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Editor

Plant Microtubules

Development and Flexibility

Second Edition

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Plant Microtubules

Development and Flexibility

2nd Edition

Volume Editor: Peter Nick

With 39 Figures and 2 Tables

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Editor

Peter Nick studied biology in Freiburg and St. Andrews and obtained his Ph.D. in 1990 at the University of Freiburg on signal responses of plant microtubules. He then joined the Frontier Research Program of the Riken Institute (Wako-shi, Japan) with a fellowship from the Japanes Science and Technology Agency to search for cytoskeletal rice mutants. 1992 he shifted to the Institut de Biologie Moléculaire des Plantes (IBMP-CNRS) in Strasbourg with a fellowship from the Human Frontier Science Program Organization to develop approaches for the isolation of signal-dependent microtubule-associated proteins. 1994 he returned to Freiburg with a habilitation fellowship of the German Research Council and habilitated 1996 in Botany. After two years as assistant professor, he became leader of a research group on Dynamics of the Plant Cytoskeleton funded by the Volkswagen Foundation. In 2003, he accepted a professor position at the the University of Karlsruhe, and since 2005 he is director of the Botanical Institute 1 at the University of Karlsruhe. Since 2003, he is editor-in-chief of PROTOPLASMA. His major research interests are the cytoskeletal functions in plant morphogenesis and development.

Preface to the Second Edition

Life is not easy. There are basically two strategies to cope with this: run away (that is basically, what animals do) or adapt (the plant strategy). Plants with their photosynthetic life style had to develop an architecture, where the area of external surface is maximized leading to the consequence that considerable mechanical load has to be balanced. The result is a sessile lifestyle that shapes plant life down to the level of individual cells. Plant cells with their rigid cell walls cannot move and therefore had to evolve alternative mechanisms to respond to the challenge posed by the environment. They adapt morphogenetically by adjusting their axis during cell division and by controlling the expression of this axis during subsequent expansion. In addition, although plant cells are immobile as entities, they are nevertheless highly dynamic with respect to their intracellular architecture. Plant microtubules have evolved into a versatile tool that allows plant cells to regulate their axis and architecture in a very flexible manner and in concert with the signals and challenges they perceive from the environment. What qualifies microtubules for this task? They are endowed with nonlinear dynamics with growing and shrinking states and a pronounced competition for free tubulin heterodimers. Self-amplification in combination with mutual inhibition are classical traits of reaction-diffusion systems that spontaneously lead to patterned outputs and display surprising properties such as proportional regulation, and high sensitivity at simultaneously robust outputs. In other words it is the specific dynamic properties of microtubules that have shaped them into a kind of molecular toolkit for cellular morphogenesis.

The book will highlight the morphogenetic potential of plant microtubules from three general viewpoints. The first part gives a survey on microtubular functions during plant development such as establishment of a division and expansion axis, interaction between microtubules and actin filaments, and control of cell-wall texture. The second part is dedicated to the interaction between microtubules and environmental challenges including fungal pathogens, viral attacks, and abiotic stress. The third part draws attention to the evolutionary processes that have led to the unique organization of plant microtubules and covers the intricate regulation of tubulin expression as well as a phylogenetic view on mitosis.

Plant-specific microtubule arrays, such as the preprophase band and phragmoplast define axis and symmetry of cell division and thus set the framework for subsequent cell expansion. The chapter "Control of Cell Axis" attempts a synthesis of classical research with recent developments on this topic. During recent years, our understanding of two central enigmas of plant-microtubule organization has substantially advanced: The deposition of the phragmoplast and cell plate has long been known to correlate with the localization of the premitotic preprophase band. However, the premitotic microtubular arrays disappear at the time when the spindle appears. It was therefore unclear, how the preprophase band can determine the phragmoplast. An endosomic belt is deposited prior to mitosis and "read out" by exploratory microtubules in anaphase. The reorientation of cortical microtubules, central to the adjustment of cell expansion, has now been analyzed by means of live-cell imaging. Direction-dependent microtubule lifetimes, spatial patterns of post-translational modifications, and new mutants with deviating orientation of microtubules shed light into a network of highly dynamic, nonlinear interactions that are endowed with pattern-generating properties.

Although, by tradition, the research communities dealing with microtubules and actin filaments are mostly separate, it is implicitly assumed that these two elements of the plant cytoskeleton are mutually interdependent. This interaction is only rarely explicitly discussed, though. The chapter "Crossed-Wires: Interactions and Cross-Talk between the Microtubule and Microfilament Networks in Plants" will bridge this gap. The cross-talk between microtubules and actin filaments does not only include the long-known colocalization between microtubules and microfilaments, but implies more indirect, possibly regulatory, interactions as well. From pharmacological studies, mutant analysis and genetic manipulation, the intensity of this cross talk has emerged for organelle movement, organelle morphogenesis, cytoplasmic streaming and localized cell expansion. The elucidation of the molecular players that link or cross-regulate the two cytoskeletal systems has made quite some progress and has pinpointed the Rop-signalling pathway as a central element.

Cortical microtubules are not only central regulators of the cell axis, but define the texture of the secondary wall. Bundles of microtubules mark the sites where cell-wall thickenings are going to be formed. Especially the texture of cellulose in the central S_2 layer of the secondary wall is important and will set the spatial framework for lignification to proceed and thus influence the mechanical properties of wood as described in the chapter "Microtubules and the Control of Wood Structure".

Microtubules respond sensitively to environmental challenges and thus represent key factors for the resistance of plants to pathogen attack as pointed out in the chapter "Microtubules and Pathogen Defence". When microtubules and actin filaments are eliminated by inhibitors, this will specifically affect the defence to fungal pathogens, allowing nonpathogenic fungi to penetrate successfully into nonhost plants. On the other hand, several plant pathogens

produce anti-cytoskeletal compounds to suppress the cytoskeletal response. Viral pathogens, in contrast, usurp the microtubules of the host plant to spread from the infection site through the plant. As reviewed in the chapter "Microtubules and Viral Movement", the spread of viral infection depends on specialized virus-encoded movement proteins that are targeted to plasmodesmata to facilitate viral movement from cell to cell.

Microtubules as targets of numerous signalling chains have now been well established, which is especially important in plants, where morphogenesis is under tight control of a broad panel of environmental cues. However, they are not only targets for signalling, but participate very actively in signal transduction itself. The chapter "Microtubules as Sensors for Abiotic Stimuli" reviews the role of microtubules in the sensing of abiotic stimuli that alter the mechanical properties of biological membranes. Focussing on the stimuli touch, osmotic pressure, gravity and cold it is proposed that by their nonlinear dynamics, microtubule assemblies are robust and sensitive signal amplifiers able to sense even minute mechanic stimuli. Using gravi- and cold-sensing as examples, it is shown, how this mechanism can be used very efficiently to detect abiotic stimuli and to adapt to even harsh environments.

Microtubules as versatile morphogenetic tools are, as to be expected, subject to intensive evolution. This evolution acts on the molecular level and has produced a complex and highly flexible regulatory system that is explored in the chapter "Plant Tubulin Genes: Regulatory and Evolutionary Aspects". The regulation of tubulin expression depends on the status of the cell, genetic background and external stimuli, and acts at the level of transcription, translation, folding, post-translational modification, and assembly into microtubules unfolding a real cosmos of interactions that ensure balance and functional diversity of microtubules and exert tight control on the abundance of free dimers, which is a prerequisite for a morphogenetic tool that relies upon the competition of different nucleation sites for free tubulins.

The final chapter "Microtubules and the Evolution of Mitosis" reviews evolution on the level of microtubule organization, which deviates considerably in higher plants, and tries to understand these structural deviations in their evolutionary context. The microtubular divergence between plants and animals can be traced back to their prokaryotic ancestors, when the walled eubacteria are compared to the mycoplasms that lack a cell wall. The complex situation in lower eukaryotes can be understood as variations of this theme. The development of the mitotic structures found in higher plants is laid down already in the Chlorophyta, but the phylogenetic analysis has remained ambiguous, due to possible convergent developments, and due to our still incomplete knowledge of the phylogenetic relationships in many taxa of the algae. At the time when plants shifted to a terrestrial lifestyle, the microtubule arrays we know from higher plants have already been worked out. This was accompanied by a progressive reduction of centriolar functions and the increasing predominance of acentriolar microtubule organization that, during recent years, could

not only be followed on the structural level, but also by a redistribution of microtubule-nucleating proteins such as γ-tubulin. Indicative of the evolutionary processes towards the highly divergent microtubular cytoskeleton of higher plants there exist still curious evolutionary footprints that are difficult to interpret merely in terms of cellular function. These include the cytoplasmic occurrence of the tubulin ancestor FtsZ in mosses or the recent discovery of intranuclear tubulin. These phenomena, at first glance, appear to be exotic and are difficult to understand, if one merely attempts to explain them in terms of current function, but can be readily interpreted as rudiments from a long evolutionary path that was driven by the necessity to divide cells that are surrounded by rigid cell walls.

Since the first edition of this book appeared seven years ago, the field has experienced substantial advances that are basically due to the progress in life-cell imaging and the availability of large-scale tools spinning off from the various high-throughput endeavours. However, these past years have also led us to new questions that could not have been asked previously because our knowledge on plant microtubules was too limited. Plant microtubules are still far from being elucidated and especially the extension of our (rather narrow) set of model organisms to more distantly related plants such as mosses and algae is expected to uncover still many surprises and mysteries.

Karlsruhe, December 2007 Peter Nick

Preface to the First Edition

Manipulation of plant architecture is regarded as a new and promising issue in plant biotechnology. Given the important role of the cytoskeleton during plant growth and development, microtubules provide an important target for biotechnological applications aiming to change plant architecture. The scope of this book is to introduce some microtubule-mediated key processes that are important for plant life and amenable to manipulation by either genetic, pharmacological or ecophysiological rationales.The first part of the book deals with the role of microtubules for plant morphogenesis. Microtubules control plant shape at three levels:

1. Control of cell expansion: cortical microtubules define the orientation of newly synthetized cellulose microfibrils and thus the mechanical anisotropy of the cell wall. Transverse microtubules are a prerequisite for stable cell elongation, whereas oblique or longitudinal microtubules favour a shift in the growth axis towards lateral growth.

2. Control of cell division: the microtubular preprophase band defines axis and symmetry of the ensuing cell division. It marks the site where, after completion of chromosome segregation, the new cell plate will be laid down. This is the cellular basis for the control of branching patterns and phyllotaxis.

