proposed to act as metabolically redundant route to secondary phytosterols/triterpenols. Although such a sterol synthase has not been found so far in *W. somnifera*, some derivatives of lanosterol have been recently reported to occur in Ashwagandha (Mishra et al. 2012). Considering that withanolides are based on an ergostane skeleton, their origin through such a redundant pathway should be investigated (Fig. 4).

5.1.4 Synthesis of Withanolide Progenitors and Diversified Withanolides

The nature of parental progenitor metabolites synthesised from the predicted intermediates (Sangwan et al. 2008) like 24-methylene lophenol, 24-methylene cholesterol, and campesterol is still not known. However, it seems quite certain that it involves some critical steps to generate the ergostane skeleton and additional functional groups; these include (i) C_1 oxidation to a hydroxylation followed by, for most (>90 %) of the withanolides, reduction of the hydroxyl group into a keto (>C = O) function. Thereby, it implicates participation of a cytochrome P_{450} hydroxylase and an oxidoreductase (dehydrogenase). The structural features of the molecules suggest that oxidative transformations may involve hydroxylation at C_{22} as well as C_{26} followed by conversion of a C_{26} alcohol to an aldehyde and, in most cases, conversion of the aldehyde (−CHO) group into an acid (−COOH) function. These catalytic reactions can be predicted to recruit appropriate hydroxylases and other oxidoreductases. Another step is formation of a lactone (mostly) or hemiacetal (few cases) ring by closure between a C_{22} hydroxy group and a C_{26} acid group, respectively. These biochemical reactions are yet to be identified and characterised to understand the events of metabolic commitment that generate the parental metabolite for withanolide.

A myriad of withanolides that number in hundreds are derived by further functionalisation in a position- and region-specific manner. These functionalisations include epoxidation, hydroxylations, and dehydrogenations. In fact, all positions of the C_{28} molecule except C_8, C_9, and C_{10} can be hydroxylated individually as well as in combinations reflecting the possibility that many more withanolides can be generated than those existing in nature. Nevertheless, a substantial number of withanolides can be grouped into clusters defined by common conjugation. Accordingly, we could define two major classes of withanolides based on a conjugated hydroxy and epoxy function around C_5 to C_7. These are class

1 withanolides containing a 4β-hydroxy 5,6 β-epoxy group with representative major withanolides like withaferin A and withanolide D and class 2 withanolides containing a 5α,6,7,α epoxy group with representative major withanolides like withanone and withanolide A (Fig. 3). Biosynthetically, these classes may record the existence of specific cytochrome P_{450} hydroxylases to generate these hydroxyl and epoxy functionalities of respective orientation due to their catalytic specificity for the carbon position in functionalisation and the stereospecificity of the functional groups. From these observations and analyses, a large number of enzymes and genes can be predicted able to confer catalytic novelties with potential commercial/industrial applications. The forthcoming availability of Ashwagandha root and leaf transcriptomes under the New Millennium Indian Technology Leadership Initiative in India would provide a working platform for advanced genomics and systems biology support to accelerate the deciphering of the metabolic pathway and withanolide metabolomics.

5.1.5 Terminal Conjugative Transformations

Acylations (particularly acetylation) and glycosylations (mainly single or multiple glucosylations) are the major conjugative metabolic transformations of withanolides observed so far. Major derivatives acetoxy withanolides, withanosides, and sitoindosides are produced by the reactions that are catalysed by acyltransferases and glycosyltransferases, respectively. Several glycosyltransferases have been isolated and characterised from *W. somnifera* leaf and root, including cloning of the respective genes and elucidation of gene functions. An enzyme catalysing the formation of 4-acetoxy and 27-acetoxy withaferin A from withaferin A has been isolated and characterised from the leaves of *W. somnifera* (Chaurasiya 2007). The enzyme contains an HXXD motif as proteomic characteristic and thus belongs to the so-called BAHD family of plant acyltransferases. The family is named according to the first letter of the first four biochemically characterised enzymes of this family, namely, benzyl alcohol O-acetyltransferase (*B*EAT) from *Clarkia breweri*, anthocyanin O-hydroxycinnamoyltransferase (*A*HCT) from *Gentiana triflora*, anthranilate N-hydroxycinnamoyl/benzoyltransferase (*H*CBT) from *Dianthus caryophyllus*, and deacetylvindoline 4-O-acetyltransferase (*D*AT) from *Catharanthus roseus* (D'Auria 2006).

5.2 *Cellular Metabolic Cross Talk for Secondary Metabolite Production*

Chemoinformatic and biogenetic comparison of triterpenes and sterols with withanolides implies that withanolides may originate through a complex and yet unknown pathway, from either 24-methylene lophenol or 24-methylene cholesterol

or campesterol, or the upstream intermediates of the triterpene pathway, or analogous metabolites of the lanosterol route (Sangwan et al. 2008). However, since the stem pathway at least up to squalene is shared, the synthesis of isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) might control the withanolide pathway through governing the flux of the isoprene units. In plants, IPP and DMAPP occur by two independent routes: the classical mevalonate (MVA) pathway in the cytosol and a non-mevalonate pathway (also called deoxy xylulose pathway (DOXP) or methyl erythritol pathway (MEP)) proceeding in plastids (Lichtenthaler 1999; Rohmer 2003; Sato et al. 2003). It is considered that the synthesis of monoterpenoids and diterpenoids involves the DOXP pathway while that of triterpenes (sterols) and sesquiterpenes recruits the MVA pathway (Lichtenthaler 1999; Rohmer 2003; Bouvier et al. 2005; Cordoba et al. 2009). This generalisation is based on early studies on the relative contributions of the MVA and DOXP pathways for various classes of isoprenoids. However, the validity of this strict categorisation is challenged by more recent evidences. Normal levels of sterols (sitosterol, stigmasterol) can be synthesised through the MVA pathway (Rohmer 2003; Bouvier et al. 2005). But the sterol hyper-producing green callus of *Croton stellatopilosus* has been shown to recruit DOXP pathway as well in the biosynthesis of isoprenoids (De-Eknamkul and Potduang 2003). However, many sesquiterpenoid compounds, such as artemisinin, are of mixed (DOXP and MVA) or exclusively of DOXP origin (Towler and Weathers 2007). Some compounds are of mixed origin such as carrot β-caryophyllene (Hampel et al. 2005), chamomile and snapdragon flower sesquiterpenes (Adam and Zapp 1998; Dudareva et al. 2005), strawberry fruit sesquiterpenes (Hampel et al. 2005), and the *Anthemis cotula* allergenic anthecotuloide (Van Klink et al. 2003) are derived from both pathways. The relative carbon contribution from the MVA and DOXP pathways to withanolide biosynthesis was determined through quantitative NMR by measuring position-specific enrichment of ^{13}C labels on individual carbons of withaferin A. $^{13}C_1$-glucose fed to in vitro microshoots of *W. somnifera* has revealed significant contribution of the DOXP pathway to withanogenesis. Thus, withanolide biosynthesis utilises both the DOXP and the MVA pathways adding to the growing list (Chaurasiya et al. 2012), where the generalised dichotomy of isoprenoid class-specific recruitment of the two pathways is blurred. Possibly, it might be the physiological context of metabolism rather than isoprenoid class that determines the relative contribution of the two isoprenogenic routes (MVA and DOXP).

5.3 Developmental Dynamics of Withanolides

Two major withanolides, withaferin A and withanone, are accumulated in prodigal amounts in Ashwagandha leaves. However, no information is available about the developmental physiology of withanolide biogenesis. The studies on ontogenic

dependence of withanolide biogenesis are highly relevant: (1) to understand the chemo-ecophysiological function of these secondary molecules, (2) to optimise harvest of bioactive phytochemical(s) from this crop, (3) to define the parameters for quality management of these herbal nutraceutical and therapeutic products, and (4) to define physiological states in studies dealing with the comparative genomics of withanogenesis. The accumulation of withaferin A and withanone was followed by TLC profiling and HPLC quantification through five stages of leaf ontogeny from very young, young, premature, mature, till senescent (Chaurasiya et al. 2007). The temporal patterns of accumulation were compared with parallel quantifications of biosynthesis using radiolabeled acetate as precursor. The levels of radioactive incorporation reported de novo biosynthesis that was found to be induced from initiating leaf development and increasing in the young leaf, such that maximal levels had accumulated by the time of leaf maturity (full expansion). The terminal degenerative and senescent phases of ontogeny seem to involve catabolic decay of withanolides, perhaps for the relocation/mobilisation of withanolide carbon not longer required for defence in tissues that are going to die anyway (Tuli and Sangwan 2010). Based on the assumption of a putative role in defence, a number of hypotheses link the production of secondary metabolites with leaf growth. For example, the optimal defence theory balances the risk for attack or damage against the costs of biosynthesis. In contrast, various resource-based theories assume that this biosynthesis is constrained by the external availability of resources and an internal trade-off in allocations between growth and defence (Chaurasiya et al. 2007).

Developmental patterns can also affect the profile of withanolide accumulation (Sidhu et al. 2011). By using NMR technology, the concentration of withanolides was found to be highest during initial stages of fruit development, whereas withanamides increased substantially during maturation of the fruits. This age-dependent shift from withanolides towards withanamides is relevant for application purposes. For instance, the mature fruit would be highly suited as antioxidant (Jayaprakasam et al. 2004), which would be beneficial in the treatment of tumours and inflammation (Jayaprakasam et al. 2003). This example illustrates that the type and developmental stages of source tissues are relevant to optimise their usage for drug and nutraceutical purposes.

6 Withanolide Pathway Genomics

Genes for the withanolide biosynthetic pathway related to upstream segment and regulatory steps of IPP synthesis (isoprenogenesis) through the MVA and DOXP pathways, the shared stem route of isoprenoid pathway, and conjugative steps of withanolide glycosylation have been cloned and characterised from *W. somnifera* (Table 3). The detailed biochemical and genomic significance is discussed below.

6.1 Deoxy Xylulose-5-Phosphate Synthase (DXS)

DXS constitutes the first step for the DOXP pathway of isoprenogenesis. Ashwagandha DXS gene (WsDXS) encoding a 717 amino acid polypeptide has been cloned and found to possess a similar exon–intron structure as found in the tomato homologue (Gupta et al. 2013). The gene was differentially regulated in different tissues with highest level of expression in flowers and young leaves. Moreover, the pattern differed between chemotypes. The abundance of the *WsDXS* transcripts parallels the content of the major withanolides withaferin A, withanone, and withanolide A. This reflects the significant contribution of DXS to deliver the IPPs required for withanolide biogeneration via the triterpenoid pathway. The expression of this gene was also induced by mechanical injury and salicylic acid and methyl jasmonate (Gupta et al. 2013).

6.2 Deoxy Xylulose Phosphate Reductase (DXR)

The reaction catalysed by DXR constitutes the next step in the DOXP pathway of isoprenogenesis. The gene has been cloned from Ashwagandha as a reading frame coding for a 475 amino acid polypeptide and, similar to DXS, harbours a putative plastid-targeting signal (Gupta et al. 2013). The comparison of WsDXS and WsDXR expression in three different chemotypes containing varying contents of withanolides, in various tissues and leaf ontogenic stages, as well as in response to chemical stimuli (SA and MeJA) and mechanical injury, indicates that WsDXS and WsDXR control the flux of carbon required for withanolide biosynthesis, for instance, during leaf development (Chaurasiya et al. 2007).

6.3 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGR)

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) catalyses an irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) into mevalonic acid constituting the key regulatory step for the synthesis of IPP and its isomer, DMAPP, as progenitors of homologous series of isoprenoids (Chappell 1995). HMGR is the rate-limiting enzyme of the MVA pathway and thus also for withanolides. Therefore, regulatory and functional characteristics of this gene are relevant for withanolide biosynthesis. From sequence homology of *WsHMGR* with other plant HMGR, conserved motifs probably corresponding to the active centre can be identified. Our observations on WsHMGR suggest that its expression is responsive to wounding and exposure to methyl jasmonate and salicylic acid. Interestingly, the rapid and steady accumulation of *WsHMGR*

transcripts in response to mechanical injury was paralleled by an identical response of Ws*FPPS*, the gene involved in the downstream stem pathway of isoprenoid progenitor prenyl phosphate generation. WsHMGR is the first gene of Ashwagandha isoprenoid/secondary metabolite biosynthesis that has been functionally validated by pathway complementation in *E. coli*. It has been demonstrated that *E. coli* expressing *WsHMGR* and pAC-BETA accumulated significantly higher saffron yellow coloured β-carotene (more than twofold) as compared to the bacteria transformed with the empty vectors. This suggests that *WsHMGR* promoted the accumulation of β-carotene, corroborating its significance for withanolide biosynthesis (Akhtar et al. 2013).

6.4 Farnesyl Diphosphate Synthase

Similar to the initial two genes of DOXP pathway, DXR and DXS, farnesyl diphosphate synthase (WsFPPS) is much more abundantly expressed in young leaves as compared to mature leaves (Gupta et al. 2011). This gene is involved in sesquiterpene prenylphosphorylation. The abundant expression of WsFPPs in the leaf indicates that root may be less relevant for DOXP-dependent isoprenoid biosynthesis. Generally, roots and leaves can synthesise withanolides independently, but leaves are more active (Sangwan et al. 2008; Sabir et al. 2012).

6.5 Squalene Synthase

Squalene synthase (WsSQS) is a key enzyme involved in the biosynthesis of isoprenoids and catalyses a branch point diverging carbon flux from the main isoprenoid pathway towards sterol and triterpene biosynthesis. Squalene synthase causes a dimerisation of two farnesyl diphosphate (FPP) molecules into squalene. Since this gene is important for biosynthesis and regulation of withanolides, this enzymatic step has been subject of investigation in *W. somnifera*. A genomic fragment of 1,765 kb has been cloned and characterised recently (Bhatt et al. 2012) comprising a 1.236 bp ORF for squalene synthase. Expression of this gene was maximal in the leaf. The promotor harbours several putative regulatory cis-acting elements. Similar to DXS and DXR, squalene synthase has been shown to be upregulated by different signalling components including methyl jasmonate, salicylic acid, and 2, 4-D (Bhat et al. 2012). Meanwhile, the catalytic activity of a recombinantly expressed C-terminally truncated WsSQS could be demonstrated (Gupta et al. 2012). WsSQS transcripts are found in two open reading frames of 1,236 and 1,242 bp, respectively, corresponding to a length difference of only 2 amino acid residues, and encode 412 and 414 amino acid polypeptides, respectively (Gupta et al. 2012). A correlative investigation on WsSQS expression in a wide range of tissues demonstrates a linear correlation between transcript

abundance and withanolide levels (Sabir et al. 2013). Specifically, expression of the SQS is elevated with the onset of tissue differentiation from callus to shoots matching an increase of withanolide content. In a recent study, squalene synthase has been overexpressed in *W. somnifera* using *Agrobacterium tumefaciens*-mediated transformation (Grover et al. 2013). The transgenic suspension cultures displayed a four-fold increase of SQS catalytic activity and a concomitant 2.5-fold enhancement in withanolide A content. Additionally, these transformed cell suspension cultures produced withaferin A, contrary to the non-transformed control cultures (Grover et al. 2013). These studies show clearly a direct function of SQS for withanolide biosynthesis (Gupta et al. 2012; Sabir et al. 2013; Grover et al. 2013).

6.6 Squalene Epoxidase

Squalene epoxidase (EC. 1.14.99.7) catalyses the production of 2, 3 oxidosqualene from squalene. The oxidosqualene possesses anaplerotic significance by serving as the common precursor for several triterpene hydrocarbons, triterpene alcohols, sterols, withanolides, and brassinosteroids. The gene has been cloned from *W. somnifera* (Razdan et al. 2013) as a 1.965 kb cDNA containing a 1.596 kb ORF (531 amino acids). A flanking fragment of 513 bp of the promotor region could be obtained through genome walking and contains several cis-elements putatively mediating responses to various biotic and abiotic stresses. Similar to the transcripts for other upstream genes of the pathway, expression of the WsSQE gene is higher in leaves as compared to shoot and root tissues (Razdan et al. 2013).

6.7 Phytosteroid Glycosyltransferases (SGT): Family Members and Functions

Sterol glycosyltransferases (SGTs) catalyse the transfer of a sugar moiety from activated (uridine diphosphate-conjugated sugars like UDP-glucose) donors to diverse acceptor molecules (aglycones) of steroidal nature. This family of genes is important for Ashwagandha, since several withanolides are found as glycosidic conjugates like withanosides and sitoindosides. Sterols and their modified counterparts are not only medically important but probably participate in stress adaptation of plants (Tuli and Sangwan 2010). The withanosides mainly comprise withanolides with one or more glucose units attached to the C-3 or C-27 positions. In a sequence of studies on *W. somnifera* sterol glycosyltransferases, it has been shown that the plant harbours a battery of SGTs that differ not only in their characteristic catalytic features but also in their size and intracellular localisation (Madina et al. 2007a, b; Sharma et al. 2007). Briefly, a sterol glucosyltransferase

specific for the 3β-hydroxy position purified from the leaves of *W. somnifera* has a catalytic specificity to glycosylate both phytosterols and steroidal sapogenins (Madina et al. 2007a). A different sterol glucosyltransferase capable of transferring glucose from UDPG to the C-3 position (Sharma et al. 2007) possessed a transmembrane domain (Sharma et al. 2007). Originally, no enzyme for the glycosylation of sterols/withanolides at positions other than C-3 had been identified, though several 27-O-glucosylated pharmacologically important metabolites, like sitoindosides IX and X, are present in *W. somnifera*. In fact, a novel 27β-hydroxy glucosyltransferase activity has been identified and characterised that can glucosylate the C-27 hydroxy position in withanolide as well as some other sterols with a hydroxyl function at higher position. This C-27 hydroxy group-specific SGT has been suggested to function in defence responses of the plant (Madina et al. 2007b). The purified enzyme showed activity with UDP-glucose but not with UDP-galactose as sugar donor and exhibited broad sterol specificity by glucosylating a variety of sterols/withanolides with β-OH groups at C-17, C-21, and C-27 positions. An enzyme with comparable catalytic activity has not been reported earlier from plants. Both the C-3 and C-27 hydroxy group-specific glucosyltransferases follow an ordered sequential bi-substrate reaction mechanism, in which UDP-glucose binds first followed by binding of the sterol (Madina et al. 2007a, b). The catalytic activities with withanolides as substrates suggest a role for this enzyme in secondary metabolism. Results on peptide mass fingerprinting of the purified enzyme revealed their resemblance with glycuronosyltransferase-like proteins. The catalytic levels of these enzymes in the leaves of *W. somnifera* were enhanced, but in different levels, by application of salicylic acid and heat stress (Madina et al. 2007b). Recently, three more members of the SGT gene family have been identified in Ashwagandha (Chaturvedi et al. 2012) with amino acid sequence homology in the range of 45–67 % compared to other known plant SGTs. The transcript expression can be induced up to tenfold depending on the organ or triggering external stimulus. Recently, in addition to SGTs, a new flavonoid-specific GT was found in *W. somnifera* (Jadhav et al. 2012). Based on the crystal structure of plant UGTs, a structural model for this flavonoid-specific glycosyltransferases (WsFGT) could be constructed. The model predicts a GT-B-type fold (Jadhav et al. 2012) and interaction of amino acids in a conserved plant secondary product glycosyltransferase (PSPG) box with the sugar donor, while His18, Asp110, Trp352, and Asn353 are probably important for catalytic function. This structural information on the docking will be useful to understand the glycosylation mechanism of flavonoid glucosides (Jadhav et al. 2012).

7 Towards Withanolide Production In Vitro

The demand for withanolides is considerably increasing not only due to academic interest but also for the production of valuable medicinal products. Commercial cultivation of *W. somnifera*, however, can yield only limited amounts of

withanolides for a series of reasons. These include (1) relatively long period between planting and harvesting, (2) chemotypic and developmental variations, (3) seasonal and somatic variations, (4) infections by microorganisms and herbivore attacks, and (5) environmental pollution. The comparison of *W. somnifera* from different geographic locations has revealed a variety of chemical profiles that are different in quantity and even quality and that are correlated with genetically distinct clusters (Sangwan et al. 2004a, b; Chaurasiya et al. 2009). Medicinal preparations from such nonuniform and non-characterised plant materials may compromise the efficacy of phytotherapy (Kushwaha et al. 2012; Mondal et al. 2010). Therefore, the establishment of efficient in vitro regeneration systems and the development of cellular technology are prerequisites to produce plant material of standardised high quality (see also chapter by Opatrný in this volume). The use of tissue culture could be an alternative method for shortening the time to obtain true-to-type plantlet regeneration and obtaining a stable production of withanolides. Reports on in vitro cultures using different Ashwagandha explants are available in the literature (Rani et al. 2003; Sabir et al. 2007; Nayak et al. 2013) and include the regeneration of plants from callus. Tissue culture of *W. somnifera* for the in vitro production of withanolides has been achieved elsewhere (Yu et al. 1974; Heble 1985) and also in our laboratory. Various in vitro-based approaches for the development of callus, cell suspensions, and shoot and root cultures have been developed for Ashwagandha (Table 4; Fig. 5c–i).

7.1 Induction and Proliferation of Multiple Shoot Cultures

Shoot induction and proliferation of *W. somnifera* and the second important Ashwagandha species, *W. coagulans*, have been achieved (Singh et al. 2005; Sabir et al. 2007, 2008, 2013; Sharada et al. 2007). A protocol utilising a hormone combination for direct induction and regeneration of shoots was developed which was successful on several *W. somnifera* chemotypes (Sabir et al. 2007) and was validated in several studies involving the use of shoot cultures (Sabir et al. 2008, 2012; Mishra et al. 2012; Chaurasiya et al. 2012; Sabir et al. 2012).

Phytochemically, these multiple shoot cultures exhibited the characteristic withanolides such as withaferin A and withanolide A (Sabir et al. 2008). The accumulation of both withaferin A and withanolide D could be enhanced when 4 % sucrose was added to the medium (Table 4). Shoot cultures accumulated the rare withanolides I, G, and D, while these could not have been observed in unorganised callus (Roja et al. 1991). Shoot cultures of *W. somnifera* from Italy accumulated withanolide J after 20 days of in vitro growth, whereas hairy root systems of the same plant failed to produce detectable withanolides (Vitali et al. 1996). Glycoderivatives of withanolides, e.g. withanoside IV (WSG-3), withanoside VI (WSG-3A), physagulin D (WSG-P), and withastraronolide (WSC-O), were isolated from in vitro multiple shoot cultures of *W. somnifera* (Ahuja et al. 2009).

Table 4 Withanolide production from in vitro cultures

In vitro cultures	Withanolide produced	References
Direct shoot regeneration		
Nodal explants, shoot tip, germinating seeds, apical bud, axillary buds, leaf, axillary meristem, cotyledonary nodes	Withanolide A, withanolide B, withanone, withaferin A, withanolide D, withanoside IV −3, withanoside 3A, physagulin D and withastraronolide (glycowithanolide), withanolide J, withanolide I, G, and D	Sabir et al. 2007, 2008; Singh et al. 2005; Sivanesan 2007; Fatima and Anis 2011; Ghimire et al. 2010;
Indirect shoot regeneration via callus phase		
Cotyledonary leaves, axillary shoots, hypocotyls, seed, internodes, nodal segment	Withaferin A, 12-deoxywithastramonolide and withanolide A	Rani et al. 2003; Supe et al. 2006; Siddique et al. 2004; Manickam et al. 2000; Sabir et al. 2008
Callus and suspension cultures		
Shoot tips of seedling, leaf, hypocotyl, rhizogenic roots	Withanolide A, withanone, withaferin A, withanolide D	Sharada et al. 2007; Sabir et al. 2007, 2013
Elicited suspension cultures		
Hypocotyl, leaf, (dual elicitor treatment-copper sulfate and *Verticillium dahliae* extract)	Withaferin A, withanolide A	Ciddi 2006; Sabir et al. 2008, 2011; Baldi et al. 2008; Nagella and Murthey 2010
Hairy root cultures induced from shoots, leaf, cotyledons	Withaferin A, withanolide D, withanolide A	Murthy et al. 2008; Nagella and Murthy 2010; 2012 Kumar et al. 2005; Bandopadhyay et al. 2007
Somaclonal variant (from organogenic calluses)	12-Deoxywithastramonolide	Rana et al. 2012

7.2 Callus and Cell Suspension Cultures

A fast and efficient protocol for callus induction and proliferation suitable for almost all chemotypes of *W. somnifera* has been established using stem, leaf, buds, and roots as explants (Fig. 5c; Sabir et al. 2008, 2009, 2012). Callus derived from axillary shoots has been demonstrated to have the best capacity for regenerating shoots (Rani et al. 2003). Only cytokinins (2.0 mg L^{-1} BAP alone or in association (at 1 mg L^{-1}) with 2.0 mg L^{-1} Kin) have been found suitable to induce the formation of nodular green calluses (see also chapter by Šmehilova and Spíchal and Opatrný in this volume) with nodal segments as explants (Siddique et al. 2004). Field-cultivated leaves as well as seeds have demonstrated to serve as the best starting material for the induction of calluses appropriate to achieve high frequency (81 %) of shoot regeneration (Supe et al. 2006; Rout et al. 2011).

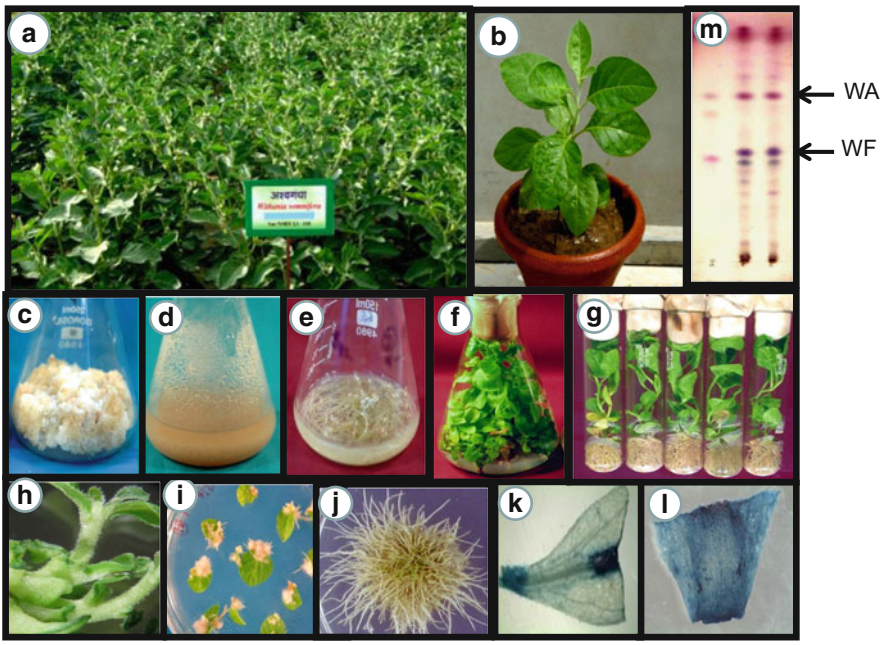

Fig. 5 Tissue culture and genetic transformation of Ashwagandha. (**a**) Superior Ashwagandha variety growing in the CSIR-CIMAP experimental farm; (**b**) young Ashwagandha plant; (**c**) profuse callus proliferation in optimised MS medium; (**d**) synchronised suspension culture; (**e**) Agrobacterium rhizogenes-mediated hairy roots of Ashwagandha; (**f**) multiple shoot cultures; (**g**) root induction in shoot cultures of Ashwagandha; (**h**) shoot bud viewed under microscope; (**i**, **j**) direct rhizogenesis; (**k**, **l**) localisation of histochemical GUS expression; (**m**) thin layer chromatography of Ashwagandha extract isolated from control plant and transformed plant; *WA* withanolide A, *WF* withaferin A

Indirect regeneration from leaf explants and a comparative analysis of withaferin A, 12-deoxywithastramonolide, and withanolide A productions in in vitro systems or greenhouse were also investigated (Dewir et al. 2010). High yields of transformed *W. somnifera* calluses were achieved from the infection of hypocotyls with *A. rhizogenes* strain MTCC-2250 (Ray and Jha 1999). We also established a protocol for regenerating tissues from root calluses (Sabir et al. 2012). Moreover, we developed a protocol for genetic transformation of *W. coagulans* to assess for understanding the functions of genes utilising this regeneration system with higher frequency of transformation (Mishra et al. 2012; Pandey et al. 2010).

Cell suspension cultures of *W. somnifera* were established, being proved to be effective in the production of withaferin A (Fig. 5d). The established cultures produced up to 25 ± 2.9 mg withaferin A L^{-1} when cells were elicited with 750 μM salicin compared to non-elicited cells which produced only 0.47 ± 0.03 mg withaferin A L^{-1} (Ciddi 2006). Leaf-derived friable cell suspension cultures were grown in highly synchronised conditions, and generated suspension cultures (see also chapter by Opatrný et al. in this issue) were also shown to possess the capacity

of accumulating withanolides (Sabir et al. 2008) and also possess biotransformation potential (Sabir et al. 2011). Various abiotic elicitors (arachidonic acid, methyl jasmonate, calcium chloride, and copper sulphate) and biotic elicitors (cell extracts and culture filtrates of *Alternaria alternata*, *Fusarium solani*, and *Verticillium dahliae*) were tested for the ability to stimulate the accumulation of withaferin A in suspension culture of transformed cells (Baldi et al. 2008).

7.3 Root Cultures

The development of fast growing root culture system would offer a unique opportunity for producing root phytochemicals in laboratory without depending on field cultivation. Several reports are available for direct root regeneration from leaves of *W. somnifera* (Fig. 5i, j). Direct rhizogenesis of Ashwagandha has been reported (Sabir et al. 2013; Wasnik et al. 2009; Wadegaonkar et al. 2005). Indirect regeneration of rhizogenic roots from leaf explants and the production of withanolide were studied (Wadegaonkar et al. 2005; Wasnik et al. 2009; Sabir et al. 2013). Production of withanolide A from adventitious root cultures of *W. somnifera* has been reported (Nagella and Murthy 2010). Elicitation by methyl jasmonate and salicylic acid enhanced withanolide production in adventitious root cultures of *W. somnifera* (Sivanadhan et al. 2012a). Chitosan-mediated enhancement of withanolide (withanolide A, withanolide B, withaferin A, withanoside IV, and withanoside V) production in adventitious root cultures of *W. somnifera* (L.) has also been observed (Sivanadhan et al. 2012b).

7.4 Hairy Root Cultures

The establishment of hairy root cultures (Fig. 5e) of Ashwagandha and the investigation of withanolide accumulation have been reported (Kumar et al. 2005; Bandopadhyay et al. 2007; Murthy et al. 2008; Praveen and Murthy 2013). Among the different strains of *A. rhizogenes,* strain A4 had the highest efficiency to induce hairy roots, while strain LBA 9402 produced different morphological responses such as callus and rooty callus (Bandopadhyay et al. 2007). Accumulation of major withanolides, withaferin A, withanolide D, and withanolide A has also been found to occur in transformed root lines of *W. somnifera* (Ray and Jha 1999; Kumar et al. 2005; Murthy et al. 2008). The effect of growth media on root biomass accumulation (Ahuja et al. 2009; Murthy et al. 2008) and the effect of carbon sources and pH on the synthesis of withanolide A in hairy root cultures have been investigated (Nagella and Murthy 2012). Methyl jasmonate and salicylic acid were found to elicit the production of withaferin A in the hairy root cultures of *W. somnifera*, indicating that the accumulation and biogenesis of secondary metabolites – withanolides – in Ashwagandha are under tight regulation.

