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

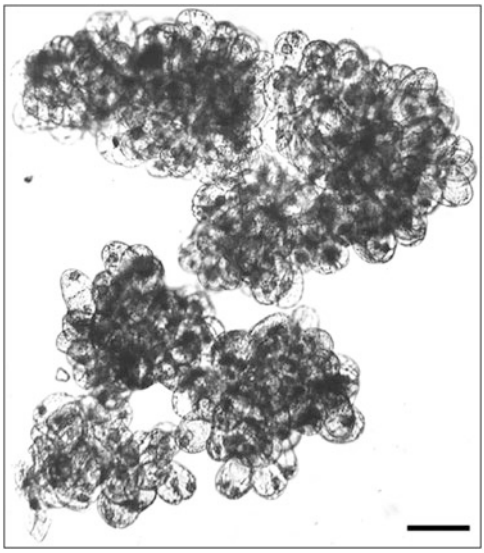

Fig. 1 Sycamore cell strain. Multicellular aggregates of either isodiametric or slightly prolonged cells, typical for the exponential phase of the subculture interval of cell suspension culture. Scale bar: 100 μm (Image taken by Opatrný in 1975)

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

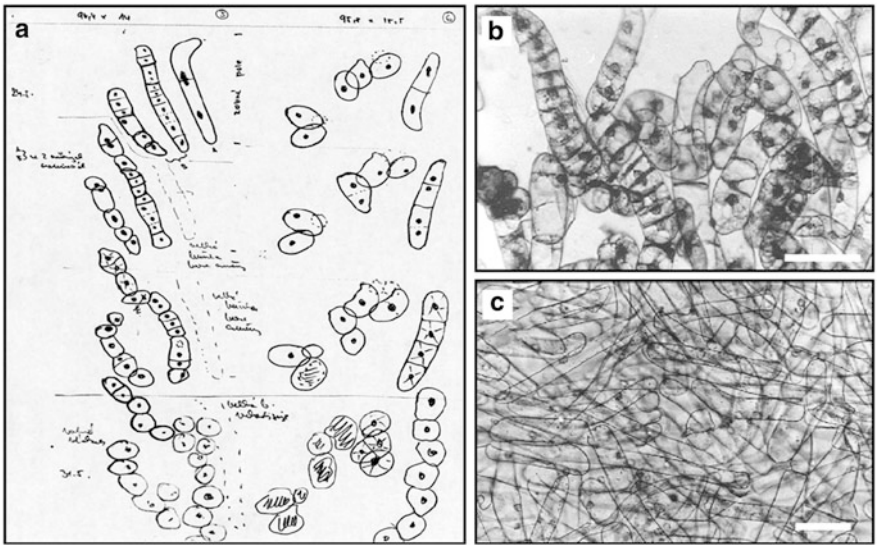

Fig. 2 VBI-0 cell strain. (**a**) Original time-lapse drawings of the formation and dissociation of polarised cell files. Cell suspension of initial density ~10^4 cell/ml was cultured in glass microchambers. Observed by light microscopy (obj. 10×) at regular time intervals (May 24, 27 and 31, 1970). 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

