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A GLORIOUS HALF-CENTURY OF MICROTUBULES

Microtubules, signalling and abiotic stress

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SUMMARY

Plant microtubules, in addition to their role in cell division and axial cell expansion, convey a sensory function that is relevant for the perception of mechanical membrane stress and its derivatives, such as osmotic or cold stress. During development, sensory microtubules participate in the mechanical integration of plant architecture, including the patterning of incipient organogenesis and the alignment with gravity-dependent load. The sensory function of microtubules depends on dynamic instability, and often involves a transient elimination of cortical microtubules followed by adaptive events accompanied by subsequent formation of stable microtubule bundles. It is proposed that microtubules, because of their relative rigidity in combination with their innate nonlinear dynamics, are pre-adapted for a function as mechanosensors and, in concert with the flexible actin filaments and the anisotropic cell wall, comprise a tensegral system that allows plant cells to sense geometry and to respond to fields of mechanical strains such that the load is minimized. Microtubules are proposed as elements of a sensory hub that decodes stress-related signal signatures, with phospholipase D as an important player.

Keywords: cold sensing, gravisensing, mechanosensing, osmosensing, phospholipase D, plant microtubules.

STRESS ADAPTATION IN PLANTS: MANY SIGNALS, ONE QUALITY?

Life is not easy – there are basically two ways to respond to its challenges: run away or adapt. Locomotion has been the major strategy of animals; because of their sessile lifestyle, plants had to evolve developmental flexibility to face adverse environmental conditions. As a central element of this strategy, plants must integrate the signalling evoked by different stress factors into a balanced and appropriate response. The specificity of stress signalling might be achieved by specific molecular players and events that convey the signal to the downstream targets. Alternatively, signalling might use common, quite 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. At first sight, these players appear almost trivial in their simplicity. Their specificity derives from the spatiotemporal pattern (the so-called signature) rather than from their molecular nature. 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 a review, see McAinsh and Hetherington, 1998). For reactive oxygen species, it is their subcellular distribution that confers specificity in drought and salinity signalling (for a review, see Miller *et al.* 2010), and, 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 a review, see Kazan and Manners, 2008). Hence, the specificity of stress signalling seems to rely on the specific combination of relatively general primary signals.

What is the quality of these primary signals? One of the most ancient, general and fundamental stress signals is mechanical load. Life requires buffering of internal homeostasis against fluctuations of the external environment (Lintilhac, 1999). A central element of this homeostasis is the dynamic establishment of chemical gradients: the gradients are protected by membranes that control the flow of different molecules, such that osmotic forces act upon the membrane (and in the case of ions are complemented by electrostatic forces). These forces represent the primary signals that allow a cell to sense differences in chemical composition between the internal and the external environment.

Already in prokaryotes, forces upon the membrane are used as signals for osmoregulation (for a review, see Kung, 2005). However, it should be kept in mind that not only numerous environmental factors, including gravity, wounding, wind, touch or pathogen attack, but also factors modulating membrane fluidity, such as cold and heat, can be sensed as a mechanical load upon the membrane (for a review, see Los and Murata, 2004).

Furthermore, plants use mechanical information not only to screen many aspects of their environment, but also to integrate their open morphogenesis. To compensate the considerable mechanical load produced by gravity on their outwardly extending body, plants have to allocate load-bearing elements (woody vessels and fibres) parallel with the direction of mechanical strain. This self-referring mechanism allows plants to adjust their architectural response to mechanical load in a flexible manner with minimal investment of energy and biomatter. Mechanical load has shaped plant architecture down to the cellular level. In contrast to terrestrial animals, where the multicellular organism provides an environment that is kept strictly isotonic, the cells of terrestrial plants are surrounded by hypotonic medium. As a consequence, the plasma membrane experiences continuous tension from the expanding cytoplasm. This turgor pressure is counterbalanced by a cellulosic cell wall. On the organ level, considerable tissue tensions develop that can be used to steer morphogenesis depending on mechanical integration. For instance, the ordered pattern of leaf primordia can be modelled by the stress–strain pattern in the expanding apical meristem (for a review, see Green, 1980; Hamant *et al.* 2008), and the expansion of aerial organs is controlled by the extensibility of the epidermis (for a review, see Kutschera, 2008). The impact of mechanical tension on plant morphogenesis extends to a degree where the shape of individual cells in a plant tissue can be described as a manifestation of minimal mechanical tension (Thompson, 1959).

It thus seems that many of the primary stress signals can be reduced to, or at least deduced from, mechanical signals. As with other physical stimuli, mechanosensing requires some kind of transformation of physical input into a signalling output of biochemical quality. It is generally believed that the original inputs are minute changes in geometry of the membrane, where the perception mechanism is located. In other words: the energy of the primary input is extremely small and therefore has to be efficiently amplified. This general problem of mechanosensing is even accentuated in plants, because plant cells are subject to continuous pressure from the inside (produced by the expanding protoplast) and the outside (produced by the tissue tension from their expanding neighbours). These pressures are in the range of several bars, and provide large background forces against which the minute changes of mechanical energy must be discriminated. A further challenge to mechanical sensing peculiar for plants is the lack of specialized sensory organs: sensing is diffusely spread over a large number of cells. Thus, each individual cell has to cross the threshold for sensing without support from its neighbours. The maximally possible input energies are therefore limited and hardly exceed thermal noise.

To address the mechanisms underlying the sensing of abiotic signals, it is important to introduce a conceptual discrimination that had originally been developed to understand gravity sensing (Björkman, 1988). In contrast to the sensing of a chemical ligand, the sensing of a physical stimulus must proceed in two steps. In the first step, physical energy is transformed into a chemical signal in a process that has been named 'susception'. As a second step, perception in the strict sense is triggered by this transformed input. This 'susception' can be a relatively passive event. For instance, during gravisusception, heavy particles (statoliths, amyloplasts) press upon stretch-activated ion channels and trigger fluxes of calcium that induce biochemical signalling cascades. When the function of a potential event in abiotic stress sensing is to be understood, it is central to discriminate between a role as sensory susceptor from actual perception.