3. Control of cell-wall structure: cortical microtubules are bundled at those sites, where cell-wall thickenings are going to be formed. The orientation of cortical microtubules will therefore define the direction of these cell-wall thickenings and thus the spatial framework for lignification. This influences the mechanical properties of wood.

The second part of the book covers the role of microtubules in response to environmental factors. The focus is on three aspects of this vast field:

4. Control of the response to biotic stress: microtubules seem to be involved in the migration of the nucleus towards the infected site upon pathogen attack. The spread of plant viruses such as the tobacco mosaic virus between cells of infected plants seems to utilize actin microfilaments and microtubules. Re-orientation of microtubules that are aligned over several cells accompanies

wound healing and the establishment of new vessel contacts. Formation of mycorhiza and *Rhizobium*-induced root-nodules are further topics in this context.

5. Control of the response to metals: metal ions, such as aluminum or cadmium, limit crop yields in about 40% of the world's arable lands. They cause swelling of root cells and a loss of cell axis. For aluminum a direct interaction with tubulin dynamics has been discovered, opening the possibility to analyze and control the cytoskeletal response to this metal.

6. Control of the response to low temperature: microtubules depolymerize in response to chilling. In plants, the cold sensitivity of microtubules is well correlated with the chilling tolerance of the whole plant. It is possible to manipulate the cold sensitivity of microtubules by ecophysiological rationales and/or certain growth regulators. Moreover, various tubulin isotypes seem to exist that differ in cold sensitivity.

The third part of the book deals with the tools that can be used for biotechnological manipulation:

7. Tubulin genes: in all plant species tested so far there exist several tubulin genes corresponding to several tubulin isotypes with subtle differences in charge, tissue expression, temporal expression and signal inducibility. The corresponding tubulin isotypes seem to confer altered responses of microtubules to cold, herbicides and hormones. These isotypes could either be used directly to manipulate the behaviour of microtubules and thus the response of the plant to stress, or on the other hand, the promotors for these genes could be utilized to drive the expression of other genes of interest with a specific, possibly inducible, spatiotemporal pattern of expression.

8. Cytoskeletal mutants: an increasing panel of mutants becomes available that has been selected either for altered resistance to cytoskeletal drugs or for a changed pattern of morphogenesis.

9. Cytoskeletal drugs: several herbicides act either directly on microtubule assembly or indirectly on microtubule dynamics by interfering with signal chains that control microtubule dynamics. In addition, several growth regulators exert their effect via the microtubular cytoskeleton. There exist species and cultivar differences in drug sensitivity that could be used for weed control as well as for the control of crop growth.

The scope of the book is twofold: it gives a comprehensive overview of the numerous functions of microtubules during different aspects of plant life, and it proposes to make use of the potential of microtubules to influence fundamental aspects of plant life such different as height and shape control, mechanical properties of wood or resistance to pathogens or abiotic stress.

Freiburg, Germany, March 2000 Peter Nick

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Control of Cell Axis

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Abstract Cell movement constitutes a basic mechanism in animal development, for instance during gastrulation or during the development of neural systems. Plant cells with their rigid cell walls cannot move and therefore had to evolve alternative mechanisms to organize their *Bauplan*. In plants, morphogenesis is controlled by the initiation of a cell axis during cell division and by the expression of this axis during subsequent cell expansion. Axiality of both division and expansion is intimately linked with specific microtubular arrays such as the radial array of endoplasmic microtubules, the preprophase band, the phragmoplast, and the cortical cytoskeleton. This chapter will review the role of microtubules in the control of cell axis, and attempt a synthesis of classical research with recent developments in the field. During the last few years, our understanding of two central enigmas of plant microtubule organization has been advanced substantially.

It had been observed for a long time that the spatial configuration of the phragmoplast was guided by events that take place prior to mitosis. However, the premitotic microtubular arrays disappear at the time when the spindle appears. It was therefore unclear how they could define the formation of a phragmoplast. The deposition of an endosomic belt adjacent to the phragmoplast, in combination with highly dynamic exploratory microtubules nucleated at the spindle poles, provides a conceptual framework for understanding these key events of cell axiality.

The microtubule–microfibril concept, which is central to understanding the axiality of cell expansion, has been enriched by molecular candidates and elaborate feedback controls between the cell wall and cytoskeleton. Special attention is paid to the impact of signalling to cortical microtubules, and to the mechanisms of microtubule reorientation. By means of live-cell imaging it has become possible to follow the behaviour of individual microtubules and thus to assess the roles of treadmilling and mutual sliding in the organization of microtubular arrays. Direction-dependent microtubule lifetimes, spatial patterns of post-translational modifications, and new mutants with deviating orientation of microtubules shed light on a complexity that is still far from being understood, but reveals a network of highly dynamic, nonlinear interactions that are endowed with pattern-generating properties. The chapter concludes with potential approaches to manipulation of the cell axis either through cell division or through cell expansion.

1 Cell Axis and Plant Development

During the growth of any organism, volume increases with the third power of the radius. Surface extension, however, increases only with the second power and thus progressively lags behind. In order to balance these two processes, the surface has to be enlarged substantially, either by internal or external exten-

sions. Due to their photosynthetic lifestyle, plants must increase their surface in an outward direction. As a consequence, plant architecture must be able to cope with a considerable degree of mechanical load. In aquatic plants, this is partially relieved by buoyancy, allowing considerable body sizes even on the base of fairly simple architectures. The transition to terrestrial habitats, however, required the development of a flexible and simultaneously robust mechanical lattice, the vessel system. The evolutionary importance of the vessel is emphasized by a large body of evidence. For instance, the so-called telome theory (Zimmermann 1965) had been quite successfully employed to describe the evolution of higher land plants in terms of a modular complexity based on load-bearing elements (the telomes) that are organized around such vessels.

The architectural response of plant evolution to the challenges of mechanical load had a second consequence, namely, a completely sessile lifestyle. This immobility, in turn, determined plant development with respect to its dependence on the environment. During animal development, body shape is mostly independent of the environment. In contrast, plants have to tune their *Bauplan* to a large degree to the conditions of their habitat. Morphogenetic plasticity thus has been the major evolutionary strategy of plants to cope with environmental changes, and fitness seems to be intimately linked to plant shape (Fig. 1).

Mechanical load shapes plant architecture, reaching down to the cellular level. Plant cells are endowed with a rigid cell wall and this affects plant development very specifically and fundamentally. The morphogenetic plasticity of a plant is therefore mirrored by a plastic response of both cell division and cell expansion with respect to axiality. In this response, cell division has to be placed upstream of cell expansion because it defines the original axis of a cell and thus the framework in which expansion can proceed. The deposition of the new cell plate determines the patterns of mechanical strain that, during subsequent cell expansion, will guide the complex interplay between protoplast expansion. This is mainly driven by the swelling vacuole, with the

Fig. 1 Adaptive response of morphogenesis in a tendril of *Vicia faba*. In response to the mechanical stimulus, upon contact with the support, cell elongation becomes arrested in the flank facing the support, whereas it continues at the opposite flank. The resulting growth differential causes a bending response towards the support and will, eventually, result in spiral growth of the tendril around the support. The time-course of the figure covers 24 h

cell wall as a limiting and guiding counterforce. It is even possible to describe the shape of individual cells in a plant tissue as a manifestation of minimal mechanical tension (Thompson 1959), emphasizing the strong influence of mechanical load on plant development.

When plants are challenged by mechanical load, they respond by changes in architecture that will allocate load-bearing elements (vessels and fibres on the organ level, cellulose microfibrils and lignin incrustations on the cellular level) in such a way that mechanical strains are balanced in an optimal fashion at minimal investment of energy and biomatter. This response of architecture is fundamental and involves changes on different levels of organization, from the spatial arrangement of macromolecules up to the allocation of biomatter to different organs.

Mechanical load affects architecture and the composition of the cell wall during cell elongation and subsequent cell differentiation. For instance, mechanical compression leads to a suppression of certain layers of the cell wall (the so-called S_3-layer) in conifer tracheids (Timell 1986; Yoshizawa 1987). Conversely, mechanical tension causes a shift in orientation of cellulose in the gelatinous layer of the challenged wood fibres in such a way that the mechanical strain is optimally buffered (Prodhan et al. 1995).

However, the effect of mechanical load by far exceeds these responses on the subcellular level. Plant cells can respond to a mechanical challenge by acute changes of cell axiality. It is even possible to demonstrate this directly: When protoplasts are embedded into agarose and the agarose block is subsequently subjected to controlled mechanical load (Lynch and Lintilhac 1997), the division planes of the embedded cells will then be aligned either perpendicular or parallel to the principle stress tensors (Fig. 2).

On the level of whole-plant physiology, mechanical stress can cause so-called thigmomorphogenesis, i.e. alterations of growth that result in adaptive changes of shape. For instance, unidirectional stem flexure of young pines (as produced, for instance, by exposure to wind) induced a larger biomass allocation to the roots parallel to the plane of flexing, which in turn resulted in an increased mechanical resistance within the plane of bending stress (Mickovski and Ennon 2003). In other words, the mechanical stimulus altered root architecture in an adaptive way to ensure optimal resistance to the triggering mechanical stress. The losses in yield that are caused by wind are conspicuous – estimates range between 20 and 50% for Graminean crops and reach up to 80% for certain apple varieties (Grace 1977). In addition to the allocation of lateral roots, it is the the angle between the primary root and the branch roots that defines the uprooting resistance of a root system to wind stress (Stokes et al. 1996).