8 *Agrobacterium tumefaciens*-Mediated Genetic Transformation

Plant transformation mediated by *A. tumefaciens* has become the main method for the introduction of foreign genes into plant tissues and the subsequent regeneration of transgenic plants (see also chapter by Opatrný in this volume) and has been successfully employed to transform several medicinal plants (Gomez et al. 2007). *A. tumefaciens*-mediated transformation of *W. somnifera* was firstly attempted by Ray and Jha (1999) using a wild-type strain of *A. tumefaciens*, but only yielding shoot-like teratomas. Successful genetic transformation up to plant level of *W. somnifera* through *A. tumefaciens* has been reported for the first time by Pandey et al. (2010) using leaf explants (both from tissue culture and greenhouse) as starting material. *A. tumefaciens* strains LBA4404 and EHA101, containing the binary vector pIG121Hm, possessing *npt*II gene under the control of P*nos* (nopaline synthase promoter), and hygromycin resistance (*hptII*) as selective marker, as well as *gus*A genes under the control of the cauliflower mosaic virus (CaMV) 35S promotor (Ohta et al. 1990), have been used for the development of transformation protocol (Fig. 5k, l). Leaf sections were co-inoculated in *A. tumefaciens* suspension for 10–30 min with gentle shaking, blotted dry, and placed on cocultivation medium and kept in the dark for 5 days. Several parameters such as position of leaf, age of seedling, infection time, and cocultivation duration were adjusted to improve transformation efficiency. Several distinct chemotypes of *W. somnifera* differing in their withanolide profile were tested and found to yield maximal frequencies of up to 77.3 % for transient and up to 1.7 % for stable transformation (Pandey et al. 2010). Although this protocol enabled for the first time genetic engineering of Ashwagandha to understand and improve the withanolide pathway, its efficiency was limited to some chemotypes. A protocol efficient for all chemotypes is still urgently required for applications in phytopharming and functional genomics. Recently, a highly efficient system for the *A. tumefaciens*-mediated transformation of the closely related withanolide-yielding *W. coagulans* has been developed to cater these requirements (Mishra et al. 2012).

8.1 Development of Efficient Protocols for Transformation of Ashwagandha

The successful and efficient transformation of plants essentially requires optimisation of several prerequisite including the competence of target cells or tissues for transformation and regeneration, the efficiency of DNA delivery, the stringency of selection, and the ability to recover fertile transgenic plants. During the past two decades, these prerequisites have been optimised leading to protocol for the improved *A. tumefaciens*-mediated genetic transformation of aromatic and medicinal plants (Ray and Jha 1999). Many of these factors have been evaluated in our

study to generate stable transgenic plants of *Ashwagandha* species. The leaf from in vitro multiple shoot cultures was identified as a suitable explant for the *A. tumefaciens*-mediated transformation and regeneration of stable transformants of *Ashwagandha* species (Mishra et al. 2012). Also leaf explants have been used effectively for the transformation of other aromatic and medicinal plants (Kumar and Gupta 2008). Therefore, margins of the leaf lamina were removed in Ashwagandha to expose cut surfaces for infection, and small pieces (2–3 mm) of leaf explants (apical, middle, and basal portion) were used for infection. *A. tumefaciens* strain *LBA* 4404 with plasmid pIG121Hm was used in this study to optimise various factors like the effect of bacterial density, duration of co-inoculation, length of cocultivation, and acetosyringone concentration for *Ashwagandha* species transformation (Fig. 5k, l). Histochemical assays showed GUS expression in all cocultivated explants (100 % transient transformation frequency) and in different parts of plant in stem, petiole, veins, and leaf lamina of putative transformant during different developmental stages (Fig. 5k, l). The presence of the transgenes (*npt*II and *gus*A) was verified and confirmed in putative transformants by genomic PCR amplifications with genomic DNA. Withanolide (withanolide A, withanone, and withaferin) patterns as assayed by TLC showed similar patterns for the putative *W. somnifera* transgenic shoots to those obtained with native non-transformed shoots of in vitro shoot cultures (Fig. 5m). Morphologically, the transformed plants were identical to their untransformed counterparts. To safeguard against false positives that may result from expression of the gene in *A. tumefaciens*, the *gus*A transgene harboured an intron. Therefore, the positive *GUS* assay accompanied by genomic PCR analysis using *gusA* and *nptII* primers in the putative transformants provided molecular proof for the integration of the *gus*A gene into the plant genomic DNA and ascertained the stable genetic transformation *in planta* (Mishra et al. 2012). Thus, the protocol developed can be reliably used for the transfer of genes of interest to design transgenic *W. somnifera* plants possessing favourable traits of agronomic advantages, phytopharmaceutical chemical specialty (production of improved levels of minor, but most potent, withanolides), and other applications for metabolic engineering applications including production of heterologous proteins. Transformation also provides a powerful tool to study the function of candidate genes related to the variety of steroidal transformations unique to this medicinal plant such as the metabolic origin of withanogenesis from the central triterpenoidal route, but also to investigate the role of these genes in plant growth and development and to utilise these genes for pathway engineering in *W. somnifera*.

9 Summary and Future Prospects

The medicinal and pharmaceutical importance of Ashwagandha crude extracts as well as pure withanolide compounds has been tremendously increasing during recent years as evidenced by a huge increase in patents and publications on

Ashwagandha in recent years. Newly discovered pharmacological roles for withanolides are being explored for modern drug development programmes, in addition to the well-known importance of Ashwagandha in traditional systems of Indian medicine and Ayurveda. In our lab, attempts have been made in a systematic manner to provide more concise and clear information about biotechnological aspects for improving quality and yield of the plant material. Also the biotechnologically generated material is assessed for further exploitation for phytotherapeutical purposes and in drug research. Earlier information particularly related to individual withanolide constituents from Ashwagandha requires further investigation and specification in terms of specific phytomolecules being associated with particular remedy, as well as establishment and definition of distinct chemotypes for sustainable utilisation. To determine precisely the phytochemical nature of the active compounds of this herb is of utmost importance to provide the appropriate doses and desired molecules for treating or preventing negative side effects of withanolide molecules or extracts. Recent efforts including those of our research group have provided new insights on how the yields of active phytomolecule (s) from Ashwagandha can be increased in a more standardised and efficient manner. The protocols for generating in vitro tissue systems, especially creating micro-clones of the developed elite varieties and hybrids, have improved leading to a unified protocol applicable to diverse geographic material for high-throughput micropropagation to take a particular accession to the field level. Various systems from unorganised or organised cells were also generated with considerable success in terms of withanolide productivity. Although in vitro-raised shoots exhibited higher amounts of withanolide A, in vitro-cultured roots exhibited lower levels of withanolide A and withanone than those of field-grown mother plants. However, certain root morphotypes accumulated withanolide A and withanone in a manner comparable to that exhibited by roots of field-grown *W. somnifera*. Thus, we have developed various tissue systems of *W. somnifera* with the ability to biosynthesise withanolides of pharmaceutical importance. Various stress conditions were studied on shoot, callus, and suspension cultures and were shown to induce the accumulation of various withanolides to a different extent. The developed tissue cultures provide also experimental models to understand the withanolide biosynthetic pathway under a convenient and controlled manner at the organ, tissue, and cellular levels, a prerequisite for large-scale production of valuable bioactive withanolides. The major withanolides were characterised in *W. somnifera* by using spectral and analytical techniques such as TLC, HPLC-UV photodiode array detection (PAD), evaporative light scattering detection (ELSD), and nuclear magnetic resonance (NMR). The compounds were identified as withanone, withaferin A, withanolide A, and withanolide D. Earlier reports as well as ours have suggested that the production of withanolides is closely associated with morphological differentiation and a tissue-specific pattern of withanolide production in cultures with relative expression of some of the pathway genes associated with withanolide biosynthesis. Highly efficient *Agrobacterium*-mediated transformation protocols for *W. somnifera* from *A. tumefaciens* or *A. rhizogenes* have been developed. Both systems can be used for the validation of gene functions by using genomic

approaches such as gene silencing or overexpression. These culture systems also provide deep insights for the determination of metabolic structural pathways with the binary vectiors, hairy roots (transient system for initial screening) of Ashwagandha plants can be used for the expression of foreign genes or overexpression of endogenous genes for increasing withanolide production. Hairy roots could be valuable for producing withanolides in amounts sufficient for medicinal purposes and clinical trials. Since the integration of T-DNA is random, hairy roots may provide novel compounds, which are structurally diverse. Although hairy roots are versatile for different applications, they have the drawback to require maintenance in small culture flasks. The design of bioreactors for large-scale culture of hairy root systems will allow for the commercial production of withanolides of pharmacological interest. Additionally, the use of low concentration of elicitors can improve the efficiency of withanolide production. By optimising a series of parameters, cellular biomass and withanolide concentration can be increased in different developed in vitro cultures/systems. This requires a more detailed molecular understanding of withanolide biosynthesis and therefore the characterisation of the pathway genes for deciphering complete biosynthetic pathway through high-throughput metabolic profiling and sequencing. Further studies with regard to the withanolide/isoprenoid pathway-related genes of this plant would not only be useful in identifying such conjoint genes but also directly contribute to improve the biotechnological utilisation of this medicinal plant.

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Metabolic Engineering of Wood Formation

Armin Wagner and Lloyd Donaldson

Abstract Wood is one of the most abundant composite materials on earth consisting primarily of cellulose, noncellulosic polysaccharides and lignin. It is a sustainable and quite versatile natural resource that can be processed into many useful products including biofuels, biochemicals, wood pellets, pulp and paper, fibres, biocomposites and timber. Transgenic studies targeting wood formation have in the past largely been of exploratory nature to better understand the molecular basis of wood formation. However, metabolic engineering approaches of woody biomass designed to enhance the quality and quantity of desired end products started to emerge in recent years. A substantial number of studies have recently been published on improving the generation of bioenergy or biofuels from lignocellulosic material, reflecting this new trend in the utilization of woody biomass. A sizable body of literature also exists on metabolic engineering strategies designed to improve the production of pulp and paper, which are important traditional products derived from lignocellulosic material. All these product streams are influenced by the structure and content of cell wall polymers that constitute wood, justifying the current research effort on this topic. Metabolic engineering experiments trying to increase the formation of woody biomass itself have also gained momentum in recent years. Metabolic engineering strategies designed to improve the quality of wood or to enhance the production of nontraditional bioproducts from wood seem also to be feasible but have received little attention to date. This might in part reflect our insufficient knowledge on biochemical and cellular processes that govern wood formation.

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1 Introduction

The worldwide rising demand for raw materials and energy combined with diminishing fossil fuel resources and climate change has stimulated global efforts to develop more sustainable approaches for the production of biofuels, platform chemicals, energy, fibres and biocomposites. Forests are important in this context, as they represent one of the most abundant resources for biomaterials on the planet. Woody biomass could serve as feedstock for many intermediate or end products currently generated by processing fossil fuels. The importance of wood as a sustainably produced feedstock will grow, as traditional uses for forest trees such as firewood, lumber and pulp production are likely to be complemented by the generation of nontraditional bioproducts (Pu et al. 2011; Tilbrook et al. 2011). It can be expected that the perception on how forest tree species can and should be utilized will change in future and that metabolic engineering of wood might play an important role in this change of thinking.

Wood is an inexpensive, versatile and sustainable resource that can be processed into many end products including fuels, wood pellets, paper, fibres, pulp, biocomposites and solid wood products. Using lignocellulosic biomass from trees as a resource for the production of biomaterials has a number of advantages over using annual crops. Tree harvest is independent of season, predictable and provides a constant supply of biomass, all features important for the operation of industries processing this resource. The carbon footprint of forest plantations is often lower than that of annual crops, and the potential of soft- and hardwood species to be grown on marginal lands can reduce competition with crop plants (Mizrachi et al. 2012). Forests can help to maintain biodiversity, protect land and water resources and mitigate climate change resulting from increasing CO_2 levels and also provide significant social and environmental benefits (Harfouche et al. 2011).

High-yielding plantation forests can produce more than 50 m^3 of lignocellulosic biomass/ha p.a., which outcompetes the productivity of natural forests many fold (Fenning et al. 2008; Mizrachi et al. 2012). This explains why forest plantations have become increasingly important for a biomaterial-based economy, where they provide sustainably produced lignocellulosic biomass and can reduce the need to utilize natural stands, thereby helping to sustain native species (Harfouche et al. 2011). Internationally, the demand for fast-growing, short-rotation plantation forestry is on the rise. Eucalyptus and poplar plantations that are ready for harvest within a few years as biomaterial feedstock are now reality (Hinchee et al. 2009). The product range to be generated from woody biomass is likely to broaden in future. This calls for the establishment of forests dedicated to particular end uses, as the generation of one product stream might compromise another. The strategy to establish plantation forests as a resource for lignocellulosic material can – if desirable – be combined with metabolic engineering experiments, as some of the propagation methods such as somatic embryogenesis, required for clonal plantations, are also used for transformation purposes.

Metabolic engineering of trees can provide economic benefits, as it offers the potential to enhance wood formation itself, can facilitate the processing of lignocellulosic biomass derived from wood and can improve the quality, quantity and range of products generated from this resource. However, significant limitations in what can be achieved to date still exist, as the ease with which wood is converted to products such as biofuels, chemicals and biomaterials depends on our knowledge of wood structure and our ability to manipulate cell wall polymers.

Wood formation has often been studied in the past by using wood-forming model systems, and experimental data obtained from such models have been invaluable in guiding metabolic engineering experiments of tree species (Zhang et al. 2011). However, model systems do not necessarily have the same cellular composition, source-sink relationships and transcriptional networks regulating cell wall biosynthesis as trees (Zhong et al. 2011). This can limit their utility and makes it essential to experimentally verify results from model systems in target tree species (Mizrachi et al. 2012). Recent advances in genetic transformation technologies, gene discovery, transcript profiling and genome sequencing have improved the ability to genetically engineer forest trees. A concerted effort is required to overcome our still limited biochemical and molecular understanding of wood formation in tree species to broaden the portfolio of metabolic engineering approaches possible to modify wood and wood formation.

2 Wood Anatomy and Composition

The design of metabolic engineering experiments to modify wood formation is likely to depend on the target species, as the cellular and biochemical composition of wood from different tree species can differ significantly. In the context of this review, it is very useful to differentiate between coniferous gymnosperms (softwoods) and arborescent angiosperms (hardwoods), as both groups of plants have a quite distinctive wood structure (Core et al. 1979). Conifer wood consists primarily of tracheids which are long fibrous cells with lignified secondary cell walls. Softwood tracheids serve two functions: to support the crown of the tree and to conduct water from the roots to the needles. Hardwoods have a more complex structure resulting in the divergence of function to different cell types. Hardwoods contain vessels which conduct water and fibres which support the crown (Fig. 1a). In softwoods, parenchyma cells are found in rays, in resin canals or as isolated strands known as axial parenchyma (Core et al. 1979). Parenchyma cells may have thin unlignified cell walls or thick lignified secondary walls depending on the species (Donaldson 2001). Some parenchyma cells in resin canals may have primary walls that become suberized (LaPasha and Wheeler 1990). Similar structures occur in hardwoods but with a much greater diversity of size and anatomy (Evert 2006). Parenchyma tissues function as stores for starch and in production of extractives and often remain living and physiologically active within the sapwood (Jane 1970).

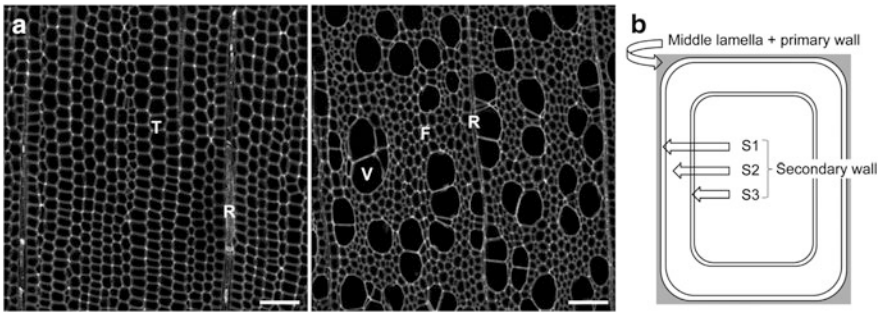

Fig. 1 (**a**) Confocal fluorescence images illustrating the main anatomical differences between the wood of softwoods (*Pinus radiata*; *left image*) and hardwoods (*Populus tremuloides*; *right image*). Wood of softwood species consists largely of tracheids (*T*) and wood rays (*R*), whereas wood of hardwood species consists mainly of vessel elements (*V*), wood fibres (*F*) and wood rays (*R*). Scale bar = 100 μm; (**b**) a diagrammatic representation of cell wall layers typically found in tracheary elements

Most of the woody biomass in trees consists of the lignified secondary cell walls of tracheids or fibres. Lignification of secondary cell walls reduces the water content, making them largely impenetrable for solutes and enzymes. Secondary cell walls of tracheary elements typically have three layers known as the outer S_1 layer, the middle and generally thickest S_2 layer and a thin layer lining the lumen known as the S_3 layer (Fig. 1b). These layers are characterized by variations in composition and microfibril orientation (Donaldson 2001, 2008). Secondary walls are surrounded by a compound middle lamella/primary wall. The outer middle lamella region is the most highly lignified wall layer, although lignin distribution changes significantly in reaction wood of both softwoods and hardwoods (Donaldson 2001). In softwoods, the outer part of the S_2 layer becomes highly lignified, and the middle lamella is less lignified in compression wood, while in hardwoods, the inner part of the secondary wall is unlignified or very weakly lignified, forming a gelatinous or g-layer rich in cellulose (Donaldson 2001; Lehringer et al. 2009). The organization of cell wall layers in tension wood varies among species where the g-layer may replace the S_2 layer or it may be an extra layer. In both compression wood and tension wood, the S_3 layer is typically absent.

Wood is mainly composed of cellulose, noncellulosic polysaccharides and lignin with residual amounts of protein, extractives and inorganic material (Fengel and Wegener 1984). The most abundant cell wall polymer is cellulose (40–44 %), followed by lignin (18–35 %) and noncellulosic polysaccharides (15–35 %). The biochemical cell wall composition in softwood and hardwood species differs, as lignin levels tend to be higher in softwoods (26–34 %) compared to hardwoods (23–30 %). Cellulose (40–44 %) and noncellulosic polysaccharide (15–35 %) levels are comparable in both groups.

Cellulose is a linear homopolymer consisting of (1-4)-β-D-glucopyranosyl units that has a much higher degree of polymerization (DP) than the noncellulosic polysaccharides contained in wood. Cellulose chains have a strong tendency to

form intra-and intermolecular hydrogen bonding via the hydroxyl groups on the glucan chain, which promotes cellulose aggregation. Aggregated wood cellulose has a high degree of crystallinity (50–70 %), a key factor contributing to the recalcitrance of cellulose to enzymatic deconstruction.

Noncellulosic polysaccharides differ in many molecular aspects from cellulose. They tend to be heteropolymers with a DP much lower than that of cellulose, frequently have side chains and are essentially amorphous in structure. The composition of noncellulosic polysaccharides in softwoods and hardwoods is different. Hardwoods contain predominantly glucuronoxylan, while galactoglucomannan and arabinoglucuronoxylan are more prominent in softwood species. Glucuronoxylan and arabinoglucuronoxylan have the same backbone consisting of (1-4)-linked β-D-xylopyranosyl units but differ in their branching and substitution pattern. Hardwood glucuronoxylan is highly acetylated at C_2-and C_3-positions of the xylopyranosyl units and has a branched structure with small amounts of (1-2)-linked pyranoid 4-O-methyl-α-D-glucoronic acid units. Galactoglucomannan is a heteropolymer of randomly arranged (1-4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl units with (1-6)-linked α-D-galactopyranosyl units attached to mannopyranosyl units. The hydroxyl groups at C_2-and C_3-positions of the mannopyranosyl units are partially substituted by acetyl groups. The arabinoglucuronoxylan in softwoods is not acetylated but branched with (1-2)-linked pyranoid 4-O-methyl-α-D-glucoronic acid and (1-3)-linked-α-L-arabinofuranosyl units. Galactoglucomannan is more abundant than arabinoglucuronoxylan in softwood species, contributing 15–20 % of the wood dry mass (Pu et al. 2011).

Lignin is an aromatic, heterogeneous cell wall polymer derived primarily from hydroxycinnamyl alcohols via combinatorial radical coupling reactions (Ralph et al. 2004; Wagner et al. 2007). The main units in lignin, p-hydroxyphenyl, guaiacyl and syringyl units, are derived from the corresponding monolignols p-coumaryl, coniferyl and sinapyl alcohol. Lignin forms a three-dimensional network within the cell wall in which cellulose and noncellulosic polysaccharides are embedded (Vanholme et al. 2012). Lignin is frequently covalently linked with noncellulosic polysaccharides contained within the cell wall. The nature of these lignin carbohydrate complexes (LCCs) is largely influenced by the cell wall composition in different wood types (Timell 1986). Sugar residues believed to be covalently linked to lignin in conifers include galactose, glucose, mannose, arabinose, xylose and rhamnose (Timell 1986).

3 Modifying Cell Wall Polymers

Polysaccharides and lignin are the principle cell wall polymers making up woody material, and both structure and content of these components impact on yield and efficiency of pulp and paper production, the generation of biofuels from woody biomass and solid wood and fibre properties. Metabolic engineering of wood

through manipulation of content, composition and structure of cell wall polymers is therefore a promising avenue to improve processing and product performance.

Unfortunately, many enzymatic steps involved in the biosynthesis of polysaccharides have still not been identified unambiguously, which is particularly true for pectic polysaccharides. For example, of the more than 60 transferases estimated to be involved in the biosynthesis of pectic polysaccharides, only very few have unambiguously been identified to date (Liwanag et al. 2012). This knowledge gap currently limits the potential to engineer content and structure of cell wall polysaccharides (Mast et al. 2009; Ye et al. 2011; Liwanag et al. 2012). The same applies to the formation of LCCs, which can have a negative impact on the processing of lignocellulosic biomass (Grabber 2005). However, detailed structural information on LCCs from soft- and hardwood species has been generated by high-resolution NMR recently (Balakshin et al. 2011). The situation in the case of lignin biosynthesis is much better. Our molecular understanding of lignin biosynthesis, polymerization and structure is quite detailed due to two decades of intense research efforts on lignification. The long-lasting effort to elucidate lignification reflects the adverse role lignin plays during pulp and paper production and saccharification of woody biomass (Vanholme et al. 2012; Wagner et al. 2012). This is a key reason why literature on metabolic engineering experiments of wood is currently still dominated by efforts to modify lignin content, composition and structure in woody plants.

3.1 Lignin Modifications

Lignin is a heterogeneous aromatic polymer created through the oxidative coupling of p-hydroxycinnamyl alcohols (monolignols) or related compounds (Ralph et al. 2004). It is particularly abundant in cells that undergo secondary cell wall thickening such as vessel elements, wood fibres in hardwoods and tracheids in softwoods (Harris 2006). In hardwood species, lignin is derived from the polymerization of p-coumaryl, coniferyl and sinapyl alcohols, producing p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively. Softwoods are incapable of synthesizing sinapyl alcohol and consequently do not contain S-units in lignin (Harris 2006). The biosynthesis and structure of lignin in hard- and softwood species have been studied intensively in the last decades and is now relatively well understood (Vanholme et al. 2012; Wagner et al. 2012).

Lignin fulfils vital functions *in planta* as it reinforces plant cell walls, facilitates water transport, provides compressive strength to conducting tissues and acts as a mechanical barrier to pathogens (Boudet 2007). Lignin is covalently linked to noncellulosic polysaccharides through LCCs, which help to reinforce the cell wall matrix. This reinforcement provides biological functionality but at the same time adds to the recalcitrance of woody biomass to be refined by biological or chemical processes. Covalent carbon-carbon linkages within the lignin polymer involving the aromatic moiety of monolignols (e.g. phenylcoumarans (β-5),

biphenyls (5-5) and dibenzodioxocins (5-5/β−O−4)) are difficult to cleave during biochemical and chemical processing. In addition, lignin can also interfere with biological conversion processes by adsorbing and inactivating enzymes involved in the hydrolysis of polysaccharides. The negative impact lignin can have on processing lignocellulosic material has made it a prime target for metabolic engineering experiments.

From a metabolic engineering perspective, it is important to point out that the process of lignification is, despite decades of research, still not fully understood. Aspects of lignification that require more research include regulatory cascades that trigger lignification, metabolic connections between monolignol biosynthesis and other metabolic pathways, the cellular biology of monolignol biosynthesis, the transport of monolignols to the apoplast, the role of monolignol glucosides in lignification, the process of lignin initiation and the interaction of lignin with other cell wall polymers such as noncellulosic polysaccharides (Wagner et al. 2012).

3.1.1 Changing Lignin Content

Depending on the product to be generated from woody biomass, it could be of benefit to either increase or decrease lignin content in lignocellulosic biomass. An increase in lignin content could be of interest, if the lignocellulosic material is destined to be converted into solid fuel, as the calorific value of lignin exceeds that of polysaccharides. Increasing lignin content could also improve some wood quality parameters including wood stability and hardness, as lignin reinforces the cell wall by cross-linking with other cell wall polymers, in particular noncellulosic polysaccharides. However, metabolic engineering strategies to increase lignin content have not been a focus in the past and could prove challenging, as the timing of lignification during xylogenesis needs to be well coordinated with other cellular processes such as cell division and cell elongation to avoid compromising plant development. Changes in the expression of lignin-related genes and transcription factors can lead to ectopic lignification which should be avoided as it can interfere with plant growth (Rogers et al. 2005; Zhong et al. 2000).

The vast majority of published metabolic engineering studies are focused on reducing lignin content due to the negative effect of lignin on pulp, paper and biofuel production (for review, see Pu et al. 2011; Ye et al. 2011; Wagner et al. 2012). Experimental evidence suggests that reductions in lignin content can be beneficial for processing woody material. For example, lignin reductions in poplar promoted decomposition of the cell wall matrix during mild acid hydrolysis (Ragauskas et al. 2006). However, lignin reductions can also become a ‘balancing act’, as lignin fulfils important functions *in planta*. This also explains why reductions in lignin content can compromise plant growth both in hard- and softwood species (Voelker et al. 2011; Wagner et al. 2009, unpublished). Reductions in lignin content can trigger complex stress responses in plants and/or changes in cell wall integrity that can interfere with plant development (Besseau et al. 2007; Dauwe

et al. 2007; Wagner et al. 2009). Potentially negative phenotypes include dwarfing, collapsed xylem and in extreme cases developmental arrest (Bonawitz and Chapple 2010). The potential for undesirable, pleiotropic effects when reducing lignin content makes it essential to undertake a thorough phenotypic investigation of the transgenic lines and to grow them in different environments/conditions to discover potential undesirable effects of lignin reductions. Currently, there is insufficient data on how transgenic trees with reduced lignin content perform under field trial conditions, an issue that needs to be addressed. Reductions in lignin content can be achieved by interfering with the transcriptional control of lignification or by the manipulation of monolignol biosynthesis itself. The transcriptional regulation of lignification involves mainly MYB, LIM, NAC and KNOX transcription factors and is complex in part because lignin biosynthesis can be triggered by a variety of cues including gravitropism, wounding, pathogen challenge, sugar content, plant hormones and circadian rhythm (Zhao and Dixon 2011). The complexity of this regulatory cascade can lead to undesirable pleiotropic effects when manipulating transcription factors associated with lignification, and care has to be taken not to compromise plant health and fitness with such a molecular approach (Zhao and Dixon 2011).

In the majority of published studies, lignin content was reduced by suppressing enzymes involved in monolignol biosynthesis including L-phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), p-hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferase (HCT), p-coumarate 3-hydroxylase (C3H), caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), coniferaldehyde 5-hyroxylase (CAld5H) and 5-hydroxyconiferaldehyde O-methyltransferase (CAldOMT) (for review, see Vanholme et al. 2012; Wagner et al. 2012). The extent to which lignin reductions can be tolerated by woody plants seems to differ between soft- and hardwood species. Hardwood species such as poplar seem to tolerate reductions in lignin content better than softwood species. For example, suppression of 4CL in poplar resulted in a 50 % reduction in lignin content without apparent negative impact on plant growth (Li et al. 2003). On the contrary, softwood species such as *Picea abies* and *Pinus radiata* seem to be compromised in their growth by less than 20 % reduction in lignin content (Wadenbäck et al. 2008; Wagner et al. unpublished). This observation might explain the relatively narrow range in lignin content found in natural conifer populations (Campbell and Sederoff 1996). Such a difference in tolerating reductions in lignin content could reflect anatomical differences between hard- and softwood. The functional diversification of structural and water-conducting elements in hardwoods does not exist in coniferous softwoods (Fig. 1). Lignin reductions will impact on tracheids, the water-conducting elements in conifers, which may then collapse under water tension leading to modified growth and development (Wagner et al. 2009).

3.1.2 Changing Lignin Composition and Structure

Plant species seem to have a remarkable, unanticipated potential to adapt to changes in lignin composition caused by changes in the abundance of naturally occurring monolignols or the supply of novel phenolic monomers (Bonawitz and Chapple 2010; Vanholme et al. 2012). One example for the ability of plants to accept novel phenols represents the incorporation of monolignols with a truncated phenylpropanoid side chain into lignin. Overexpression of a bacterial hydratase-lyase created a lignin polymer with unusual C6-C1 monomers in *Arabidopsis thaliana*. The lignin polymer formed under these conditions was structurally modified and reduced in its molecular weight, modifications in lignin structure that enhanced saccharification of the lignocellulosic biomass (Eudes et al. 2012). It is also possible to reduce the molecular weight of the lignin polymer in conifers by increasing the incorporation of dihydroconiferyl alcohol (Ralph et al. 1997). This can be accomplished by suppression of CAD (Wagner et al. 2005) or potentially by overexpression of a double-bond reductase that can modify the phenylpropanoid side chain of coniferyl alcohol (Kasahara et al. 2006).