SENSORY TENSEGRITY: THE PLANT CYTOSKELETON

Biological architecture combines the very parsimonious use of material with maximal robustness and dynamicity through the use of self-supportive structures, where stiff elements (that can transmit compression forces) are linked with tensile elements (that can transmit traction forces). Based on the first so-called Equilibrist Studies of the the Soviet artist Karl Joganson, this biological principle was systematically exploited by the architect and engineer Richard Buckminster Fuller, who later also coined the term 'tensegrity', from 'tension' and 'integrity', for these structures (Robby, 1996). For the mobile cells of metazoan animals, it could be shown later that it is tensegrity that maintains their shape (for reviews see Ingber, 2003a,b). Here, actin microfilaments play the role of the tensile elements, as they are contractile, and at the same time can transmit considerable forces because of a Young's modulus comparable with that of silk (Gittes *et al.* 1993). The compression forces instead are transduced by microtubules that, by their peculiar structure as hollow cylinders, are much more rigid, and can be approximated mechanically as very delicate glass fibres. As actin filaments are also linked through integrins, with the extracellular matrix as supporting scaffolding, the resulting tensegral structure can maintain shape even under the dynamically changing conditions of a migrating cell. Here, the motive force at the leading edge has been shown to depend on the assembly of actin (Pollard and Borisy, 2003), but this will only lead to a locomotive force when there is a counterforce. This counterforce is provided by the adhesion plaques, in which the

actin cytoskeleton is tethered through transmembrane integrins with the extracellular matrix.

The situation in plant cells differs fundamentally. Here, there is no locomotion, and shape is maintained by the cell wall, where elongate load-bearing elements (cellulose microfibrils) are embedded in an amorphous matrix (hemicelluloses, pectins, proteins). In these cells, the interphasic plant cytoskeleton is not directly required to support cell shape, and is therefore free to adopt additional functions.

The tensegrity principle provides maximal mechanical stability at minimal use of resources, and, in addition, can adapt continuously to the changes induced by growth and development. The tensegral structures are continuously remodelled to align them with the field of stress and strain, establishing a minimal level of potential energy: a phenomenon that is impressively manifest during the formation of tension or compression wood (Funada, 2008). Tensegral integration requires a feedback of mechanical forces and strains upon cytoskeletal organization. Thus, the tensegral cytoskeleton is not only a device for architectural stability, but can also be used to sense and integrate patterns of stress and strain. This *sensory tensegrity* is especially important in walled plant cells that are subject to continuous turgor pressure, and use this pressure for regulated expansion. In animal cells, this *sensory tensegrity* is partially obscured by the role of the cytoskeleton for the maintenance of cell shape. In plants, the presence of a stable cell wall has promoted a functional shift of the cytoskeleton from architectural towards sensory tensegrity.

Two molecular mechanisms have been discussed for the perception of mechanical stimuli (Figure 1). The stretching of anisotropic molecules will change their conformation and create or mask domains for interaction with associated proteins (for reviews, see Janmey and Weitz, 2004; Orr *et al.* 2006). Alternatively, mechanosensitive ion channels can respond directly to forces within the lipid bilayer. Such channels will open when the plasma membrane is deformed or when the channel is pulled by a tether (for a review, see Kung, 2005). Mechanosusception, in both cases, is based on changes of protein conformation, and it is mainly in the second step, perception, that they differ: in the case of anisotropic molecule stretching it is the differential binding of the associated molecules that triggers the signalling, and for ion channels it is the changed ionic composition that triggers the signalling.

It should be kept in mind that mechanosusception can convey a signal over considerable distances, i.e. the actual perception is not necessarily at the cellular site, where the mechanical stimulus is acting. For instance, direct propagation via the cytoskeleton seems to be most rapid signal to the nucleus in animal cells (Wang *et al.* 2009).

Anisotropic molecule stretching is well documented for the adhesion sites of mammalian cells, where the conformation of actin filaments is transduced by associated proteins and integrins (for instance, see Ehrlicher *et al.* 2011). The transfer of this mechanism to plant cells is not straightforward, because the molecular components differ considerably (for a review, see Baluška *et al.* 2003): the extracellular matrix of mammalian cells is replaced by the cell wall. Moreover, despite considerable effort, canonical integrins could not be identified in plants. Central components linking actin to focal contacts in mammalian cells, such as talin, do not exist either; however, similar to animal cells, the plant cytoskeleton is tethered to transmembrane proteins that span through the membrane to the extracellular face, and therefore anisotropic molecule stretching is conceivable for plants as well (for a review, see Telewski, 2006). It should be kept in mind that in addition to filamentous protein structures, the lipids in the deformed membrane itself could act in signalling: by mechanical stress they might be more accessible to attack by the lipases acting in signalling.

Mechanosensitive channels have been discovered by patch-clamp studies in specialized plant cells, where a touch stimulus can induce an action potential such as the seismonastic leaves of *Mimosa pudica*, or the giant

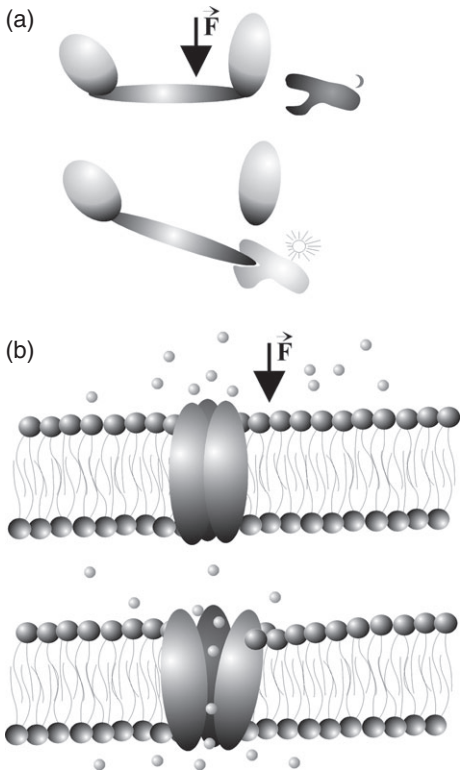

Figure 1. Principle mechanisms for sensing mechanical stimuli.
(a) Stretching of anisotropic molecules provides mechanosusception, such that differential sets of interacting molecules are recruited and mechanoperception is brought about through the changed activity of these recruited molecules.
(b) Stretch-activated ion channels open in response to alterations of membrane curvature, and perception arises from the changed cytoplasmic concentration of ions.

internodal cells of *Chara* (for a review, see Jaffe *et al.*, 2002). These touch-sensitive channels mediate an influx of calcium, which could be demonstrated for instance by aequorin-expressing plants (Knight *et al.* 1991). A causative role of calcium fluxes is supported by the finding that calmodulin genes of touch-insensitive Arabidopsis mutants are often affected (Braam and Davis, 1990). A mechanosensitive calcium channel was demonstrated for epidermal cells of *Allium cepa* (onion; Ding and Pickard, 1993), but the molecular identity of mechanosensitive calcium channels has remained elusive. This may result from the difficulty in transferring patch-clamp measurements to physiological function. Removal of the cell wall, as a precondition for the Gigaohm seal required for patch clamping, and the suction by the holding electrode create conditions where most ion channels meet the criteria for mechanosensitivity (Gustin *et al.* 1991).