The economic impact of thigmomorphogenesis is tremendous, but very often overlooked. Repetitive mechanical stimulation, e.g. by wind, will cause a redistribution of growth towards lateral expansion. Again, this thigmomorphogenetic response is clearly of adaptive quality. The resistance of a plant to

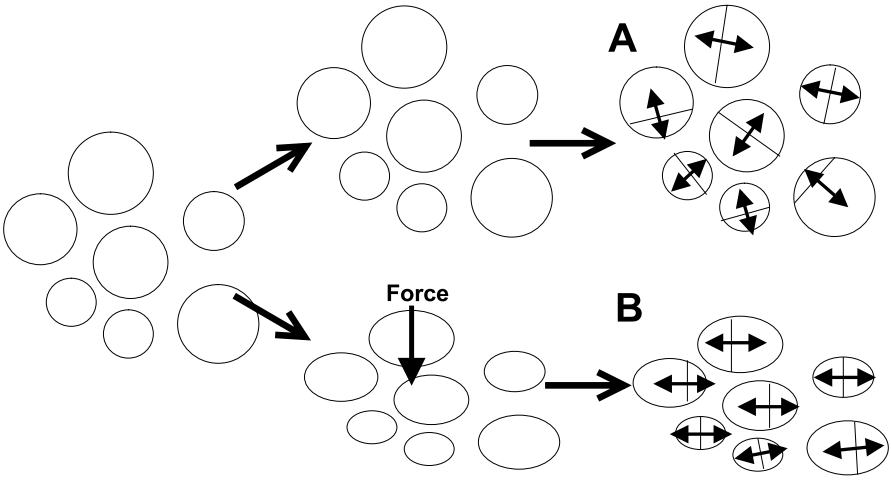


Fig. 2 Alignment of cell division in response to mechanical tension. Protoplasts that are embedded into agarose will divide randomly upon regeneration of the cell wall (**A**). However, when they subjected to mechanical tension, the direction of the subsequent division will be aligned (**B**)

windbreak and lodging is inversely related to plant height (Oda et al. 1966):

$$L_R = \frac{W \cdot M}{L^2\, w},$$

with W = fresh weight, M = bending momentum at breaking, L = shoot length and w = dry weight of the shoot. Thus, lodging resistance will increase parabolically with decreasing plant weight, and a repartitioning of growth from elongation to thickening is a very efficient strategy for increasing lodging resistance, because fresh weight W is kept constant, while the reduction of the shoot length by a given factor will contribute with the second power of this factor.

Lodging is of enormous importance for agriculture and accounts for yield losses up to 10–50% in wheat (Laude and Pauli 1956; Weibel and Pendleton 1961), up to 60% in barley (Schott and Lang 1977; Knittel et al. 1983) and 20–40% in rice (Basak 1962; Kwon and Yim 1986; Nishiyama 1986). The increase of lodging resistance therefore has been a traditional target for agricultural technology over several decades, especially in Graminean crops. This includes genetic approaches, where dwarfing genes are introduced into high-yield cultivars (Borner et al. 1996; Makela et al. 1996; Mcleod and Payne 1996), as well as the application of growth regulators such as chlormequat chloride or ethephone (Schott and Lang 1977; Schreiner and Reed 1908; Tolbert 1960).

The success of these strategies is limited by the specific environment generated by modern agriculture, such as high nutrient influx and high canopy densities. These conditions stimulate internode elongation and thus increase

the susceptibility of the crops to lodging and windbreak (Luib and Schott 1990). Most crop plants are typical sun plants, i.e. they exhibit a pronounced shade-avoidance response when grown in dense canopies (Smith 1981). They are able to sense their neighbours through subtle changes in the ratio between red and far-red light utilizing the photoreversible plant photoreceptor phytochrome. They respond to this change in red/far-red ratio by enhanced stem and petiole elongation. The shade-avoidance response is supposed to protect these plants against overgrowth by neighbouring plants. Indeed, this has been confirmed in field trials, where photoreceptor mutants of *Arabidopsis thaliana* that were not able to trigger shade avoidance were monitored under field conditions and found to be less competitive as compared to the respective wild type (Ballaré and Scopel 1997). As useful as this response may be for the survival of a weed like thale cress in a canopy, it is undesired for a crop plant. In the dense canopy of a wheat field, for example, shade avoidance will increase the risk of lodging. In fact, field trials with tobacco plants that overexpress phytochrome and are thus incapable of sensing the reflected light from their neighbours demonstrated that the suppression of shade avoidance allows for increased yield (Robson et al. 1996).

A classical example of thigmomorphogenesis is the barrier response of young seedlings. Upon contact with a mechanical barrier, the major axis of growth tilts from elongation towards stem thickening. This barrier response is triggered by the ethylene that is constantly released from growing stems and accumulates in front of physical obstacles (Nee et al. 1978). The increase in diameter improves the mechanical properties of the seedling, for instance the flexural rigidity, and thus allows the seedling to remove the barrier.

These examples may suffice to illustrate the impact of cell axis on growth, architecture and eventually on the performance of the plant under challenge by the environment. There are basically two mechanisms that define and contribute to the axis of a plant cell: first, the basic geometry of a cell is defined by the axis of cell division; and second, the manifestation of this geometry depends on the axis of subsequent cell expansion. The next two sections will therefore survey the mechanisms that control the axiality of division and expansion.

2 Control of Cell Division

The spatial control of cell division employs specialized populations of microtubules that are unique to plant cells: cortical microtubules, preprophase band (PPB) and phragmoplast (Fig. 3). The cortical microtubules prevailing in interphase cells are usually arranged in parallel bundles perpendicular to the main axis of cell expansion (Fig. 3a). They are involved in the directional control of cellulose deposition and thus in the axiality of cell growth and will

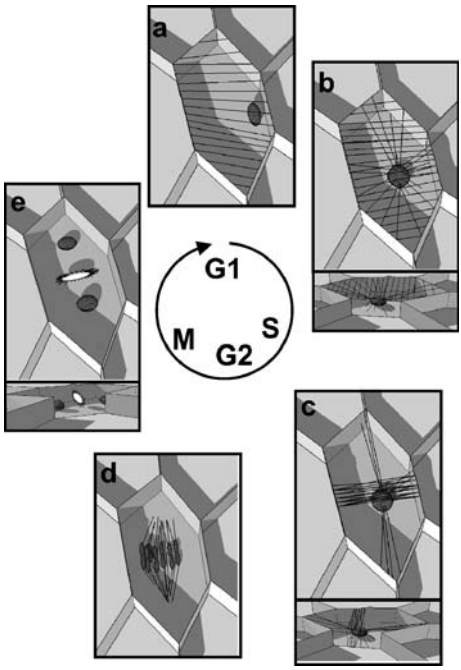


Fig. 3 Microtubular arrays during the cell cycle of higher plants. **a** Elongating interphase cell with corticale microtubules. The nucleus is situated in the periphery of the cell. **b** Cell preparing for mitosis seen from above and from the side. The nucleus has moved towards the cell centre and is tethered by radial microtubules emanating from the nuclear envelope. **c** Preprophase band of microtubules. **d** Mitosis and division spindle. **e** Cell in telophase with phragmoplast that organizes the new cell plate extending in centrifugal direction

be discussed in more detail in Sect. 3. When a plant cell prepares for mitosis, this is heralded by a migration of the nucleus to the site, where the prospective cell plate will form. The nucleus is surrounded by a specialized array of actin microfilaments, the phragmosome (for review see Lloyd 1991; Sano et al. 2005). This phragmosome is, in fact, responsible for the correct positioning of the nucleus (Katsuta and Shibaoka 1988). At the same time, the cortical microtubules are progressively replaced by a new structure, the radial or endoplasmic microtubules that emanate from the nuclear envelope and often merge with the cortical cytoskeleton (Fig. 3b).

Concomitantly with the eclipse of cortical microtubules a band of microtubules emerges at the cell equator. This preprophase band (Fig. 3c) is laid down in parallel to the direction of cortical microtubules and is connected with the nucleus by the radial microtubules and by the phragmosome. The preprophase band (PPB) marks the site and orientation of the prospective cell plate. However, it disappears with the formation of the division spindle that is usually organized in an axis perpendicular to the PPB, whereby the

spindle equator is situated in the plane heralded by the PPB (Fig. 3d). Once the daughter chromosomes have separated, a new array of microtubules, the phragmoplast, emerges at the site of the ensuing cell plate (Fig. 3e). The phragmoplast targets vesicle transport to the periphery of the expanding cell plate. Microtubules seem to pull at tubular-vesicular protrusions emanating from the endoplasmatic reticulum (Samuels et al. 1995). The phragmoplast consists of a double ring of interdigitating microtubules that grows in diameter with progressive extension of the cell plate. New microtubules are organized along the outer edge of the expanding phragmoplast (Vantard et al. 1990).

These observations assign to nuclear migration a central role in the control of division symmetry. Nuclear migration can be blocked by actin inhibitors such as cytochalasin B (Katsuta and Shibaoka 1988), suggesting that the phragmosome forming the characteristic "Maltesian cross" seen in premitotic vacuolated plant cells is, in fact, moving and tethering the nucleus and thus ultimately defines the site where the new cell plate is formed. However, microtubules also seem to be involved in nuclear positioning, since antimicrotubular compounds such as colchicine (Thomas et al. 1977) or pronamide (Katsuta and Shibaoka 1988) have been found to loosen the nucleus such that it can be displaced by mild centrifugation.

At the end of the S-phase, formation of the PPB begins (Gunning and Sammut 1990), which faithfully predicts the symmetry and axis of the ensuing cell division. This is impressively illustrated by asymmetric divisions, for instance during the formation of guard cells (Wick 1991) or in the response of root tissue to wounding (Hush et al. 1990). It has been under debate whether the PPB is more than just a true indicator for the spatial organization of mitosis.

In classical studies, Murata and Wada analysed the functions of the nucleus and PPB in the formation of the ensuing cell plate by means of centrifugation at different time points prior to mitosis (Murata and Wada 1991). As experimental system, they used protonemata of the fern *Adiantum* and elegantly exploited the advantages of these cells. Since they are very long, it is possible to displace the nucleus over a considerable distance leading to clear outcomes. To avoid migration of the displaced nucleus back to its original position (due to the tethering cytoskeletal network), they first induced a phototropic bending and subsequently centrifuged the nucleus into the curved part of the protonemata such that it was prevented from shifting back to the apex. Upon centrifugation prior to the formation of the PPB, the nucleus induced a new PPB in the new (basal) position, where later the new cell plate was formed, suggesting that it is the nucleus that defines the position of the PPB. When the nucleus was displaced from the apex somewhat later (when a PPB had already been laid down), the nucleus induced a second, somewhat smaller, PPB in its new position in the base of the cell. If the centrifugation occurred even later, the nucleus had already lost the ability to induce a second PPB, leading to a situation where an isolated PPB was observed near the apex, whereas the nucleus was found void of a PPB in the cell base. This situ-

ation allowed logical discrimination of the functions of nucleus and PPB in the orientation of cell division. In those cells, the new cell plate was established at the site of the nucleus (i.e. in the cell base) and not at the site of the PPB (i.e. in the cell apex) demonstrating unequivocally that it is the nucleus and not the PPB that determines the position of the ensuing cell plate. However, the cell plate in those cells was laid down randomly with respect to its orientation. Thus, the PPB is responsible for the correct orientation of the ensuing cell plate.