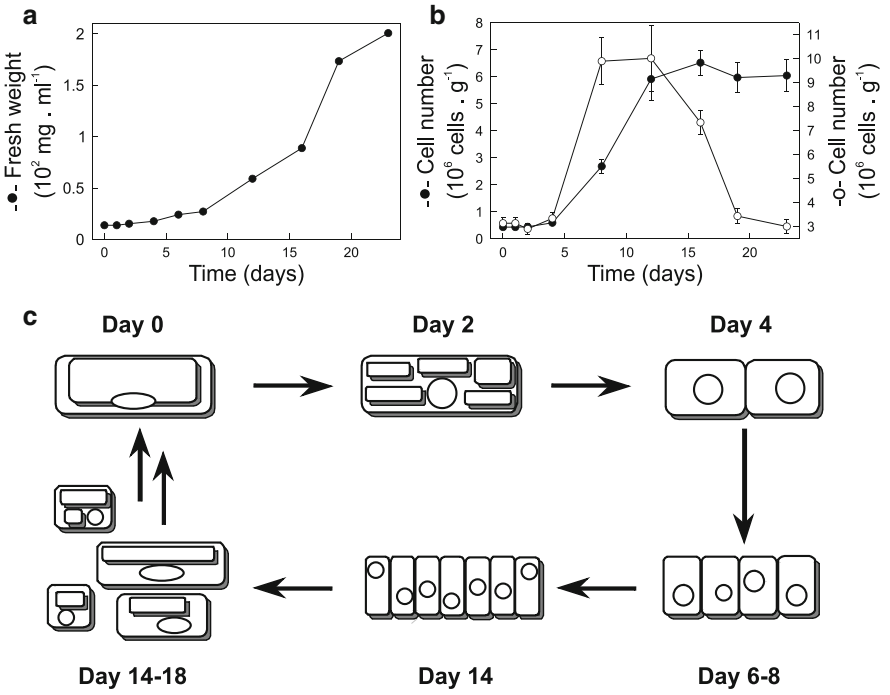

Fig. 3 Growth parameters and schematic representation of a standard life cycle for the VBI-0 cell strain. (**a**) Fresh weight (-●-). (**b**) Cell number per ml of the culture medium (-●-) and g of fresh weight (-○-). Values represent arithmetic means, SE, n = 10 samples (for fresh weight) or counting 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

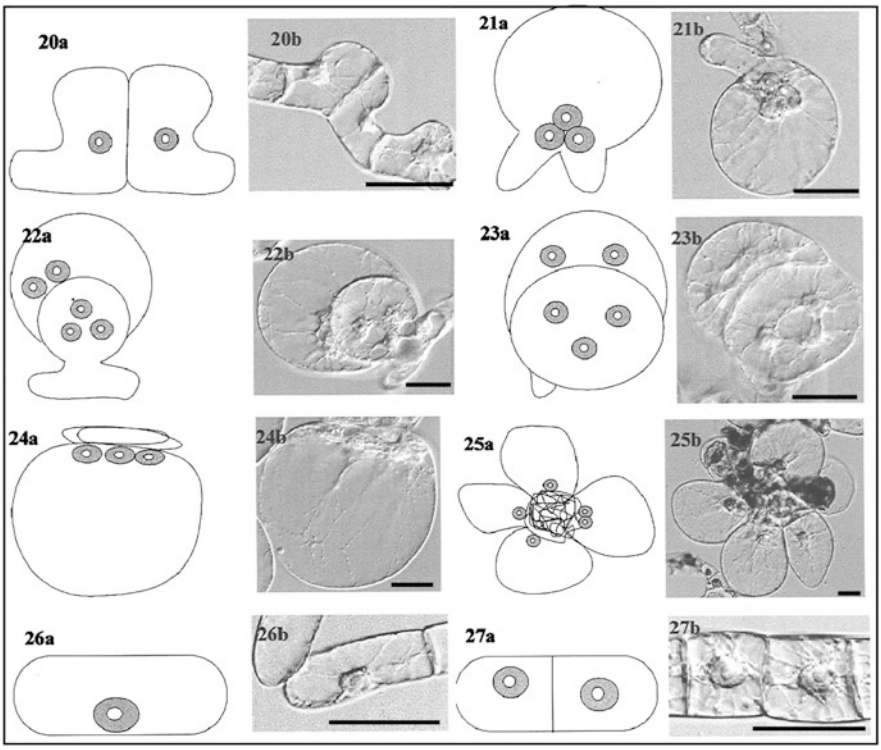

Fig. 4 Cell strains as models in the studies on cell shape determination. The effect of compounds interacting with cell wall formation. BY-2 cell strain, effect of ANC. 20–25 cells form samples containing various concentrations of ANC, 26–27 control cells. Scale bars: 50 μm (Bořík ová 2002)

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

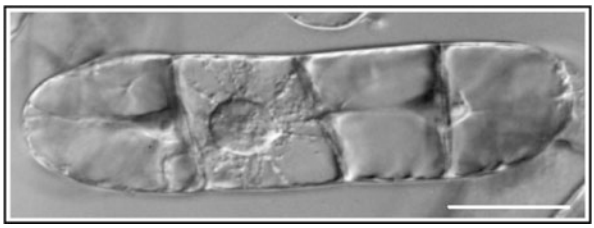

Fig. 5 VBI-0 cell strain, nonequal functional differentiation of the stepwise formed polar cell file already during the early exponential phase. Three of the daughter cells of the tetrade originated as the result of two successive divisions of "mother" cell remain in telophase, but one is again passing 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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