Both mechanisms are not mutually exclusive, and it seems that in plants they are linked into devices capable of *sensory tensegrity*. The so-called cytoskeleton–plasma membrane–cell wall continuum (Wyatt and Carpita, 1993; Pont-Lezica et al. 1993; Baluška *et al.* 2003) is linked with the reticulate structure of the plasma membrane, termed plasmalemmal reticulum (for review see Pickard, 2008), an integrative structure comprising both adhesive components (such as arabinogalactan proteins and wall-associated kinases) and functional analogues of integrins, as well as mechanosensory calcium channels. Evidence for this functional unit has been demonstrated in *Nicotiana tabacum* (tobacco) BY-2 cells (Gens *et al.* 2000; Pickard and Fujiki, 2005). The plant integrin analogues have been proposed to connect microtubules, plasma membrane, actin filaments and stretch-activated membrane channels (for a review, see Telewski, 2006). Such a network could convey and focus mechanical forces upon stretch-activated channels. But it might also induce conformational changes leading to altered decoration, with the associated proteins as trigger for further biochemical signalling. The necessity for stress to be focused can be concluded from the activation energies: mechanosensitive channels require around 1 mN m^{-1} for activation (Sachs and Morris, 1998), which is not far below the lytic tension of plant membranes, estimated to be approximately 4 mN m^{-1} (Kell and Glaser, 1993).

Through its interaction with both the cell wall and the plasma membrane, the plant cytoskeleton is therefore ideally suited as a susceptive structure to collect, integrate and process mechanical stimuli. These sensory functions of the cytoskeleton have therefore to be seen in context with the mechanical field collected and shaped by the cell wall.

THE CELL WALL: A SENSORY EXTENSION OF SENSORY MICROTUBULES?

During the cell cycle, plant microtubules are dynamically reorganized into different arrays. These arrays convey different cellular functions. In the interphase, microtubules form parallel bundles oriented perpendicular to the axis of preferential cell expansion. These so-called cortical microtubules control the direction of cellulose deposition, and reinforce axial cell expansion (reviewed in Geitmann and Ortega, 2009). Cortical microtubules can change their orientation in response to a broad range of signals. Thus, the microtubule-guided deposition of cellulose provides a versatile mechanism to tune plant morphogenesis with the challenges of the environment, and therefore is also of agronomical importance (for a review, see Nick, 2012). It is actually this phenomenon that stimulated the current special issue (as described in more detail by the contribution of Hepler *et al.*): in a thin-walled cylinder under tension the laws of physics should promote lateral expansion. The search for a mechanism that 'reinforces' longitudinal expansion through anisotropic extensibility of the cell wall led Paul Green (1962) to predict the existence of 'microtubules' even before they were actually discovered 1 year later by Ledbetter and Porter (1963). Microtubules were conceived as tracks guiding the movement of motors pulling cellulose synthase complexes through the fluid membrane, leaving a 'trace' of crystallizing cellulose behind them (Heath, 1974). This 'monorail' model was later challenged by observations where the cellulose synthase complexes were found 'in gap' between adjacent microtubules (Giddings and Staehelin, 1988), leading to a 'guard rail' model, assuming that microtubules induce small protrusions in the plasma membrane that confine the movement of the enzyme complexes driven by the crystallizing cellulose itself.

During the last decade, the classical 'monorail' model has regained support from evidence for a central role of kinesins and microtubule binding proteins in cell-wall deposition (recently reviewed by Cai and Cresti, 2012). For instance, fluorescently tagged cellulose synthases were shown to move in tracks adjacent to the subtending cortical microtubules (Paredez *et al.*, 2006). Moreover, a protein interacting with cellulose synthase (CSI1) was found to bind microtubules directly (Li *et al.*, 2012). Additional molecular players in the microtubule 'monorail' complex have been identified through a screen for reduced mechanical resistance in *Arabidopsis thaliana*. These *fragile fiber* mutants were specifically affected in wall texture. One of these mutants, *fragile fiber 2*, allelic to the mutant *botero*, was altered in the microtubule-severing protein katanin, leading to swollen cells and increased lateral expansion (Burk and Ye, 2002). 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 strongly suggested that the FRA1/KIF4 motor is a component of the 'monorail' complex (Zhong *et al.*, 2002).

However, the 'monorail' model suffers from a couple of weaknesses, and calls 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 a 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-organization that was sustained by microtubules during cell elongation by constraining the secretion of non-cellulosic 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-organization of cellulose synthesis. The microfibrils that are already laid down could act as templates for the synthesis of new microfibrils (for a review see Mulder *et al.*, 2004). As a consequence, microtubules would not be required during all stages of cellulose deposition.

Last but not least, there is clear evidence for an effect of the cell wall upon cortical microtubules. As membrane-associated structures, cortical microtubules are connected with the extracellular matrix through transmembrane proteins that so far have not been identified but seem share certain analogies with animal integrins (Baluška *et al.*, 2003; Pickard, 2008). This link seems to stabilize cortical microtubules. For instance, removal of the cell wall renders microtubules more cold-sensitive in tobacco cells (Akashi *et al.*, 1990). Moreover, cobtorin, compound identified from a screen that specifically disturbs the parallelity of microtubules and microfibrils (Yoneda *et al.*, 2007) has meanwhile been found to target to cell wall pectins (Yoneda *et al.*, 2010).

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

Thus, the cell wall can be conceived as an extension of cortical microtubules that, despite the chemical stability of the β-1,4-glucose chains, is endowed with certain textural dynamics.

SENSORY MICROTUBULES AND ARCHITECTURAL INTEGRATION

Mechanical tension is used to integrate the architecture of the expanding plant. In terrestrial plants, the considerable lever forces from branches and leaves are not compensated for by buoyancy, and require the compensatory deposition of supportive structures. Mechanical force can instantenously reach 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 from the expanding protoplasts upon the counteracting cell wall. The turgor of individual cells accumulates to considerable tissue tension on the organ level, and this hydraulic principle seems to be used as signal for the integration of the body plan (Niklas and Spatz, 2004). The pattern of tissue tension will locally increase when new organs emerge, a phenomenon that has been intensively studied and modelled for phyllotaxis by Paul Green and co-workers (Green, 1980). This model stimulated experiments in which the cell wall was locally softened by beads coated with expansin (Fleming *et al.*, 1997), which could induce appendices that resembled primordia.