This guiding function of the PPB is supported by evidence from *Arabidopsis* mutants, where the PPB has been reported to be absent. In these so-called *tonneau* or *fass* mutants, the ordered pattern of cell divisions that characterizes the development of the wild type is replaced by a completely randomized pattern of cross walls (Traas et al. 1995; McClinton and Sung 1997). It should be mentioned, however, that, during meiosis, the division plane can be controlled in the absence of a PPB (Brown and Lemmon 1991), suggesting that there exist additional mechanisms of spatial control.

The organization of the PPB is accompanied by a phosphorylation of proteins. Some of these phosphorylated proteins reside in the nucleus (Young et al. 1994), whereas the cell-cycle-dependent protein kinase $p34^{cdc2}$ localizes to the PPB (Colasanti et al. 1993). The formation of the radial array of endoplasmic microtubules can be triggered in interphase cells by cycloheximide, a blocker of protein synthesis (Mineyuki et al. 1994). This suggests that the radial array represents a kind of default state, whereas the cortical microtubules have to be actively maintained by the synthesis of proteins with a relatively short lifetime. Interestingly, the formation of the PPB was not inhibited by cycloheximide, indicating that it is independent from these rapidly cycling proteins.

An intriguing question has been how the PPB can guide the formation of the phragmoplast, since it disappears completely at the time when the nuclear envelope breaks down. Recently, this mystery was at least partially unveiled by in-vivo microscopy. In a beautiful study, Dhonukshe et al. (2005) followed the behaviour of individual microtubules during mitosis of tobacco BY-2 cells. Using the plus-end marker EB1, they observed that the radial microtubules that emanate from the premitotic nucleus are indeed oriented with their plus-ends pointing outwards. They observed further that during the formation of the PPB, a belt composed of endosomes is laid down adjacent to the PPB probably produced by joint action of microtubule- and actin-driven transport. This belt persists during mitosis. Upon completed separation of the chromosome, a new set of microtubules emerges from the spindle poles and "explores" the cell periphery in different directions. Hereby the lifetime of microtubules that hit the endosomal belt is enhanced over that of microtubules that fail to interact with the endosomes and are therefore prone to undergo catastrophic decay. As a consequence, microtubules will be enriched at the site where the PPB was located prior to mitosis. In principle, the individual

"exploratory" microtubules are bound to compete for a limited pool of soluble dimers and thus are linked by mutual inhibition. This system is nothing other than a realization of a reaction-diffusion system, in the Turing sense (1952), combining self-amplification with lateral inhibition. Such systems are capable of self-organization and will rapidly produce a clear output pattern even in a situation of variable and noisy inputs.

Thus, the persistent "trace" that is laid down by nucleus, radial, endoplasmic microtubules and the PPB seems to be an endosomal belt. This "trace" is "read" by "exploratory" microtubules after mitosis, employing their self-organizing properties. As a consequence, the phragmoplast will be formed at the site heralded by the PPB. Thus, the PPB represents the earliest manifestation of the division axis known so far. The spindle is always established strictly in a direction perpendicular to the PPB. However, in small cells (e.g. precursors of the guard cells), it can become secondarily tilted or distorted to oblique orientations as a consequence of limited space (Mineyuki et al. 1988). This does not result in the formation of an oblique phragmoplast or an oblique cell plate, though, indicating that the formation of the spindle must be seen as a bypass of the morphogenetic processes that link nuclear migration, the formation of the PPB and the induction of the phragmoplast.

The PPB decides over the division plane. For the symmetry of division, however, it is nuclear migration and the nuclear envelope that are the decisive factors. They define where the radial microtubule network and the PPB is organized, they define the position of the spindle, and they mark the site where phragmoplast and cell plate will develop. The decisive questions remain to be solved – how is the nuclear movement directed towards the prospective plane of division? How is the nuclear surface differentiated into an equatorial region that can organize a PPB and two polar domains that seem to lack this ability?

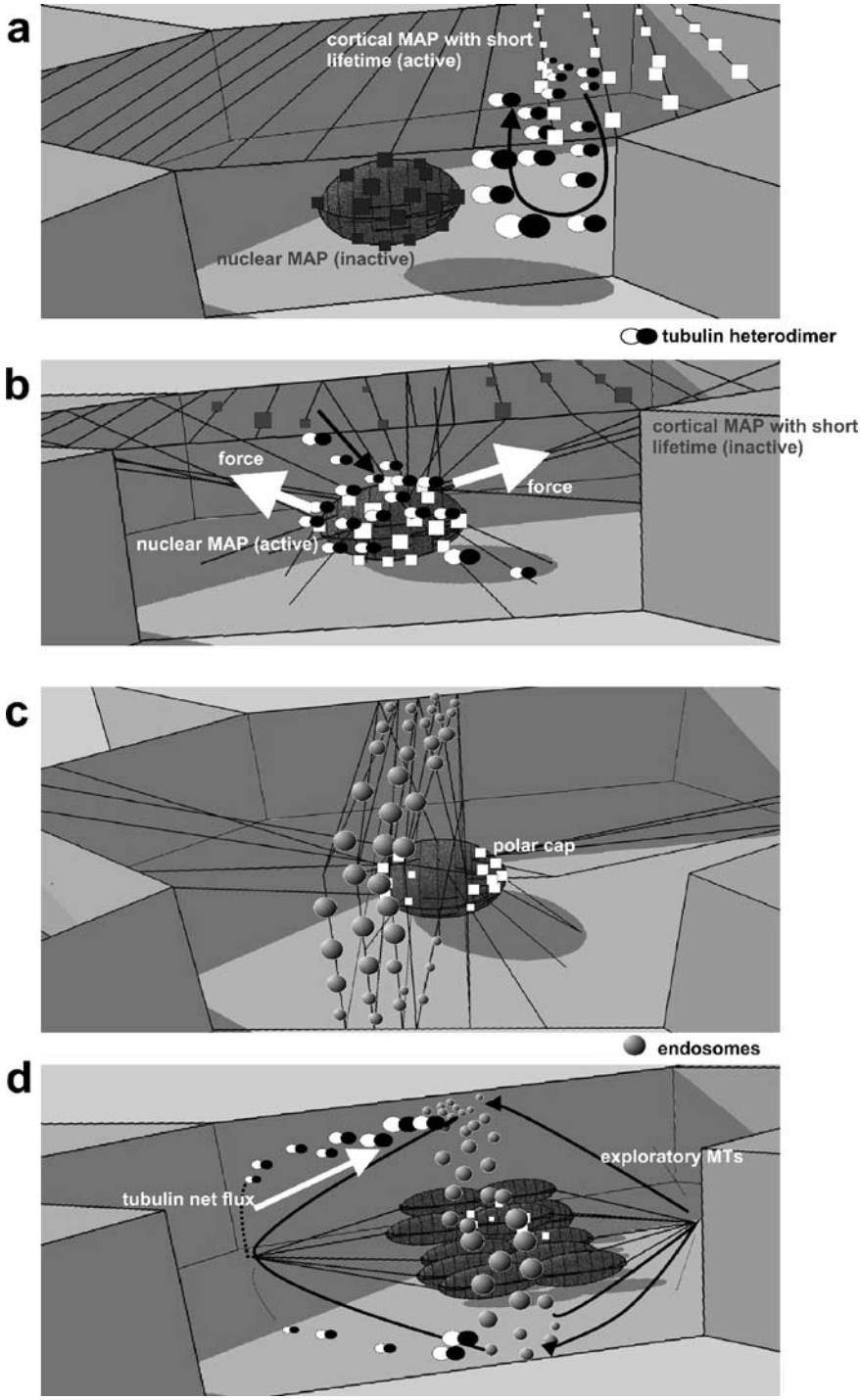
The function of the nucleus as the ultimate organizer of division symmetry is supported by its ability to nucleate microtubules. Whereas spindle microtubules are nucleated from centrosomes in animal and algal cells (Wiese and Zheng 1999), they emerge from rather diffuse microtubule-organizing centres (MTOCs) in the acentriolar cells of higher plants (Baskin and Cande 1990; Shimamura et al. 2004). However, the major MTOC of higher plants seems to be the nuclear envelope (for review see Lambert 1993). In addition, the kinetochores of both animal and plant cells are endowed with a microtubule-nucleating activity (Cande 1990). The nucleating activity of plant MTOCs is mirrored by their molecular composition. For instance, γ-tubulin, a minus-end nucleator of microtubule assembly, is found in centrosomes as well as in MTOCs (Pereira and Schiebel 1997; Stoppin-Mellet et al. 2000), and is also enriched in the nuclear envelope (Liu et al. 1994). The same holds true for CCT, a chaperone that specifically folds nascent tubulin (Himmelspach et al. 1997; Nick et al. 2000). Even during the G_2 phase, i.e. prior to the disintegration of the nuclear envelope, γ-tubulin is imported into the nucleus (Binarová et al. 2000). Interestingly, the breakdown of the nuclear envelope coincides with the

formation of the spindle, suggesting that microtubule-nucleating components of the nuclear envelope might be used to organize spindle microtubules (for review see Nick 1998). In fact, RanGAP1, an accessory protein of the small GTPase Ran involved in nuclear transport, not only localizes to the nuclear envelope, but also decorates spindle microtubules (Pay et al. 2002). The same protein can co-assemble with tubulin into microtubules, but only if the interaction takes place in extracts from cycling (not from non-cycling) cells. The specific role of the nuclear envelope is possibly linked with the presence of proteins or protein domains that are specific for plants. For instance, the plant homologues of RanGAP1 share an N-terminal extension that is absent from their animal counterparts. Conversely, the nuclear-rim protein MAF1 (present at the site where the microtubules of the preprophase band are nucleated) is not found in animals at all (Patel et al. 2004).