Changing the supply of naturally occurring monolignols can be a very successful strategy to facilitate processing of lignocellulosic material without compromising plant fitness. For example, transgenic studies in *A. thaliana, Nicotiana tabacum* and *Populus tremula* × *alba* have proven that it is possible to increase syringyl lignin content in angiosperms to over 90 % without compromising plant fitness or performance (Franke et al. 2000; Sibout et al. 2002; Li et al. 2003; Huntley et al. 2003; Stewart et al. 2009). S-type-rich lignin has high levels of β-aryl ether bonds (β−O−4) which are easily degradable using alkaline pulping or acidolytic methods. In general, the molecular size of the lignin polymer declines with increasing S-type content, and the linearity of polymer increases (Brunow and Lundquist 2010; Ralph 2010). These structural features of the modified lignin polymer help its removal from the lignocellulosic cell wall matrix. The S-type-rich lignin produced in transgenic angiosperms enabled all cell types contained within woody tissue to function normally, including the naturally G-lignin-rich vessel elements. Huntley et al. (2003) demonstrated that an increase of S-type lignin from 65 % to 93 % in *P. tremula* × *alba* could increase throughput at pulp mills by more than 60 %, enhance product quality and yield and at the same time reduce the consumption of harmful chemicals. In addition, high syringyl lignin content in hardwoods improved biofuel production from lignocellulosic material (Stewart et al. 2009; Studer et al. 2011). Softwood lignin is naturally rich in so-called 'condensed' interunit linkages such as phenylcoumarans (β-5), biphenyls (5-5) and dibenzodioxocins (5-5/β−O−4), which reflects the fact that softwood lignin consists of H-and G-type monolignols (Brunow and Lundquist 2010). The absence of S-type lignin in softwoods increases branching and polymer size and reduces the abundance of cleavable β-aryl ether in the lignin polymer (Brunow and Lundquist 2010; Ralph 2010). A metabolic engineering approach to introduce S-type lignin in softwoods could therefore be quite beneficial. Such a strategy might be possible, as

experimental evidence shows that softwood species can incorporate nontraditional monolignols into lignin (Wagner et al. 2011, 2013).

Recently, experiments have been initiated to completely redesign the lignin polymer in plants by creating a biologically functional polymer that contains chemically labile linkages (Grabber et al. 2008; Ralph 2010). One such experiment is based on the incorporation of coniferyl ferulate (or more generally monolignol ferulates or sinapates) into the lignin polymer. In vitro polymerisation studies using peroxidases provided experimental evidence that it is possible to generate a lignin polymer derived from coniferyl alcohol and coniferyl ferulate (Grabber et al. 2008, 2010). Ferulate behaved like a normal monolignol in this process and participated in the lignification process in the usual combinatorial manner (Ralph 2010). The newly formed lignin polymer contained ester linkages that could easily be hydrolyzed chemically. This led to significant improvements in processing efficiency including enhanced alkaline delignification and higher fibre yields (Grabber et al. 2008; Ralph 2010).

A small number of plants naturally produce coniferyl ferulate (Ralph 2010, Ralph et al. unpublished), and the gene responsible for the biosynthesis of coniferyl ferulate in *Angelica sinensis* has been identified recently, which now enables this lignin design concept to be tested in plant species.

3.2 Polysaccharide Modifications

Cell walls of tracheids, wood fibres and vessel elements contain significant amounts of cellulose and noncellulosic polysaccharides within primary and secondary cell walls and significant amounts of pectic polysaccharides in the compound middle lamella. Cellulosic and noncellulosic polysaccharides in the cell walls of these wood-forming cells are synthesized in different cellular compartments. Cellulose is synthesized by cellulose synthase complexes at the plasma membrane, whereas noncellulosic polysaccharides are synthesized in the Golgi and transported to the cell wall environment via secretory vesicles. Cellulose is the most abundant polysaccharide both in hard- and softwood species and provides strength and rigidity to the stem through its predominantly crystalline structure in woody tissue. Noncellulosic polysaccharides are essentially amorphous in structure and interact with cellulose through hydrogen bonding. The most abundant noncellulosic polysaccharides in hard- and softwood species are arabinoxylans and galactoglucomannans, respectively (Pauly and Keegstra 2010). Insufficient knowledge on biosynthetic and regulatory processes of polysaccharide production currently limits our ability to engineer cell wall polysaccharides (Pauly and Keegstra 2010; Liwanag et al. 2012). Metabolic engineering studies targeted to cell wall polysaccharides in tree species are therefore at present still fairly uncommon. Existing literature focuses primarily on strategies to improve biofuel production from lignocellulosic biomass. A number of potential avenues to accomplish this have been proposed or experimentally verified. This includes (a) molecular approaches

to 'loosen' the cell wall matrix to make it more accessible to hydrolytic enzymes, (b) approaches to increase the relative proportion of easily fermentable polysaccharides, (c) strategies to reduce hydrogen bonding between polysaccharides and (d) transgenic approaches to enhance the content of polysaccharides relative to other cell wall polymers, in particular lignin (Pauly and Keegstra 2008, 2010).

As in the case of lignin, care has to be taken when trying to manipulate cell wall polysaccharides in woody tissue owing to the multiple functions of cell walls. Polysaccharide biosynthesis is complex involving various processes and regulators, and our understanding of gene-trait relationships is far from complete (Mizrachi et al. 2012). Plants have developed mechanisms to monitor cell wall integrity, and interference with polysaccharide biosynthesis can upset or trigger defence-related responses. Cell walls are known to contain signalling molecules that allow plants to recognize pathogen attack. Such molecules might inadvertently be released by metabolic engineering of cell wall polysaccharides, thereby activating plant stress responses (Skjøt et al. 2002). Attempts to manipulate pectic polysaccharides have proven difficult, as these polysaccharides have a crucial role in cell adhesion. Manipulation of these polysaccharides can be lethal or compromise the potential to withstand mechanical stresses (Krupková et al. 2007). In addition, it seems not to be possible to raise the relative content of a polysaccharide simply by overexpressing the gene responsible for the synthesis of the polymer backbone (Naoumkina et al. 2008).

3.2.1 Changing Polysaccharide Content

Only a few studies have been published on the modification of cell wall polysaccharides in tree species to date, and those that are available are largely focused on strategies to enhance the yield of biofuels from hardwood species such as poplar (Pauly and Keegstra 2008, 2010; Mizrachi et al. 2012; Ye et al. 2011). One way of achieving this is by raising the relative proportion of polysaccharides within the cell wall matrix that can readily be converted to biofuels. This means primarily an increase of polysaccharides that consist of hexoses such as glucose, mannose and galactose, which are easily fermented by most microorganisms (Pauly and Keegstra 2008). In poplar and other plant species, this has been accomplished indirectly by manipulating lignin biosynthesis, as this polymer competes for carbon allocation with polysaccharides within woody tissue. The combination of 4Cl suppression with CAld5H overexpression in poplar resulted in a 30 % increase in cellulose content in transgenic trees (Li et al. 2003). A more direct metabolic engineering strategy capable of raising cellulose content in tree species relies on manipulating enzymes involved in sucrose synthesis and transport. The biosynthesis of cellulose involves the cellulose synthase complex with its catalytic subunits (CesA) and alignment of certain isoforms of sucrose synthase (SuSy), invertase, phosphate synthase (SPS) and UDP-glucose pyrophosphorylase (UDPase). Recent research has shown that it is possible to alter carbon partitioning and to slightly increase cellulose production in *Populus alba* × *grandidentata* by manipulating

UDP-glucose pyrophosphorylase, sucrose synthase or sucrose phosphate synthase (Coleman et al. 2007, 2009; Park et al. 2009). An alternative strategy to enhance cellulose content involves overexpression of transcription factors that have the potential to impact on cellulose biosynthesis (Ambavaram et al. 2011). For example, overexpression of SHN, a transcription factor that activates the expression of genes associated with cell wall polysaccharides in a coordinated fashion, increased cellulose content by 34 % in rice (Ambavaram et al. 2011). Another strategy to enhance saccharification of lignocellulosic biomass is to increase hexose-containing polysaccharides other than cellulose. This might be achievable by manipulating nucleotide sugar conversions, e.g. by changing the expression pattern of epimerases involved in this conversion process (Reiter 2008).

3.2.2 Changing Polysaccharide Composition and Structure

Plant cell walls have evolved to resist mechanical and microbial breakdown processes, the very processes needed for the efficient production of biofuels from woody biomass. Polysaccharides can, based on their molecular structure (e.g. cellulose crystallinity) and interactions with other cell wall polymers (e.g. hydrogen bonding, LCCs), contribute to this recalcitrance. Metabolic engineering approaches have been developed to 'loosen' the cell wall matrix to improve access for hydrolyzing enzymes required during pretreatment and fermentation processes. One way of accomplishing this might be by increasing the proportion of highly water-soluble polysaccharides within the cell wall matrix at the expense of less water-soluble polysaccharides (Pauly and Keegstra 2008, 2010). However, care might have to be taken in such an approach, so as not to compromise water and solute conduction through vascular elements by making the cell wall environment too hydrophilic. An alternative strategy to loosen the cell wall matrix could involve the addition of side chains to noncellulosic polysaccharides such as glucuronoxylan, which would then prevent hydrogen bonding with cellulose (Pauly and Keegstra 2008). Demonstration of manipulations of noncellulosic polysaccharides improving digestibility of woody material can be seen in the results from transgenic experiments in poplar. A reduction of glucuronoxylan content in *P. alba* × *tremula* caused by suppression of a glycosyltransferase belonging to the GT47 family reduced the recalcitrance of poplar wood to cellulose digestion (Lee et al. 2009).

A further way of loosening the cell wall matrix involves the disruption of cellulose microfibrils (see also chapter by Nick in the current volume), which can be accomplished by the overexpression of proteins containing a cellulose-binding module such as expansins (Cosgrove 2001). Such proteins are likely to disrupt cellulose microfibrils without hydrolyzing the glucan chains, a molecular strategy proven to substantially enhance sugar yield after cellulose digestion (Cosgrove 2001). Overexpression of CEL1 – an endoglucanase – promoted cell wall relaxation in poplar by cleaving cross-linkages between xyloglucan and cellulose. This genetic modification had the additional benefit of accelerating plant growth (Levy

et al. 2002; Shani et al. 2004, 2006). A similar result was obtained by Park et al. 2004, who overexpressed a xyloglucanase in poplar, which resulted in cell wall loosening, cell expansion, the accumulation of cellulose and an increase in plant growth. Yet another way to loosen the cell wall matrix is based on reducing the level of cellulose crystallinity by increasing the amorphous proportion of the polymer. Such a structural change to cellulose is expected to promote enzymatic deconstruction of lignocellulosic material, but it has yet to be determined to what extent this property can be manipulated using metabolic engineering (Pauly and Keegstra 2008).

Despite the current, almost exclusive focus on biofuel production from lignocellulosic biomass, it should not be overlooked that polysaccharide composition and content in wood can also affect solid wood properties. This makes polysaccharides interesting targets from a wood quality perspective. One example where modifications of polysaccharides could promote product performance is wood durability. Many plantation forestry species, including most poplar and pines, produce wood that is not naturally durable. One successful chemical strategy to make wood extremely durable is based on the acetylation of hydroxyl groups within the wood matrix using acetic anhydride (Tullo 2012). Acetylation of free hydroxyl groups in wood greatly reduces the water content in wood and makes polysaccharides less amenable to fungal degradation. It might be possible to simulate this chemical treatment procedure with metabolic engineering by promoting polysaccharide acetylation *in planta*. However, a better molecular understanding on the enzymes involved in the acetylation of polysaccharides in tree species needs to be established to enable such an approach.

4 Modifying Wood Formation, Structure and Composition

The move from exploiting native forests to the establishment and utilization of intensively managed plantation forests was and is essential to enhance forest productivity and to preserve native forests in times of population growth and climate change (Fenning et al. 2008). There is experimental evidence that metabolic engineering offers the opportunity to further enhance the productivity of plantation forests by promoting plant growth (Hirsch 2012). The promotion of plant growth and wood formation using metabolic engineering might be of particular relevance for forest plantations that are dedicated to the generation of products other than solid timber, as large growth increments can compromise wood quality. This has been well established for *P. radiata* plantations, where wood strength, stiffness, stability and density can be compromised in fast-growing genotypes. In addition, the proportion of juvenile wood, which has undesirable wood characteristics in *P. radiata*, increases dramatically (Cown 1992; Cown et al. 2004). Metabolic engineering of wood could be used to combat some of these negative trends on wood quality associated with plantation forestry. For example, it might be possible to reduce the prevalence of juvenile wood in plantation forests using metabolic

engineering. Juvenile wood in conifers has disadvantageous wood properties such as low density and high microfibril angle, as well as issues such as spiral grain and resinous defects. Candidate genes associated with juvenile wood formation in *P. radiata* have been identified recently (Li et al. 2012).

The underpinning molecular and biochemical mechanisms governing wood quality traits such as juvenile wood formation or dimensional stability are complex and not very well understood at present. Systems genetics strategies might be useful in this context to connect complex traits (e.g. transcript, protein and metabolite levels) with measurable phenotypic traits (e.g. cell dimensions and wood properties), to generate more insight into gene-trait and trait-trait relationships and to identify and prioritize candidate genes for functional genomics studies (Mizrachi et al. 2012).

Trees have been and are still being used as a resource for the production of bioactives and platform chemicals including the generation of turpentine and rosin obtained by steam distillation. Metabolic engineering of wood offers the opportunity to substantially enhance the production of biochemicals in trees and/or to transfer biochemical pathways for desirable metabolites from slow growing native tree species to plantation forestry species. This has the potential to improve their quantity and to reduce the cost of their generation. Changing the biochemical profile of wood using metabolic engineering could also be used to modify wood properties that rely on the quality and quantity of secondary metabolites contained within it.

4.1 Promoting Wood Formation

Ongoing improvements in pretreatment and fermentation procedures (for review, see Kuhad et al. 2011; Jordan et al. 2012; Madhavan et al. 2012; Tracy et al. 2012) might make the biochemical composition of woody biomass in future less important for the production of biofuels than the quantity of feedstock available for processing. It might under this scenario become more important to accelerate the generation of woody biomass itself rather than changing the biochemical composition of the feedstock. Accelerating tree growth in plantation forestry has the additional advantage of shortening rotations and minimizing the land area required for the production of a given amount of biomass.

A substantial body of literature exists on metabolic engineering experiments that can promote biomass production in plants (for review, see Demura and Ye 2010; Harfouche et al. 2011; Nieminen et al. 2012; Pu et al. 2011). Plant hormones have proven to play a key role in cambial activity and hence wood formation in all tree species examined to date. Experimental evidence suggests that wood formation can be promoted by manipulating the biosynthesis, transport, storage and catabolism of plant hormones involved in cambial activity, in particular auxins (see also chapter by Skůpa et al. this volume), gibberellins, cytokinins and ethylene (Nieminen

et al. 2012). It is also possible to manipulate the responsiveness of cambial tissue to plant hormones involved in cell division and elongation (Nieminen et al. 2012).

A significant number of transcription factors (TF) have been identified in recent years that are involved in regulating xylogenesis-related processes (for review, see Du and Groover 2010; Yamaguchi and Demura 2010; Zhao and Dixon 2011; Zhong and Ye 2012). The potential of most of these transcription factors for enhancing wood formation in trees has not been experimentally verified to date. However, it is not difficult to imagine that TF involved in regulating cambial activity, secondary cell wall formation, the morphology and abundance of tracheary elements and programmed cell death might have the potential to benefit wood formation in trees (McCarthy et al. 2010; Mitsuda et al. 2007; Muñiz et al. 2008; Ohtani et al. 2011; Yamaguchi et al. 2011; Zhong et al. 2006).

An alternative strategy to enhance tree growth involves the manipulation of the cell wall matrix itself. Enzymatic cleavage of linkages between cellulose and noncellulosic polysaccharides using hydrolytic enzymes such as CEL1 resulted in taller poplar trees with increased stem diameter, wood volume and dry weight (Shani et al. 2004). These data suggest that the linkages between cellulose and noncellulosic polysaccharides within cell walls can be a rate-limiting factor for cell elongation and that cleavage of these linkages can have phenotypic effects beyond the extension of cell dimensions.

An important factor for plant growth and therefore wood formation in long-living plant species such as trees is nutrient uptake and utilization. Nutrients such as nitrogen and phosphorus can become growth-limiting factors, especially in forest trees, due to their extended lifespan. Metabolic engineering of key enzymes involved in nitrogen metabolism such as glutamine synthetase have proven to promote plant growth in poplar (Jing et al. 2004). The acquisition and redistribution of phosphorus in forest trees could potentially be enhanced by the expression of enzymes such as purple acid phosphatase (Lim 2012).

4.2 Changing Wood Structure

Metabolic engineering of wood structure and composition could be beneficial for the generation of a range of products generated from lignocellulosic biomass including biofuels, pulp and paper and solid timber.

Currently, literature on metabolic engineering of tree species is dominated by the desire to enhance the production of biofuels from woody biomass, most likely reflecting the commercial importance of this emerging market. Structural elements within wood that can have negative impacts on biofuel production from lignocellulosic biomass include LCCs, making them targets for metabolic engineering (Grabber 2005). One possible avenue to reduce/minimize cross-linking between polysaccharides and lignin might lie in the manipulation of the monolignol population that contributes to lignin formation *in planta*. Monolignols with an ortho-diphenol structure readily form benzodioxanes during β-O−4 coupling reactions,

which can suppress the formation of LCCs (Vanholme et al. 2012). Lignins derived from monolignols with an ortho-diphenol structure have recently been discovered in seed coats (Chen et al. 2012). LCCs can, depending on their molecular structure, also be targeted by overexpressing hydrolytic enzymes capable of cleaving such linkages. An example for this represents the expression of esterases in the apoplast, a strategy which improved the release of fermentable sugars (Akin 2007). Another successful metabolic engineering approach focusing on biofuel production from wood is based on the introduction of cleavable peptides into the cell wall matrix. Experimental evidence shows that introduction of tyrosine-rich cell wall proteins can increase sugar release from lignocellulosic biomass during enzymatic treatments (Liang et al. 2008).

Metabolic engineering experiments of the wood structure could potentially also be used to improve the properties of solid wood. This might be of particular relevance for plantation forestry, where wood quality can be compromised based on increased growth rates. It should, however, be noted that changes to the wood structure that might assist in the production of high-quality timber might hinder the production of alternative products such as biofuels and pulp and paper from lignocellulosic material. This calls for the establishment of forest plantations dedicated to the production of certain end products. For example, increased cross-linking between cell wall polymers that interferes with biofuel production has the potential to improve mechanical properties of wood. Wood hardness, stiffness and stability can be promoted by cross-linking cell wall polymers (Torr et al. 2006). This is currently accomplished by postharvest modification of wood, e.g. by infiltrating timber with a combination of chemical cross-linking agents (Yasuda and Minato 1994). Unfortunately, this practice increases the weight of the product, which can be undesirable. Generating a light-weight product with superior mechanical properties might be advantageous for a number of applications. It is conceivable that metabolic engineering could be used to increase cross-linking within the cell wall matrix to enhance mechanical wood properties once more enzymes involved in cross-linking cell wall polymers have been identified.

Another process that could benefit from metabolic engineering of wood structure is the industrial production of pulp and paper. For example, the quality of pine pulp produced by the traditional Kraft process depends on a number of key parameters such as fibre length and coarseness (Kibblewhite et al. 2003). The establishment of early rotation pine plantations has substantially increased the proportion of juvenile wood, which tends to have shorter tracheids than mature wood. Metabolic engineering experiments have shown that it is possible to change fibre dimensions in plants. For example, overexpression of hydrolytic enzymes such as endoglucanases or xyloglucanases increased cell elongation in hardwood species such as poplar and promoted plant growth at the same time (Park et al. 2003, 2004). An increase in cell elongation has also been observed when enzymes associated with gibberellin biosynthesis were overexpressed. Cell elongation was accompanied with loosening and remodelling of the cell walls (Carpita 2012). Similar phenotypic trends might be achievable in conifer species such as pine, as the same gibberellin species are biologically active in hard- and softwood species (Macdonald and Little 2006).

4.3 Changing Wood Biochemistry

Traditionally, forest trees have mainly been used for firewood, timber and pulp and paper production. However, with the impending shortages in water and arable land, population growth and climate change, forests are expected to increasingly be used as sources of nontraditional bioproducts (Tilbrook et al. 2011). This could include the production of biodiesel, bioplastics and useful biochemicals from woody biomass. Wood naturally contains a number of biochemicals such as sugar alcohols, fatty acids, oils, phenols, resin acids, stilbenes, flavonoids, lignans and terpenes that find use in industrial applications such as sweeteners, solvents, lubricants, fragrances, flavour chemicals, adhesives, preservatives, tanning agents and antioxidants. Metabolic engineering experiments could be used to enhance the production of desirable secondary metabolites in woody tissue to generate additional product streams from forest plantations. The production of secondary metabolites with useful industrial, medical or pharmaceutical properties in plantation forestry species could also help to preserve natural forests and/or make such compounds more affordable. For example, recent experimental data suggest that it is possible to manipulate the biosynthesis of stilbenes in *P. radiata* by overexpression of stilbene synthase in woody tissue (Wagner et al. unpublished). Another strategy to change the biochemical composition of wood represents the mass production of terpenes in *Pinus taeda*, which can be used for the production of biodiesel and aviation fuel (Peter 2012). Terpenes represent an excellent source for biofuel based on their high-energy density and the existence of simple methods to efficiently convert them to drop-in fuels (Peter 2012). Terpene production could be enhanced in conifers by manipulating the conserved biosynthetic pathway and/or by promoting the formation of resin ducts, the cell types required for terpene production in conifers. Resin duct formation in conifers can be triggered by treatment with the plant hormones methyl jasmonate and ethylene (Hudgins and Franceschi 2004). Genes involved in ethylene production in conifers have been identified (Barnes et al. 2008; Hudgins et al. 2006).

Changing the biochemical composition of wood using metabolic engineering could also benefit the performance of traditional wood products such as timber. An example for this is seen in timber durability, which often reflects the biochemical composition of wood. Many important soft- and hardwood species used in plantation forests today produce nondurable wood, which limits their use in challenging conditions such as contact with soil or the marine environment. More and more chemical treatment options to enhance wood durability (e.g. copper chrome arsenate) are being phased out or are already banned due to the hazardous nature of the chemicals used in such processes. Many tree species produce secondary metabolites that make wood resistant to fungal degradation. One such class of bioactives are tropolones, terpene derivatives that are naturally produced in many *Cupressus*, *Juniperus* and *Thuja* species. Tropolones can have fungicidal properties similar to that of commercially available fungicides (Baya et al. 2001). Metabolic engineering of wood could be used to introduce bioactives such as tropolones into tree species

that produce naturally nondurable wood, which includes many pine species. However, more work on the biosynthesis of tropolones in plants is required to do so (Bentley 2008).

5 Conclusions

Experimental evidence from existing literature proves that metabolic engineering of wood can be successfully used to enhance the quantity, yield and quality of products generated from woody biomass. Our ability to devise well-designed molecular approaches to modify wood formation is likely to improve in years to come, provided sufficient effort is dedicated to enhance our knowledge on biochemical, cellular and regulatory aspects of xylogenesis, the biosynthesis of wood-related secondary metabolites, and cell wall polymers, in particular noncellulosic polysaccharides. New developments in genome manipulation such as zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) technologies are likely to be applied to forest trees to further enhance the precision of metabolic engineering experiments. The combination of emerging knowledge and superior technologies will enable metabolic engineering of wood to make more meaningful contributions to the sustainable generation of biochemicals, biofuels, bioenergy and solid wood products required in future.

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Part IV
The Cell Biology Toolbox: New Approaches

Flow Cytometry in Plant Research: A Success Story

Jan Vrána, Petr Cápal, Martina Bednářová, and Jaroslav Doležel

Abstract Flow cytometry is a powerful technique with numerous applications in biomedical research, including immunology, haematology, oncology and other fields. It has also found important applications in plant science where it accompanied scientists for almost four decades. Without its invaluable outcomes, some areas of plant research would not be in the position where they are now. This chapter focuses on exploitation of this state-of-the-art technology for studying plants at cellular and subcellular level, first providing a general overview and then focusing on nuclei and nuclear DNA content – by far the most frequent and most important application of flow cytometry in plant science. We review applications of the method from the early days to recent advances and discuss its applied aspects.

1 Introduction

Since its inception and, in particular, its commercialization in the late 1960s and the early 1970s, flow cytometry (FCM) played important roles in many areas of the life sciences. The field where this technique had the deepest impact is without any doubt biomedical research. However, it did not take plant biologists a long time before they realized the potential of the new technology. The main advantages, which attracted plant science and biotechnology, were (a) high throughput (hundreds or even thousands of particles can be analysed per second) providing statistically relevant data; (b) high accuracy and resolution; (c) negligible destructiveness (only small tissue samples are required); and (d) low operating costs per sample (once the initial instrument purchase costs have been invested). One publicly acclaimed disadvantage of FCM is the lack of visual information when compared

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to laser scanning cytometers and image analysers. This is true for all but one type of flow cytometer (Basiji et al. 2007). Nevertheless, the speed of analysis together with the growing army of fluorescent dyes, fluorescent proteins, fluorescently labelled antibodies and other reagents makes flow cytometry a formidable force capable of analysing over 15 parameters simultaneously in each particle (Chattopadhyay et al. 2008; Perfetto et al. 2004).

Flow cytometry requires samples in a form of aqueous suspension of single particles and this imposes some limits on its use in plant biology. Plant cells are tied together by a complex extracellular matrix in various tissues and organs and it may be difficult to isolate single cells. The cells have rigid cell walls, which may be autofluorescent, bind fluorescent probes nonspecifically and hamper staining of intracellular components. Due to irregular shape, plant cells disturb laminar flow in the narrow liquid stream compromising the precision of analysis. Moreover, many plant cell types may be larger than the diameter of the orifices in the flow chambers (typically 100 μm). One way how to prepare plant cells with regular shape suitable for flow cytometry is to remove their cell walls by digestion with hydrolytic enzymes and obtain protoplasts. Various projects relied on the analysis of protoplasts using flow cytometry (Galbraith 2007). Unfortunately, protoplast preparation is time-consuming, and as the tissues are exposed to various compounds and digestion represents a stress factor, protoplasts cannot be considered a good surrogate for intact cells. As with intact cells, protoplast analysis is complicated by their size; protoplast fragility and laborious preparation pose further constraints. Last but not least, protoplasts can be prepared only from some species and limited types of tissues.

Given the difficulties to analyse intact cells and/or protoplasts, it comes as no surprise that the most frequent application of flow cytometry in plant science has been the analysis of subcellular organelles – mitochondria, plastids, nuclei and chromosomes. Among the organelles, nuclei have been the most studied. This may be due to the fact that, unlike intact cells and protoplasts, they are small and can be relatively easily analysed. The nuclei harbour the majority of cellular DNA, and their analysis provides data on ploidy, genome size and cell cycle, to name just a few. The analysis of nuclei by flow cytometry and the application of their analysis are the main topics of this chapter.

Probably, the first paper ever on FCM in plants was published in 1973 (Heller 1973), but as it seems from today's perspective, plant biology was not ready for the revolution as the technique did not gain much attention and was largely overlooked, perhaps also because the paper was written in German. No papers on this topic were published for the long 10 years after this report. All changed in 1983, when David Galbraith and his colleagues at the University of Nebraska published a breakthrough paper, which brought FCM to a wider audience thanks to a simple protocol to prepare suspensions of intact nuclei suitable for flow cytometric analysis (Galbraith et al. 1983). This paved the way for this technology, and the number of plant flow cytometric papers has been steadily increasing.

Currently, flow cytometry has been used both in basic and applied plant research, as well as industrial applications, including breeding. It has become the

number one tool for quick, reliable and reproducible analysis of nuclear genome size and ploidy level. It has quickly found its way into taxonomy where it has been used to study speciation processes and population dynamics. The gender of plants and even the gender of the determining pollen grains can be determined using this method. This chapter discusses the most important applications of flow cytometry in plant biology with a special emphasis on the estimation of nuclear DNA content.

2 Principles of Flow Cytometry

Flow cytometry is an analytical and preparative technique offering high throughput and precision. Its unique feature is the ability to analyse multiple optical properties of single particles at a rate of several hundreds or thousands per second. Although flow cytometers are sophisticated instruments utilizing fluidics, lasers, optics and electronics, the basic principles are simple (Fig. 1). By virtue of a fluidic system, individual particles of the sample are brought to flow in a single file in a core of a narrow stream of liquid and pass individually through a beam of light (a typical light source is laser). Optical signals arising from the interaction between the particles and light (light scatter and fluorescence) are then steered by elements of the optical system to spectrally separated detectors (photomultiplier tubes or photodiodes), where optical pulses are transformed into electrical pulses which are then processed by electronic processing. The results are displayed as monoparametric frequency distribution histograms, biparametric (2D) dot plots or multiparametric dot plots.

A special group of flow cytometers, called sorters, can physically isolate particles of interest for further analyses, and they can do it simultaneously with the analysis and at high speed. There are two basic sorting systems: fluid switching and droplet, with the latter being the most common and efficient sorting type (Fig. 1). In this system, the fluid stream emerging from the flow chamber is subdivided into small droplets (the place where this occurs is called break off point). When a particle of interest is detected, the instrument waits until the particle reaches the break-off point and electrically charges the droplet containing the particle. Further downstream, the charged droplet is electrostatically deflected and collected in a tube.

Flow cytometers were originally developed to analyse blood cells but later were found suitable for the analysis of cell organelles (e.g. nuclei, mitochondria, plastids, chromosomes) and even particles as small as bacteria and viruses. Although it may be surprising, a plethora of physical and physiological parameters and processes could be inferred (e.g. size, internal complexity, total DNA content, pH and viability, to name just few) from the analysis of optical parameters of particles (light scattered at different angles, total fluorescence, fluoresce pulse profile).

Apart from the number of scientific publications, the popularity of flow cytometry can be judged from the increasing number of companies, which produce flow cytometers and sorters and the number of models on the market. The

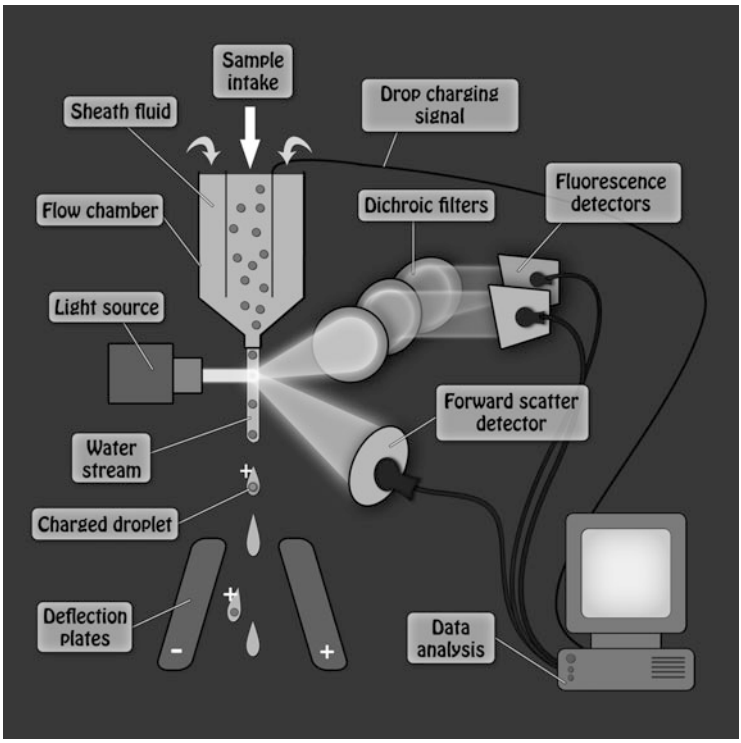

Fig. 1 Schematic view of a flow cytometer and sorter with one excitation light source, one detector of forward light scatter and two detectors of fluorescence signals. This type of instrument can sort two different populations of particles simultaneously

instruments differ in the number of functions, technical design and price tag. The range of flow cytometers is broad, starting with basic small footprint analysers capable of measuring just one or a few parameters, continuing with a strong middle class of cell analysers usually equipped with automatic sample loaders for quick analyses in multi-user facilities and finally ending with high-end multiparametric analysers and sorters which are often customized for particular needs. The reader is referred to the recent paper of Picot et al. (2012), which provides a qualified overview. The progress that flow cytometry has made since its beginnings (and a lot of useful information) can be found in Shapiro's excellent "Practical flow cytometry" book series (Shapiro 1985, 1988, 1995, 2003).