Their work could demonstrate that stress–strain patterns derived from the buckling of the pre-existing older primordia predicted the positions where incipient primordia would be laid down. Interestingly, the first cellular event of primordial initiation is a reorientation of cortical microtubules that reorient perpendicularly with respect to the microtubules of the non-committed neighbouring cells. This difference is at first sharp, but is later smoothed 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 live-cell imaging with fluorescent microtubule markers in *Arabidopsis thaliana* (Hamant *et al.*, 2008), accompanied by the modelling of stress–strain patterns. This approach revealed that cortical microtubules align in the direction of maximal mechanical stress in a transcellular pattern. By ablation of the outer meristem layer, the resulting changes of stress pattern could be modelled and, in fact, microtubules were then observed to reorient accordingly, leading to a compensatory bulging of the apex. The impact of cortical microtubules is further corrobated by recent evidence for a role of the microtubule-severing protein katanin for meristem patterning (Uyttewaal *et al.*, 2012). However, not only cortical microtubules can respond to mechanical load, but also other microtubule arrays. Three decades ago it could already be demonstrated that new cell plates (structures that are guided by the phragmoplast microtubules) in callus subjected to compression forces align with the force vector (Lintilhac and Vesecky, 1984). Mechanosensing by microtubules has also been demonstrated using the alignment of cell divisions parallel with the force vector after mild centrifugation (Wymer *et al.*, 1996).

Architectural integration allows plants to optimize the arrangement of force-bearing elements in space in a manner such that they provide optimal mechanical support, but simultaneously consume minimal biomass and are as light as possible. This task requires the orientation of architecture with gravity. When the orientation of a plant is changed with respect to gravity, the plant will respond by bending in such a way as to restore the original orientation, and thus to minimize mechanical stress (gravitropism). When new organs develop, they are often adjusted with respect to gravity (gravimorphosis). To sense the direction of gravity, high sensitivity is required. Whereas the direction of light in phototropism is sensed by measuring the gradient of light over the sensing organ (Buder, 1920; Nick and Furuya, 1996), the difference in the gravitational field over the two flanks of a tilted plant organ is certainly too small to be perceived. Gravity must therefore be sensed by individual cells. Thus, the maximal energy available for stimulation is the potential energy of the sensing cell itself. If it were not focused in small areas, this energy would barely exceed that of thermal noise. These considerations stimulated research on a potential role of microtubules as amplifiers of gravitropic perception. In fact, gravitropism can be blocked by antimicrotubular drugs in the rhizoid of *Chara* (Hertel and Friedrich, 1973), as well as in moss protonemata (Schwuchow *et al.*, 1990; Walker and Sack, 1990) or in the coleoptiles of *Zea mays* (maize; Nick *et al.*, 1991) and *Oryza sativa* (rice; Godbolé *et al.*, 2000; Gutjahr and Nick, 2006), at concentrations that leave the machinery for growth and bending essentially untouched. Conversely, when the dynamics of microtubules are reduced as a consequence of either a mutation (Nick *et al.*, 1994) or treatment with taxol, this results in a strong inhibition of gravitropic responses (Godbolé *et al.*, 2000; Gutjahr and Nick, 2006).

Microtubules participate in the bending response to gravity: in the upper flank of a gravitropically stimulated organ the originally transverse cortical microtubules are replaced by longitudinal arrays, whereas microtubules remain transverse in the lower flank, and thus support efficient cell elongation (Nick *et al.*, 1990; Himmelspach *et al.*, 1999). It is therefore crucial to discriminate a 'sensory' function of microtubules from their role in the downstream response to gravity. This can be achieved by a dose–response series with antimicrotubular compounds. These compounds act by sequestering tubulin heterodimers from being integrated into assembling microtubules, such that microtubules will be eliminated depending on the degree of their innate turnover. At low concentrations of these drugs, gravitropic perception is blocked, whereas growth (as monitored, for instance, by the ability to respond to a phototropic stimulus) still proceeds (Schwuchow *et al.*, 1990; Nick *et al.*, 1991; Gutjahr and Nick, 2006). Moreover, lateral auxin transport, an event upstream of curvature, is also blocked by antimicrotubular compounds, and interestingly also by taxol, a drug that blocks their disassembly and thus impairs reorientation (Godbolé *et al.*, 2000).

It may seem trivial that roots form at the lower pole of a plant organ, but this is actually a manifestation of gravimorphosis. Although a considerable volume of phenomenological work was dedicated to this problem at the turn of the 19th century (Vöchting, 1878; Sachs, 1880; Goebel, 1908), the underlying mechanisms remain far from being understood. This is partially because of the use of adult organs, where polarity has already been fixed and persists upon inversion; however, in germinating fern spores, the first asymmetric division that separates a larger, vacuolated rhizoid precursor from a smaller, denser thallus precursor can be oriented by gravity (Edwards and Roux, 1994). This first cell division is clearly of formative character: when it is rendered symmetric by treatment with antimicrotubular herbicides (Vogelmann *et al.*, 1981), the two daughter cells both give rise to thalloid tissue. When this spore is tilted after the axis of the first division has been determined, rhizoids will grow in the wrong direction and cannot adjust for this error (Edwards and Roux, 1994). Prior to division, at the time when the spore is competent to the orienting influence of gravity, a vivid migration of the nucleus towards the lower half of the spore is observed. This movement is not a simple sedimentation process, because it is oscillatory in nature and interrupted by short periods of active sign reversal, indicating that the nucleus is tethered to a motive force (Edwards and Roux, 1997). The action of antimicrotubular compounds strongly suggests that this guiding mechanism is based on microtubules that probably align with the gravity vector, resembling the determination of the grey crescent in amphibian eggs (Gerhart *et al.*, 1981), where the prospective dorsoventral axis is determined by an interplay between gravity-dependent sedimentation of yolk particles, sperm-induced nucleation of microtubules and self-amplifying alignment of newly formed microtubules that drive cortical rotation (Elinson and Rowning, 1988).

In summary, a specific subpopulation of microtubules that is highly sensitive to antimicrotubular compounds (indicating a high turnover of these microtubules) is involved in the perception of mechanical forces, and can be clearly discriminated from microtubules that are involved in the downstream growth responses. This function of microtubules in mechanosensing participates in architectural integration, minimising mechanical tension either produced by outgrowth of new organs (as in the case of phyllotaxis) or gravity (as in the case of gravitropism and gravimorphosis).