Although many of the molecular components organizing cell division in time and space are unknown, it is possible to build first models on the sequence of events (Fig. 4):

1. The cortical array of microtubules is actively maintained in interphase cells by proteins that have to be synthesized continuously (Mineyuki et al. 1994). If the activity of these proteins decreases, this will result in a rapid deterioration of the cortical array. The efficiency of this transition will depend on the lifetimes of individual microtubules. These have been assessed either by microinjection of fluorescent tubulin (e.g. Yuan et al. 1994; Himmelspach et al. 1999) or by expression of GFP-fusions of plant tubulins (e.g. Shaw et al. 2003) and found to be in the range of 30–60 s. Under these conditions, cortical arrays are expected to deteriorate within minutes if their active maintenance becomes arrested.
2. The nuclear envelope contains proteins that are able to nucleate new microtubules (Liu et al. 1994; Stoppin et al. 1994; Himmelspach et al. 1997), and it seems that this nucleating function is actively suppressed during

Fig. 4 Possible mechanisms for the control of division axis and symmetry. During interphase, tubulin dimers are partitioned into cortical microtubule arrays (**a**) due to the activity of a rapidly cycling cortical MAP, whereas the nucleation activity of the nuclear envelope is low. In premitotic cells, the nucleus is moved to a central position (**b**), and the MAPs at the nuclear envelope are activated or unmasked. The activity and/or synthesis of the cortical MAP is reduced such that a net flux of tubulin towards radial microtubules occurs that interacts with the force-generating system (probably actomyosin) that drives and tethers the nucleus. The formation of the preprophase band is accompanied by an endosomal belt in the cell equator (**c**). The microtubule-organizing activity of the nuclear envelope is spatially organized into different domains such as the polar caps. From late anaphase, the endosomal belt is "read" by exploratory microtubules (**d**) that emanate from the spindle poles and differ in lifetime depending on their contact with the endosomal belt, resulting in a net flux from incorrectly oriented microtubules towards those microtubules that are correctly oriented ►


a
cortical MAP with short lifetime (active)
nuclear MAP (inactive)
tubulin heterodimer
b
cortical MAP with short lifetime (inactive)
force
force
nuclear MAP (active)
c
polar cap
endosomes
d
exploratory MTs
tubulin net flux

interphase. In the simplest case, the inhibition of microtubule nucleation at the nuclear envelope might be the direct consequence of elevated nucleation activity in the cortical plasma if both sites compete for a limited number of free tubulin dimers (Fig. 4a).

3. At the onset of G_2, this suppression is released (possibly by weakening the active maintenance of nucleation in the cortical cytoplasm leading to an increase of tubulin dimers available for nucleation elsewhere). New microtubules will form spontaneously at the nuclear envelope with their growing ends pointing outwards (Fig. 4b; Dhonukshe et al. 2005).
4. These microtubules, probably in joint action with the microfilaments of the phragmosome, organize the PPB along with a belt of endosomal vesicles in the symmetry plane of the prospective division (Dhonukshe et al. 2005). The detection of cell-cycle regulators such as $p34^{cdc2}$ in the PPB (Colasanti et al. 1993) suggests that these events involve the activity of associated proteins that are under cell-cycle control. An important aspect that is often ignored is the partitioning of the nuclear envelope into different domains (Fig. 4c). Confocal sectioning of the nucleus in *Arabidopsis* cells that express GFP-tagged RanGAP1 reveals that the nuclear surface is not labelled uniformly, but in large patches (Pay et al. 2002). It might be possible that similar types of partitioning could define different regions that differ in their nucleating activity and thus contribute to a regionalization of the nuclear envelope, contributing to the definition of a division plane.
5. The spindle seems to be established independently of the PPB and represents a bypass to the causal chain between radial, endoplasmic microtubules, endosomal belt, PPB and phragmoplast. This is evident from situations where the spindle is secondarily tilted or distorted with respect to the orientation of the PPB due to space limitations (e.g. during the formation of guard cells), but nevertheless the cell plate is deposited correctly, parallel to the PPB (Mineyuki et al. 1988). Moreover, when, in wheat roots, the dissolution of the PPB was blocked by treatment with taxol, an inhibitor of microtubule disassembly, a spindle was formed although the PPB persisted (Panteris et al. 1995). This spindle, although being multipolar and aberrant, demonstrated clearly that it can be formed independently of the PPB.
6. Following the separation of chromosomes, highly dynamic microtubules emanate from the spindle poles in various directions (Fig. 4d). Those that touch the endosomal belt deposited prior to mitosis are stabilized such that a net redistribution towards this belt is achieved (Dhonukshe et al. 2005). This self-organization requires a high dynamics of microtubules, because misoriented microtubules have to disassemble in order to reach this net redistribution. Consequently, taxol should block the formation of a phragmoplast such that microtubules will be trapped in the spindle. This has indeed been shown for tobacco BY-2 cells (Yasuhara et al. 1993).

7. The phragmoplast will then organize the cell plate by means of motor proteins that are able to bind and transport vesicles containing cell wall material. Phragmoplasts could be purified from synchronized tobacco BY-2 cells and yielded a microtubule-associated protein that binds microtubules dependent on ATP (Yasuhara et al. 1992). A dynamin-like protein, termed phragmoplastin, binds to the newly formed cell plate and is supposed to recruit exocytotic vesicles to the growing cell plate (Gu and Verma 1995). Additional candidates for microtubule-bound cargo have been identified from genetic screens, for instance the KNOLLE protein, a syntaxin that decorates the phragmoplast.

The control of cell axis during cell division is a central element of plant morphogenesis. During the past few years our understanding of this process has advanced quite a bit, although many molecular components still remain to be identified. However, the underlying mechanisms are beginning to emerge. It has become clear that the mother cell does not transmit cell axis in form of a fixed structure. It rather transmits surprisingly vague spatial cues that will guide the self-assembly of microtubular arrays on the background of a high level of stochastic noise. The "exploratory" microtubules, for instance, which emanate from the spindle poles and eventually establish the phragmoplast, grow initially in various directions. Their final orientation is brought about by mutual competition of these highly dynamic microtubules for free tubulin heterodimers. Those microtubules that *by chance* hit the endosomal belt laid down prior to mitosis are stabilized over other microtubules that are misoriented. In a recent conceptual review, the classical view of the cell as a complex type of clockwork was confronted with the findings from live-imaging. This leads to a more dynamic and flexible view of the cell (Kurakin 2005) and the conclusion that cells are not organized in a "Watchmaker" fashion, but mainly by self organization. The way that the cell axis emerges during the division of plant cells provides an excellent illustration of this view. The ultimate tool for this self-organization is the nonlinear nature of microtubules, which can switch rapidly between growth and catastrophe and mutually compete for free dimers.

3 Control of Cell Expansion

Organisms grow by increasing the number of cells (division growth) or the volume of individual cells (expansion growth). In plants, division growth is confined to specific tissues or developmental states, e.g. to embryogenesis or the apical meristems (Steeves and Sussex 1989). During most of their life-cycle, plants grow predominantly by cell expansion. In some organs, such as hypocotyls (Lockhart 1960) or coleoptiles (Furuya et al. 1969; Nick et al. 1994), the growth response is even carried by cell expansion alone.

Plant cells expand by increasing the volume of the vacuole, which accounts for more than 90% of total cell volume in most differentiated cells. The driving force for this volume increment is a gradient of water potential from the apoplast towards the cytoplasm and vacuole, where the potentials are more negative (Kutschera et al. 1987). The expansion of the vacuole would eventually result in infinite swelling and a burst of the cell were it not limited by rigid cell walls. The importance of the cell wall for the integrity of plant cells can be impressively demonstrated when protoplasts are placed in a hypotonic medium (Fig. 5a).

Most plant cells derive from isodiametric meristematic cells, but assume approximately cylindrical shapes during differentiation, especially pronounced in expanding tissues such as hypocotyls, internode, petioles or coleoptiles. This cylindrical shape is usually lost upon removal of the cell wall;

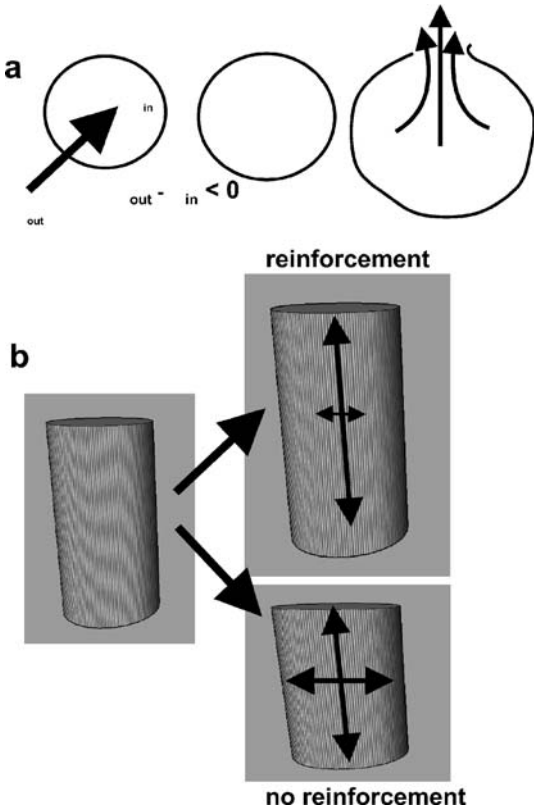


Fig. 5 Role of the cell wall for the axis of cell expansion. **a** Swelling and burst of protoplasts in the absence of a cell wall due to a gradient in water potential between the environment and cell interior. **b** Corroboration of cell axiality (*upper cell*) when expansion is not actively maintained anisotropic by a reinforcement mechanism (*lower cell*)

protoplasts, with very few exceptions, are spherical. This simple fact already illustrates the importance of the cell wall for the control of cell shape.

In cylindrical cells, cell expansion is expected to occur preferentially in a lateral direction, which should progressively corroborate the axiality of these cells (Fig. 5b). This means, on the other hand, that cylindrical cells must be endowed with some kind of reinforcement mechanism to maintain their original axiality during expansion (Green 1980). This reinforcement mechanism seems to reside in the cell wall and was first described for the long internodal cells of the green alga *Nitella* (Green and King 1966). In these elongate cells, the cellulose microfibrils were demonstrated by electron microscopy to be arranged in transverse rings, especially in the newly deposited inner layers of the wall. It should be mentioned that, much earlier, the birefringency of the cell wall had been discovered by polarization microscopy in growing tissue and interpreted in terms of an anisotropic arrangement of cellulose (Ziegenspeck 1948). However, the functional significance of this observation had not been recognized at that time. It is evident that the transverse arrangement of microfibrils can account for the reinforcement mechanism that maintains longitudinal expansion in cylindrical cells (Fig. 5c). The tight correlation between transverse microfibrils and cell elongation has been confirmed in numerous studies and has been discussed in several reviews (Robinson and Quader 1982; Kristen 1985; Giddings and Staehelin 1991; Smith 2005). As expected, reorientations in the axis of growth are accompanied either by a loss or by a switch in the anisotropy of cellulose deposition (Green and Lang 1981; Hardham et al. 1981; Lang et al. 1982; Hush et al. 1990).