3 Plants and Flow Cytometry: An Uneasy but Fruitful Marriage

With only a few exceptions represented by the pollen grains of seed plants and other microspores of some nonvascular plants such as algae, plants are constituted of complex solid multicellular tissues, where individual cells are tied together in complex 3D matrix. Therefore, analysing intact plant cells using flow cytometry is not straightforward and found its applications in only a few areas of research. One of them is aquatic science, as most of phytoplankton exists as single cells. Phytoplankton analysis using flow cytometry is used mostly in marine ecology and biodiversity studies (reviewed in Dubelaar et al. 2007). The analysis in situ often requires customized (semi)automated instrumentation (Dubelaar et al. 1999). Another application of flow cytometry with plant cells is metabolic engineering. Plants themselves work as bioreactors; they synthesize a wide range of secondary metabolites, which may be used in pharmaceutical and food industry. In order to achieve higher yields of these compounds, cell cultures with standardized growth conditions have to be used (see also the chapter by Opatrný et al., this volume). Flow cytometry can be applied to analyse a broad range of plant products and culture properties, provided a desired trait correlates with fluorescence or light-scattering parameters that can be measured by FCM. The technique can be used for counting cells in culture, estimating their viability, growth potential and, most importantly, for detection of the desired metabolite accumulation (Gaurav et al. 2010). Moreover, flow sorting permits selection of subpopulations from cell cultures to establish highly producing cell lines (Schulze and Paulz 1998, Gaurav et al. 2010). Flow cytometry has also been used in plant pathology for detection, characterization and quantification of pathogens (reviewed by D'Hondt et al. 2011). The pathogens (viruses, bacteria, fungi and oomycetes) have been analysed for their presence and abundance, genome size and their biological status (viability, gene expression).

Cells bereft of cell walls are called protoplasts. Their advantage over whole cells is a round shape and hence regular passage in a laminar flow, but other disadvantages limit the usefulness of protoplasts for flow cytometry. The first is connected to lengthy preparation as plant tissues must be treated with cell wall-digesting enzymes. Thus, protoplasts are generally not suitable to study dynamic processes. Protoplasts may be large (up to 150 μm), depending on the tissue and species (Fox and Galbraith 1990), and therefore not compatible with most of commercial flow cytometers where nozzle diameters are typically in the range of 70–120 μm. The use of larger nozzles puts restrictions on the speed of analysis and sorting (Galbraith 1994). Without cell wall, plant protoplasts are fragile, which may limit their use and they cannot be prepared from all species and tissue types (Galbraith 2007). Since nuclei are located near plasma membrane and not in the centre of protoplasts, the protoplasts are generally not suited for estimation of DNA content as the nuclei are irregularly positioned against the focus of excitation light, resulting in a variability of signals (Galbraith 1990; Ulrich et al. 1988). Despite the difficulties, there have

been interesting applications of flow cytometry involving plant protoplasts. The most important uses of protoplasts include the analysis of gene expression (Birnbaum et al. 2003) and sorting of heterokaryons after protoplast fusion for production of somatic hybrid plants (Liu et al. 1995). Other applications of plant protoplast analysis include estimation of protein contents (Naill and Roberts 2005), measurement of physiological parameters such as pH (Giglioli-Guivarc'h et al. 1996) and analysis of apoptosis (Yao et al. 2004; Watanabe et al. 2002).

Apart from whole cells, also cell organelles can be analysed by flow cytometry. While nuclei are the most studied organelles using flow cytometry, flow cytometry was used to some extent to study chloroplasts and mitochondria. Analysis of isolated intact chloroplasts discriminated two populations of chloroplasts based on different chlorophyll fluorescence intensities (Kausch and Bruce 1994). Pfündel and Meister (1996) succeeded in discriminating chloroplast thylakoids obtained from mesophyll and bundle sheath cells of maize based on their differences in fluorescence spectra and sorted them for further analysis. In order to investigate the process of DNA depletion in chloroplasts during leaf development, Rowan et al. (2007) developed a flow cytometric method for detection of DNA in chloroplasts. Unfortunately, only a few studies have been undertaken on isolated plant mitochondria using flow cytometry. Petit et al. (1986) measured the binding of concanavalin A to mitochondria of potato, and Petit (1992) successfully monitored changes in membrane potential using Rhodamine 123. The only application of flow cytometry in plant research which can rival nuclear analysis and sorting in its importance and abundance is flow cytometric analysis and sorting of plant metaphase chromosomes. Flow-sorted plant chromosomes found many applications in cytogenetics and genomics. The recent article by Doležel et al. (2012) provides an extensive overview of this field.

4 Plant Nuclei in Focus

Nuclei are important cellular organelles where the majority of hereditary information is stored and important processes related to genome replication, repair and response to various stimuli and transcription into a range of RNA species take place. These processes impact the destiny of a cell, tissue and the whole organism as well as its progeny. Details of these processes and their significance are far from understood and flow cytometry has been revealing and certainly will continue revealing many of the enigmas of Mother Nature.

But before flow cytometry of cell nuclei could become a useful tool in plant biology, many problems and obstacles have to be solved and new approaches and protocols developed. The main difficulties were and to some extent still are associated with preparation of samples accessible for this method. The obstacles are numerous due to plant cell structure and physiology mentioned above – rigid cell wall, presence of secondary metabolites in cytosol, auto-fluorescence from

photosynthetic pigments and others, which will be dealt with in following paragraphs.

But all these difficulties are worth to be overcome as flow cytometry is able to give important results. The two most frequent applications of FCM on plant nuclei are the measurement of genome size in absolute units and the estimation of ploidy level. Other applications include cell cycle analysis, tissue-specific gene expression analysis and nuclear sorting.

5 Principles and Methodology

5.1 Sample Preparation

Flow cytometry requires samples in a form of aqueous suspensions of particles. Plants are not very cooperative in this regard as their cells have rigid walls and it may not be easy to release intact nuclei. The oldest method is based on enzymatic treatment of plant tissues in order to obtain wall-free cells – protoplasts – from which the nuclei are released into the isolation buffer by osmotic lysis (Heller 1973). This method is laborious and time-consuming and therefore impractical for wider use. However, as the nuclei are released gently, this method yields histograms of DNA content with excellent resolution and minimum of debris background (Ulrich and Ulrich 1991). The fact that protoplasts isolation takes time makes this method unsuitable for the analysis of cell cycle kinetics.

To date, the most frequently used method for preparation of nuclear suspensions is the ingeniously simple method developed by Galbraith et al. (1983). The method relies on homogenization of fresh tissue using a razor blade in a small volume of isolation buffer into which the cellular contents are released. Filtering the crude homogenate through a nylon mesh removes large tissue debris to avoid blockage of sample tubing and nozzle. The main advantages of this method are the speed and the need of only small amounts of practically any plant tissue (leaves, roots, stems, flowers, etc.). Silva et al. (2010) went so far that they developed a protocol for successful isolation of intact nuclei from only a single root meristem. The only drawback of the chopping method is the difficulty to automatize sample preparation for high-throughput applications. Therefore, Roberts (2007) adapted the so-called bead-beating method, routinely used for releasing DNA from cells for genomic studies (Haymes et al. 2004; Harmon et al. 2006), where small amount of plant tissue and isolation media are shaken together with zirconia/silica beads (2.5 mm in diameter) and intact nuclei are released. Several samples could be prepared at the same time using this method. The authors concluded that this method was suitable not only for fresh material but also for dried herbarium specimens and pollen. Cousin et al. (2009) took advantage of this sample preparation method, and using a cytometer equipped with an automatic sample loader, they created a high-throughput pipeline where one operator could analyse several hundred samples

per working day. One of few exceptions, where razor chopping was not very successful, is pollen grains, and therefore, Kron and Husband (2012) developed a method for isolating pollen nuclei by inducing bursting of pollen through a nylon mesh filter.

5.2 Nuclear Isolation Buffers

Chemical composition of the nuclear isolation buffers is important and a correct choice of a buffer may be the key to successful flow cytometric analysis. A number of isolation buffers has been published, differing in chemical composition, but they all should facilitate the release of nuclei free of cytoplasm and, in sufficient quantities, maintain the integrity of isolated nuclei, protect DNA against nucleases and facilitate stoichiometric DNA staining (Doležel 1991). Generally, the isolation buffers contain substances that stabilize nuclear chromatin such as magnesium ions (Galbraith et al. 1983; Arumuganathan and Earle 1991; Pfosser et al. 1995) or spermine (Doležel et al. 1989), chelating agents which serve as nuclease cofactors such as EDTA (Doležel et al. 1989; Marie and Brown 1993) or sodium citrate (Galbraith et al. 1983; Marie and Brown 1993) and inorganic salts (KCl, NaCl) to adjust appropriate ionic strength. Detergents (e.g. Triton X-100 or Tween 20) are included to facilitate nuclear release from the cytoplasm and to prevent nuclei from aggregation. Doležel and Bartoš (2005) and Greilhuber et al. (2007) list popular isolation buffers and discuss their advantages and limitations. Loureiro et al. (2006a) conducted a series of tests for the four most popular buffers. Although some buffers performed better than others, the authors concluded that there is no universal buffer suitable for every species and tissue due to diversity of plant tissues in structure and chemical composition, and thus, suitable buffers must be tested individually for a given material. Based on these findings, Loureiro et al. (2007a) developed two improved buffers. One performs well in species relatively free of cytosolic compounds (general purpose buffer (GPB)), while the other is more suitable for more problematic tissues (woody plant buffer (WPB)). They claim that WPB works better in problematic tissues/species than other nuclear isolation buffers and therefore is recommended as the first choice.

5.3 Parameters Analysed on Plant Nuclei

Fluorescence has been the most common optical parameter in flow cytometric analysis of plant nuclei. Although light scatter is helpful in detection and determination of single nuclei populations and also in detection of effects of cytosolic compounds on estimation of DNA amounts (Loureiro et al. 2006a, b), their use in flow cytometric analysis of plants is rather scarce. Fluorescence is a physical phenomenon caused by absorption of light energy by a molecule (fluorochrome)

and subsequent emission of light with longer wavelength. The fluorochromes can be either innate to the examined particle (so-called auto-fluorescence) or they are added artificially (this is the usual case). The choice of fluorochromes is very rich these days and new dyes are added to the list continuously. One can choose dyes for a wide range of structural or physiological features of the nuclei, including nucleic acid dyes (e.g. DAPI, propidium iodide, acridine orange), protein dyes, which can be used as fluorescent "tags" (e.g. FITC, PE, APC) and fluorescent proteins to monitor gene expression (e.g. GFP, YFP, DsRed). The most frequently used fluorochromes to analyse plant nuclei are nucleic acid dyes.

5.4 Nuclear Staining

The most common dyes used to stain plant nuclear DNA are DAPI and PI (Doležel et al. 1992a). DAPI (4′,6-diamidino-2-phenylindole) is a minor groove-binding molecule with selectivity for AT bases. It is used in non-saturating concentrations, typically 2–4 μg/ml (Pfosser 1989; Ulrich et al. 1988). DNA-bound DAPI is best excited by near UV light (358 nm excitation maximum), and therefore, it has become popular among plant researchers who preferred cheaper arc lamp-based instruments. DAPI binding to DNA is mostly independent of chromatin structure and the peaks on histograms of nuclei fluorescence are characterized by low variability. As DAPI is base selective and therefore not suitable for absolute DNA measurements (Doležel et al. 1992a), it is used predominantly for estimation of relative amounts to determine ploidy levels. PI (propidium iodide) is an intercalating dye and has no apparent base specificity. PI is used in saturating concentrations, typically 50–100 μg/ml (Taylor and Milthorpe 1980). Its most frequent use is the measurement of absolute DNA amounts, but as it also binds to double-stranded RNA, samples must be treated with RNAse. PI provides a broad range of excitation wavelengths (325–568 nm), but the most common wavelengths for PI excitation in commercial instruments are blue (488 nm) and green (532 nm) laser lines.

The progress in chemistry offers new fluorochromes, which may replace the traditionally used PI. For instance, Clarindo and Carvalho (2011) used SYBR Green 1 for measurement of genome size in two *Coffea* species and compared it with results of PI-stained nuclei. The results for genome size obtained either with SYBR Green 1 or PI were statistically identical. Together with the observation that the peaks of G0/G1 nuclei stained by SYBR Green 1 had lower coefficients of variation compared to those stained with PI and the fact that SYBR Green 1 is less mutagenic than propidium iodide, these findings indicate that SYBR Green 1 can be used for flow cytometric experiments with plants. Another advantage is that the excitation maximum of SYBR Green 1, compared to PI, is closer to 488 nm, the most common laser line in most commercial analysers, and therefore, the excitation of fluorescence is more efficient.

Fluorescent antibiotics such as chromomycin A3, mithramycin and olivomycin represent a further class of DNA dyes which bind preferentially to the GC-rich

regions of DNA. Their use in plant nuclear research is limited mainly to base composition studies (Meister and Barow 2007), as described later in this chapter. Acridine orange (AO) has been used even less frequently (Bergounioux et al. 1988). The dye binds to DNA and dsRNA and yields a green fluorescence upon binding to DNA, while the RNA-bound dye produces red fluorescence. This property of AO is used in human and animal cells to estimate simultaneously DNA and RNA content and to discriminate between dividing and quiescent cells (Darzynkiewicz 1994).

5.5 Secondary Metabolites

Secondary metabolites present in cytosol can affect the accessibility of the dye to the DNA. Their effects have been recognized first by micro-spectrophotometry (Greilhuber 1986, 1988) and only later in plant FCM analyses (Noirot et al. 2000, 2002, 2003, 2005; Price et al. 2000). Interference of cytosolic compounds with staining of DNA was originally observed as fluorescence inhibition but can also be manifest as an increase in fluorescence (Noirot et al. 2003; Loureiro et al. 2006b). Loureiro et al. (2006b) showed that cytosolic compounds can change light-scattering properties of nuclei (increase in side scatter signal intensity and decrease in resolution of histograms) and termed this phenomenon "tannic acid effect". Changes in light scatter provide an opportunity to detect the interference of cytosol. To eliminate unfavourable effects of cytosolic compounds in studies on genome size, a good practice has been to carefully choose the most suitable isolation buffers, inhibitors of phenolic compounds such as mercaptoethanol and PVP (Price et al. 2000; Noirot et al. 2003, Yokoya et al. 2000) and to select tissues with lower content (or preferably even the absence) of phenolic compounds (Loureiro et al. 2007a).

5.6 Materials for Nuclear Isolation

Almost any fresh plant tissue is suitable to prepare suspensions of intact nuclei for flow cytometry using the mechanical chopping method. This, however, holds true only if the tissues are healthy as degradation of DNA results in low resolution of DNA content histograms. A problem may occur in field conditions where rapid dispatch of fresh material to the laboratory may not be possible. The difficulty may be overcome either by using a flow cytometer *on site* or by shipping preserved plant tissues to the laboratory. As the development of instrumentation continues, there are several small portable cytometers available on the market. However, their use in exotic and remote localities may be hampered by shortage of continuous supply of power and consumables. Plant tissues can be preserved in several ways. For short-term (several days) storage/transport, tissues can be bagged with moistened paper and kept at low temperatures (ca. 4°C). To ensure preservation over longer periods

of chemical fixation of the material (Doležel et al. 1992b; Suda and Trávníček 2006), dried samples (silica gel-dried samples or traditional herbarium vouchers; Suda et Trávníček 2006) or glycerol-preserved nuclei (Hopping 1993, Kolář et al. 2012) have been employed. Alternatively, dormant seeds may be used (Matzk et al. 2000; Śliwinska et al. 2005).

Chemical fixation is routinely used to store human and animal cells, but for plants it has turned out not to be practical, as the release of nuclei from cells fixed in some fixatives such as 3:1 (ethanol to acetic acid) is difficult or impossible. Importantly, fluorescence of some DNA dyes is altered by the fixation, and fixed materials are not suitable for some applications. For example, formaldehyde-fixed samples are not amenable to estimation of DNA content in absolute units, as the fixation interferes with propidium iodide staining (Becker and Mikel 1990; Overton and McCoy 1994). However, for some applications (e.g. ploidy level estimation and cell cycle analysis), combination of DAPI staining and formaldehyde-fixed nuclei is feasible (Jarret et al. 1995, Sgorbati et al. 1986). Flow cytometric estimation of ploidy levels was carried out successfully with plant tissues that have been rapidly frozen (Dart et al. 2004; Nsabimana and Van Staden 2006; Halverson et al. 2008; Cires et al. 2009). Several authors (Baranyi et al. 1996; Matzk et al. 2000; Śliwinska et al. 2005, 2009; Jedrzejcyzk and Śliwinska 2010) investigated the possibilities to estimate DNA content from dry seeds, which can be easily transported from a collection site to the laboratory. The use of seeds may be advantageous especially for species that contain cytosolic compounds in leaf cells, affecting quality of DNA content histograms (Śliwinska et al. 2005, Matzk 2007). However, this approach bears some limitations. The seeds must be collected during seed maturation season, and there are additional difficulties linked with seed germination ex situ and taxonomic complexity (Śliwinska et al. 2005, Kolář et al. 2012). Śliwinska et al. (2009) conclude that it is possible to use seeds for flow cytometric measurements of nuclear DNA content, but detailed understanding of seed biology is needed to interpret the results correctly. However, when the seeds are used up for flow cytometric analyses, no further analysis, such as karyological or molecular investigations, is possible. As an alternative approach seeds can be collected and germinated to analyse the growing plants in the laboratory (Suda et al. 2005).

Suda and Trávníček (2006) analysed nuclei isolated from herbarium and silica gel-dried material (up to 2 years old) in a set of plant groups. They concluded that as the quality of DNA histograms might be compromised (shifts in fluorescence intensity compared to that of fresh samples and decrease of uniformity of fluorescence, resulting in higher coefficients of variation (CV)), this method should be generally avoided for estimation of DNA content in absolute units. For example, Šmarda (2006) reported up to 10 % difference in estimates of DNA content between fresh and dry tissues in *Festuca*. On the other hand, dehydrated vouchers of mosses were suitable even for genome size estimation in absolute units (Voglmayr 2000). In contrast to genome size estimation, herbarium and silica gel-dried materials proved suitable for large-scale ploidy screening (Šmarda et al. 2005, Šmarda and Stančík 2006, Schönswetter et al. 2007, Suda and Trávníček 2006, Whittemore and Olsen 2011). But the idea of using silica gel-dried material for genome size studies

is still pursued, and Bainard et al. (2011a) discussed criteria and conditions under which this method might be used more broadly. Kolář et al. (2012) revisited a method to preserve suspensions of isolated nuclei in glycerol published by Chiatante et al. (1990) and Hopping (1993). This method is suitable even for genome size estimation and Kolář et al. (2012) state that this method, although requiring sample preparation in situ, is probably the most reliable way to preserve plant material for all flow cytometric applications. The same authors predict that the shipping of cooled fresh material for short-term and rapid silica-gel drying for long-term field trips, respectively, will still remain the methods of choice for the near future.

5.7 DNA Flow Cytometry

To correctly interpret the results of flow cytometric analysis of DNA content, one needs to consider its changes during cell cycle. Cycling cells undergo cyclical changes in nuclear DNA content, which can be described using C-values. In G_0/G_1 phase, the nuclei of somatic cells have an identical DNA amount – 2C (i.e. two copies of nuclear genome) – while nuclei in G_2/M phases have twice as much DNA described as 4C. During DNA synthesis (S) phase, cellular DNA content varies between 2C and 4C. As typical result of FCM analysis of DNA content histograms for nuclei in G1 and G2 phase yield narrow peaks, while the nuclei in S show a distribution ranging from 2C to 4C levels (Fig. 2). The resolution of flow cytometric measurements is influenced by many factors as discussed above. Quality of peaks is characterized by a coefficient of variation (CV), which is defined as standard deviation of the peak divided by the mean value of the peak. The lower the CV, the more accurate is the measurement. Other factors influencing reproducibility of histograms are the presence of debris and aggregates. Debris can be removed from histograms by combination of electronical thresholding and good gating strategies. Doublets and other aggregates are usually gated out from populations of single nuclei using 2-D (biparametric) histograms where one parameter is total fluorescence (DNA content) and the other is the width of fluorescence pulse (aggregates are usually larger/longer than singlets).

6 Biological Aspects: Analysis of Nuclei

6.1 Genome Size

Nuclear genome size (DNA amount representing one copy of nuclear genome) is an inherent and unique attribute of every eukaryote and its knowledge is critical for many areas of research, including taxonomy, ecology and evolutionary biology.

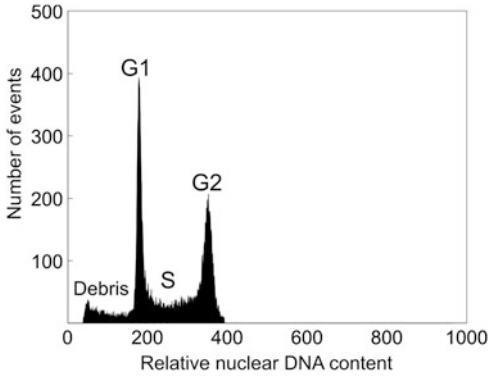

Fig. 2 Distribution of nuclear DNA content in a population of cycling cells. Histogram of relative DNA content obtained after the analysis of DAPI-stained nuclei isolated from actively growing root tips of bread wheat. As cells progress from G_1 to G_2 phase of cell cycle, their DNA content doubles. This situation reflected on the histogram by two peaks representing G_1 and G_2 nuclei with 2C and 4D DNA amounts, respectively. The distribution between the two peaks represents nuclei in S phase with DNA amounts ranging between 2C and 4C. The distribution to the left of G_1 peak represents cellular and nuclear debris

Data on genome size are essential for genomic studies as sequencing programmes need this information to estimate costs and manage the complexity of individual projects (Cardoso et al. 2012). Yet, despite all efforts, it was estimated in 2007 that only about 1.8 % of angiosperm plants have been analysed for genome size (Leitch and Bennett 2007). The most recent report lists DNA content estimates for 6287 angiosperm species (Bennett and Leitch 2011). A high-throughput method is thus needed to estimate genome size in a majority of plants, and flow cytometry has been a major player in this area.

Historically, the first method to estimate nuclear DNA content was chemical extraction (Schmidt and Thannhauser 1945), but it was laborious and time-consuming, therefore impractical for wider use. A further disadvantage was that it provided average values for the population and the results were compromised by the occurrence of cells in S and G2 phases of cell cycle. The most popular method before the advent of FCM was Feulgen microdensitometry (Typas and Heale 1980; Voglmayr and Greilhuber 1998). This method estimates DNA amount by measuring absorption of monochromatic light by the stained nucleus and comparing it to known standards. A modern version of densitometry is the so-called image cytometry (ICM), relying on CCD camera and image analysis software (Hardie et al. 2002). Although very reliable, the main disadvantages of microdensitometry are the rather limited speed of analysis and a need for mitotically active tissues (Greilhuber 2008). In contrast, FCM offers both speed and reliability, does not require dividing cells and provides greater statistical accuracy. These may be the reasons why this method is today by far the most popular (Bennett and Leitch 2005; Leitch and Bennett 2007; Greilhuber et al. 2007). The trend is clearly visible in the compilations of plant C-values made by the Bennett group; while in 2005 the

percentages of first estimates made by FCM were some 58 % (Bennett and Leitch 2005), in the most recent release of C-value data compilation, they reached almost 85 % (Bennett and Leitch 2011). Several studies (e.g. Doležel et al. 1998; Vilhar et al. 2001) verified that results obtained by both FCM and Feulgen densitometry are reliable and comparable and can be used in parallel.

DNA amounts of eukaryotic organisms are expressed as C-values (C for constant); this term was first used by Swift (1950) based on a concept first proposed by Boivin et al. (1948) that within any tissue of an organism, the actual amount of DNA per somatic nucleus is constant. The DNA amount in the unreplicated gametic nuclear chromosome complement (n) is 1C, while DNA amount of a diploid (somatic) nucleus is 2C. Ambiguities in terminology may occur in polyploid organisms, and therefore, Greilhuber et al. (2005) proposed a unified terminology, where 1C value refers to half of somatic DNA content (2C), while 1Cx is 2C value divided by the ploidy level of the respective organism. 1C and 1Cx values correspond to the holoploid and monoploid genome sizes, respectively. These two values are the same only in diploids. A more complete version of the terminology on genome size which deals with various cytogenetic conditions, life cycle segments and nuclear phases was proposed by Greilhuber and Doležel (2009).

In order to report on absolute DNA amounts, C-values are quoted either in picograms (pg) or base pairs (bp), with the conversion formula being 1 pg DNA = 978 Mbp (Doležel et al. 2003). The first compilation of published data on plant DNA C-values was published by Bennet and Smith (1976), and since then, new C-values have been reviewed on a regular basis (Bennett et al. 1982, 2000; Bennett and Leitch 1995, 1997, 2005, 2011; Zonneveld et al. 2005). From 1997 onwards, the C-values database is available online (http://data.kew.org/cvalues/). But, as some published data for the same species could vary significantly, Bennet and Smith (1976) stressed already in the first release of their database and others agreed later (Temsch and Greilhuber 2000, 2001; Doležel et al. 1998) that some results should be critically re-evaluated as not all data were obtained in experiments carried out in the right way. Problems with standardization, wrong choice of fluorochromes and ignorance of the effects of secondary metabolites can lead to misleading results.

Estimation of DNA content in absolute units is based on comparison of relative DNA amounts of the unknown sample with a sample with known C-value (reference standard):

$$\begin{aligned}&\text{2C value of unknown sample} = \\ &[\text{G}_0/\text{G}_1 \text{ peak mean of unknown sample}/\text{G}_0/\text{G}_1 \text{ peak mean of reference standard}] \\ &\times \text{2C value of reference standard (pg)}.\end{aligned}$$

There are two principal ways of standardization: external and internal. External standards are measured separately from the sample, while the internal standard is processed together with the sample. In order to avoid errors due to variation in sample preparation and staining, internal standardization has been recommended (Doležel et al. 1992a, Greilhuber 2008). Doležel et al. (1998) compared results of

genome size estimations in four different laboratories and concluded that flow cytometry is a reliable method, when a set of reference standards and their calibration are adopted. The issue of standardization has been dealt with in more detail by several authors (e.g. Doležel et al. 1992a; Johnston et al. 1999; Bennet et al. 2003; Doležel and Bartoš 2005; Greilhuber et al. 2007; Suda and Leitch 2010; Praça-Fontes et al. 2011).

In general, a reference standard must have well-defined genome size, preferably not too distant from the examined sample (Bennett et al. 2003; Doležel et al. 1992a; Doležel and Bartoš 2005) but not too close to avoid that the peaks of G_1 nuclei of sample and standard would overlap (Greilhuber et al. 2007), and the standard must be biologically similar, i.e. plant standards should be used for plant samples (Suda and Leitch 2010), it must be cytologically stable, it must contain low (or no) level of secondary metabolites and it must be generally available. As 1C DNA amounts in higher plants range from 0.065 pg in *Genlisea margaretae* (Greilhuber et al. 2006) to 152.2 pg in *Paris japonica* (Pellicer et al. 2010), which corresponds to a difference of approximately 2,400-fold, it is impossible to use a single standard for all species. Doležel et al. (1998, 2007) give a list of recommended standards with 1C genome sizes ranging from 1.1 to 34.89 pg DNA. These standards were calibrated using human leukocytes considering 2C values of 7 pg DNA (Doležel et al. 1998), a value recommended by Tiersch et al. (1989) as reference value for estimation of genome size in animals. The standards are freely available from the Doležel lab (Doležel et al. 2007). Ideally, 1C values of reference standards should be known exactly. Unfortunately, due to difficulties in assembling repetitive parts of genomes, the exact genome size is not known for humans and also not for plants that would be otherwise suitable as reference standards (Doležel and Greilhuber 2010).

To conclude, some of the most important critical preconditions for successful and reliable estimation of DNA content in absolute units are (a) precisely aligned instrument, (b) awareness of possible interference of secondary metabolites with the staining, (c) use of intercalating DNA stains and RNAse treatment of nuclei before staining, (d) proper standardization procedure and suitable reference standards and (e) other factors, such as buffer composition and dye concentration (Bainard et al. 2010). Moreover, it has been recommended that measurements have to be performed with at least three different plants from the same species or population and that the analyses are repeated at least three times, ideally on different days (Lysák et al. 1999).

6.2 Ploidy

Probably the most common use of FCM in plants has been the estimation of nuclear DNA content in relative units to determine ploidy levels. The number of chromosome sets is especially important especially for plant taxonomy and plant breeding programmes (Doležel 1997; Eeckhaut et al. 2005; Ochatt 2008). The most reliable

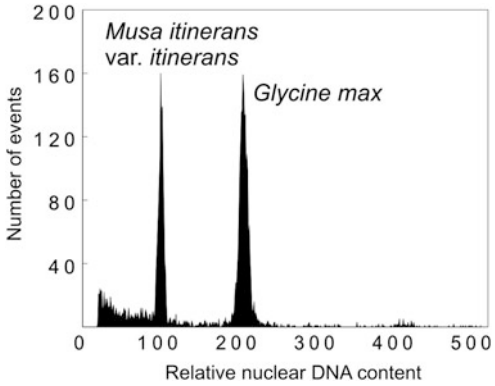

Fig. 3 Estimation of nuclear DNA amount in absolute units. Histogram of relative DNA content was obtained after simultaneous analysis of propidium iodide-stained nuclei of a banana species *Musa itinerans* var. *itinerans* and soybean (*Glycine max*). The soybean with 2C = 2.50 pg DNA was used as internal reference standard to estimate DNA amount of the banana species according to the formula:
2C value of *Musa* = (2.5 × G_1 peak mean of *Musa*)/(G_1 peak mean of *Glycine*)
The estimated 2C amount of *Musa* was 1.217 pg DNA. The mass of DNA in pg can be converted to the number of base pairs considering 1 pg DNA = 0.978 × 10^9 bp (Doležel et al. 2003)

method to determine ploidy levels is chromosome counting. However, this is laborious and time-consuming and should be done by experienced cytologists (especially in species with numerous and small chromosomes). Moreover, this approach requires actively dividing cells. There are indirect techniques for ploidy estimation including the estimation of leaf stomatal density and size (van Duren et al. 1996), determination of chloroplast number in guard cells and pollen diameter (Mishra 1997), but none of them is reliable enough for routine use. Thus, flow cytometry has become the method of choice in ploidy level estimation (Fig. 3).