YIELD TO PERSIST: SENSORY MICROTUBULES AND OSMOADAPTATION

Life is based on the ability to maintain an internal space that is chemically different from the environment. This

function is provided by lipid bilayers that are semipermeable and therefore subject to osmotic challenges. Multicellular animals circumvent osmotic forces by establishing an isotonic cellular environment using specialized excretion organs. In contrast, the cells of bacteria, fungi and plants have to cope with variable osmotic conditions. When these organisms are sufficiently supplied with water, the plasma membrane is subject to considerable pressure from the cell interior that is balanced by a cell wall. However, as a consequence of drought or salinity, the protoplast shrinks and is detached from the cell wall. In order to maintain a functional metabolism, the cells must sense osmotic changes and adjust their volume actively. It is therefore not surprising that prokaryotes have already evolved mechanisms to sense osmotic forces at the plasma membrane (reviewed in Kung, 2005). Osmosensing is then followed by active changes in volume (reviewed in Koivusalo *et al.*, 2009). Several decades ago wall-free plant protoplasts were shown to swell or shrink considerably within seconds in response to fluctuations of osmotic potential, without changing their spherical shape (Wolfe *et al.* 1986). As the plasma membrane is a lipid bilayer, its capacity for areal expansion is limited. Elastic stretching of plasma membranes is confined to around 2% during the first second after transfer into hypo-osmotic medium (Wolfe *et al.* 1985). Swelling or shrinkage of round protoplasts must therefore be caused either by the insertion of additional membrane material (in the case of swelling) or by the internalization of excess membrane material (in the case of shrinking). This is different from the situation in animal cells, where large proportions of the plasma membrane are folded into filopodia, ruffles and other protrusions, such that considerable increases in cell volume can be accommodated without the need for adjusting the cell surface (Koivusalo *et al.*, 2009).

Prokaryotes respond to osmotic stress by the active transport of ions, as shown for the mechanosensitive channel of large conductance, MscL, in *Escherichia coli* (Kung, 2005). In animal cells, such concentration changes remain minute (Koivusalo *et al.*, 2009), and are therefore thought to be of minor impact as compared with the yielding of membrane protrusions and ruffles. As animal cells lack a cell wall, and as the membrane itself cannot provide a mechanical barrier, the actin cytoskeleton has been proposed to control shape after osmotic challenge in animal cells (for a review, see Papakonstanti *et al.*, 2000). The bundling of actin has also been reported for plant cells subjected to a hyperosmotic shock (Komis *et al.*, 2002b).

A plant-specific variation of the theme is a strong and transient response of microtubules to hyperosmotic stress: microtubules first disappear, but soon are replaced by massive bundles that have been termed macrotubules (Komis *et al.*, 2002a). The formation of macrotubules can be suppressed by oryzalin, which at the same time blocks osmoadaptation, demonstrating that this microtubule response is not a byproduct of adaptation but represents an essential event. A pharmacological study on this phenomenon revealed that inhibitors of phospholipase D, such as *n*-butanol or *N*-acetylethanolamine suppress both macrotubule formation and osmotic adaptation (Komis *et al.*, 2006). Phospholipase D generates phosphatidic acid, and this enzymatic product can rescue the inhibition by *n*-butanol. The impact of phospholipase D is also supported by the observation that a T-DNA insertion into the PLD gene affects the response of *A. thaliana* to drought stress (Hong *et al.*, 2008).

Interestingly, phospholipase D had originally been identified as a membrane linker of plant microtubules (Gardiner *et al.*, 2001), suggesting phospholipase D as a signalling hub controlling the interaction between the plasma membrane and the 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.

The inhibition of phospholipase-D signalling by *n*-butanol is explained by the fact that *n*-butanol can activate phospholipase, but simultaneously acts as an acceptor for the cleavage product phosphatidic acid, which is thus consumed (Munnik *et al.*, 1995). In contrast, *sec*-butanol can activate phospholipase D without accepting phosphatidic acid, which is considered as the relevant signal (for a recent review, see Testerink and Munnik, 2011). Therefore *n*-butanol will block signalling, whereas *sec*-butanol should not be active. In fact, *n*-butanol, but not *sec*-butanol, can disorganize cortical microtubules in *A. thaliana* (Gardiner *et al.*, 2003), consistent with a model that transphosphatidylation is required for the effect. This was explained by a model in which the transfer of the phosphatidyl moiety to phospholipase would cleave the membrane interaction of microtubules, detaching them from the membrane (Dhonukshe *et al.*, 2003). However, this model was challenged by reports that (relatively high concentrations of) *n*-butanol can disrupt microtubules *in vitro*, and that some microtubules remain attached to the plasma membrane of tobacco protoplasts after treatment with *n*-butanol (Hirase *et al.*, 2006). Also, in stramenopile cells, *sec*-butanol can induce microtubule depolymerization, indicating that an alternative pathway independent of phosphatidic acid can convey the signal from phospholipase-D activation to cytoskeletal downstream targets (Peters *et al.*, 2007).

The signalling that activates osmoadaptation in plants is linked with the activity of the phospholipase-D signalling hub. In addition to the canonical signalling, running through the production of phosphatidic acid, there seem to exist alternative pathways. 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). An attractive possibility to be explored would be a modulation of the phospholipase-D signalling hub, depending on the 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 and causing, among other responses, the formation of stable macrotubules. This mechanism would explain why microtubules must yield in order to persist.

SECONDARY MEMBRANE STRESS: MICROTUBULES AND COLD ADAPTATION

Touch, wounding and osmotic challenge impose a direct mechanical stress upon the membrane. However, there exist additional abiotic stress factors that affect membranes in a more indirect manner, with cold stress being most prominent. 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). Consistent with this model, the overexpression of desaturases can modify the chilling sensitivity of plants (Murata *et al.*, 1992). The signal relevant for cold perception seems to be increased membrane rigidity (Los and Murata, 2004). In fact, the input of low temperature can be mimicked by chemical compounds that increase rigidity, such as dimethylsulfoxide, whereas cold signalling is blocked by benzyl alcohol, a compound that increases membrane fluidity (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). Inhibitor studies (Monroy *et al.*, 1993) confirmed that this calcium spike is not only a byproduct of the cold response, but is necessary to trigger cold hardening. A study using a cold-responsive promotor–reporter system identified microtubules as additional components of cold sensing. Using this reporter, both disassembly of microtubules by oryzalin or treatment with the calcium ionophore A23187 were found to mimick 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).