In intact organs, the control of growth axiality is not necessarily maintained actively by each cell individually, but is sometimes confined to specific tissues. These tissues, the epidermis in most cases, are responsible for growth control of the entire organ. This can be demonstrated by a very simple experiment in which stem sections are split and subsequently allowed to grow in water. They will then curl inside out because the inner tissues expand faster than the epidermis. If growth-promoting agents such as auxins are added, the sections begin to curl outside inwards, because now it is the epidermis that exceeds the inner tissues in growth. This curling response is so sensitive that it had been used as a classical biotest for auxin (Schlenker 1937). Biophysical measurements confirmed later that, in fact, auxin stimulates the elongation of maize coleoptiles by increasing the extensibility of the epidermis such that its constraint upon the elongation of the compressed inner tissues is released (Kutschera et al. 1987).

As outlined above, the preferential axis of cell expansion is linked with a preferential orientation of cellulose microfibrils. The cell wall in those cells is formed by apposition of cellulose to the inner surface of the cell wall. Specialized cells such as root hairs or pollen tubes, in contrast, grow by intussusception of cell wall material into the cell poles and follow different mechanisms, which have been reviewed elsewhere (Taylor and Hepler

1997; Geitmann and Emons 2000) and will not be considered here. Cellulose is synthesized by specialized enzyme complexes that, in freeze-fracture preparations, appear as rosettes of six subunits of about 25–30 nm diameter surrounding a central pore (e.g. Kimura et al. 1999). These so-called terminal rosettes are integrated into the membrane of exocytic vesicles and, upon fusion of the vesicle, are then inserted into the plasma membrane. UDP-glucose is transported towards the central pore and polymerized in a β-1,4 configuration. Each subunit has been inferred to produce around six cellulose chains that will be integrated by hydrogen bonds yielding a long and fairly stiff cellulose microfibril. These enzyme complexes are thought to move within the fluid membrane and leave a "trace" of crystallizing cellulose behind them. This movement will thus decide the orientation of cellulose microfibrils and thus the anisotropy of the cell wall. It is at this point that the microtubules come into the play.

Even before they were actually discovered microscopically by Ledbetter and Porter (1963), cortical microtubules were predicted to exist and to guide cellulose deposition (Green 1962). During subsequent years, an intimate link between cortical microtubules and the preferential axis of growth has been proposed by a number of studies:

1. Cortical microtubules are closely associated with the plasma membrane, and upon plasmolysis a direct contact between cortical microtubules and newly formed cellulose microfibrils could be demonstrated by electron microscopy (for review see Giddings and Staehelin 1991; Smith 2005).
2. Parallel bundles of thick microtubules mark the prospective sites of cell wall thickening in differentiating cells (Fukuda and Kobayashi 1989; Jung and Wernicke 1990).
3. Changes in the preferential axis of cell expansion are accompanied by a switch in the preferential axis of cellulose deposition, and are preceded by a corresponding reorientation of cortical microtubules (ethylene response, Lang et al. 1982; auxin response, Bergfeld et al. 1988; gibberellin response, Toyomasu et al. 1994; wood formation, Abe et al. 1995; for review see Nick 1998).
4. When cortical microtubules are eliminated by antimicrotubular compounds, this results in a progressive loss of ordered cellulose texture and the axiality of cell expansion, leading, in extreme cases, to lateral swelling and bulbous growth. This effect was first discovered in the green alga *Nitella* (Green 1962), but was later observed in higher plants as well (Hogetsu and Shibaoka 1978; Robinson and Quader 1981; Kataoka 1982; Bergfeld et al. 1988; Vaughan and Vaughn 1988; Nick et al. 1994; Baskin and Bivens 1995; Hasenstein et al. 1999). This phenomenon is even of importance for application, since the mode of action of some herbicides, such as the phenyl carbamates or the dinitroanilines, is based on the elimination of cortical microtubules and the subsequent inhibition of

elongation growth. Especially impressive are the effects of colchicine on differentiating xylem elements, where the characteristic cell wall thickenings do not form at all in presence of the drug (Pickett-Heaps 1967; Robert and Baba 1968; Barlow 1969; Hepler and Fosket 1971; Hardham and Gunning 1980).

The striking parallelity between cortical microtubules and newly deposited cellulose microfibrils has stimulated the proposal of two alternative models:

The original model postulated that cortical microtubules adjacent to the plasma membrane guide the movement of the cellulose-synthesizing enzyme complexes and thus generate a pattern of microfibrils that parallels the orientation of microtubules (Heath 1974). The differences in length between microtubules and microfibrils would be explained by an overlap of individual microtubules that are organized in bundles. The driving force for the movement of cellulose synthases in this "monorail" model would be active transport through microtubule motors (Fig. 6a).

Alternatively, the interaction between microtubules and cellulose-synthases could be more indirect, whereby the microtubules act as "guard rails" that induce small folds of the plasma membrane that confine the movement of the enzyme complexes (Herth 1980; Giddings and Staehelin 1991). The driving force for the movement would result from the crystallization of cellulose. The solidifying microfibril would thus push the enzyme complex through the fluid plasma membrane and the role of microtubules would be limited to delineate the direction of this movement (Fig. 6b).

The practical discrimination between these two models is not trivial because experimental evidence was mostly based on electron microscopical

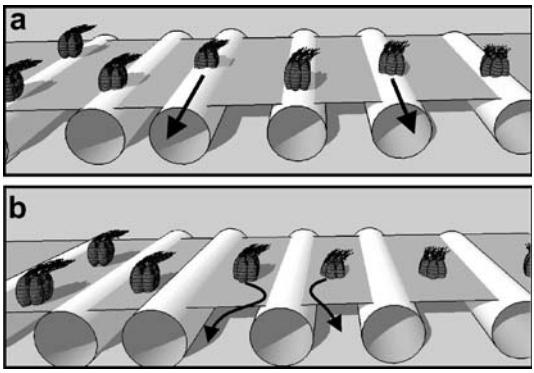


Fig. 6 Models on the guidance of cellulose synthesis by cortical microtubules. **a** Monorail model, where the cellulose-synthesizing complexes are moved along microtubules driven by a microtubule-dependent motor. **b** Guardrail model, where the cellulose-synthesizing complexes are moved by the force from the crystallizing cellulose, but are confined to the troughs between individual microtubules

observation and thus was prone to fixation artifacts, and great luck was required to locate the right section. For instance, the newly synthesized cellulose microfibrils formed after a treatment with taxol were found to be directly adjacent to individual microtubules in tobacco BY-2 cells (Hasezawa and Nozaki 1999), favouring the monorail model. On the other hand, the cellulose synthase complexes were observed "in gap" between adjacent microtubules in the alga *Closterium* (Giddings and Staehelin 1988), which was difficult to reconcile with a monorail mechanism.

The situation is further complicated by situations where the orientation of microtubules and cellulose microfibrils differ (for instance Emons and Mulder 1998; Himmelspach et al. 2003; for review see Baskin 2001; Wasteneys 2004). Some of these inconsistencies may depend on the choice of the system – for instance, the root hair of *Equisetum hyemale* with its helicoidal wall texture deviating from the orientation of cortical microtubules (Emons et al. 1992) is a cell endowed with tip growth and differs from a tissue cell that is expanding in a diffuse manner and is subject to considerable tissue tensions. In addition, the orientation of cellulose microfibrils is shifted and distorted when the wall lamella gradually shift from the plasma membrane to the periphery of the apoplast during the apposition of the subsequent lamellae. The contribution of these older lamellae to the reinforcement of growth vanishes progressively. It had been estimated for *Nitella* that only the innermost fifth of the wall is responsible for the majority of reinforcement (Green and King 1966). It is not trivial to determine the cellulose texture of the innermost lamellae of a cell wall (Robinson and Quader 1982; Kristen 1985). Moreover, the orientation of microtubules as well as the orientation of cellulose can change rhythmically (Zandomeni and Schopfer 1993; Mayumi et al. 1996; Hejnowicz 2005) leading to transitional situations where the microtubules have already assumed a new orientation and the time elapsed since this transition has not been sufficient to deposit a significant number of microfibrils in the new direction.

Despite these caveats in the interpretation of apparent differences between microtubule and microfibril orientation, they have led to a debate on the role of microtubules in the guidance of cellulose synthesis. This debate stimulated a key experiment exploiting the potential of live-cell imaging in *Arabidopsis thaliana* (Paredez et al. 2006). A component of the terminal rosette, the cellulose synthase subunit A6 (CESA6), was expressed as fusion with the yellow fluorescent protein under the native promotor in the background of a *cesa6* null mutant, such that overexpression artifacts could be excluded. The resulting punctate signal was observed to be localized adjacent to the plasma membrane and to move along parallel pathways that resembled cortical microtubules. By crossing this line into a background, where one of the α-tubulins was expressed as fusion with a blue fluorescent protein, it became possible to follow this movement under simultaneous visualization of CESA6 and microtubules. This dual-image approach demonstrated very

clearly that CESA6 was moving along individual microtubule bundles. Moreover, in a very recent publication, a central problem of the monorail model, i.e. the existence of polylamellate walls with layers of differing microfibril orientation, could be plausibly explained by a rotary movement of groups of microtubules (Chan et al. 2007).

The original monorail model postulated a microtubule motor that pulls the cellulose synthase complex along the microtubules. If this motor were defective, a situation would result where microtubules were arranged in the usual transverse arrays, whereas cellulose microfibrils were deposited deviantly. A screen for reduced mechanical resistance in *Arabidopsis thaliana* yielded a series of so-called *fragile fiber* mutants (Burk et al. 2001; Burk and Ye 2002) that were shown to be completely normal in terms of cell wall thickness or cell wall composition, but were affected in wall texture. One of these mutants, *fragile fiber 2*, allelic to the mutant *botero* (Bichet et al. 2001), was affected in the microtubule-severing protein katanin, leading to swollen cells and increased lateral expansion. A second mutant, *fragile fiber 1*, was mutated in a kinesin-related protein belonging to the KIF4 family of microtubule motors. As expected, the array of cortical microtubules was completely normal; however, the helicoidal arrangement of cellulose microfibrils was messed up in these mutants. This suggests that this KIF4 motor is involved in the guidance of cellulose synthesis and might be a component of the monorail complex.