The first report on estimation of ploidy levels using FCM was published by de Laat et al. (1987) and has been followed by an ever-increasing number of reports. As the amount of data on ploidy levels obtained by flow cytometry increases, Doležel (1991) and later Suda et al. (2006) appeal for consistent terminology first proposed by the Committee on Nomenclature, Society of Analytical Cytology (now International Society for Advancement of Cytometry; Hiddemann et al. 1984), distinguishing between the results obtained by conventional chromosome counting using microscopy (ploidy) and data obtained by flow cytometric analysis (DNA ploidy). As the main reason for this terminological distinction, Suda et al. (2006) list and discuss several cases in which discrepancies between the results from karyology and flow cytometry could arise.

A prerequisite for reliable estimation of ploidy levels using flow cytometry is a correlation between chromosome number and nuclear DNA amount. The results must be calibrated against a sample with known number of chromosomes as standard (Suda et al. 2006), which must be an individual from the same species. Unlike in the estimation of genome size, it is generally accepted to use external

standards in ploidy screening. However, as internal standardization eliminates potential errors due to variation in sample preparation and instrumental "drifts", internal standards may be preferred, especially in cases where aneuploidy is expected. Samples for ploidy screening are often stained by DAPI instead of PI or ethidium bromide (EB), because its binding to DNA is less affected by chromatin structure, and DNA peaks show lower variation. Moreover, DAPI can be used in lower concentrations, does not bind to RNA and can be excited by mercury lamps, which were common in older models of benchtop instruments popular among botanists.

Apart from the estimation of the number of complete chromosome sets, under certain conditions FCM may be used to detect minor changes in nuclear DNA amounts due to gain or loss of a few or even only a single chromosome (aneuploidy). Bashir et al. (1993) and Pfosser et al. (1995) analysed genome sizes of several wheat-rye addition lines and confirmed that flow cytometry was able to detect differences in DNA content as small as 1.8 %, corresponding to the presence of single telocentric rye chromosomes on the background of an entire wheat chromosome set. Roux et al. (2003) used flow cytometry for rapid detection of aneuploidy in triploid *Musa*, and the results were confirmed by conventional chromosome counting. Flow cytometry was also successful in detection of monosomy and trisomy in hops (Šesek et al. 2000).

In many plant species, differentiated tissues comprise cells with DNA contents higher than 2C (i.e. 4C, 8C). This phenomenon is called endopolyploidy and is usually caused by endoreduplication, which involves repeated rounds of DNA synthesis without intervening mitoses, leading to chromosomes with 4, 8, 16 and more chromatids. These cells do not divide, and flow cytometry is probably the best method to identify them and establish the frequency of individual levels of endopolyploidy. Endopolyploidy is common in angiosperms (Barow 2006; Barow and Jovtchev 2007) and mosses (Bainard and Newmaster 2010) but is rare in gymnosperms and ferns (Barow and Jovtchev 2007; Barow and Meister 2003) and even absent in liverworts (Bainard and Newmaster 2010). Although the biological significance of endoreduplication is poorly understood, it was proposed that it plays an important role in plant cell and tissue growth and differentiation (reviewed by Chevalier et al. 2011). In flow cytometric experiments, endopolyploidy is manifest in DNA content histograms as series of distinctive peaks, each corresponding to nuclei with different level of endoreduplication (Fig. 4). The degree of endopolyploidy (mean value of endopolyploidization) in different samples (organism, organ or tissue level) has been quantified as mean C-level and cycle value (Barow and Jovtchev 2007; Barow and Meister 2003).

6.3 Base Composition

Base composition (ratio of AT to GC base pairs) in nuclear DNA differs between species, may have a biological relevance and can be used as additional parameter in

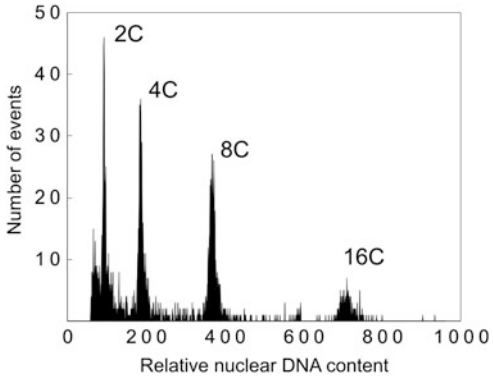

Fig. 4 Analysis of endoreduplication. Histogram of relative DNA amount was obtained after the analysis of DAPI-stained nuclei isolated from 14-day-old plants of *Arabidopsis thaliana*. The histogram comprises three peaks – one representing G_1 phase cells with 2C DNA content, one representing G_2 phase cells with 4C DNA amount, while the third peak represents nuclei with 8C DNA amounts which resulted from one round of endoreduplication and fourth peak stands for nuclei that experienced two rounds of endoreduplication and thus have 16C DNA content

taxonomic studies. DNA base content can be estimated using physicochemical methods such as chromatography, density centrifugation, DNA temperature melting analysis and UV absorbance and, more recently, by complete genome sequencing (Meister and Barow 2007; Šmarda et al. 2011). Nevertheless, flow cytometry offers comparable results for a fraction of time, cost and from the smallest amount of material as compared to other methods.

Flow cytometric measurements of base composition exploit DNA dyes with different affinity for DNA bases: intercalators with no base preference (typically propidium iodide) are compared to AT-specific dyes (e.g. DAPI, Hoechst dyes) or fluorescent antibiotics with GC preference (e.g. chromomycin A3, mithramycin A and olivomycin). Sample and standard are processed together and their fluorescence intensity profiles are measured independently with both the intercalating dye and the base-specific dye (either AT or GC specific). AT or GC content (depending on the base-specific dye used) is then calculated using the formula of Godelle et al. (1993). However, the fluorescence intensity found for the base-specific dyes does not correlate with base composition in a linear manner, and dye binding to DNA is influenced by the binding length (minimum number of consecutive complementary bases necessary to bind one dye molecule) and by biased base distribution over the length of DNA molecule (Godelle et al. 1993; Barow and Meister 2002). Therefore, all estimations of AT (or GC content) by flow cytometry should be taken as approximates. Barow and Meister (2002) conducted measurements of genome sizes and base contents for 54 plant species and concluded that there is no general correlation between genome size and AT/GC ratio in higher plants. The latest compilation of base composition values (Meister and Barow 2007) included 215 species. Since then, new estimates were published (Favoreto et al. 2012; Šmarda et al. 2008, 2011) and to date, there are data for about 300 species. These

numbers contrast with the number of data on genome size or DNA ploidy estimates. The main reasons for the scarcity of data may be higher laboriousness of the method (the need to consecutively measure the samples with two different dyes) and the need for internal standard with known C-value and AT/GC ratio, as well as uncertainties for some input parameters (binding length of the dye, nonrandomness of base distribution). Several studies tried to find some correlation between base composition and other biological parameters. For example, Vinogradov (1994) found a high correlation between GC content and genome size in angiosperms. However, other studies did not confirm these results (Cerbah et al. 2001; Ricroch et al. 2005), or even found opposite correlations (Barow and Meister 2002).

6.4 Cell Cycle

Basic information about the distribution of cells over the various phases of cell cycle can be obtained from a single parametric analysis of DNA content. However, there are several drawbacks to this method: cycling cannot be distinguished from quiescent cells, mathematical algorithms must be applied to resolve all major phases and lastly, this method is not amenable for kinetic studies and not suited for disturbed populations. Monoparametric flow cytometric cell cycle analyses were employed, for example, in studies on cell cycle activity and microtubule organization in seeds (Fujikura et al. 1999; Pawlowski et al. 2004), or in roots (Binarová et al. 1993), or to evaluate the effects of various drugs on the cell cycle (Binarová and Doležel 1993; Binarová et al. 1998a, b).

More refined approaches involve simultaneous analysis of DNA content and several key processes or molecules involved in the cell cycle (e.g. DNA synthesis, cyclin-dependent kinases). There are several methods which rely on detection of DNA synthesis, such as incorporation of the thymidine analogue BrdU (bromodeoxyuridine) into newly synthesized DNA and its detection either using fluorescently labelled antibodies against BrdU, or quenching fluorescence of Hoechst-DNA complex. In plants, this approach has been utilized by several groups (Glab et al. 1994; Lucretti et al. 1999; Sgorbati et al. 1991; Yanpaisan et al. 1998) but was not adopted widely, probably due to complicated sample preparation. For a long time, the BrdU-based assay was considered gold standard in flow cytometric cell cycle studies in humans and animals, but recently a new assay (Buck et al. 2008) based on incorporation of the thymidine analogue EdU (5-ethynyl-2′-deoxyuridine) and its detection using click chemistry started to replace it (Diermeier-Daucher et al. 2009; Sun et al. 2012). Kotogány et al. (2010) were the first to verify the feasibility of this new method in plants. They compared the EdU-based assay with traditional BrdU-based assay using cultured cells and root meristems of several plants and concluded that the EdU assay was superior, considerably faster, simpler, did not require digestion of the cell wall nor denaturation of the DNA and can be used in different plant systems.

6.5 Gene Expression

A further attractive but still underutilized application of flow cytometry is the analysis of tissue-specific transcription. Flow-sorted nuclei can be used as a source of transcripts for gene expression analysis in particular tissue types. This approach, developed by Macas et al. (1998), assumes that the transcript levels within the karyoplasm reflect the state of gene expression more accurately than total RNA samples whose majority is comprised of cytoplasmic RNA. For this purpose, intact nuclei are released by a quick tissue homogenization on ice (Galbraith et al. 1983) which preserves transcriptional state in the moment of homogenization. The method utilizes transgenic plants expressing fusion protein, comprising GFP marker and nuclear localization sequence under control of tissue-specific promoter (Zhang et al. 2005). Tissue-specific nuclei can then be sorted based on the GFP signal (Zhang et al. 2008), and RNA is extracted followed by gene expression analysis on microarrays or by next-generation sequencing (Zhang et al. 2008; Macas et al. 1998). This approach overcomes drawbacks of whole-cell sorting, namely, the risk of changing gene expression during protoplast isolation. Flow cytometry was also used to study transcriptional activity in nuclei with different levels of endoreduplication and showed positive correlation of increased transcription and endoreduplication level (Bourdon et al. 2012). This work demonstrated for the first time in plants that endoreduplication correlates with elevated transcriptional activity.

7 Examples for the Application of DNA Flow Cytometry in Plant Research

Information gained on plant nuclei using flow cytometry has been exploited in several fields of plant biology and applied research and industry, including plant taxonomy (e.g. Suda et al. 2007a, Jersáková et al. 2013), ecology (Leitch and Leitch 2012; Herben et al. 2011), evolutionary and population biology (reviewed by Kron et al. 2007), breeding (reviewed by Ochatt 2008; Ochatt et al. 2011) and cell biology (e.g. Binarová et al. 1993, 2000, Petrovská et al. 2012).

7.1 Taxonomy

Until recently, taxonomy has relied on classical morphological approaches. This has changed and molecular methods are being increasingly used. Flow cytometry complements these molecular methods as an invaluable tool and has been contributing significantly to taxonomic research. Genome size and ploidy estimations are important in this regard. Although ploidy studies can be conducted based on

karyological methods, the main advantage of FCM is a possibility to analyse many individuals in a short time and using almost any tissue as sample.

Flow cytometry is ideal to study variation in ploidy levels within the same species (intraspecific cytotype diversity) as it facilities large-scale sampling (cf. Suda et al. 2007b). Ploidy variation can be assessed among the analysed populations, including the occurrence of aneuploidy, and absolute DNA contents can be estimated. For example, analysis of 59 populations of *Cardamine* species in Japan and South Korea revealed large cytotype diversity (Marhold et al. 2010). The patterns of cytotype distribution differed between species, and while some were strictly uniform, containing only one cytotype, multiple cytotypes were present in populations of other species. Thus, *Cardamine yezoensis* was found to comprise six cytotypes ranging from common hexaploids to rare dodecaploids, while *C. amaraeiformis* comprised only one tetraploid cytotype. Similar studies were carried out in *Allium oleraceum* (Šafářová et al. 2011), *Centaurea phrygia* (Koutecký et al. 2012a) and *Odontites vernus* (Koutecký et al. 2012b) where spatial distribution of different cytotypes was observed.

Apart from studying differences in genome copy number, flow cytometry has been used in taxonomic and ecological research on homoploid plants (reviewed in Loureiro et al. 2010). Differences in genome sizes obtained by flow cytometry were useful for distinguishing between subspecies with the same ploidy level, for example, in genus *Festuca* (Loureiro et al. 2007b), *Equisetum* (Obermayer et al. 2002) and *Taraxacum* (Záveský et al. 2005). Flow cytometry was also successful in detection of homoploid hybrids as documented for the genera *Amaranthus* (Jeschke et al. 2003) and *Hieracium* (Morgan-Richards et al. 2004).

7.2 Evolution of Plant Genomes

Evolution of genome size in plants is dynamic and has been accompanied by increases and decreases within lineages (e.g. Leitch et al. 2005). As a consequence, there is at least 2,400-fold variation in genome size among angiosperms. Flow cytometry has become an important tool to study this variation and the underlying mechanisms. According to Bennett and Leitch (2005), the most important components needed to understand the evolution of plant genome sizes include distributional patterns of variation within and among taxa, historical trends that generated current patterns, mechanisms of genome size changes and phenotypic consequences influencing both taxonomic and geographical distribution for the variation of genome size. While the amplification of transposable elements and polyploidization events are the main forces behind increasing genome size, mechanisms of genome reduction are still poorly understood. Data on genome size in different plant groups together with improved phylogenetic knowledge allow us to understand genome size diversity in a phylogenetic context (Leitch et al. 1998). The first group of plants, where genome size values were superimposed onto phylogenetic trees, were the angiosperms (Leitch et al. 1998; Soltis et al. 2003). This allowed to

reconstruct ancestral genome size of angiosperms, which was assumed to be small (1C $\leq$ 1.4 pg). Similar studies followed the suite taking advantage of the ever-increasing data on genome sizes, including other groups of land plants (Leitch et al. 2005; Leitch and Leitch 2013).

7.3 Ecology and Plant Population Biology

Genome size is known to correlate with cell size and length of the cell cycle which in turn affect other physiological and phenotypic traits of plants (Knight and Beaulieu 2008). Therefore, ecological preferences of a species might be predicted based on their genome size. Leitch and Bennett (2007) and more recently Greilhuber and Leitch (2013) discuss interesting implications of genome size on phenotypic traits of plants. For example, Herben et al. (2012) examined variation in genome sizes, several plant traits and regional abundance in more than 400 herbaceous species of the Central European flora and found a weak but significant correlation between genome size and the characteristics of regional abundance for a given species. Similarly, Beaulieu et al. (2007) used data from more than 1,000 species and confirmed correlations between genome size and seed mass. After a comparison of genome sizes of weed and non-weed species, Bennett et al. (1998) concluded that weed species are characterized by small genome sizes. Suda et al. (2010) used genome size as a reliable marker of invasiveness in knotweed species and found that naturalized and invasive plants harbour also significantly smaller genomes than their non-invading relatives. Several studies tried to correlate genome size with climate (temperature, precipitation and length of growing season) connected to altitude and latitude, but the results were not always consistent (Knight et al. 2005). Temsch et al. (2010) and Vidic et al. (2009) studied relationships between heavy-metal soil pollution and genome size of surviving plant species. Both studies concluded that all surviving plants had on average smaller genome sizes.

7.4 Plant Sex and Reproduction

DNA flow cytometry has been used to determine the gender of plants already in early stages of growth and to characterize the mode of reproduction in plants, both of which can influence ecological behaviour. Flow cytometry can be used to distinguish the two genders of dioecious plant species when gender is determined by heteromorphic sex chromosomes. Using high-resolution flow cytometry, Doležel and Göhde (1995) analysed nuclear DNA contents for the two dioecious species *Melandrium album* and *Melandrium rubrum* and were able to discriminate between male and female nuclei based on small differences in DNA amounts. It is possible to reveal this difference very early in the development, and one can even

tell whether the pollen nuclei are male- or female-determining (Stehlik et al. 2007). FCM can thus serve in plant sex determination as an alternative to classical approaches – sex-specific molecular markers or cytology (Stehlik and Barrett 2005).

The use of flow cytometry for testing the mode of reproduction was termed flow cytometric seed screen (FCSS) and to a large extent substituted formerly used approaches (Matzk et al. 2000). Sporophytic or gametophytic mutants in sexual species can be evaluated by flow cytometry to distinguish between purely sexual, obligatory apomictic and facultative apomictic species. Each pathway of seed formation is characterized by different combination of DNA amounts in embryo and endosperm (Matzk et al. 2000), which can be estimated using FCM. Sexually raised seeds show ratios of embryo to endosperm DNA of 2C:3C, while apomictic seeds show 2C:4C. In case of facultative apomixis, more complicated histograms comprising several peaks representing different C-values are observed. Diploid nuclei isolated from leaf tissue are usually used as a standard for these measurements. The method remains popular and has been improved by mathematically estimating the male and female genomic contributions to the embryo and endosperm independent of the mode of gametophyte formation and ploidy of parental plants (Dobeš et al. 2013).

7.5 Cell Biology

One of the popular applications of DNA flow cytometry in cell biology is the analysis of cell cycle activity and dynamics in heterogeneous plant cell suspensions (Yanpaisan et al. 1998; Lee et al. 2004) and the cellular response to different stresses or cell cycle inhibitors. Glab et al. (1994) analysed effects of olomoucine, an inhibitor of cdc2/cdk2 kinases activity, on plant cells and found that this compound blocked cell cycle transitions at G_1 to S and G_2 to M phases. Other studies investigated the effects of oxidative stress and drought on plants and used flow cytometry to determine the cell cycle phases which were the most affected (Reichheld et al. 1999; Bagniewska-Zadworna 2008). Flow cytometric analysis was also the critical tool to verify the function of plant Aurora kinases that participate in the switch from meristematic cell proliferation to differentiation and endoreduplication (Petrovská et al. 2012). Similarly, the function of nitrilase 1 plant homologues in the regulation of cell cycle exit was studied using flow cytometry (Doskočilová et al. 2013). Combination of flow cytometry with immunofluorescence helped to understand the role of several important cytoskeletal proteins in cell division (Pawlowski et al. 2004; Binarová et al. 1993, 2000; Fujikura et al. 1999).

Also the analysis of cell and tissue differentiation in connection with endopolyploidy was advanced by flow cytometry. In order to get insight into the incidence of endopolyploid cells, Barrow and Meister (2003) probed for differences in ploidy status at organ and tissue levels. Zhang et al. (2005) used *Arabidopsis thaliana* roots as a model and confirmed, using simultaneous analysis of cell-specific transgenic

reporter lines and DNA content measurements, that different cell types differ in nuclear ploidy. The biological significance of endopolyploidy is not clear, although it has been linked to cell expansion (Cookson et al. 2006, Jovtchev et al. 2006, Gendreau et al. 1998, Melaragno et al. 1993), metabolic activity (Larkins et al. 2001, Vilhar et al. 2002), fruit size (Sugimoto-Shirasu et al. 2003) and the response abiotic stress (Ceccarelli et al. 2006). Cheniclet et al. (2005) studied variability of tomato fruit size and demonstrated a correlation of increased ploidy levels with the size of pericarp cells. Subsequently, Bourdon et al. (2011) sorted nuclei from tomato pericarp tissues and used them as template for BAC-FISH. Using this method, they were able to establish a ploidy map of the tomato fruit pericarp in intact tissues. The extent of endoreduplication was also used by Rewers and Śliwinska (2012) as a marker for seed developmental stages in five species of Fabaceae. Bainard et al. (2011b) analysed 37 species from 16 angiosperms families and found that mycorrhizal symbiosis positively stimulates endoreduplication.

7.6 Plant Breeding

FCM has been used extensively in plant breeding. According to Eeckhaut et al. (2005), the most important applications of flow cytometry in this field are the identification and characterization of parent plants suitable for breeding programmes, screening of the offspring and determination of ploidy levels after haploidization and/or polyploidization. To characterize of parent plants, there are many germplasm collections from which suitable candidates for breeding can be chosen. Screening for ploidy levels and genome sizes of different accessions using flow cytometry is a useful tool, and this procedure has been used for many species, e.g. bananas (Nsabimana and van Staden 2006; Pilay et al. 2006; Doleželová et al. 2005), agave (Palomino et al. 2003), grass pea (Ochatt et al. 2001) and water yam (Egesi et al. 2002). In some breeding programmes it might be of agronomic importance to screen for genotypes of specific gender (e.g. in asparagus; Ozaki et al. 1998). In plants where gender is determined by heteromorphic sex chromosomes differing in either size or base pair content, flow cytometry can discriminate between male and female plants based on their slightly different DNA content or AT/GC ratios, as successfully shown for White campion (Doležel and Göhde 1995) and date palm (Siljak-Yakovlev et al. 1996).

DNA flow cytometry has also been useful to characterize the progeny from interspecific hybridizations. The method facilitated identification of interspecific hybrids based on intermediate DNA contents in onion (Keller et al. 1996) and coffee (Barre et al. 1998). Triploidy is often connected with low fertility and seedlessness, which is a much sought character in a number of crops. In hops, seedless triploids are sought after for their better brewing qualities (Beatson et al. 2003). In citruses, triploid plants are produced after diploid x tetraploid hybridization (Aleza et al. 2012). In these cases, flow cytometry is an ideal tool to verify ploidy as it can quickly and efficiently screen large numbers of plants.

Doubled haploid (dihaploid) plants are useful in breeding to shorten breeding cycles. As a common practice dihaploids are generated by polyploidizing haploids produced in vitro from immature anthers. However, plants may regenerate not only from immature pollen but also from diploid somatic anther tissues. Flow cytometry is an effective tool to select haploid regenerants, as shown, for example, in chickpea (Grewal et al. 2009). Alternatively microspores can be cultured in vitro to produce haploids, and also in this case flow cytometry is invaluable to confirm the haploid status of regenerated plants (Weber et al. 2005). Some breeding programmes require development of autopolyploids, which are bigger and more robust compared to their diploid parents and have bigger flowers and fruits. Autopolyploids are also needed to produce triploids after crossing with diploids. Polyploidization is typically induced by exposing multicellular explants in vitro to mitotic spindle poisons. As some cells in the explant are not cycling and hence are not polyploidized, regenerated plants may be diploid, mixoploid (chimaeric) or tetraploid. Identification of solid tetraploids during early stages is best achieved using DNA flow cytometry (Awoleye et al. 1994, van Duren et al. 1996). The avoidance of chimaeras may be a problem, and Roux et al. (2001) used flow cytometry to follow dissociation of chimaeras after repeated cycles of in vitro propagation.

Rapid propagation of new and superior genotypes is often needed and micropropagation in vitro is often the method of choice (see also the chapter by Opatrný, this volume). Micropropagation has been applied without proper genetic verification in many commercial operations to supply planting material to producers and farmers. However, depending on the source of the material (e.g. shoot tip, embryo culture, callus culture), the culture may be prone to genetic instability, also called somaclonal variation (Neelakandan and Wang 2012), leading to high variability in culture-derived individuals. Deviations among the regenerants and the frequency of these aberrations have been assessed by various methods, and DNA flow cytometry enabled identification of plants differing in ploidy (e.g. Zhao et al. 2012). For example, genome stability of six medicinal plants that were propagated in vitro for a year was assessed by Sliwinska and Thiem (2007), and true-to-typeness of zygotic embryos, somatic embryos and somatic embryogenesis-derived plantlets of *Pinus* was verified to exclude major changes in ploidy level in this economically significant species (Marum et al. 2009). Screening for culture-induced variation is of enormous significance in forest trees and woody plants, as they have long life cycles (Rani and Raina 2000; Loureiro et al. 2005). When hundreds of accessions have to be screened, the speed of FCM analysis becomes the major advantage. It took some time to screen ploidy in the world's largest banana germplasm collection located in Leuven (Belgium), comprising more than a thousand accessions maintained in vitro under slow growth conditions (Doleželová et al. 2005), but no other method could achieve this goal so efficiently.

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Photo-Convertible Reporters for Selective Visualization of Subcellular Events and Interactions

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Abstract Photo-convertible fluorescent proteins are a recent addition to the cell biological tool box available to plant biologists. Here, we provide a brief overview of the use of green-to-red photo-convertible proteins such as monomeric EosFP, Kaede and Dendra2 for understanding subcellular behaviour in plants. A general description of the differential colouring technique using mEosFP for discriminating within an organelle population and tracking interactions between similar organelles is provided. This review aims to provide baseline standards and guidelines for pursuing studies using these novel proteins.

1 Introduction

Plant cells are encased within a thick wall that protects them from the myriad abiotic and biotic stresses in the environment. However, the presence of the cell wall has long hindered the visualization of the subcellular environment of the plant cell. Historically, the study of subcellular structures in plant cells has been carried out through tissue maceration and sectioning on chemically fixed and dead samples. Though effective, these techniques prevent the observer from gaining an appreciation of the amazingly dynamic nature of the living plant cell. This can be counteracted to some extent by the use of light microscopy and differential contrast methods. Usually the only organelles that can be clearly distinguished with these techniques are the bulky nucleus and organelles such as plastids and vacuoles that might display a natural pigmentation. Other organelles blend together within the cytoplasm to form a strikingly motile but otherwise indistinguishable mass. Thus, the trade-off for live cell visualization using incident light is an inability to recognize individual organelles.

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The development of vital dyes and stains went a long way towards enhancing our understanding of cell dynamics (Fricker et al. 2001; Swanson et al. 2011; Lichtscheidl and Url 1990), but very often these dyes are toxic and allow only a short time period for observation. The cloning of the *g*reen *f*luorescent *p*rotein (GFP) and its subsequent expression in plant cells truly revolutionized the field of cell biology (Prasher et al. 1992; Haseloff et al. 1997). Single-coloured fluorescent proteins (FPs), which are now available in colours that span the entire visible spectrum, allow the selective highlighting of proteins and organelles (Giepmans et al. 2006). They have proven to be an indispensible tool for studying organelle movement, interactions and response to stimuli, as well as for tracking protein expression, localization and diffusion (Hawes et al. 2001). As of 2007, probes have been available that highlight almost every subcellular structure in the plant cell, often with several hues of FP from which to choose (Mathur 2007). The targeted fluorescent protein probes can be readily observed using epifluorescence, confocal laser scanning or multiphoton microscopes. When combined with the ease of cloning, simple methods of introducing fluorescent protein genes into plant cells and their tolerance by the living cell (Wroblewski et al. 2005; Clough and Bent 1998), fluorescent protein fusions have achieved the status of routine tools for investigating the functions of newly discovered genes and for observing organelles.

Despite their usefulness and prominence in recent studies, conventional fluorescent proteins such as GFP have certain limitations when it comes to studying many of the more dynamic cellular processes. A novel class of fluorescent proteins, the photo-convertible fluorescent proteins, can overcome many of these limitations due to their inherent ability to alter their colour or fluorescence intensity.

2 Photo-Convertible Fluorescent Proteins: Advantages and Characteristics

Photo-convertible proteins, also known as ‘optical highlighters’, are often separated into three categories: photo-activatable, photo-switchable and photo-convertible. Photo-activatable proteins are those capable of going from minimally to brightly fluorescent state in response to a specific wavelength of light. Photo-convertible and photo-switchable fluorescent proteins both shift their fluorescence emission in response to a trigger, but differ in that photo-convertible FPs do so irreversibly while photo-switchable are capable of back and forth conversion (Ai et al. 2006; Shaner et al. 2007; Wiedenmann et al. 2009). Here, we use the term photo-convertible to span all three categories, describing all those fluorescent proteins (FPs) that are capable of changing their fluorescent properties in response to a specific trigger, most often a specific wavelength of light.

2.1 Why Do We Need Photo-Convertible FPs?

Though conventional single-coloured fluorescent proteins have proven to be excellent tools for visualizing subcellular behaviour, they have limitations that preclude or diminish their usefulness in some situations. Organelles that have been highlighted by fluorescent proteins can be seen to flit about the cell in all directions with widely varying velocities. This can be easily observed with single-coloured FPs. However, in a homogeneous population of rapidly moving organelles, keeping track of a single one is a near impossible task. When being tracked using epi-fluorescent microscopy, two or more organelles can easily come to occupy the same position along the x, y axes but at different z-axis depths for a brief period of time. When these organelles separate, one has no way of conclusively determining which is the singular organelle originally being tracked. Additionally, when time-lapse videos are being recorded the issue of organelles moving in and out of the range of focus is compounded by the time between frames, during which an organelle can move a considerable distance.

Single-coloured FPs also have limitations when studying extremely dynamic structures such as the actin cytoskeleton or the endomembrane system. They do not allow for the observation of rapid and often transient changes in specific regions of these systems, which can take the form of changes in organization, protein movement or interaction with other organelles. In the latter case, interactions can be difficult to conclusively demonstrate as most forms of light microscopy lack the resolution to use the terms contact or touch to describe adjacent organelles. The Abbe limit prevents the resolution of distinct objects separated by less than half the wavelength of visualization (Heintzmann and Ficz 2006), which typically translates to an inability to resolve spaces of less than 200–300 nm. Fluorescent molecules can also create a 'blooming effect' that further inhibits resolution, as especially with epifluorescence microscopy, out of focus or overlapping organelles can produce a halo that extends the apparent diameter of an organelle beyond its actual size (Heintzmann and Ficz 2006; Waters 2009). The ability to show that an association between two organelles was sustained over time would support the idea of an interaction even if direct contact cannot be concluded, but the issues of organelle tracking mentioned before come into play. Several research groups have successfully employed strategies that involved the use of two or more distinct FPs to highlight multiple organelle varieties (Dhonukshe et al. 2005; Mathur et al. 2002; Sampathkumar et al. 2011), but when dealing with interactions among an organelle population the use of more than one FP is not possible.

Similar difficulties are encountered when using single-coloured FPs to study protein trafficking or diffusion, long-term plant development or when attempting to establish the continuity or independence of organelles. Photo-convertible FPs provide a solution to many of these limitations as, among other possibilities, they give the opportunity to single out one organelle among hundreds of near-identical companions by converting it to a differently coloured form.

2.2 The Photo-Convertible FP Spectrum

Since the creation of a photo-activatable GFP (PA-GFP) (Patterson and Lippincott-Schwartz 2002), many FPs have been found that change their fluorescent properties in response to a specific trigger. They can be broadly characterized by whether they are initially fluorescent or nonfluorescent. A summary of many of the available photo-convertible FPs can be found in Table 1. PA-GFP is one of the most commonly used photo-activatable FPs and converts irreversibly from an initially nonfluorescent form to a highly fluorescent green form upon irradiation with violet light. It has found applications in tracking protein dynamics (Runions et al. 2005), but the inability to visualize it prior to conversion makes it difficult to identify cells expressing the protein, as well as to visualize specific organelles for targeting photo-activation without the use of a secondary probe. Photo-activatable proteins also exist in red fluorescent forms and include PA-mRFP1, PA-TagRFP and PA-mCherry1 (Verkhusha and Sorkin 2005; Subach et al. 2009, 2010), but they have not been used in plant cells to date (Lukyanov et al. 2005).