As microtubules disassemble in the cold, they have long been discussed as direct sensors for low temperature. The cold sensitivity of microtubules is subject to evolutionary change and correlates with the cold sensitivity of a species. Whereas mammalian microtubules already disassemble at temperatures below +20°C, the microtubules from cold-water fish remain intact far below that temperature (Modig *et al.*, 1994). In plants, the cold stability of microtubules is generally more pronounced compared with animals, reflecting their higher developmental plasticity. However, the critical temperature at which microtubule disassembly occurs varies between different plant species, which is correlated with differences in chilling sensitivity (Jian *et al.*, 1989). At the same time abscisic acid, which can induce cold hardiness (Irving, 1969), stabilizes cortical microtubules against low temperature (Sakiyama and Shibaoka, 1990; Wang and Nick, 2001). In fact, antimicrotubular compounds alter cold hardiness (Kerr and Carter, 1990). Transgenic tobacco lines in which microtubules are more cold-stable, because of the expression of an activation tag, show cold-resistant leaf expansion (Ahad *et al.*, 2003). Conversely, the destabilization of microtubules by assembly blockers such as colchicine or podophyllotoxin increased the chilling sensitivity of cotton seedlings, an effect that could be rescued by the addition of abscisic acid (Rikin *et al.*, 1980). Gibberellin, a hormone that has been shown in several species to reduce cold hardiness (Irving and Lanphear, 1968; Rikin *et al.*, 1975), renders cortical microtubules more susceptible to the cold (Akashi and Shibaoka, 1987).

Cold-resistant species are able to sense low temperature and to respond by an adaptive response, termed as cold hardening. It is possible to increase the cold resistance of an otherwise chilling-sensitive species by precultivation at moderately cool temperatures. This cold hardening can also be detected on the level of microtubules. Microtubules of cold-acclimated cells cope better with a freezing shock [*Spinacia oleracea* (spinach) mesophyll, Bartolo and Carter, 1991a; *Secale cereale* (rye) roots, Pihakaski-Maunsbach and Puhakainen, 1995; *Triticum* spp. (wheat) roots, Wang and Nick, 2001; Abdrakhamanova *et al.*, 2003] . Cold hardening proceeds slower after treatment with taxol (Kerr and Carter, 1990; Bartolo and Carter, 1991b), indicating that microtubules must disassemble to a certain degree in order to trigger this adaptive response to cold.

Thus, the plant thermometer seems to comprise membrane fluidity on the one hand and microtubule disassembly on the other. Calcium influx is the third player responding to membrane rigidification. As to be expected from this set-up, the activity of cold-triggered calcium channels is negatively modulated by pharmacological stabilization of microtubules, but is amplified by microtubule elimination (Mazars *et al.*, 1997). The resulting signal cascade will activate cold-hardening as an adaptive response to cold stress. Interestingly, as a consequence of this cold hardening, microtubules will acquire cold stability (Pihakaski-Maunsbach and Puhakainen, 1995; Abdrakhamanova *et al.*, 2003), which in turn should result in a reduced

activity of the calcium channels that respond to membrane rigidification. Thus, microtubules would not only mediate cold sensing with high sensitivity, but, in addition, would downregulate sensitivity upon prolonged stimulation, leading to adaptation, a key requirement for any biological sensory process. The analogy to the macrotubules observed during osmotic adaptation is evident.

This microtubular thermometer function is of eminent agronomical importance. In temperate regions, temperature poses major constraints to crop yield. Attempts to increase photosynthetic rates by conventional breeding programmes, although pursued over a long period, were not very successful, which indicates that evolution has already reached the optimum (Evans, 1975). However, optimal photosynthetic rates can only be reached when the leaves are fully expanded. The cold sensitivity of growth is much more pronounced than that of photosynthesis. This means that, in temperate regions, productivity is mostly limited by the cold sensitivity of leaf growth (Watson, 1952; Monteith and Elston, 1971), a conclusion supported by the finding that in cool climates the production of biomass is not source-limited, but is sink-limited (Warren-Wilson, 1966). The major target seems to be the root: it has been known for a long time that root temperature defines the velocity of shoot development (Atkin *et al.*, 1973). Moreover, the thermometer activating the adaptation protecting against chilling damage to the leaves could be located in the root also (Suzuki *et al.*, 2008). The development of winter cereals with efficient cold acclimation allowed us to sow in autumn, and thus to advance the harvest by several weeks in the following year, because the hibernating seedlings with their fully expanded leaves were able to use light more efficiently during the early spring. The earlier harvest bridged the sensitive period before the new crops had been brought in, and the resources from the preceding year had been used up. Thus, cold hardening was a central factor fueling the industrial revolution.

Microtubules play a central role for the cold hardening of winter wheat. This was investigated in three cultivars of winter wheat that differed in freezing tolerance (Abdrakhamanova *et al.*, 2003). During pre-cultivation at chilling temperatures roots became progressively resistant to a challenging freezing shock that would impair growth irreversibly in non-acclimated roots. When microtubules were monitored during cold hardening, a rapid but transient partial disassembly was observed in cultivars that were freezing-tolerant, but not in a cultivar that was freezing-sensitive; however, transient treatment of seedlings with the antimicrotubular herbicide pronamide was able to induce freezing tolerance in the sensitive cultivar. This demonstrates that a transient, partial disassembly of microtubules was necessary and sufficient to trigger cold hardening, which represents a second analogy with the sensing of osmotic stress. In this context, it should be noted that abscisic acid, generally known to increase microtubule stability (for instance, Wang and Nick, 2001), has recently been shown by live-cell imaging to cause a transient destabilization of microtubules that is later followed by stabilization (Seung *et al.*, 2012). The analogy between cold and osmotic stress even reaches the molecular details. The activation of phospholipase D falls under the earliest events triggered by a cold shock (Ruelland *et al.*, 2002), and genetic engineering of phospholipase D can confer freezing resistance in *A. thaliana* (Li *et al.*, 2004), suggesting that the microtubule phospholipase-D signalling hub not only processes osmotic stimuli but also processes cold stress. To discriminate between these two sensory qualities, a mechanism is needed that can decode their individual signatures.