Thus, the original monorail model for the microtubule guidance of the terminal rosettes (Heath 1974) experienced a rehabilitation after more than three decades of dispute. However, the microtubule–microfibril model is still far from complete. In addition to the discordant orientations of microtubules and microfibrils discussed above, there are cell wall textures that are difficult to reconcile with a simple monorail model. For instance, cellulose microfibrils are often observed to be intertwined (for instance Preston 1981). This has stimulated views that claim that microtubules are more or less dispensable for the correct texture of microfibrils. The self-organization of cellulose synthesis would be sufficient to perpetuate the pattern because the geometrical constraints from microfibrils that are already laid down would act as templates for the synthesis of new microfibrils (Emons and Mulder 1998; for review see Mulder et al. 2004). This view ignores the fact that microtubules and microfibrils *are* parallel in most cases, at least if cells in a tissue context are analysed. It also ignores the disruption of microtubules either by inhibitors (see above) or by mutations that impair the formation of ordered microtubule arrays, causing a progressive loss of ordered cell wall texture and a loss of growth axiality (Burk et al. 2001; Bichet et al. 2001 for katanin; Whittington et al. 2001 for *mor1*).

However, the focus on the self-organizing properties of cellulose synthesis forces the original microtubule–microfibril model to be extended by a feedback control of microfibrils upon cortical microtubules. A mounting body of evidence shows that the cell wall acts to stabilize cortical microtubules. For instance, removal of the cell wall results in enhanced cold sensitivity of cortical

microtubules in tobacco cells (Akashi et al. 1990). When, in the same cells, the incorporation of UDP-glucose into the cell wall was blocked by the herbicide isoxaben (Fisher and Cyr 1998), this impaired the axiality of cell expansion resulting in isodiametric cells and disordered cortical arrays of microtubules. This suggests that the mechanical strains exerted by the cellulose microfibrils during axial expansion provide directional cues for the alignment of microtubules. The fact that microtubules are able to sense mechanical stimuli will be discussed in detail in Sect. 6.

At this point it should be pointed out that this mechanosensory function will close a feedback loop between cell wall and cytoskeleton. Since expansion is reinforced in a direction perpendicular to the orientation of microtubules and microfibrils, biophysical forces will be generated parallel to the major strain axis. These forces are then relayed back through the plasma membrane upon cortical microtubules that are aligned with relation to these strains. In other words, microtubules and microfibrils constitute a self-reinforcing regulatory circuit. Since individual microtubules mutually compete for a limited supply with tubulin-heterodimers, and since the number of microfibrils is limited by the quantity of cellulose synthase rosettes, this regulatory circuit should be capable of self-organization and patterning.

In fact, microtubule–microfibril patterns that transcend the borders of individual cells have been reported in early work on plant microtubules in apical meristems (Hardham et al. 1980). Here, the formation of new primordia is suppressed by the older primordia. The tissue tension present in an expanding meristem would yield considerable mechanical stresses resulting from buckling from the older primordia. In fact, models of stress–strain patterns could perfectly predict the position of incipient primordia (for review see Green 1980). One of the earliest events of primordial initiation is a reorientation of cortical microtubules that are perpendicular with respect to the microtubules of their non-committed neighbours. This difference is sharp, but later it is smoothed by a transitional zone of cells with oblique microtubules, such that eventually a gradual, progressive change in microtubular reorientation emerges over several rows of cells. A similar supracellular gradient of microtubule orientation was reported upon wounding of pea roots (Hush et al. 1990), heralding corresponding changes of cell axis and cell divisions that align such that the wound is efficiently closed. A curious case of microtubule patterning was discovered in the *Arabidopsis* mutants *spiral*, *lefty* and *tortifolia* (Furutani et al. 2000; Thitamadee et al. 2002; Buschmann et al. 2004). In these mutants, microtubules are obliquely aligned over many cells in the distal elongation zone of the root (*spiral* and *lefty*) or the petiole (*tortifolia*), accompanied by twisted growth. In contrast, in the temperature-sensitive mutant *radially swollen 6* (Bannigan et al. 2006) microtubule arrays of individual cells are ordered and parallel, but arrays between neighbouring cells deviate strongly, suggesting that this mutant is affected in the supracellular patterning of microtubule arrays.

The twisted growth phenotype of these mutants is conventionally explained on the base of uniformly oblique arrays of microtubules (and consequently microfibrils). In the *spiral*, *lefty* and *tortifolia* mutants it is the microtubular cytoskeleton that is affected by these mutations. Moreover, spiral growth can be phenocopied in the wild type by inhibitors of microtubule assembly (Furutani et al. 2000). As pointed out above, the microtubule–microfibril circuit is endowed with self-amplification linked to mutual inhibition. A typical systemic property of such a self-organizing morphogenetic system is an oscillating output (Gierer 1981). Any factor that alters the lifetime of microtubules will alter the relay times within this feedback circuit. Since neighbouring cells are mechanically coupled by tissue tension, even a weak coupling will result in a partial synchronization of the individual circuits (Campanoni et al. 2003). The degree of synchrony will depend on the velocity of the feedback circuit. Thus, mutations in an associated protein such as the *tortifolia* gene product (Buschmann et al. 2004), mutations in tubulin itself, as in case of *lefty* (Thitamadee et al. 2002), or treatment with microtubule inhibitors (for review see Hashimoto and Kato 2006) are expected to enhance synchrony leading to the observed oscillations of growth. Interestingly, the mutant *root swollen 6*, where microtubule arrays of individual cells are completely uncoupled, is reported to be endowed with increased resistance to microtubule inhibitors suggesting that microtubule lifetimes are increased in this mutant (Bannigan et al. 2006).

The spatial control of cell expansion is a central element of the developmental flexibility crucial for survival in organisms with a sessile lifestyle. The past few years have seen a surprising rehabilitation of the classical ideas on the mechanisms driving this control. However, the original straightforward model of microtubules as guiding tracks for cellulose synthesis has been extended by elaborate feedback controls from the microfibrils upon microtubules. This means that the self-organizing properties of microtubules are combined with the self-organizing properties of cellulose synthesis, constituting a patterning system that is composed of oscillators (the microtubule–microfibril circuits of individual cells) that are coupled through mechanical strains. Thus, in analogy to the spatial control of cell division, the nonlinear properties of microtubules are utilized to generate and maintain a flexible, but nevertheless defined, axis of cell expansion.

4
Signal-Triggered Reorientation of Microtubules

The previous two sections have described microtubules as central players in the definition of cell division and cell expansion. Both phenomena have to be flexibly tuned with the environment. This means that plant microtubules must be able to reorganize in response to signals.

In fact, this has been observed in numerous cases (for review see Nick 1998). A classical example is the ethylene response of growth: When angiosperm seedlings encounter mechanical obstacles, they display a characteristic barrier response that involves a shift of the growth axis from elongation towards stem thickening. The trigger for this response is ethylene (Nee et al. 1978), which is constantly released by the elongating shoot and accumulates in front of physical obstacles. It is, by the way, this ethylene-induced block of internode elongation accompanied by a thickening of the stem by which the growth regulator ethephone increases lodging resistance (Andersen 1979).

Using this ethylene-triggered switch of the growth axis, Lang et al. (1982) succeeded in demonstrating that environmental signals probably control growth through the microtubule–microfibril pathway. Electron microscopy in pea epicotyls showed that the cortical microtubules reorient from their original transverse orientation into steeply oblique or even longitudinal arrays. This reorientation is followed by a shift of cellulose deposition from transverse to longitudinal, and a thickening of the stem.

During subsequent years, similar correlations between growth, microfibril deposition and cortical microtubules could be shown for other hormones as well. In coleoptile segments of maize, where elongation is under the control of auxin and limited by the epidermal extensibility (Kutschera et al. 1987), microtubules and microfibrils were oriented longitudinally when the segments had been depleted of endogenous auxin (Bergfeld et al. 1988). However, they became transverse when exogenous auxin was added. In parallel, elongation growth was restored. Interestingly, this response is confined to the outer epidermal cell wall, and it is exactly this cell wall where auxin has been shown to stimulate growth by increasing the extensibility of cell walls.

With the adaptation of immunofluorescence to plant cells (Lloyd et al. 1980) it became possible to follow the dynamics of reorientation and to investigate the factors that trigger a reorientation of microtubules. These studies identified various plant hormones such as auxin (Bergfeld et al. 1988; Nick et al. 1990, 1992; Nick and Schäfer 1994), gibberellins (Mita and Katsumi 1986; Nick and Furuya 1993; Sakiyama-Sogo and Shibaoka 1993; Shibaoka 1993; Toyomasu et al. 1994) and abscisic acid (Sakiyama-Sogo and Shibaoka 1993) as triggers of microtubule reorientation, but also physical factors such as blue light (Nick et al. 1990; Laskowski 1990; Zandomeni and Schopfer 1993), red light (Nick et al. 1990; Nick and Furuya 1993; Zandomeni and Schopfer 1993; Toyomasu et al. 1994), gravity (Nick et al. 1990; Godbolé et al. 2000; Blancaflor and Hasenstein 1993; Himmelspach et al. 1999; Himmelspach and Nick 2001), high pressure (Cleary and Hardham 1993), mechanical stress (Zandomeni and Schopfer 1994), wounding (Hush et al. 1990) or electrical fields (Hush and Overall 1991).

However, only in a few cases has the dynamics of microtubule reorientation been analysed in direct comparison with signal-induced changes of growth. In maize coleoptiles, microtubules were observed to reorient rapidly from

transverse to longitudinal upon phototropic stimulation (Nick et al. 1990). This reorientation was confined to the lighted flank of the coleoptile and clearly preceded the onset of phototropic curvature. The time-course for the auxin-dependent reorientation in the same organ supported a model (Fig. 7)