Photo-switchable proteins are capable of back and forth conversion between a nonfluorescent and fluorescent state or between two different hues. These include IrisFP, Dronpa, Kindling FP1 and rsCherry fluorescent proteins (Habuchi et al. 2005; Chudakov et al. 2003; Shaner et al. 2007; Stiel et al. 2008). RsCherry is available in two forms, one that is initially red fluorescent and can be dimmed and another that is initially nonfluorescent and can be switched on. As they have not frequently been used in plants, and have been well reviewed elsewhere (Shaner et al. 2007; Wiedenmann et al. 2009), the focus here will be placed on photo-convertible FPs for whom the conversion is irreversible.

The most commonly used set of photo-convertible FPs in plants are those that irreversibly convert from one colour to another. These are primarily green-to-red photo-convertible, and the family consists of mClavGR2, Kaede, KikGR, Dendra2 and Eos, as well as their derivatives (Shaner et al. 2007). Other photo-convertible proteins possess different colour spectrums and include the cyan-to-green photo-switchable CFP (Chudakov et al. 2004) and the orange-to-far-red PSmOrange (Subach et al. 2011).

Kaede (Ando et al. 2002) has been targeted in plants to mitochondria, peroxisomes, plastids and Golgi bodies (Arimura et al. 2004; Brown et al. 2010). It is important to take note of the fact that Kaede is a large tetrameric protein whose expression can result in aggregates of sufficient size that they may be confused for small organelles. Additionally, although it has been used for the creation of a protein fusion, it is thought that its large size and tetrameric tendencies could interfere with proper function of its fusion partner (Brown et al. 2010), so care must be taken when using it for this purpose. KikGR was engineered from the green fluorescent kikG using knowledge of Kaede's conversion mechanism (Tsutsui et al. 2005). The original KikGR is also a tetrameric protein, but a monomeric derivative, mKikGR, has since been created (Habuchi et al. 2008). Both KikGR and mKikGR have primarily been used in mammalian systems to date.

Table 1 Characterized photo-convertible fluorescent proteins

Protein	Emission λ (nm) before conversion	Trigger	Emission λ (nm) after conversion	Reversible	Structure	Reference
Irreversibly photo-convertible probes						
EosFP	Green, 516 nm	390 nm	Red, 581 nm	No	Tetramer	Wiedenmann et al. 2004
Kaede	Green, 518 nm	350–400 nm	Red, 582 nm	No	Tetramer	Ando et al. 2002
kikumeGR	Green, 517 nm	350–420 nm	Red, 593 nm	No	Tetramer	Tsutsui et al. 2005
d1EosFP and d2EosFP	Green, 516 nm	390 nm	Red, 581 nm	No	Dimer	Wiedenmann et al. 2004
mEos2	Green, 519 nm	390 nm	Red, 584 nm	No	Monomer Some dimeric tendency	McKinney et al. 2009
mEosFP	Green, 516 nm	390 nm	Red, 581 nm	No	Monomer	Wiedenmann et al. 2004
Dendra	Green, 505 nm	405 nm or intense 488 nm	Red, 575 nm	No	Monomer	Gurskaya et al. 2006
mKikGR	Green, 515 nm	405 nm	Red, 591 nm	No	Monomer	Habuchi et al. 2008
mClavGR2	Green, 504 nm	405 nm	Red, 583 nm	No	Monomer	Hoi et al. 2010
mMaple	Green, 505 nm	405 nm	Red, 583 nm	No	Monomer	McEvoy et al. 2012
Photo-switchable CFP	Cyan, 468 nm	405 nm	Green, 511 nm	No	Monomer	Chudakov et al. 2004
PSmOrange	Orange, 565 nm	540 nm	Far red, 662 nm	No	Monomer	Subach et al. 2011
Photo-activatable GFP (PA-GFP)	Dim to nonfluorescent	413 nm (~400 nm)	Bright green, 517 nm	No	Monomer	Patterson and Lippincott-Schwartz 2002
Photo-activatable mRFP1	Dim to nonfluorescent	365 nm	Red, 605 nm	No	Monomer	Verkhusha and Sorkin 2005
PA-TagRFP	Dim to nonfluorescent	370–410 nm	Red, 595 nm	No	Monomer	Subach et al. 2010
PA-mCherry1	Dim to nonfluorescent	405 nm	Red, 595 nm	No	Monomer	Subach et al. 2009
PA-mKate	Dim to nonfluorescent	405 nm	Far red, 628 nm	No	Monomer	Gunewardene et al. 2011
KillerRed	Red, 610 nm	540–580 nm	Produces ROS, kills host cell	No	Dimer	Bulina et al. 2005

(continued)

Table 1 (continued)

Protein	Emission λ (nm) before conversion	Trigger	Emission λ (nm) after conversion	Reversible	Structure	Reference
Reversibly photo-convertible probes						
Dronpa	Dim to nonfluorescent	405 nm to green; 488 nm to dim	Green, 518 nm	Yes	Monomer	Ando et al. 2004
Padron	Dim to nonfluorescent	488 nm to green; 405 nm to dim	Green, 522 nm	Yes	Monomer	Andresen et al. 2008
IrisFP[a]	Dim to nonfluorescent green IrisFP	405 nm to green; 488 nm to dim	Bright green, 516 nm	Yes	Tetramer	Adam et al. 2008
	Bright green, 516 nm	405 nm	Bright red, 580 nm	No		
	Bright red, 580 nm	532 nm to dim; 440 nm to red	Dim to nonfluorescent red IrisFP	Yes		
mIrisFP[a]	Dim to nonfluorescent green IrisFP	405 nm to green; 488 nm to dim	Bright green, 516 nm	Yes	Monomer	Fuchs et al. 2010
	Bright green, 516 nm	405 nm	Bright red, 578 nm	No		
	Bright red, 578 nm	561 nm to dim; 473 nm to red	Dim to nonfluorescent red IrisFP	Yes		
rsCherry	Dim to nonfluorescent	550 nm to red; 450 nm to dim	Red, 610 nm	Yes	Monomer	Stiel et al. 2008
rsCherryRev	Red, 608 nm	450 nm to red; 550 nm to dim	Dim to nonfluorescent	Yes	Monomer	
Kindling FP1 (KFP1-Red)	Dim to nonfluorescent	532 nm high intensity	Red, 600 nm	No	Tetramer	Chudakov et al. 2003
	Dim to nonfluorescent	532 nm, low intensity to red; 458 nm to dim	Red, 600 nm	Yes		
Select probes with non-light-induced conversion						
Flash-Pericam	Dim green, 514 nm	Ca^{2+}	Bright green, 514 nm	Yes	Monomer	Nagai et al. 2001
Inverse-Pericam	Bright green, 516 nm	Ca^{2+}	Dim green, 516 nm	Yes	Monomer	

Ratiometric-Pericam	Dim green, 517 nm	Ca^{2+}	Bright green, 511–517 nm	Yes	Monomer	
Yellow Cameleon 2.1 and 3.1	Cyan, 476 nm	Ca^{2+}	Bright yellow green, 528 nm	Yes	Heterodimer	Miyawaki et al. 1999
HyPer-GFP	Dim green, 516 nm	H_2O_2 or white light	Bright green, 516 nm	Yes	Monomer	Belousov et al. 2006
CaspeR3-GR	Green, 505 nm	Caspase-3-mediated apoptosis	Red, 584 nm	No	Monomer	Shcherbo et al. 2009

[a]IrisFP switches irreversibly from green to red but can also switch reversibly between 'on' and 'off' states of either green or red

Dendra2 has a couple of advantages over the tetrameric Kaede. It is monomeric and thus better suited to protein expression studies, and it has been promoted as being photo-convertible with the more commonly available 488 nm Argon laser rather than the 405 nm laser recommended for other green-red photo-convertible FPs (Gurskaya et al. 2006). Dendra2 requires prolonged exposure, often up to several minutes, and additionally requires an intensity of light much above that of normal imaging (Gurskaya et al. 2006; Koizumi et al. 2011). This prolonged exposure can cause damage to the plant cell, including the bleaching of chlorophyll, indicating that photo-conversion may compromise normal cellular activity. It has been found by several groups that Dendra2 expresses at a low level in plant cells, but the reason for this has not yet been determined. Despite its limitations, it has been successfully used to study transcription factors in plant cells (Koizumi et al. 2011).

The Eos fluorescent protein, which naturally occurs as a tetramer, has been engineered to create a bright green monomeric form that converts to a red fluorescent state upon irradiation with a 405 nm centred violet-blue light (Fig. 1a; Wiedenmann et al. 2004). Though mEosFP does not mature well at higher temperatures and is thus not considered appropriate for mammalian studies (McKinney et al. 2009), it has proven to be an excellent reporter for the plant system and is currently the most targeted photo-convertible FP in plant cells (Mathur et al. 2010). Methods for the quantification of colour change in green-to-red photo-convertible probes, as well as techniques for the use of mEosFP in plants, have been well described (Schenkel et al. 2008; Mathur et al. 2010, 2012).

Photo-convertible FPs also have the advantage of being able to work in tandem with a wide variety of single-coloured FPs. Due to their ability to switch between two colours, most notably green and red, they can often be used in conjunction with cyan, green, red or yellow fluorescent proteins. This enhanced flexibility allows them to be incorporated quite easily within a laboratory's existing fluorescent protein toolbox. Table 2 summarizes the organelles that have been highlighted in plants to date using photo-convertible FPs.

2.3 Basic Properties of Photo-Convertible FPs

Fluorescent proteins get their fluorescent capabilities from a chromophore within their structure. The basic chromophore structure remains fairly constant between fluorescent proteins from many naturally occurring sources, but minor alterations, either artificial or due to differences among species, are what have given rise to the wide range of fluorescent colours available today (Wiedenmann et al. 2009).

Kaede, Dendra2 and mEosFP all share a His-Tyr-Gly tripeptide chromophore that gives them their initial green fluorescence as well as their photo-convertibility (Shaner et al. 2007). The photo-conversion of these proteins results from light-induced cleavage of the protein backbone and is thus irreversible (Shaner et al. 2007; Wiedenmann et al. 2004). Photo-switchable proteins in contrast

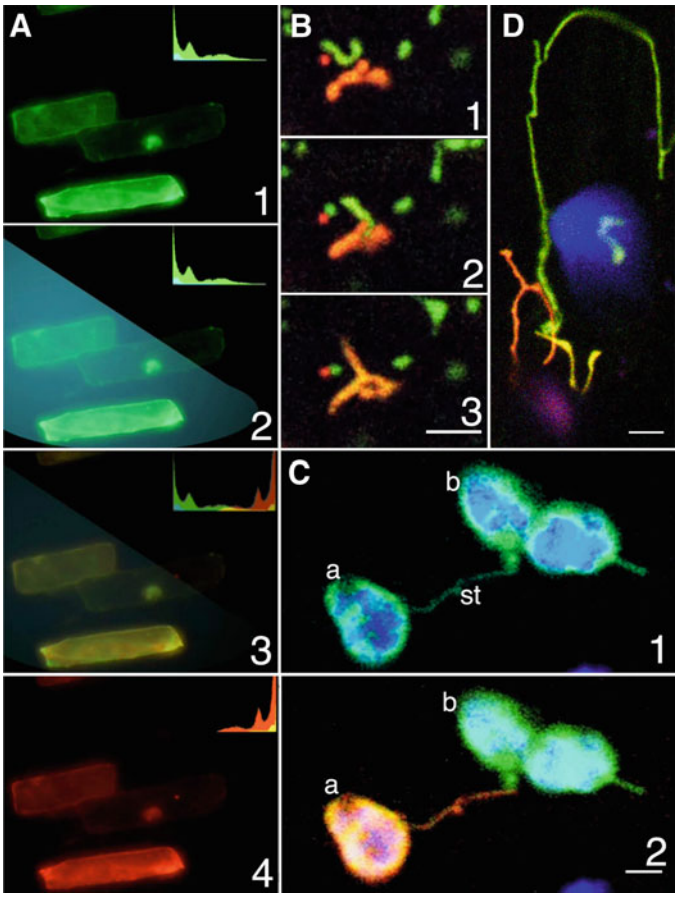

Fig. 1 The use and colours of the green-to-red photo-convertible mEos fluorescent protein. (**a**) Panels 1 to 4 show onion epidermal cells expressing cytosolic mEosFP to provide a semi-diagrammatic depiction of the different colours and derivative colour histograms that are observed due to different states of the protein. Panel 1 shows the green pre-photo-converted form; 2 depicts the general process of photo-conversion using violet-blue epi-fluorescent lighting for 3–10 s. Panel 3 represents an intermediate yellow-orange colour that is produced through partial photo-conversion and a mixing of green and red forms, whereas panel 4 shows the fully photo-converted red form of mEosFP. (**b**) Differential colouring is useful for observing the behaviour of similar organelles and is illustrated by mitochondrial fusion shown here in panels 1 to 3. Green and red mitochondrial subpopulations have been created (panel 1) through photo-conversion of a few mitochondria. Two interacting mitochondria are observed in panel 2 and ultimately fuse to produce one long mitochondrion with an altered morphology and an intermediate orange colour (size bar = 2.5 μm). (**c**) Differential colouring can also be used to conclude organelle independence as seen in panels 1 and 2. Panel 1 with single green colour fluorescence suggests that plastid 'a' and 'b' might be interconnected via a stromule (st). Panel B, however, clearly differentiates between the two plastids and shows that their fluorescent protein-labelled stromal contents do not get exchanged (size bar = 2.5 μm). (**d**) A single image of a leaf cell in the *Arabidopsis arc6* mutant transformed with tpFNR:mEosFP shows the representative colours that can be observed in a three-channel (RGB) scan using a confocal laser scanning microscope. Green being the non-photo-converted form; red, the photo-converted form; and yellow, the partially

Table 2 Published targeted photo-convertible FP probes for use in plants

Name	Target	Targeting sequence	Reference
Monomeric Eos-based probes			
mEosFP-cytosolic	Cytosol	None	Mathur et al. 2010
mEosFP:PIP1	Plasma membrane	At3g1430 CDS	Mathur et al. 2010
mEosFP:aTIP1	Tonoplast	At1g73190 CDS	Mathur et al. 2010
CX:mEosFP	ER membrane	At5g61790 membrane-targeting domain of Calnexin1	Mathur et al. 2010
Mito-mEosFP	Mitochondria	*N. plumbaginifolia* ATP2-1 N-terminal 261 bp	Mathur et al. 2010
mEosFP-2xFYVE	Endosomes/MVB/PVC	FYVE domain from HGF-regulated tyrosine kinase substrate protein	Mathur et al. 2010
mEosFP:GONST1	Golgi bodies	At2g13650 CDS	Mathur et al. 2010
mEosFP-PTS1	Peroxisomes	PTS1 C-terminal tripeptide SKL	Mathur et al. 2010
mEosFP:MBD-MAP4	Microtubules	Mammalian MAP-4 microtubule-binding domain	Mathur et al. 2010
LIFEACT:mEosFP	F-actin	17 aa of yeast Abp140p	Mathur et al. 2010
mEos:FABD-mTn	F-actin	F-actin-binding domain of mammalian Talin	Schenkel et al. 2008
FNR:mEosFP	Plastid stroma	Ferredoxin NADPH oxidoreductase	Schattat et al. 2012
H2B:mEosFP	Histone (nuclei)	At3g45980 CDS	Wozny et al. 2012
PIN2-mEosFP	Plasma membrane receptors, endosomes	PIN2 auxin efflux carrier	Dhonukshe et al. 2007
PIP2-mEosFP	Plasma membrane	PIP2 water channel	Dhonukshe et al. 2007
SHR-tdEosFP	Cytosol and nucleus	At4g37650	Wu et al. 2011
Kaede-based probes			
Kaede-Mito	Mitochondria	ATPase ∂ subunit	Arimura et al. 2004
Kaede-Peroxi	Peroxisomes	C-terminal tripeptide SKL – PTS1	Arimura et al. 2004
Kaede:Plastid	Plastid stroma	N-terminus of chloroplast ribosomal protein	Arimura et al. 2004
CardA-Kaede	Vacuole	Cardiosin A	Brown et al. 2010

(continued)

Fig. 1 (continued) photo-converted form of mEosFP. The vivid blue has been created by false colour allocation to chlorophyll, whereas a pink colour (*bottom*) is created through overlap of blue chlorophyll and the red form of mEosFP (size bar = 5 μm)

Table 2 (continued)

Name	Target	Targeting sequence	Reference
ST-Kaede	Golgi bodies	Transmembrane domain of rat sialyltransferase	Brown et al. 2010
Kaede-ABCB4	Plasma membrane and endosomes	ATP-binding cassette B4 (auxin efflux transporter)	Cho et al. 2012
Dendra-based probes			
D2-cytosolic	Cytosol	None	Wu et al. 2011
CPC-D2	Nucleus	At2g46410	Wu et al. 2011
At4g00940-D2	Cytosol	At4g00940	Wu et al. 2011
SHR-NL-D2	Cytosol	At4g37650	Wu et al. 2011
Photo-activatable GFP-based probes			
CX:PA-GFP	ER membrane	At5g61790 membrane-targeting domain of Calnexin1	Runions et al. 2005

typically gain their conversion abilities from reversible isomerization (Wiedenmann et al. 2009).

2.4 Photo-Convertible Proteins for Super-Resolution Microscopy

The inability of conventional light microscopy to resolve structures or spaces of less than 200 nm in the x-y direction or 500 nm in the z direction limits the biologists' ability to properly image nanometre scale structures such as membranes, cytoskeletal elements and the sub-organellar structure of mitochondria and chloroplasts (Sengupta et al. 2012). The field of super-resolution microscopy has been developed to help fill this gap in visualization and has the ability to resolve structures of 10 nm or less (Henriques et al. 2011). The mechanism of image acquisition however currently limits its ability to create fast-scale time-lapse reconstructions of the cell, though the rapid development of the field may make this a possibility in the future (Henriques et al. 2011). Indeed, techniques and fluorescent probes are already being generated that could allow time-lapse images to be taken with frame rates of 10s or less depending on the size of the region being imaged, though some loss of resolution is currently the trade-off (Grotjohann et al. 2012).

The general principle of single-molecule-based super-resolution approaches relies on the stepwise localization of individual fluorescent molecules through computational techniques. These molecules can be either fluorescent dyes or genetically encoded fluorescent proteins but regardless require that they have photo-activatable, photo-switchable or photo-convertible properties. In each image, a number of fluorophores are selectively activated, recorded and then inactivated. In theory, this results in a sequence of images, each highlighting a different subset of the fluorescent molecules that when processed and combined produce a high-resolution image of the structure in which the FP is localized. It is

this requirement for many images per frame that can limit acquisition times and has to date limited its use to live imaging of slowly moving structures or to the study of the movement of individual molecules (Henriques et al. 2011; Sengupta et al. 2012). Genetically encoded fluorescent probes have advantages over the available dyes, as the latter typically require buffers that can be cytotoxic (Henriques et al. 2011). A variety of fluorescent proteins have been used for this technique to date and include mEos2, PA-mCherry, PA-mKate, tdEosFP, Dronpa and PS-CFP2 (McKinney et al. 2009; Gunewardene et al. 2011; Shroff et al. 2007). The availability of probes with distinct emission spectra, especially those in the red-to-far-red range, allows for the super-resolution co-visualization of two or more molecular species (Gunewardene et al. 2011).

3 Photo-Convertible FPs for Visualizing Organelle Dynamics

The organelles in the cell interior, as well as their membranes and their contents, are constantly in motion. This motion is easily seen with standard fluorescent proteins but is often difficult to track, monitor or quantify. The colour-changing ability of photo-convertible FPs opens up a whole new world of opportunities for studying organelle dynamics, as they allow the selective targeting of both individual organelles and specific regions within them. Through this selective colouring, one can observe and track the movement of organelles within the cells as well as the movement of organellar contents or components.

3.1 Tracking Organelle Movement and Dynamics

Organelles such as the cytoskeleton and the endomembrane system undergo constant transient alterations that can be difficult to study and quantify. The endomembrane system in particular is extremely variable both in terms of shape, movement patterns and membrane dynamics. It was shown with mEosFP probes labelling endosome, pre-vacuolar and vacuolar membranes that membrane fusion could be observed between photo-converted and non-photo-converted probes (Mathur et al. 2010), indicating the usefulness of photo-convertible FPs in studying membrane fusion events.

In addition to the study of membrane fusion, photo-convertible FPs can provide a novel method for studying protein flow within an organelle membrane. The endoplasmic reticulum (ER) is a large organelle that stretches throughout the cell. It can take many forms, including cortical mesh-like ER, spindle-shaped ER bodies and flattened sheetlike cisternae. These various structures are constantly rearranging, and their membranes are always in flux within a healthy cell. Mathur et al. (2010)

created a CX:mEosFP fusion protein that targeted to the ER membrane. After photo-conversion of a region of the ER, the red colour became diluted over time as green protein moved back into the area, and red protein spread out. As the photo-conversion of mEosFP, as well as many other photo-convertible probes, is irreversible, the dispersal of the red colour indicates the flow of the membrane rather than a reversion to the green form. By analyzing the ratio of green to red over time, it is thus possible to use a photo-convertible FP to track membrane flow. This provides a quantifiable mechanism of measuring the rate and direction of movement in a membrane in response to various stimuli, as was demonstrated by the significantly slower dispersal of the red colour in response to inhibitors of ER motility (Mathur et al. 2010). The same concept has previously been demonstrated using PA-GFP in plants (Runions et al. 2005); however, mEosFP and other green-red FPs provide the advantage of being able to see the entirety of the organelle highlighted in green as well as the highlighted red region, while PA-GFP is initially nonfluorescent. A similar methodology was applied to the study of filamentous actin, a component of the cytoskeleton. Actin harbours polarity that is indicated by its direction of extension, and it was demonstrated that once a region was photo-converted, the red fluorescence spread out in the direction of filament extension (Mathur et al. 2010).

Thus far, the usefulness of mEosFP in studying organelles that have a dynamic membrane or structure has become apparent, but its use also extends to studying the movement of more discrete organelles, such as vesicles, mitochondria, peroxisomes and chloroplasts. In a population of highlighted organelles that all possess similar shapes and sizes, it can be almost impossible to track a single or group of organelles. This is especially apparent when they are moving rapidly throughout the cell and often in and out of the plane of focus. By allowing the selective colouring of individual organelles, photo-convertible FPs provide a tool for identifying either a single organelle or a subset of them, and the irreversibility of the colour change allows for them to be tracked over an extended time period. In a study of mEosFP-labelled endosomes, it was noticed that a subset of the vesicles displayed abnormal movement characteristics. When these were specifically photo-converted and tracked over time, they were seen to have a very distinctive behaviour. These vesicles could elongate into tubules, which would entangle and eventually fuse with other vesicles, and they represented a subpopulation of vesicles not seen previously. The tubules have since been considered to represent autophagosomes that encircle other endosomal vesicle (Mathur unpublished). Without the ability to track an organelle of interest, the connection between the abnormally moving vesicles and the tubules would likely not have been made (Mathur et al. 2010).

3.2 Organelle Interactions

When observing a plant cell, the sense that the organelles within it are interacting is readily obtained. It is often difficult, however, to determine whether interaction is actually occurring. In many studies, fluorescence overlap has been given as

evidence of interaction between organelles. This overlap can be between two similarly coloured organelles or result in the formation of an intermediate colour in regions where the fluorescent signals of two differently highlighted organelles meet. In many cases, samples have been pressed in order to increase image quality, which can bring together objects dispersed through the z-axis and give the appearance of overlap. As z-axis resolution information is rarely provided, it is often assumed that the two organelles are on the same plane when interaction is concluded. It is thus important to take great care when assessing interactions based on overlap and to ensure that the conclusions are well supported with other lines of evidence.

Prolonged association between two or more organelles can support the idea that an interaction is occurring. In some studies, the association between organelles is shown only through snapshots that show close apposition, but in many of these cases prolonged observation would have shown that they were simply briefly passing each other by as they streamed around the cell. Time-lapse imaging is thus necessary to demonstrate that the association continues over time. For the study of different organelles, this can be readily accomplished by labelling with differently coloured fluorescent probes, but in a single-coloured group of organelles the same issue arises as in organelle tracking: one cannot be confident that the same organelles are being viewed throughout the period of observation. Photo-convertible proteins are thus extremely useful in showing prolonged association between organelles as they allow the investigator to give organelles different colours, facilitating the continuous observation of a specific organelle. Prolonged association of organelles, though it may be one piece of evidence supporting an interaction, is alone insufficient to conclude interactivity between organelles.

The first irrefutable visual evidence for inter-organelle connectivity and interaction was seen using mitochondrial targeted Kaede that demonstrated fusion between two differently coloured organelles subpopulations (Arimura et al. 2004). The same was later demonstrated for Golgi bodies (Brown et al. 2010). The use of photo-convertible fluorescent proteins to highlight organelles in the same population with different colours has been called the ‘differential colouring’ technique (Schattat et al. 2012) and can be used to demonstrate the presence or absence of protein exchange within the population. Using mEosFP in the differential colouring technique, the photo-convertible FP highlighted organelles are at first homogeneously green, so a subpopulation is photo-converted to red. Over time, the natural streaming of the cell causes the red organelles to disperse among the green. Using mitochondria as an example, two differentially coloured organelles will associate for a brief period of time before fusing, which results in the mixing of green and red proteins to form an intermediate orange colour and often causes a morphological alteration (Fig. 1b panels 1 to 3). It is often possible to observe organelles that associate over the same period of time but do not fuse to exchange protein. Photo-convertible FPs thus provide a mechanism by which interconnectivity and protein exchange between organelles can be tested. Care must be taken however in cases of organelle overlap. Two differentially coloured organelles may appear to fuse in a snapshot as their overlapping fluorescence gives an intermediate hue, but if viewed for a longer period of time, they will eventually

move apart, having maintained their separate green and red forms. This overlap and subsequent separation has been seen for peroxisomes, which are known not to fuse (Arimura et al. 2004; Schattat et al. 2012), as well as for chloroplasts (Schattat et al. 2012).

3.3 Using Photo-Convertible FPs to Demonstrate Organelle Independence

In single-coloured images, the overlap of organelles or organelle extensions can give the impression of continuity. In these cases, whether one is dealing with several independent organelles or a single continuous network can be unclear. The differential colouring technique has recently been used with mEosFP to demonstrate the distinction between independent and elongated plastids (Schattat et al. 2012). That plastids extend tubular projections, termed 'stromules', has been demonstrated in several studies using fluorescent proteins (Köhler et al. 1997; Köhler and Hanson 2000; Gray et al. 2001). Very often, stromules extended by individual plastids will intersect and overlap, which with single-coloured FP gives a strong sense of their connectivity and continuity. It has become commonly assumed that at least a part of stromule function is to allow the exchange of contents between plastids (Gray et al. 2001; Hanson and Sattarzadeh 2011). Indeed, GFP has been shown to flow through narrow regions of irregularly shaped root leucoplasts (Köhler et al. 1997), while other studies focusing on independent plastids contradicted the observations and did not observe protein exchange (Köhler and Hanson 2000). Schattat et al. (2012) aimed to confirm the assumption of stromule fusion and subsequent protein exchange but found something contrary to what was expected. Their observations of differentially coloured plastids showed that each plastid remained independent at all stages of observation and fluorescent protein exchange did not take place (Fig. 1c panels 1, 2). In this case, photo-convertible FPs were used to demonstrate organelle independence and the importance of not concluding connectivity without clear evidence of protein exchange.

Several organelles undergo rapid expansion or elongation in response to various stimuli or cellular conditions. In the case of mitochondria, this process may involve a morphological change due to fusion of two organelles or the elongation of a single organelle, while peroxisomes often elongate to form 3 to 5 μm long beaded tubules before fission (Sinclair et al. 2009). Photo-convertible fluorescent proteins were useful in demonstrating that the beaded structure results from the elongation of a single organelle rather than the fusion of several different peroxisomes. This was accomplished by photo-converting some peroxisomes and then allowing the photo-converted and non-photo-converted populations to mix. The elongation was then triggered with hydrogen peroxide, and it was observed that each elongated peroxisome was either red or green, not an intermediate colour (Sinclair et al. 2009). In this way, photo-convertible fluorescent proteins can be used to understand the mechanisms of rapid organelle expansion within the cell in a manner that would not be possible with single-coloured FPs.

4 Studying Protein Synthesis and Movement

When they are designed to highlight an organelle of interest, FPs are often targeted using a portion of a protein or targeting sequence known to localize to that organelle. Another use of FPs that has become a standard procedure in many labs is to tag a full-length or partial protein, express it in the system of interest and observe its localization to determine or confirm the compartment in which it is found.

4.1 Studying Protein Diffusion

The fluorescence recovery after photobleaching (FRAP) technique is a method that uses single-coloured FPs to study protein diffusion and calculate diffusion constants (Axelrod et al. 1976; Braga et al. 2004). The technique begins with the exposure of the FP in a region to high-intensity light. This causes permanent loss of fluorescence of the FP molecules and is termed 'photobleaching'. It then becomes possible to observe whether, and how rapidly, new FP molecules move into the region. By graphing the fluorescence intensity in the region over time, it is possible to obtain the rate of diffusion of the protein of interest, through either the cytosol or within its targeted organelle. Additionally, it can be qualitatively used to observe relative rate of recovery between different treatments or conditions (Sprague et al. 2004). Though it is an effective technique and has been used in many studies, there are considerations that limit its effectiveness to some extent. The procedure makes the assumption that though the light used to photobleach is of sufficient intensity to inactivate a fluorophore, it does not damage the surrounding proteins or alter cell dynamics (Mathur et al. 2010). Additionally, with single-coloured FP experiments that rely on photobleaching, it can be difficult to determine the directionality of movement, and there is no control to confirm that recovery fluorescence is due entirely to protein diffusion rather than synthesis of fresh protein (Wu et al. 2011). Photo-convertible FPs may be used to the same end as FRAP but can additionally addresses the above-mentioned concerns. Instead of photobleaching an area of interest, the protein within that area can be permanently photo-converted. The intensity and duration of light required for photo-conversion is typically less than that of photobleaching and is thus less likely to cause damage to the cell. Additionally, as the red fluorescent form is visible it provides a control to show that the process has not altered movement in the cell. The diffusion of protein into the area can be observed by a recovery of green fluorescence and decrease of red fluorescence. The red-green-blue (RGB) scale is a standard method of identifying a colour as a triplet of numbers, each ranging from 0 to 255, representing the contribution of red, green and blue, respectively, to the colour. If the ratio of green to red is determined and graphed against time, the relative diffusion rate of FP can be

determined as with FRAP. This technique has been termed 'colour recovery after photo-conversion' (CRAP; Mathur et al. 2010).

In addition to measuring the diffusion of protein within a cell or organelle, photo-convertible proteins, specifically mEosFP and Dendra2, have been successfully employed to study the movement of transcription factors between adjacent root cells in *Arabidopsis* (Wu et al. 2011). In this study, the transcription factor fusion proteins were photo-converted in a specific file of root cells, and over time, the photo-converted protein became apparent in other cell files. In addition, photo-conversion of specific nuclei was carried out, and it was observed that as time progressed, some red fluorescence had migrated to nuclei that were not converted (Wu et al. 2011). In this case, the concern that diffusion resulted from new protein synthesis was absolved by observing the migration of the red fluorescent form, and the directionality of movement was easily established as it was moving from a red fluorescent to a non-red fluorescent area.