SENSING GEOMETRY

In plant cells that prepare for division, the nucleus migrates towards the site of the prospective cell plate. At the same time, microtubules emanate from the nuclear surface and connect with the cortical cytoskeleton, thus tethering the nucleus to its new position. Once the nucleus has reached its final destination it will organize the preprophase band. The function of the preprophase band has been investigated by a series of elegant experiments in fern protonemata, where the nucleus can be displaced over a large distance by centrifugation (Murata and Wada, 1991). These experiments demonstrated a causal relationship between the preprophase band and the orientation of the ensuing cell plate. In cells where the axis or symmetry of cell division deviates, this deviation is always predicted by a corresponding localization of the preprophase band. The division spindle is always laid down perpendicular to the preprophase band. As soon as the chromosomes have separated, the phragmoplast microtubules appear at the site that had previously been occupied by the preprophase band. Thus, through organising cell division in space, the premitotic nuclear migration controls the entire geometry of the daughter cells.

A plant-specific subgroup of the class-14 kinesin families seems to be a central player of this crucial nuclear movement. These unconventional motors are directed to the minus-end of microtubules and harbour a calponin-homology domain that can bind to actin filaments. As the nuclear positioning is sensitive to inhibitors of microtubules and microfilaments (Katsuta and Shibaoka, 1988), actin–microtubular interaction seems to be relevant. As to be expected from such a cross-linker, the KCH kinesins are dynamically repartitioned during the cell cycle (Frey *et al.*, 2009; Klotz and Nick, 2012): in pre-mitotic cells, KCH is clearly aligned in a punctate pattern along filamentous, mesh-like structures on both sides of the nucleus and on perinuclear filaments spanning and surrounding the nucleus. At the onset

of mitosis, KCH retracts to both sides of the nucleus, but does not associate with preprophase bands nor the spindle apparatus nor the division plate. During late telophase and the beginning of cytokinesis, KCH appears at the phragmoplast, repartitions towards the newly forming nuclei, and aligns the filaments that tether these nuclei to the periphery and the new cell wall (Frey *et al.*, 2010; Klotz and Nick, 2012). The cellular function of KCH was investigated in both loss-of-function and gain-of-function assays. Insertions of the *Tos17* retrotransposon into the rice *kch1* locus leads to increased cell numbers in the coleoptile, whereas overexpression of rice KCH1 in tobacco BY-2 reduced the cell number (Frey *et al.*, 2010). This effect could be attributed to a delay in pre-mitotic nuclear migration.

Two principal modes are conceivable that are not necessarily mutually exclusive. Microtubules and actin filaments might transmit forces that are generated by the KCH1 motor at the perinuclear contact sites to the cortex, such that the nucleus is either pulled or pushed, or both. Alternatively, KCH might simply anchor the perinuclear network at the cell cortex, and move the nucleus by mutual sliding of actin filaments and microtubules in the cortical cytoplasm. From studies in yeast, filamentous fungi and a variety of animal cells, the molecular mechanisms that orient and move nuclei were found to be moderately conserved, and to involve as key players dynein, dynactin and other proteins at the plus ends of astral microtubules, mediating interaction with the cell cortex and actin filaments (Morris, 2003; Yamamoto and Hiraoka, 2003). Both repulsive and attractive forces are generated by a combination of microtubule assembly and disassembly, complemented by dynein-mediated sliding of microtubules along the cell cortex (Adames and Cooper, 2000). In Angiosperms, despite transient reports of dynein homologues (King, 2002), dyneins must be considered absent (Wickstead and Gull, 2007). The mechanisms driving nuclear movement must therefore involve fundamentally different players that are able to interact with both pre-mitotic microtubules and actin filaments. Could KCH proteins be the missing links and act as functional homologues of dyneins by anchoring minus-end-directed motor activity to the cortex?

Microtubules as mechanically rigid structures can convey compression forces, whereas actin as flexible elements can convey traction forces. When both elements are linked by proteins, such as the KCH-kinesins, a tensegral network is formed that can collect and integrate mechanical forces, allowing the determination of symmetry as the nuclear position, where the forces conveyed by the tensegral microtubule–KCH–actin network are balanced. The cytoskeleton-driven searching movement of the pre-mitotic nucleus might therefore be nothing else than an exploration of cellular geometry. In fact, when regenerating protoplasts regenerate their axis and polarity *de novo* this is heralded by just such an exploratory nuclear movement (Zaban *et al.*, 2012), as is also observed during gravimorphosis of germinating fern protonemata (Edwards and Roux, 1997). Using the tensegral cytoskeleton, once it has explored geometry, the moving nucleus arranges the radial microtubules to deposit a belt of endosomes that later, upon completed mitosis, are read out by a different set of 'exploratory' microtubules that organize the phragmoplast (Dhonukshe *et al.*, 2005). Thus, microtubules in concert with actin are used to explore geometry.

WHY MICROTUBULES SHOULD BE GOOD SENSORS

As the tensegral cytoskeleton is linked to the cell wall through integrative linkers, the mechanical strains produced by cellulose microfibrils can align cortical microtubules, thus closing a self-referring circuit between cell wall and cytoskeleton. Cell expansion is reinforced in a direction perpendicular to the orientation of microtubules and microfibrils, and the resulting forces are generated parallel with the major strain axis (Fischer and Schopfer, 1998). These forces will then relay back through the plasma membrane upon cortical microtubules that are aligned in relation to these strains. As individual microtubules mutually compete for tubulin heterodimers, and as the number of microfibrils is limited by the quantity of cellulose synthase rosettes, this regulatory circuit should follow the rules of a reaction–diffusion system (Turing, 1952), and should therefore be capable of self-organization and patterning.

Microtubules are endowed with nonlinear dynamics, leading to phase transitions between growth and catastrophic shrinkage. In addition, they must compete for a limited pool of free heterodimers. Microtubules represent ideal devices to amplify the minute inputs from mechanical stimulation (small deformations of the perceptive membranes change the dynamic equilibrium between the assembly and the disassembly of microtubules at the microtubule plus end) into clear and nearly qualitative outputs that can then be processed by downstream signalling cascades. In all organisms investigated so far, tubulin synthesis is tightly regulated by elaborated systems of transcriptional and post-transcriptional controls, and the artificial accumulation of supernumerous free heterodimers is toxic in most systems (for a review, see Breviario and Nick, 2000). The reason might be that free dimers must be limited in order to couple microtubules growing in different directions by mutual competition, a prerequisite for the self-organization of patterns.

Because of their dynamic instability, microtubules themselves might sense mechanical stress. Growing microtubules are charged by considerable mechanical tension. This tension is caused by the transition of 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). Specific proteins

complexing the growing end (so called +TIP proteins, such as EB1) counteract this innate tension, and thus stabilize the growing microtubules against the catastrophic outward bending of protofilaments. The +TIP complex, including EB1, is therefore subject to mechanical tension, and is a primary target for mechanical strain on microtubules. In fact, mutations in the EB1 family render *A. thaliana* touch insensitive (Bisgrove *et al.*, 2008).

In fact, microtubules might also act as accessory machinery in concert with other mechanosensitive mechanisms. For instance, they can focus mechanical stress upon ion channels, as elucidated from the analysis of touch-insensitive mutants in *Caenorhabditis* (Savage *et al.*, 1989), and inferred from inhibitor studies with stretch-activated (Ding and Pickard, 1993) or cold-activated (Mazars *et al.*, 1997) calcium channels. In this context microtubules act as classical susceptors (*sensu* Björkman, 1988) by translating a physical input (force) into a chemical readout (calcium influx); however, in concert with phospholipase, they might also act in true perception. Because of their innate mechanical strain that has to be continuously counterbalanced by +TIP proteins, microtubules are expected (and observed, see Komis *et al.*, 2002b; Wang *et al.*, 2011) to disassemble upon osmotic imbalance acting on the membrane. This will release phospholipase D and trigger phosphatidic acid-dependent signalling. This model is consistent with the recent finding that phosphatidic acid can bind to NADPH-oxidase, thereby elevating hydrogen peroxide levels, which is then transduced by altered activities of glyceraldehyde-3-phosphate dehydrogenases in the adaptation to drought stress (Guo *et al.*, 2012).

This scintillating role of microtubules as susceptors (requiring stability) and simultaneously perceptors (requiring dynamic instability) is puzzling at first sight, but might be crucial for signature decoding (Figure 2). Signature decoding is not an instantaneous event, but is based on two preconditions: (i) temporal changes of input must be integrated and translated in bifurcations of output; (ii) the output to a certain signal quality is modulated by the presence of further signals of different quality. In other words – a signature decoder discriminates the *history* of a signal, not just its actual amplitude. To read a history, some kind of feedback of downstream signalling upon perception is required. Microtubule-based osmotic adaptation might provide a good illustration for this: reactive oxygen species such as those generated by phospholipase-activated NADPH-oxidase activity (Guo *et al.*, 2012) destabilize microtubules (Livanos *et al.*, 2012), closing a self-referring

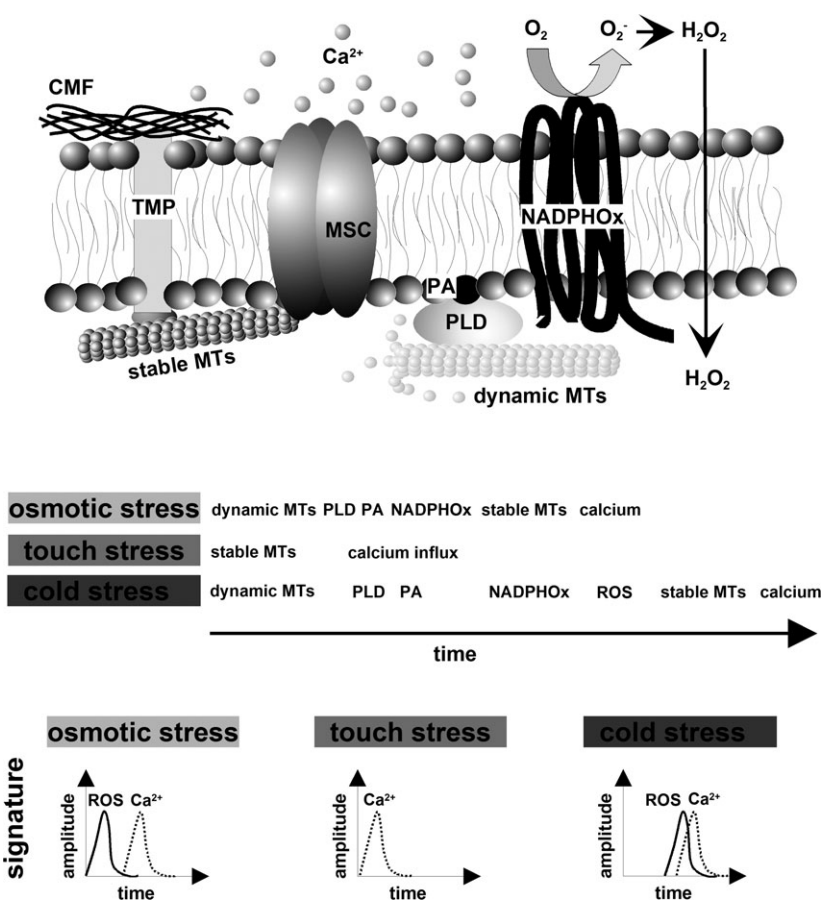

Figure 2. Working model for microtubular function in the decoding of stress signatures. Microtubules with elevated stability can act as (mechano-) susceptors, and microtubules with elevated dynamics can directly perceive mechanical stress. Osmotic stress, touch or wounding stress and cold stress are predicted to interact differently with the susceptor and perceptor populations of microtubules, leading to different temporal readouts specific for the respective quality of input.

signalling circuit. This would then modulate the susceptor function of microtubules in the gating of calcium channels (Ding and Pickard, 1993). The readout would be a first wave of reactive oxygen species followed by a second wave of calcium. In the case of mechanical interference (such as touch or wounding), the susceptor function would be affected more directly, such that a calcium wave would result without a preceding oxidative burst. In the case of cold sensing, the oxidative burst is expected to be slower than for osmotic sensing, because it involves two enzymatic activities with pronounced temperature sensitivity. Moreover, the reduced membrane fluidity limiting the diffusion of phosphatidic acid (the product of phospholipase D) is expected to delay the timing even further. As microtubules are cold-sensitive, the gating of calcium channels is expected to be released (Mazars *et al*., 1997), such that the calcium peak will occur simultaneously with an oxidative burst.

The term 'cytoskeleton' suggests a supportive lattice function of microtubules and actin filaments. The development of GFP technology and advances in fluorescence microscopy changed our concepts and revealed that the cytoskeleton should be described not as a structure, but rather as a process in dynamic equilibrium, thus predestined to adopt functions requiring dynamic change. Because of its innate nonlinearity and versatile interaction with a variety of partners, the cytoskeleton would meet all the requirements of a signature decoder. Although this idea is still speculative, it can be tested experimentally. This will require, in the first place, following stress responses at high spatial resolution in time: a challenge, but possible.

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