4.2 Observing Protein Synthesis, Uptake and Processing

Protein turnover is constantly occurring throughout the cell, with protein being both degraded and synthesized. This process can be difficult to observe in a living cell, but photo-convertible FPs provide a unique opportunity to do so. The irreversible photo-conversion of proteins like Kaede and mEosFP allows for the study of the synthesis and uptake of fresh protein, seen as the gradual reversion of an organelle from red to green.

This process has been seen in a couple of studies. In a study using Kaede targeted to Golgi bodies, the photo-converted Golgi bodies when observed over time turned green as newly synthesized fluorescent Kaede caused the reversion of colour from red to orange and yellow to green (Brown et al. 2010). As its photo-conversion is irreversible, this reversion represents the uptake of de novo FP. They were additionally able to observe that the intensity of red fluorescent remained relatively constant over the hours of observation, likely indicating that the change in colour was caused more by synthesis than turnover, and that this process was halted by treatment with Brefeldin A (Brown et al. 2010). Another study showed that photo-convertible FPs provide a method for studying histone synthesis during endoreduplication, as well as an indicator for identification of nuclei that started to undergo endoreduplication (Wozny et al. 2012). In this experiment, mEosFP was fused to histone 2B, and it was found that the change in green-red ratios of photo-converted nuclei correlated with the expected production of DNA and synthesis of new histones. In cell types that are known to undergo endoreduplication, an increase in green fluorescence was observed, whereas nondividing cells and those that do not endoreduplicate showed red fluorescent nuclei throughout the observation period (Wozny et al. 2012). Thus, photo-convertible FPs can be used to study the time frame required for the synthesis and subsequent uptake of fresh protein, as well as to monitor this process as an indicator of cellular events. It has also been successfully

demonstrated in plant cells that photo-convertible fluorescent proteins can be used to follow the endocytosis and recycling of membrane proteins. This process was studied using a fusion between the auxin efflux carrier PIN2 and EosFP, and it was shown endocytosis occurred by photo-converting only a specific portion of the plasma membrane and tracking the red vesicles that appeared over time (Dhonukshe et al. 2007).

5 Following Development and Long-Term Cell Tracking

The development of an organism is a complicated and highly coordinated process, and tracking one cell over time poses a challenge. In several vertebrate systems, photo-convertible FPs have been used to study the movement and growth of cells or groups of cells in developing embryos through the selective photo-conversion of the area of interest (Hatta et al. 2006; Kulesa et al. 2008; Lombardo et al. 2012). This same concept has been proposed for plant cells (Mathur et al. 2012) and could be used to track the fate of a cell throughout the growth and expansion of a seedling or specific organ.

6 Challenges and Caveats

Photo-convertible FPs have allowed researchers to overcome many of the limitations of single-coloured FPs, but there are nonetheless factors that must be taken into consideration when using these optical highlighters.

With most photo-convertible probes that have been used to date in plant cells, unintentional photo-conversion either does not occur or occurs at a sufficiently low rate that is not evident. In situations of overexpression however, regions of high FP accumulation may exhibit a degree of unintentional photo-conversion under the blue-white light that is standard for most plant growth chambers. This has been seen with an mEosFP probe targeted to the ER membrane, as well as photo-convertible probes that accumulate in the nucleus (Mathur et al. 2010).

Partial photo-conversion of a photo-convertible FP, where some but not all of a FP population is converted, can occur when the time of photo-conversion is insufficient and is evident through the appearance of a yellow or orange colour in the targeted region (Fig. 1d). Partial conversion at the periphery of a region can also occur, as the excitation beam that is used for photo-conversion is often most intense at the focal point and the intensity diminishes somewhat towards the edges of the region of interest, leading to a colour gradient (Mathur et al. 2010). As in many of the uses for photo-convertible FPs discussed the mixing of red and green fluorescence is used as indicative of cellular events or protein movement, it is important to ensure that a snapshot is taken immediately after photo-conversion to establish the initial green/red ratio, and that extra care is taken for co-localization studies.

Though it can lead to artefacts if a baseline is not properly established, partial photo-conversion can also be used as a beneficial technique for differentially highlighting several organelles, as it allows for the creation of yellow, orange and red subpopulations (Fig. 1d).

The time required for photo-conversion of probes is typically quite short. Despite this, it is possible that the illumination can harm the cell. The natural chlorophyll autofluorescence provides a method of monitoring the negative effect that photo-conversion or subsequent imaging has on the cell. When working with a microscope capable of imaging in multiple channels, if the chlorophyll autofluorescence is given a false blue colour, and is made to slightly overlap with the red fluorescence emission, then the appearance of a pink colour to the chloroplasts indicates that photo-damage has occurred and that imaging should not be continued on that cell. Alternative methods of observing for cell damage include observing the mitochondria or ER for morphological alterations such as expansion and aggregation.

It is important to keep in mind that like single-coloured FPs, optical highlighters are foreign to the plant cell. Overexpression can cause aggregation of dimeric or tetrameric FPs, such as Kaede and KikGR. Additionally, overexpression or simply alteration of the native protein through the addition of a fluorescent tag can cause mislocalization of the protein or impair proper function, behaviour or turnover.

7 Conclusions and Future Prospects

The fluorescent protein-based way of looking inside plants has involved constant innovation. Whereas each newly introduced fluorescent protein probe requires its own learning curve, it greatly increases our ability to delve into the living plant cell. This chapter has remained focused largely on photo-convertible FPs and their recent use in plants. However, proteins that change their fluorescent properties in response to specific metabolites, ions or other factors have also become available (Table 1, bottom) and are finding use in plant biology (Swanson et al. 2011; Meyer and Dick 2010; Costa et al. 2010). Although it will be challenging to express combinations of conventional fluorescent proteins, photo-convertible FPs and other conditionally triggered FPs simultaneously in a cell, the possibility of doing so is tremendously exciting and well within reach.

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Plant Cell Strains in Fundamental Research and Applications

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Abstract This chapter introduces plant cell strains and describes their main characteristics which render them unique experimental models to study plant morphogenesis. Although composed only of few cells, these systems are characterised by strict natural polarity of cell growth and division and high spontaneous friability of the daughter cell files. Both features are most clearly exhibited in two tobacco cell lines, Virginia Bright Italia (VBI-0) and Bright Yellow (BY-2). Owing to their ability to grow in vitro resembling animal single cell layer systems, they offer the possibility to accompany molecular or biochemical analyses of the material by a precise but nondestructive in vivo cytological observation of the cell population. Here we summarise the use of these two lines in various plant phenotyping studies including investigations of morphoregulatory signals like phytohormones or stress factors as well as organisation of cytoskeleton, endomembranes and cell wall. Although so far not frequently utilised in practical applications, some transgenic BY-2 strains show high potential for molecular farming. Last but not the least, the mechanisms responsible for the spontaneous or targeted selection of similar plant cell strains from genetically more attractive plant species are discussed.

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1 Introduction

Contemporary plant biology passes through its postgenomic era. The amount of results generated by high-throughput approaches such as genomics, transcriptomics, proteomics and metabolomics is progressively increasing. However, our understanding of the role of particular genes and their products has not kept pace with this wealth of information. The need to assign biological functions to all these newly discovered molecules has shifted phenotyping of living organisms on both whole organism and cellular level into the focus of attention. On the cellular level, parameters like cell size, cell shape, cell volume and mitotic activity can be used to characterise the processes of cell growth, division and developmental fate and, thus, define a "cellular phenotype". Undoubtedly, the success of this "phenomics" approach not only requires new sophisticated methodology but also suitable experimental models that allow noninvasive investigations. Among such systems, plant cell strains offer unique applications for numerous plant biology studies.

2 Plant Cell Suspension Cultures, Plant Cell Cultures, Plant Cell Lines and Plant Cell Strains: What Is the Difference?

2.1 History of Cell Lines and Strains: Animal and Human Models

When Harrison (1907) started his experiments with pieces of the animal tissue cultured in vitro, he registered for the first time the ability of some cells to migrate actively from the original explants and to cover progressively the surface of their supports, i.e. the glass walls of culture vessels or microscopic slides. Thanks to the contact inhibition, they did not form cell clumps or thick layers. Instead, they grew in the form of single cell layers, thus allowing convenient observation by means of light microscopy. As postulated later, this behaviour persisted in some materials only, apparently dependent with their genotype or epigenetic characteristics, and, therefore, this behaviour was characteristic of specific animal cell or tissue lines.

Massive progress in animal tissue cultures used both for animal biology and for medicine caused half of century ago the necessity to standardise both the methodology and the increasingly inconsistent terminology of this research area. For this purpose, an "Ad Hoc Committee of Terminology" was formed by the International Tissue Culture Association (TCA) in 1961. After 5 years, during the TCA Annual Meeting in San Francisco, the "fifth draft of a proposed usage of animal tissue terms" was accepted. And in the subsequent year, the chairman of this terminology committee, S. Fedoroff from the University of Saskatchewan, published the

appropriate document as base for animal tissue culture (Fedoroff 1967). This document contains both the definition of "cell lines" and also of their more precisely characterised progenies, the so-called cell strains:

> A **cell line** arises from a primary culture at the time of the first subculture. The term "cell line" implies that cultures from it consist of numerous lineages of the cells originally present in primary culture [. . .]. A cell line maybe said to have become "established" when it demonstrates to be subcultured indefinitely in vitro.

The term "cell line" is thus mostly specified operationally, by its origin, without reference to any specific characteristics. To incorporate such functional specificities, the terms "cell strain" and "substrain" were introduced:

> A **cell strain** can be derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. The properties or markers must persist during subsequent cultivation. In describing a cell strain its specific features should be defined, e.g. a cell strain with a specific marker chromosome, a cell which has acquired resistance to a certain virus or a cell having specific antigen etc [. . .]. A "**sub-strain**" can be derived from a strain by isolating a single cell or groups of cells having properties or markers not shared by all cells of the strain.

To tell the truth, this terminology has only been partially accepted and used in animal or human research and applications. For example, already the first established animal cell culture model, derived by George Otto Gey (see Gey et al. 1952; Scherer et al. 1953; Fedoroff 1971) from cervical cancer cells isolated on February 8, 1951, from Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951, is commonly termed the "HeLa cell line". Similarly to the numerous subsequently isolated, characterised and commercially sold animal or human cell lines, it is rather a "cell strain".

2.2 History of Cell Lines and Strains: Plant Models

In general, growth and morphological characteristics of plant cell and tissue cultures reflect the pronouncedly different life strategies of plants and animals. During their whole lifespan, plant cells are enclosed in a solid cell wall. The cells constituting the plant body are therefore not mobile and they are tightly connected to each other through the central lamella and plasmodesmata establishing symplasmic continuity. Plant morphogenesis is based on cell multiplications and modifications of cellular shape and size that are precisely confined in time and space. The key step defining subsequent cell differentiation, embryogenesis, histogenesis and organogenesis (see also the chapters by Opatrný, Skůpa et al. and Smertenko and Bozhkov, this volume) is the establishment of cell polarity.

To obtain the desired separation of the compact (cohesive) plant tissue into individual cells, the very first plant cell lines were originated by permanent shaking of either primary explants or pieces of callus cultures in liquid medium. Unfortunately, in these plant cell suspension cultures, the subpopulation of physically

separated cells was frequently exhibiting different stages of cell death, such that the continuous growth of such cultures was sustained by the subpopulation in the cell aggregates. As a rule, these aggregates were of pronouncedly multilayer form and irregular or spherical shape, containing several tens to hundreds of individual cells highly variable in their size, shape, age, physiological properties and position in the cell cycle. Such experimental models have unfortunately very limited use for any cytological studies and precise "phenotyping".

Paradoxically, the early reports on successful generation of plant suspension cultures were very optimistic. The first plant cell suspension cultures capable of subculture were probably those of *Tagetes erecta* and *Nicotiana tabacum* described by Muir in his Ph.D. thesis presented to the University of Wisconsin in 1953 (see Street et al. 1971). However, already Nickell (1956) described experiments on long-term (more than 48 months continuously subcultured) suspension culture of cells derived from the hypocotyl of *Phaseolus vulgaris* and even predicted their future use as equivalents to cultures of new types of microorganisms. Unfortunately, the photographic documentation consisted only from one image of a single isolated cell, and no supplementary studies had been published since then.

The wealth of methodological knowledge on early plant cell suspension cultures was summarised in brilliant publications by Prof. Street and his team, summarised in Street (1973). Their research was motivated mainly by two aims: to enrich our knowledge on the biology of plant cells cultured in vitro and, based on this, to integrate plant cell suspensions into fermentation technology for the production of biomass to synthetise or process various pharmaceutically valuable compounds.

Street perceived it necessary not only to standardise the methodology of his research but also the terminology. Already in the introductory chapter of his book he defined key terms to be used for the descriptions of various plant cell and tissue cultures with the terms *suspension culture* implying cells and cell aggregates dispersed in moving liquid medium (there were no cultures consisting entirely of separated cells) and *cell cultures* as a more general term designating any system with a lower level of organisation than tissue cultures.

Already Fedoroff (1967) emphasised that "the word "line" is used most properly with the meaning of an uninterrupted sequence." and therefore "the term "cell line" in the field of tissue culture should imply an interrupted sequence of cell growth, whereas the term "cell strain" should imply certain relationship of the cells, i.e. that they all have one or more common properties or markers for which these cells were specifically selected. . .". In most cases, just tissue cultures grown on solidified media were and are discriminated from suspension cultures grown in liquid media. Inconsistencies of nomenclature are widespread in the field. For instance, in the catalogue of the Leibniz Institute DSMZ – the largest German collection of microorganisms and cell cultures (http://www.dsmz.de/) – morphologically heterogenous experimental materials are offered under the term "plant cell lines".

Although terminological precision seems to be dispensible, when dealing with mere accumulation of biomass cultured in vitro, the experimental potential offered by real plant cell lines or even plant cell strains in the strict sense for basic research vastly exceeds that of a mere mass of dividing cells.

3 Plant Cell Strains

3.1 The Sycamore Cell Strain

As described in Street et al. (1971), sycamore culture is characterised by high spontaneous friability, and therefore it can be classified as "nonadhesive mutant of sycamore cells". It was selected through stepwise, long-term fractionation of the original suspension culture, which led to the "cell culture clone". In harmony with the terminology mentioned above, this culture was not exclusively composed of free cells, but contained morphologically uniform, mostly spherical, cell aggregates. Under standard conditions, these cultures maintained high viability and cell proliferation rates. Besides, the friability of cell aggregates exhibited regular dynamics during the subculture cycle. The sycamore cell strain allowed for the first time to correlate a particular cellular "micromorphology" and, to some extent, cellular phenotypes with individual phases of the subcultivation interval. Repeated cell division and formation of the aggregates, consisting mostly from small, spherical cells, were typical for the exponential growth phase. In contrast, the subsequent stationary phase was characterised by cell expansion and loosening of the aggregates to the degree comparable with the parameters of animal culture "single layers". Aggregates contained 10–30 cells during the stationary phase, whereas for the exponential phase, larger aggregates, consisting of even more than 50 cells, were typical (Fig. 1).

The sycamore cell strain was not optimal for more detailed light microscopical analyses, but it allowed relatively precise and noninvasive estimation of phenotypical parameters such as viability, cell number and changes of cell size, along with biochemical or histochemical parameters. These characteristics distinguished the sycamore cell strain from previously used systems like Paul's Scarlet *Rosa sp*. (Tulecke and Nickell 1959; Nickell and Tulecke 1960; Nash and Davies 1972) and *Nicotiana tabacum* (Filner 1965). Undoubtedly, Street's sycamore cell strain was the first system qualifying as "real" plant cell strain in the definition by Fedoroff (1967).

3.2 Tobacco Cell Strains

3.2.1 The VBI-0 Tobacco Cell Strain

The tobacco cell strainVBI-0 was derived in 1967 as one of the tissue culture lines originated from tobacco stem pith tissue explants and cultured on slightly modified Heller's agar medium (Heller 1953) containing the auxins 2,4-D and NAA (Opatrný 1971; Opatrný and Opatrná 1976). VBI-0 differed from all other tobacco cell/tissue cultures by its atypical micromorphology. Instead of the conventional lumps of callosic tissue, it formed very flat, mould-type colonies of soft to even mucilaginous consistence. When transferred to liquid medium, VBI-0

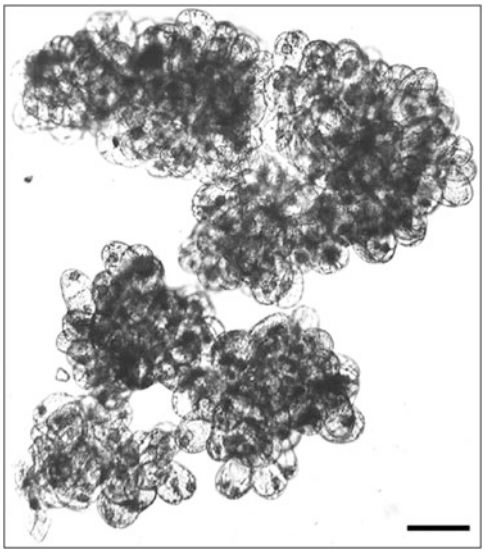

Fig. 1 Sycamore cell strain. Multicellular aggregates of either isodiametric or slightly prolonged cells, typical for the exponential phase of the subculture interval of cell suspension culture. Scale bar: 100 μm (Image taken by Opatrný in 1975)

spontaneously exhibited an extreme friability, and even upon gentle shaking, it dissociated into a very homogenous suspension, with the majority of cells being either singular or in couples. Similar to the sycamore system, this friability changed over the subculture interval. After a lag phase of 2–3 days, free cells of the stationary inoculum restored cell division and gradually formed aggregates consisting of up to 20–24 cells. During the final phase of the subculture interval, these aggregates split up again (Fig. 2). One of the characteristics absolutely unique for this strain is a permanent polarity of both cell division and growth of the individual cells within the cell files. Thus, the culture is composed of elongated free cells coexisting with files consisting of several cells. Cells of the late stationary phase are clearly senescent but still show a viability of more than 80–90 %. Mature cultures can be even composed of mostly fully isolated and very prolonged cells (width 20–40 μm, length up to 100–150 μm). In general, a set of parameters can be continuously followed and allows to define the micromorphology of these cultures for various experimental conditions (Opatrný et al. 1983). These parameters include viability; cell shape parameters such as length and width; proportionality; cell division characteristics (mitotic index, distribution of cell divisions along the cell file, orientation of division planes); architecture of the cytoplasmic network; vacuolisation; and nuclear shape, size and positioning (Fig. 3).

This morphological complexity renders VBI-0 a cell strain ideally suited for plant cell phenotyping to study the mechanisms of plant morphogenesis, growth and signalling under both control conditions and in response to various exogenous stress factors, xenobiotics and morphogenetic regulators (see Sect. 4). Calli of VBI-0 exhibit uniform growth when either inoculated as pieces of various sizes and weight directly on the agar medium or plated in melted agar or agarose. VBI-0 cells are well growing in the form of continuously agitated suspension in

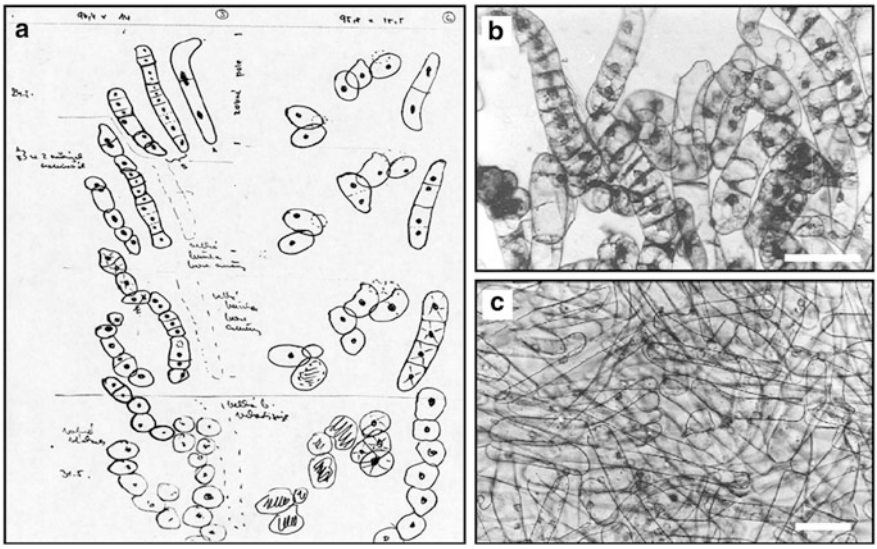

Fig. 2 VBI-0 cell strain. (**a**) Original time-lapse drawings of the formation and dissociation of polarised cell files. Cell suspension of initial density ~10^4 cell/ml was cultured in glass microchambers. Observed by light microscopy (obj. 10×) at regular time intervals (May 24, 27 and 31, 1970). Localisation of selected objects by means of cross table coordinates (here 96.4 × 14 or 95.8 × 15.5) and handmade orientative drawings. Successive polar cell division, formation of the cell files and their final dissociation or even death demonstrated. Protocols Opatrný 1972. (**b**, **c**) Phenotypical changes during the subculture interval. Cell files typical for the exponential phase, composed of repeatedly dividing, almost isodiametric cells. (**b**) Free long cells, spontaneously accumulating in late stationary phase. (**c**) Scale bars: 100 μm

Erlenmayer flasks of various size, but they can be also cultured as thin layer liquid suspensions in stationary petri dishes. This ability documents that it is not very dependent on pronounced aeration, a property that helps the line to survive technical problems such as shakers stopping over night. For quick and multivariate screening, a system of hanging drops was successfully adopted from Potrykus et al. (1979). Interestingly, upon protoplasting, the recovered protoclones preserved the original high spontaneous friability and the polar character of both cell division and growth (unpublished results), suggesting that this character is genetically or epigenetically fixed. One of the few limitations of VBI-0, as compared to the BY-2 system (see below), is a relatively low rate of cell proliferation. For this reason, synchronisation protocols show a lower efficiency, and mitotic indices after synchronisation with anti-replicative drugs do not exceed 20–25 % (Campanoni 2003).

In spite of its high and long-lasting phenotypical stability, subpopulations of VBI-0 gradually diversify, which might be due to either genetic mutations or epigenetic changes. Therefore, when various selective conditions are imposed on these subpopulations, it is possible to isolate strains that differ in growth parameters, behaviour and phenotype. Their characteristics can be adjusted for prospective applications by choosing appropriate selective conditions. Some of these derived

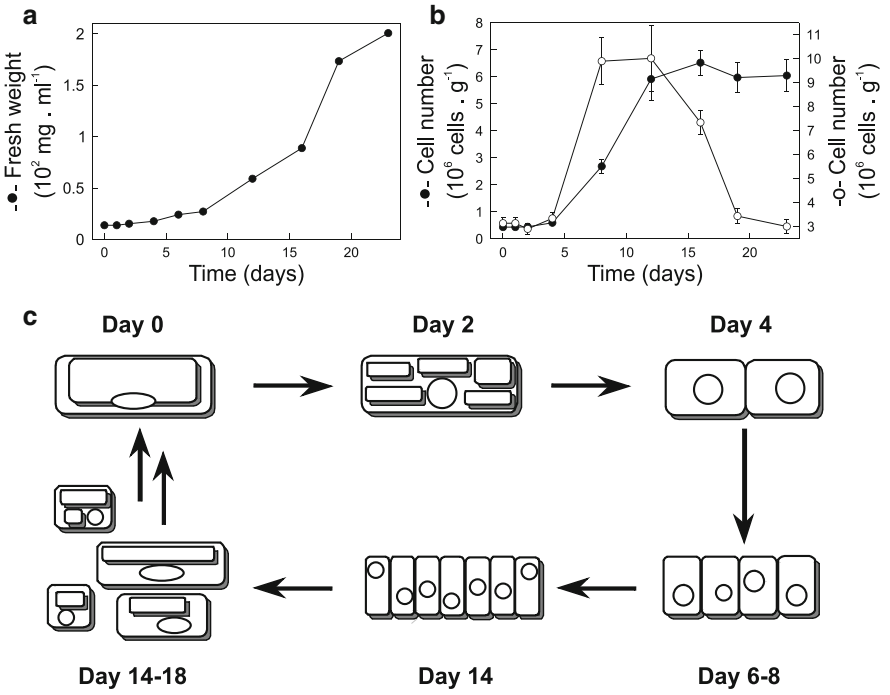

Fig. 3 Growth parameters and schematic representation of a standard life cycle for the VBI-0 cell strain. (**a**) Fresh weight (-●-). (**b**) Cell number per ml of the culture medium (-●-) and g of fresh weight (-○-). Values represent arithmetic means, SE, n = 10 samples (for fresh weight) or counting chambers (for cell numbers). (**c**) During an 18-day-long subculture interval, the VBI-0 cell strain proceeds through well-separated phases of cell division and cell elongation

strains have been cultured temporarily, whereas others have been preserved in long-term stock collections. Among the first sets, cell strains exhibiting increased tolerance to heavy metals were isolated (Domažlická and Opatrný 1989). Our collection of VBI-0 strains contains also substrains differing from the progenitor VBI-0 strain with respect to their sensitivity to exogenously applied auxin. In addition to strains that grow on tenfold lower auxin concentrations, three completely auxin-autonomous substrains were isolated (Petrášek, unpublished; Qiao et al. 2010). Upon growth in the dark, these substrains display stable phenotypes with either pronounced cellular elongation (VBI-1) or radial expansion (VBI-2). One of these strains, substrain VBI-3, even recovered the ability to synthetise chlorophyll.

VBI-0 cells can be readily transformed by particle bombardment in a transient manner (Freudenreich 1996, Nick unpublished). Recently, it has become possible to transform VBI-0 cells also in a stable manner using a modified protocol based on *Agrobacterium*-mediated gene transfer (Seifertová et al. 2013). Using this technique, a set of marker lines containing GFP-tagged auxin efflux carriers from

Arabidopsis thaliana was obtained and used for studies of endomembrane dynamics of these proteins (Marhavý et al. 2011).

3.2.2 The BY-2 Cell Strain

Nagata's BY-2 cells are undoubtedly one of the most popular models of plant cell biology. Citations of the review extensively describing this model (Nagata et al. 1992) range near 750 by now, and even an own Wikipedia page (http://en.wikipedia.org/wiki/Tobacco_BY-2_cells) as well as two exhaustive book monographs (Nagata et al. 2004, 2006) have been dedicated to this cell strain. The original characterisation of BY-2 cells as a model for basic research was published in 1992, i.e. almost quarter a century after the origination of VBI-0. However, according to Ken Matsuoka (http://mrg.psc.riken.go.jp/strc/BY-2%20references.htm), BY-2 (also sometimes called NT-1) was originated from seedlings of *Nicotiana tabacum* cv. Bright Yellow 2 already in 1968 by Dr. Kawashima at the Hatano Tobacco Experimental Station of the Japan Tobacco and Salt Public Corporation, and the first characterisation of the culture was published in 1972 (Kato et al. 1972). The first original scientific report using BY-2 as system (for the isolation of ubiquinone) appeared in 1974 (Ikeda et al. 1974). To obtain high amounts of cells, one of the most requested parameters was rapid proliferation of the population. This parameter is indeed exceptionally high in BY-2, with some 100-fold multiplication during 1 week of subcultivation (Nagata and Kumagai 1999; see also Seifertová et al. 2013). With respect to basic research, the high multiplication rates are a precondition for protocols of cell cycle synchronisation that are very efficient for BY-2 cells (Kumagai-Sano et al. 2006). In contrast to VBI-0, BY-2 cells are routinely cultured in media containing only one auxin, namely, 2,4-D (Nagata et al. 1992), but higher concentrations of nitrogen in the form of ammonium ions as well as of phosphates. However, the cellular phenotypes of VBI-0 and BY-2 are very similar. Both lines display a strict polarity of cell growth and division and a high spontaneous friability (Seifertová et al. 2013).

Thanks to its rapid proliferation, its homogeneous cellular phenotype, the existence of efficient synchronisation protocols (Nagata et al. 1992; Kumagai-Sano et al. 2006) as well as protocols for stable transformation (An 1985; Mayo et al. 2006), BY-2 has been used in numerous studies on cell cycle, phytohormones, cytoskeleton, endomembrane dynamics, cell wall biogenesis, defence responses and secondary metabolism, especially on isoprenoids and alkaloids (reviewed in Nagata et al. (2004, 2006)). However, a detailed phenotypical description of singular BY-2 cells or cell files and their development has been conducted only in few of these numerous studies (see Sect. 4). Rather, BY-2 often serves as a rich source of homogeneous biomass for molecular biology and biochemistry.

Considering the wide use of the BY-2 model, with high certainty from the "mother" strain of BY-2a, a broad spectrum of substrains has emerged in the numerous laboratories, where this model is cultivated. This "evolutionary process", as all evolutionary processes, is shaped by variability and selection as driving

forces, although this may not be evident at first glance. Variability is mainly due to the processes leading to somaclonal variability. Selection, in most cases, is not a conscious process, but mainly results from variations in subcultivation procedures between different laboratories or even between different members of the same laboratory. Due to the robust proliferation of BY-2 cells, it is very often overlooked that the details of subcultivation (cellular density of the inoculum, culture volume, relation between surface and volume, speed and mode of shaking) represent selective pressures that over time will alter the properties of the cell population in the flask giving rise to a new substrain of BY-2. As long as just proliferation is considered, such differences between substrains will go unnoticed. However, when phenotypical parameters are compared for BY-2 cells from different laboratories, clear differences between substrains are manifest (Nick and Maisch, unpublished observations). One of the crucial factors is inoculation density. When BY-2 cells are inoculated at a density that is too high, cell division rate decreases dramatically accompanied by an increase of phenotypical variability. Under optimal inoculation density, typically around 1:90–120 (Nagata and Kumagai 1999), the culture shows highly homogeneous phenotype of cells, and only such culture meet the criteria of a cell strain with long-lasting stability. However, in case of only week dilutions (1:10), frequent incidence of genetical aberrations and decreased cell viability can be observed (Kovařík et al. 2012). Unfortunately, these authors did not compare different inoculation densities. Not only genetic stability, but also the expression of introduced transgenes can vary within a population of BY-2 cells; however, this variation can be reduced by a passage of cellular cloning after plating on solid medium (Nocarová and Fischer 2009). In general, these substrains of BY-2 arise spontaneously. To our knowledge, there has been only one intentionally derived, auxin-autonomous substrain of BY-2 (Shimizu et al. 2006).

3.3 Arabidopsis thaliana *Cell Strains*

Cell strains derived from seedlings of *Arabidopsis thaliana* represent a valuable complement to the most frequently used model plant. They are derived both from Col-0 and Ler ecotypes (Axelos et al. 1992; May and Leaver 1993). Unfortunately, from the cytological point of view, they are less homogeneous and, they do not show a stable polarity of cell chains. The proliferation rates of Arabidopsis cell lines are somewhat lower as compared to the tobacco cell strains, but still sufficient to obtain reasonably high synchrony for cell cycle studies (Menges and Murray 2002).

4 Use of Tobacco Cell Strains for Fundamental Research

4.1 Cellular Level: Studies of Cell Shape and Polarity

Multicellular organisms are built up from individual cells. The immobile plant cells can predetermine as well as finalise both form and structure of the entire plant only by oriented cell growth and division. Therefore, phenomena such as formation, stabilisation and modification of cell axis and polarity, as well as axis and polarity of the cell aggregates, represent key events in plant morphogenesis. Starting from the first division of the zygote (Mayer et al. 1993), cellular polarity is tightly connected with cell differentiation, both in structure and function (for details, see also Opatrný, this volume).

As postulated in numerous reviews on the strategy of plant ontogenesis (probably most recent being Nick 2013), the basic morphogenetic unit in the plant development is the individual cell (Lintilhac 1999; Nick 2013). To put it bluntly, in animals, the organism produces cells, whereas in plants, cells produce an organism. Long-term, maybe even permanent, functional "totipotency" or at least "pluripotency" of numerous somatic plant cells (see Opatrný, this volume) connected with the indeterminate growth of the plant body offers to each individual cell a plethora of potential ways for its differentiation. However, the selected cell fate is controlled by regulatory factors of mostly external nature. To study the mechanisms and molecular players underlying the transduction of these developmental cues in the context of intact plants is a difficult task. The coexistence of different cell fates creates a complexity that is difficult to be dissected. Moreover, these cues can rarely be precisely applied to exclusively the particular cell population under investigation such that modulating crosstalk between different cell types will obscure the results even more. Therefore, simplified experimental models have been established for plant developmental biology to study how size, shape and volume of individual cells, as well as their division, differentiation, senescence or death, respond to external signals. Moss protonemata (Bonhomme et al. 2013), pollen tubes (Žarský and Cvrčková 2013), roots and root hairs (Ridge and Emons 2000) or brown algae (Bogaert et al. 2013; Hable and Hart 2010; Le Bail and Charrier 2013) provide such models. Unfortunately, all of them are in fact exhibiting a very narrow developmental potential and only limited phenotypical responses. For this reason, isolated somatic cells originating from various tissue origins were used, including the regeneration of cells from isolated protoplasts (for details, refer to the chapter by Opatrný, this volume). This technique has been developed in the late 1970s and used in plant breeding, either as the tool for somatic hybridisation or as one of the early strategies for a direct gene transfer via electroporation or PEG poration. For tobacco mesophyll protoplasts, the ratio between auxin and cytokinin was reported to be crucial for this regeneration process (Stickens et al. 1996); however, at least for protoplasts from BY-2, exogenous cytokinins seem to be dispensible (Zaban et al. 2013).

Protoplast regeneration can be used as a system to study the mechanisms underlying the induction of polarity and axis de novo. At least for the readily transformable BY-2 system, fluorescently tagged cytoskeletal markers can be integrated (Zaban et al. 2013). The regeneration system could be standardised to such a degree that quantitative data on the temporal patterns of regeneration stages could be collected leading to a model, where the microtubular cytoskeleton conveys positional information between the nucleus and the membrane controlling the release or activation of components required for cell wall synthesis. Cell wall formation in turn is then followed by the induction of a new cell pole requiring dynamic actin filaments, and the new cell axis becomes manifest as elongation growth perpendicular to the orientation of the aligned cortical microtubules. When the stability of actin filaments was manipulated at a specific time point by inducible expression of an actin-bundling protein, peculiar Siamese twins were produced. In developmental biology, the observation of Siamese twins is considered as generally accepted as evidence that polarity is generated de novo and not caused by reorientation of a hidden pre-existing polarity.

Although the protoplast system is the method of choice to study induction of cellular organisation de novo (in the absence of preset factors, "inherited" from the mother cell), a closer approximation of cellular development in a tissue context is provided by walled cells of the strains BY-2 and VBI-0.

Firstly, one can concentrate on the morphogenesis and quantify parameters like cell length and diameter, cell area or even cell volume using image analysis. Using this approach in BY-2 cells, modifications of cell growth and division were analysed and quantified in cells after the insertion of potato genes coding for hybrid proline-rich (HyPRPs) cell wall proteins (Fischer et al. 2002; Dvořáková et al. 2012). A detailed analysis of various BY-2 strains overexpressing HyPRPs with different types of N-terminal domains suggested that C-terminal domains of HyPRPs are involved in cell wall loosening, thus allowing cell expansion. However, no visible phenotypic alterations were detected when HyPRPs were overexpressed in their homologous system, i.e. potato plants, suggesting the existence of compensating mechanisms acting in planta, highlighting the usefulness of BY-2 system that allowed to uncover this function of HyPRPs.

Secondly, one can analyse the impact of various factors on the cellular structures expected to regulate cell shape such as cell wall, cytoskeleton and secretory system. For instance, the growth retardant ancymidol (ANC) was used in experiments to get insight into the role of microtubules and actin filaments. As shown in Bořík̆ová (2002) and Bořík̆ová et al. (2003), drastic cellular malformation of BY-2 cells in response to ANC (Fig. 4) was accompanied by only a partial destruction of the cytoskeleton. The primary mode of ANC action is the inhibition of *ent*-kaurene oxygenase, one of the key enzymes for the biosynthesis of gibberellin (GA). As to be expected from this proposed mode of action, the effect of ANC could be compensated by simultaneous application of GA, as it had been already demonstrated in planta (Shive and Sisler 1976; Coolbaugh et al. 1982). But, surprisingly, no GA effect on either control or ANC-treated BY-2 cells has been observed. To explain this contradiction, coordination of cell wall synthesis with the cell

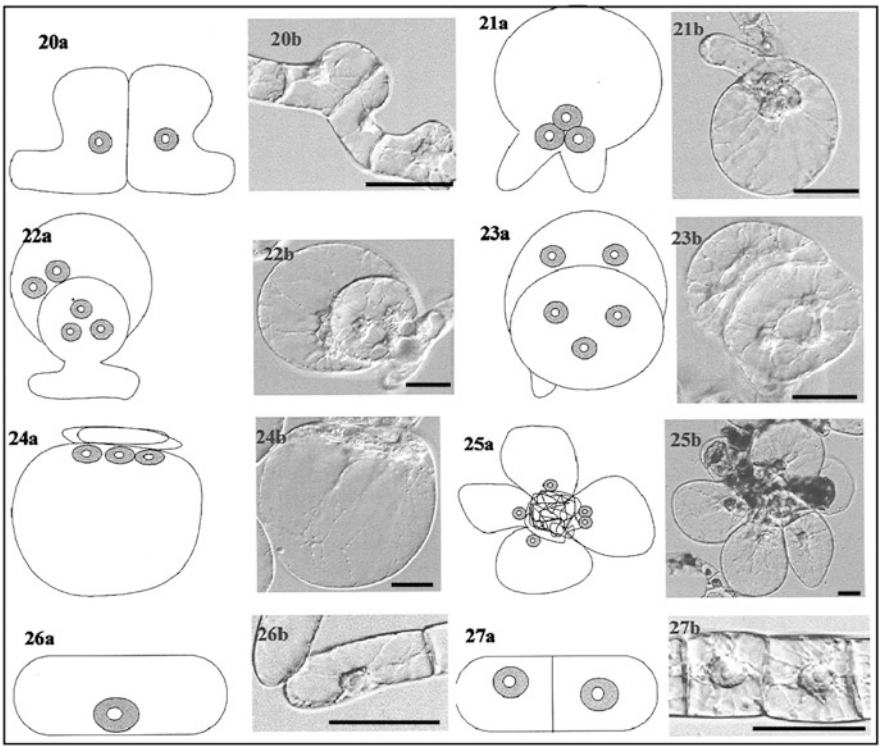

Fig. 4 Cell strains as models in the studies on cell shape determination. The effect of compounds interacting with cell wall formation. BY-2 cell strain, effect of ANC. 20–25 cells form samples containing various concentrations of ANC, 26–27 control cells. Scale bars: 50 μm (Bořík ová 2002)

expansion was tested in BY-2 cells as the potential target of ANC action not related to GA (Hofmannová et al. 2008). This novel effect of ANC was clearly shown to be similar to the action of inhibitors of cellulose synthesis and also to be dependent on the functional vesicle secretory system. This shows the capacity of the simplified model of BY-2 cells to uncover novel action of some drugs.

4.2 Intercellular Level: Tobacco Cell Lines as Minimal Organisms

During their entire life time, plants add new cells to the tip of roots and shoots, and cell differentiation in these mitotically active meristems is controlled by signals from the neighbouring, already differentiated, cells (for details, refer to Opatrný, this volume). This process is iterative, but in a fully fledged meristem, cell biological analysis is difficult, because differentiation is already channelled.

Although it is possible to manipulate this pattern by microsurgery or laser ablation, it is not possible to observe pattern formation ab initio. Thus, meristems are beautiful systems to study how patterns are perpetuated, but for the analysis of pattern induction, simpler systems are needed, where determination has not progressed that far. Cell strains as those introduced above can serve as systems of reduced complexity that allow insight into the cellular mechanisms of self-organisation in plants. However, the full potential of this approach has not been exploited so far, probably, because plant cell lines/strains are generally considered as "dedifferentiated". Based also on this view the BY-2, cells have been even termed "HeLa cells of plant biology" (Nagata et al. 1992). However, as pointed out in detail in the chapter by Opatrný in this book, the "dedifferentiation" might be a myth. Even suspension-cultured plant cell lines often preserve certain features from their source tissue, such as the ability to generate the structured cell wall thickenings characteristic for vascular cells observed in VBI-0 (Nick et al. 2000) or the ability to generate, through a series of axial cell divisions, cell files with a clear axis and polarity and a preserved responsiveness to the controlling signal, auxin. Since these files derive from singular cells, they cannot rely on positional information inherited from the mother tissue. Patterns of competence within a cell file must therefore originate de novo during the culture cycle (Fig. 5).

In tobacco, cell line VBI-0 files consisting of even numbers of cells were shown to dominate over files with uneven cell numbers (Campanoni et al. 2003; Maisch and Nick 2007). At first sight, frequency peaks of even-numbered files might occur, when the cell cycle proceeds with a precise timing. This should generate files in a sequence of

$$f(n) = 1, 2, 4, 8, \ldots 2^n$$

individual cells (with n representing the number of cell cycles). However, the length of individual cell cycles varies over a broad range, and there is, in addition to the expected peaks at 2^n, a curious frequency peak for files composed of six cells (in some cases accompanied by a smaller peak of ten cells). This peculiar feature could be simulated using a mathematical model derived from nonlinear dynamics, where elementary oscillators (cycling cells) with a high level of noise (variation in the length of individual cell cycles) were weakly coupled and where the number of these oscillators was not constant, but increased over time (Campanoni et al. 2003). In contrast to concurrent models, this weak-coupling algorithm was able to predict the observed frequency peak of hexacellular files. Moreover, this model predicted several nonintuitive properties of the experimental system, prominently, that coupling must be unidirectional, i.e. that the coordinating signal is transported in a polar fashion. The coupling becomes manifest as a phase shift in the cell cycle, i.e. a dividing cell will cause its downstream neighbour to accelerate its cell cycle such that it will also initiate mitosis. Unidirectional signalling is a diagnostic feature of auxin transport (see also the chapter by Skůpa et al., this volume). In fact, the predominance of even-numbered cell files could be eliminated by low concentrations of 1-N-naphthylphthalamic acid (NPA), a specific inhibitor of directional

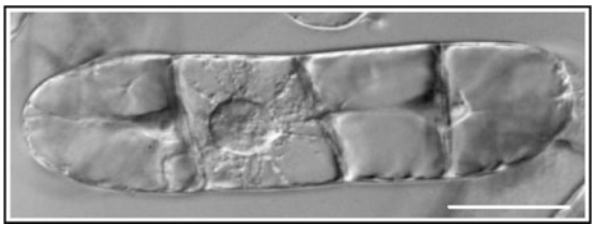

Fig. 5 VBI-0 cell strain, nonequal functional differentiation of the stepwise formed polar cell file already during the early exponential phase. Three of the daughter cells of the tetrade originated as the result of two successive divisions of "mother" cell remain in telophase, but one is again passing through new mitosis. Scale bar: 30 μm

auxin transport. Although the noise in this system was considerable, with high variation in the cycling period over the cell population, the division of adjacent cells was synchronised to such a degree that files with uneven cell numbers were rare compared to files with even numbers. Frequency distributions over the cell number per file thus exhibited an oscillatory behaviour with characteristic peaks at even cell numbers.

NPA-induced disruption of polarity of cell divisions in VBI-0 (Petrášek et al. 2002) and BY-2 (Dhonukshe et al. 2005) suggests that even in these simplified systems, auxin efflux plays a coordinative role for the establishment cell file axis. Using a protocol for tracing radioactively labelled auxin (Delbarre et al. 1996), carrier-mediated auxin transport in both VBI-0 (Petrášek et al. 2002) and BY-2 (Petrášek et al. 2003) has been shown to be NPA dependent. Moreover, BY-2 transformed with *Arabidopsis thaliana* auxin efflux carriers from the PIN and PGP families have been used to determine kinetic parameters of their auxin transporting activities (Petrášek et al. 2006; Petrášek and Zažímalová 2006). Phenotyping of cells after inducible overexpression of PIN auxin efflux carriers uncovered remarkable similarities with effects caused by auxin starvation (Winicur et al. 1998; Miyazawa et al. 1999), i.e. inhibition of cell division, amyloplast formation and cell elongation (Petrášek and |Zažímalová 2006). Therefore, extensive carrier-mediated auxin efflux seems to cause auxin starvation. This conclusion is further supported by the fact that this auxin starvation phenotype is fully rescued by NPA (Mravec et al. 2008), probably by reducing the excessive activity of the overproduced auxin transporters. Interestingly, also a simultaneous overexpression of the auxin-binding protein ABP1 in BY-2 acts antagonistic with excessive auxin transporter action consistent with a role of ABP1 in the regulation of trafficking of plasma membrane proteins including auxin carriers (Čovanová et al. 2013).

To identify the underlying cellular mechanism driving the self-organisation of these pluricellular files, transgenic derivatives of BY-2 were used. This strain shows the same patterning phenomenon as VBI-0 although at the background of higher noise (due to the more rapid cycling of the BY-2 line as compared to the slower, but more orderly, VBI-0). By scoring frequency distributions of cell number per file, it was possible to monitor and to quantify the degree of organisation. The directional

transport of the plant hormone auxin depends on transcellular gradients of auxin efflux carriers that continuously cycle between plasma membrane and intracellular compartments (see chapter by Skůpa et al. for details). This cycling has been proposed to depend on actin filaments. Therefore, the effect of excessive actin bundling was tested by overexpression of the actin-binding domain of mouse talin in tobacco BY-2 cells. The bundling can be reverted by addition of auxins, which allows to address the role of actin organisation on the flux of auxin and the division synchrony (Maisch and Nick 2007). In fact, the bundling of actin was accompanied by a disturbed synchrony of cell division, such that the difference in frequencies between even- and odd-numbered cell files was eliminated. When a normal configuration of actin was restored by exogenous auxin, also division synchrony could be recovered, demonstrating that a normal, debundled configuration of actin was necessary and sufficient for self-organisation of the pluricellular file, which is indicative of an effect of actin on polar auxin transport. The same response of auxin-induced restoration of cell division synchrony was observed in the auxin-autonomous strain VBI-3. In this line, which is responsive to light, division synchrony was being rescued by irradiation. The light-induced rescue was most pronounced for continuous far-red light, intermediate for continuous blue light and weak for continuous red light, a spectral pattern indicative of plant photoreceptor of the phytochrome A type (Qiao et al. 2010).

To demonstrate this effect of actin on auxin transport more directly, the same strategy was later transferred to rice plants overexpressing mouse talin (Nick et al. 2009). Here, polar auxin transport can be measured by feeding radioactively labelled auxin via a small block of agar to the tip of rice coleoptiles and quantifying the amount of radioactivity recovered in a receiver block at the basal end of the coleoptile. Using this approach, it could be shown that the talin overexpressing rice harboured heavily bundled actin cables and that auxin transport was reduced to only half of the value found in non-transformed wild-type seedlings. When now exogenous auxin was added, the debundling of actin could be followed in vivo by confocal microscopy, and in parallel a recovery of auxin transport to the level found in the wild type could be observed. Thus, the findings from the BY-2 cell strain could be confirmed in a “real” plant. These results were integrated into a model of a self-referring regulatory circuit between polar auxin transport and actin organisation (for review, see Nick 2010). This model of an actin-auxin oscillator was able to explain a curious phenomenon discovered almost half a century ago that so far had remained unsolved: When auxin transport had been measured at high temporal resolution in the classical coleoptile system, it turned out not to be steady, but to oscillate (Hertel and Flory 1968). These oscillations and their period of around 20 min can be explained by the oscillatory behaviour of the actin-auxin regulatory circuit. To further dissect this actin-auxin oscillator, photoactivated release of caged auxin was achieved in the mouse talin overexpressor generated in the BY-2 background. As to be expected, actin debundling was triggered by microirradiation in the cell, where auxin was released from the deactivating cage, whereas in the neighbouring cell that had not been irradiated, actin cables remained bundled (Kusaka et al. 2009). Thus, auxin gradients can be manipulated at a

subcellular level by means of optical engineering in the tobacco BY-2 line. This technology can now be used to manipulate auxin distribution within a pluricellular file of tobacco BY-2, and this approach already succeeded to manipulate the synchronisation of cell divisions by overriding the natural polarity of a cell file (Maisch et al., in preparation).

The auxin-dependent debundling of actin and the concomitant restoration of auxin transport (monitored as division synchrony) require that auxin signalling must control expression or activity of actin-associated proteins. To identify these targets for auxin signalling, a panel of actin-binding proteins was cloned from tobacco BY-2, introduced into the GATEWAY vector system, fused with GFP under control of the 35S promoter and then overexpressed in tobacco BY-2 to screen for alterations of cell division patterning (Durst et al. 2013). This strategy identified the actin-depolymerisation factor 2 (NtADF2) as crucial factor. Here, the overexpression resulted in a specific elimination of the diagnostic frequency peak of hexacellular files. When the excess of NtADF2 was sequestered by adding the phospholipid PIP2 specifically linking ADFs to the plasma membrane, the frequency peak at $n = 6$ could be recovered. Likewise, when the destabilising effect of NtADF2 on actin dynamics was counteracted by low concentrations of phalloidin, a compound trapping actin in the assembled state, this diagnostic frequency peak could be rescued. This pharmacological rescue shows that activation of actin disassembly by NtADF2 is necessary and sufficient for the auxin effect on actin and also for the synchrony of divisions in a cell file.

This case study on the actin-auxin oscillator that had been discovered using VBI-0 and BY-2 cells demonstrates that these tobacco cell strains are not a mere plant version of the famous "HeLa" cell line (Nagata et al. 1992), but represent systems capable of simple self-organisation and therefore provide experimental models, where the cellular aspects of self-organisations can be studied more conveniently as compared to a complex meristematic tissue. In other words, we should more seriously consider these tobacco cell lines as (minimal) "organisms" rather than as mere "cells". In summary, both tobacco cell lines, BY-2 and VBI-0, can be used as experimental systems to study the genesis of an organism from highly autonomous and noisy individual cells.

4.3 Supracellular Level: Tobacco Cell Strains to Study Cell Fate

One feature of biological organisms is the ability to undergo development. Although this might sound odd for a suspension cell line, there are clear indications for developmental changes on the cellular level. VBI-0 and BY-2 cell strains can simulate, depending on the particular culture conditions, not only the early, but also late phases of plant development. Although these developmental responses are also found in the more rapidly cycling BY-2 line, they are more evident in the slower,

but more "orderly", VBI-0 and its clonal derivatives (Campanoni and Nick 2005): in response to an inductive pulse of auxin (in this cell line, 2,4-D has to be accompanied by NAA), a period of rapid cycling is followed by a second stage, where cells rarely divide, but expand rapidly and directionally (parallel to the file axis), and a third phase, where the cell files decay into smaller units and eventually single cells. When the cultivation is prolonged without addition of fresh auxin, even differentiation into cells with secondary wall thickenings indicative of early stages of protoxylem formation can be observed (Nick et al. 2000). In other words, the VBI-0 line recapitulates almost the entire developmental programme of parenchymatic tissue triggered in response to auxin, a phenomenon that strongly argues against the often repeated dogma of "dedifferentiation".

In fact, under specific experimental conditions, VBI-0 can be induced to form tissue-like structures. When cultivated, growing on the surface of the agar plates, rather flat, mould-looking tissue layers are produced that even exhibit a specific endogenous structure composed of tangential tiers layers of daughter cells covering the mother inoculum. This experimental system of relatively free, but tightly communicating, plant cells recalls the behaviour of evolutionary very ancient structures such as yeast or bacterial colonies (Rieger et al. 2008; Váchová et al. 2012; Pátková et al. 2012; Čáp et al. 2012). This observation indicates that this tobacco strain is capable of a residual developmental programme, which is under control of (unknown) signals.

Among the known signals that regulate the developmental fate of a cell, auxin plays a prominent role. Through directional transport, auxin maxima and minima provide a pre-pattern for subsequent differentiation (for details, refer to the chapter by Skůpa et al. and Opatrný, this volume). Since VBI-0 requires both 2,4-D and NAA for normal development, it also provides a nice system to dissect the complex developmental signalling triggered by auxin. In addition to its role in cell expansion, auxin can induce cell division, a fact that is widely employed for tissue culture and the generation of transgenic plants (for details refer to chapter by Opatrný of this book). Investigations of lateral root formation in Arabidopsis suggested that, here, auxin regulates cell division through a G-protein-dependent pathway (Ullah et al. 2001; for review, see Chen 2001), which differs from the classical auxin stimulation of cell expansion through the auxin-binding protein ABP1. The relationship between auxin, cell division and cell expansion was dissected further in the VBI-0 cell line (Campanoni and Nick 2005) by recording dose responses of cell division and cell expansion over 2,4-D and NAA. It was found that these two auxin species affected cell division and cell elongation differentially: NAA stimulated cell elongation at concentrations that were much lower than those required to stimulate cell division. In contrast, 2,4-D promoted cell division but not cell elongation. Pertussis toxin, a blocker of heterotrimeric G-proteins, inhibited the stimulation of cell division by 2,4-D, but did not affect cell elongation. Conversely, aluminium tetrafluoride, an activator of G-proteins, could induce cell division at NAA concentrations that were otherwise not permissive for division and even in the absence of any exogenous auxin. These data suggest that the G-protein-dependent pathway responsible for the auxin response of cell division is triggered by a

different receptor than the pathway mediating auxin-induced cell expansion. The two receptors appear to differ in their affinity for different auxin species, with 2,4-D preferentially binding to the auxin receptor responsible for division and NAA preferentially binding to the auxin receptor inducing cell growth. Differential activities of NAA and 2,4-D in the stimulation of cell division and cell elongation were also observed in protoplasts regenerating from BY-2 cells (Hasezawa and Syono 1983). Moreover, the stimulatory activity of 2,4-D on cell division could be also induced with 2,4-D structural analog 2,4,5-trichlorophenoxyacetic acid in the BY-2 strain (Simon et al. 2013).

Both tobacco strains have their merits and weaknesses. BY-2 is more readily transformed, but, due to its rapid cycling, its developmental potency is quite reduced and does not extend over cell division and cell expansion. In contrast, the slower cycling VBI-0 cell line is more synchronous and in addition comprises early stages of cell differentiation, but slightly more recalcitrant to transformation. Thus, whereas BY-2 is suited to analyse cellular aspects of localisation and function of candidate genes, VBI-0 is endowed with a more extensive developmental potency and thus represents a system coming closer to a situation in a developing tissue.

Despite these differences in experimental suitability, both cell strains offer various approaches to study the mechanisms underlying both cell senescence and programmed cell death (PCD) (see also the chapter by Smertenko and Bozhkov, this volume).

For instance, using the BY-2 system, the toxic effect of Cd (see also the chapter by Martinka et al., this volume) was dissected and found to be more complex and interesting as thought before. In fact, depending on the phase of the cell cycle, Cd induced different types of death, some of which met the criteria for PCD (Fojtová and Kovařík 2000; Kuthanová et al. 2004, 2008a, b). Similarly, BY-2 was used to probe for potential PCD in response to cytokinins (Mlejnek and Procházka 2002), ethylene (Herbert et al. 2001), fungal elicitors (Kadota et al. 2004; Kadota and Kuchitsu 2006) or various genotoxic compounds, such as bleomycin (Smetana et al. 2012). However, it is again to some extent surprising, or even symptomatic, that only for the studies on Cd (Kuthanová et al. 2004, 2008a, b) and bleomycin (Smetana et al. 2012), the claim of induced PCD was verified by more detailed cytological and micromorphological analysis.

5 Limitations of Tobacco Cells: How to Originate New Plant Cell Strains of Desired Phenotype?

In spite of all their unique properties, a serious limitation of tobacco cell strains is the still quite poor description of the tobacco genome. Although it has been sequenced and partially assembled, the annotation is far from being complete. All sequences of both genomic and transcriptional origins are accessible through NCBI.

However, authors of this chapter are aware of some deep sequencing efforts in tobacco and hope that in the near future, the situation will be better.

As already mentioned in Sect. 3.2.2, Kovařík et al. (2012) uncovered genetic instability in stationary BY-2 culture grown under suboptimal conditions stimulating a dramatic heterogeneity of the cell population. To tell the truth, for any scientist experienced in the cytology or karyology of plant cell or tissue cultures, these results are not surprising. Already a long time ago it was possible by mere conventional karyology to document not only the fact of the incidence of various chromosomal changes (such as aneuploidy, polyploidy, chromosomal breaks, transpositions) but also to follow their dynamics. As a rule, chromosomal aberrations accumulate in cell populations of both tissue and cell suspension cultures during long-term (continuous) cultivation. However, many of these aberrations are of transient nature and are eliminated during subsequent cultivation, mostly without leaving any pronounced effect on phenotype or even morphogenesis (Butenko 1964; Sunderland 1973). Similarly, in clones derived from tobacco protoplasts, an early massive DNA endoreduplication was followed by stepwise deduplication reverting the cells to "ploidy normality" as revealed by flow cytometry (Valente et al. 1998; see also the chapter by Vrána et al., this volume). Thus, as long as culture conditions are kept constant, the risk of heterogeneity in the physiological response of tobacco cell strains is small. Additional evidence for mechanism maintaining genomic integrity is the fact that genes cloned from tobacco cell strains produce sequences identical to these found in planta. For instance, the entire family of tobacco homologs of PIN auxin efflux carriers is now cloned both from BY-2 cell strain and plants in our laboratory, and so far all sequences from both sources have been found to be exactly identical, i.e. in the culture there are no changes in the coding sequence of the particular genes coding for auxin efflux carriers. In addition, many genes coding for cytoskeleton-associated proteins have been cloned in our laboratories, again yielding sequences identical with published data from tobacco plants, and some of them have been even used successfully to complement corresponding mutations in *Arabidopsis thaliana*.

A challenge for future research and applications is the generation of comparable cell strains with defined and stable phenotypes for the favourite plant genetic and genomic models, such as rice, grapevine, maize, tomato or barley. Unfortunately, even after the half century of research with plant cell lines and strains, no reliable general protocol exists. Even just to obtain a well-growing plant cell line of the "sycamore type" with high friability and growth rate and polar growth and division seems to be a matter of lucky circumstances rather than targeted experimentation. Any effort to support the spontaneous friability of the clone by means of various culture regimes, applications of chelatones, enzymes that digest cell wall or central lamella turned out to yield only limited success. In those attempts, friability increased only temporarily, whereas at the same time the harmful consequences of repeated treatment on either cell viability or cell division rate turned out to be irreversible (Street et al. 1971; Street 1973).

It could be concluded that in case of BY-2 and VBI-0 cell strains, the success in their establishment was dependent on "good luck" during the spontaneous selection

of the line, expressing again only rudimentary "cell adhesivity" (a term used by Street 1973. In theory, some kind of sophisticated transgenosis, which could stimulate the enzymatic machinery of natural maceration of plant tissues (such as polygalacturonidases) similar, for example, to those participating in the fruit ripening, might be a strategy, but so far there are no reports on attempts in this direction.

6 Industrial Applications of Plant Cell Strains

As mentioned above, already starting with the first shell bean culture (Nickell 1956), the work on plant cell suspension cultures was motivated by their potential for industrial applications, mainly for the production of specific biomass or specific products of plant secondary metabolism. For such pharmaceutical applications the "cytological parameters" described in this chapter had been ignored for a long time as their characteristics are without any technological value. For the routine propagation of cell mass in a fermenter, the only features considered to be relevant were sufficient friability and cell proliferation. In numerous cases the yield of the secondary compound appeared to be inversely related with mitotic activity in the cultures. The reason is that, similarly to the in vivo situation, secondary compounds mostly accumulate in maturated or even senescing cells or in cell populations undergoing some type of functional differentiation.

The situation has pronouncedly changed when novel technologies enabled molecular farming. These are based on more sophisticated systems of transgenic cell cultures or even cell lines and cell strains (Fischer et al. 1999a, b) and are usually constructed with inducible promoters such that expression of the transgene can be controlled both in time and space. With this approach, both quantity and quality of the products have been improved. Moreover, recombinant proteins are produced by transformed cells and tailored such that they are secreted directly into the culture medium, which allows for steady-flow instead of batch fermentation, which allows for more efficient procedural engineering. It is generally desirable to produce these proteins by exponentially growing cell population, i.e. by dividing cells. Exponentially growing cells contain low level of proteases, which would degrade secreted product in contrast to stationary cells, which enter quickly into the senescence (Zhong 2001; Schiermeyer et al. 2005). In addition, the product itself, either still present in the producing cells or secreted into medium, can negatively affect the cellular metabolism as a whole, which leads to highly detrimental or even lethal phenotypes for the production cultures (Sorrentino et al. 2009; Avesani et al. 2010). Again, steady-flow strategies are superior, because they allow to remove the secreted product from the production flow by using appropriate filters. By their high proliferation rate, BY-2 is and undoubtedly will be of high interest for "molecular farmers", as exemplified by the study of Bortesi et al. (2012), who described the development of an optimised tetracycline-inducible expression system to increase the accumulation of interleukin-10 in BY-2 culture, or by the study

of Vassilev et al. (2013), who improved the production of human antibody by changing nutrient and hormonal composition of the culture media.

Economical comparisons of different systems for molecular farming (Daniell et al. 2001) show that plant-based systems are superior for smaller production volumes, whereas they fall back against mammalian and bacterial systems, when the market moves towards mass production. The future of plant molecular farming will therefore be mainly in the field of small-scale production of therapeutically valuable compounds. The major trend in medical innovation is at present the so-called personalised medicine – this trend will change the pharmaceutical markets still dominated by large-scale production of a few compounds towards a highly dynamic situation, where small-scale production has to adapt rapidly to new therapeutical trends. Plant molecular farming, in comparison with competing systems of production, might be better suited to meet this challenge, and it is therefore to be expected that plant cell strains will become a central tool for the pharmaceutical production of the future.

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