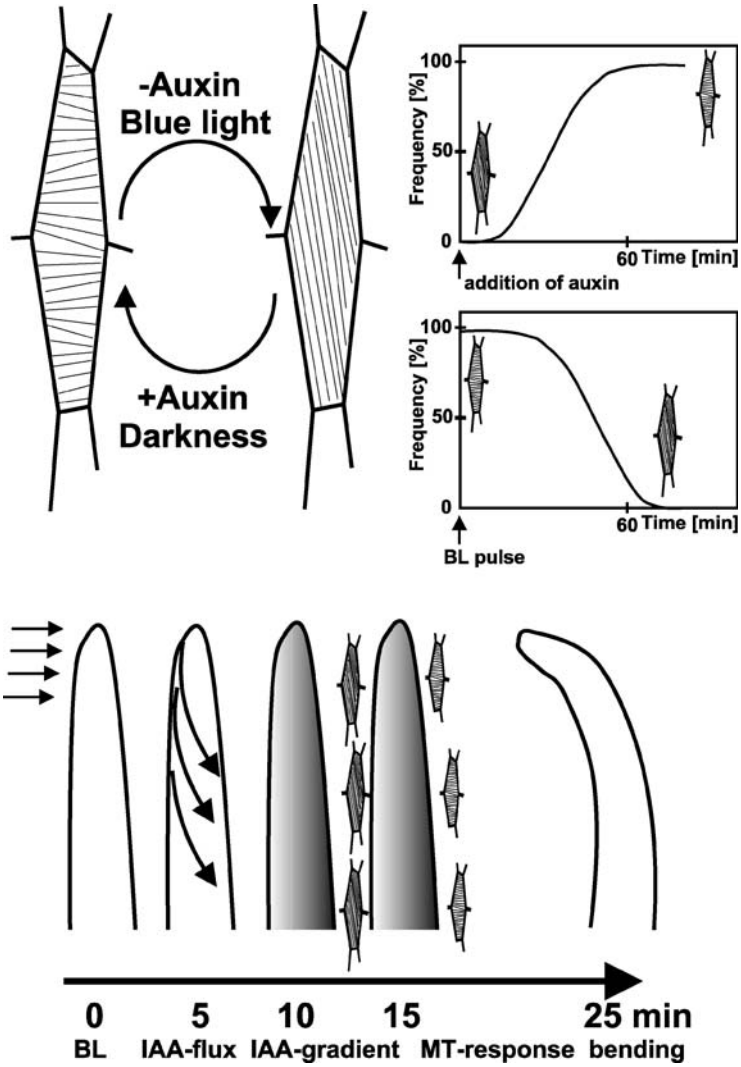


Fig. 7 Behaviour of cortical microtubules during phototropic curvature of maize coleoptiles (Nick et al. 1990). Microtubules reorient from transverse to longitudinal in response to auxin depletion or in response to phototropic stimulation. The reorientation induced by phototropic stimulation is confined to the lighted flank of the coleoptile and initiates subsequent to the auxin displacement across the coleoptile, but prior to the onset of the phototropic curvature

where photo- or gravitropic stimulation induced a shift of auxin transport from the lighted towards the shaded flank of the coleoptile. The depletion of auxin in the lighted flank subsequently stimulated a reorientation of cortical microtubules into longitudinal arrays (Nick et al. 1990), and, in parallel, a longitudinal deposition of cellulose microfibrils (Bergfeld et al. 1988). Conversely, microtubules, as well as cellulose microfibrils, remain transverse in the auxin-enriched shaded flank. The gradient of microfibril orientation would then result in a decreased longitudinal extensibility of epidermal cell walls in the lighted flank, and, as a consequence, a decrease in asymmetric growth leading to phototropic curvature towards the light stimulus.

A more detailed investigation of the phenomenon revealed, however, a more complex reality (Nick et al. 1992; Nick and Schäfer 1994; Nick and Furuya 1996). It is possible, by rotating the seedlings on a clinostat in the absence of tropistic stimulation, to generate a so-called nastic bending. This nastic response is not preceded or accompanied by a reorientation of microtubules and thus occurs without a corresponding gradient of orientation across the coleoptile cross-section (Nick et al. 1991). On the other hand, the gradient of microtubule orientation established in response to a light pulse persists, whereas the curvature vanishes due to gravitropic straightening (Nick et al. 1991). In parallel to phototropic curvature, a phototropic stimulus can induce a stable transverse polarization of the coleoptile that persists over several days. This polarity can mediate stable changes in growth rate (Nick and Schäfer 1988, 1991, 1994) and can even control morphogenetic events such as the emergence of crown-roots manifest several days after the inducing stimulus had been administered (Nick 1997). These stable changes in growth are closely related to a stabilization of microtubule orientation (Nick and Schäfer 1994) because 2 h after the inducing light stimulus, cortical microtubules had lost their ability to reorient in response to a counter-directed light pulse. At the same time, the transverse polarity manifest as stable change in growth becomes persistent. Interestingly, the microtubules lose their ability to respond to auxin as well, indicating that it is not sensory adaptation of phototropic perception that is responsible for the block of the reorientation response (Nick and Schäfer 1994). The stabilization of microtubule orientation 2 h after an inducing light pulse requires blue light, and this light effect cannot be mimicked by a mere depletion of auxin nor by gradients of auxin depletion.

These studies suggest that the microtubule–microfibril pathway is responsible for persistent changes of growth. They also suggest, however, that a second pathway can control fast growth responses independently. In most cases, both pathways seem to act in concert; it required detailed time-course studies to detect discrepancies between growth and microtubule reorientation. In this context it should be mentioned that in some cases the microtubule response has been found to be somewhat slower than the signal response of growth, for instance in the blue light-induced inhibition of growth in pea stems (Laskowski 1990) or root gravitropism in maize (Blancaflor and

Hasenstein 1993). Here, a microtubule-independent mechanism seems to be at work. The microtubule–microfibril pathway is designed for persistent changes of growth, since it requires a certain time until enough cellulose microfibrils are deposited in a new direction (Lang et al. 1982) before a corresponding change of growth can occur. When the two growth patterns have been analysed in parallel (e.g. Nick and Schäfer 1994), they were observed to act in parallel and to play complementary roles. However, it seems to be the microtubule–microfibril pathway that is crucial for the morphogenetic flexibility essential for plant survival. Thus, to understand developmental flexibility and its link to signal transduction, it is necessary to understand, how cortical microtubules reorient.

5
How Do Microtubules Change Direction?

Before the mechanism of microtubule reorientation could be seriously investigated it was necessary to visualize the plant cytoskeleton in its three-dimensional organization. Thus, our understanding of microtubules was shaped by the methodology that was available. Originally, microtubule orientation could only be inferred from the shape of the cross-sections in stacks of ultrathin sections viewed by electron microscopy, which was very cumbersome and at the edge of the impossible. The first breakthrough was therefore the combination of fluorescence microscopy with immunolabelling, which allowed for the first time observation of the microtubular cytoskeleton as an entity (Lloyd et al. 1980). When this approach was later complemented by confocal microscopy, it became possible to view microtubules in different layers of an intact tissue. However, for immunofluorescence, microtubules have to be fixed by aldehydes to preserve their structure during the preparation process. This means that the dynamics of microtubules could not be observed by this approach, and the term "cytoskeleton" evoking a more or less rigid structure was inspired by the structural appearance of fixed microtubules seen in electron micrographs and later immunofluorescence images. It was a big surprise when microtubules could be visualized in living cells, first by microinjection of fluorescent tubulin (Yuan et al. 1994), and later by the use of GFP-tagged markers such as the microtubule-binding domain of MAP4 (Marc et al. 1998) or tubulins themselves (Kumagai et al. 2001). Our understanding of microtubule reorientation represents a classical example for the interdependence of biological concepts and experimental approach.

When microtubule arrays could be visualized for the first time as an entity, it was discovered that, in elongating cells, they are arranged in helicoidal arrays along the cell periphery. This stimulated the first model for microtubule reorientation (Lloyd and Seagull 1985). This very elegant and beautiful model perceived cortical microtubules as a mechanically coupled entity that cor-

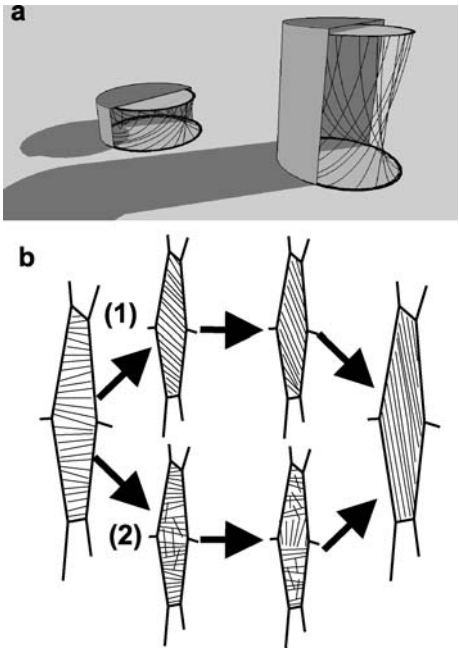


Fig. 8 Potential mechanisms for the reorientation of cortical microtubules. **a** Dynamic spring model: microtubules are organized into a mechanically coupled helicoidal array. By mutual sliding of microtubules the helix can change from a relaxed state with almost transverse pitch (*left*) to a tightened state with almost longitudinal microtubules (*right*). **b** Directional reassembly model: the equilibrium between assembly and disassembly of a given microtubule depends on its orientation with respect to the cell axis. A switch in the direction of preferential stability will result in a net reorientation of microtubules. Whereas the final result is the same as for the dynamic spring model, the transitional states are different. In the dynamic spring model (1), the transition would consist of homogenously oblique microtubules. In the directional reassembly model (2), transverse and longitudinal microtubules coexist during a transitional phase. These are coaligned to patches that subsequently move and reorient as coupled entities until a homogenous new array is established

responds to a dynamic spring. By releasing or increasing the tension in this spring (caused by mutual sliding of the constituting microtubules), the pitch of this helix would change between transverse and longitudinal (Fig. 8a). According to this model, the molecular mechanism of reorientation is expected to involve microtubule motors.

However, it became evident during subsequent years that the dynamic-spring model failed to describe microtubule reorientation:

1. In epidermal tissues, the reorientation of cortical microtubules is confined to the microtubules adjacent to the outer cell wall, leading to a situation where microtubules were transverse at the inner wall, but longitudinal at

the outer wall (Bergfeld et al. 1988; Nick et al. 1990; for review Wymer and Lloyd 1996). This difference in orientation within a single cell was difficult to reconcile with the concept of a mechanically coupled spring.

2. The transitions between transverse and longitudinal arrays of microtubules should involve situations where microtubules are homogenously oblique and then gradually change pitch until the longitudinal array is established. Although oblique microtubules can be observed, they seem to occur as a final rather than as a transitional situation (Gunning and Hardham 1982; Hush et al. 1990). In contrast, early phases of reorientation in response to strong stimuli, or incomplete reorientation in response to a suboptimal stimulation, tend to look different (Nick et al. 1990, 1992). Here, a patchwork of transverse and microtubules is observed, where transverse and longitudinal microtubules can coexist even within the very same cell (Fig. 8b).
3. Taxol inhibits microtubule disassembly and was found to suppress microtubule reorientation (Falconer and Seagull 1985; Nick et al. 1997), indicating that microtubule disassembly is required for reorientation, contrasting with the dynamic-spring model. Taxol did not inhibit, however, the coalignment of initially disordered microtubules into the parallel arrays that are observed in regenerating protoplasts (Wymer et al. 1996) suggesting that a disassembly-independent mechanism contributes to the organization of cortical microtubules.
4. Cortical microtubules were initially thought to be relatively inert lattices. However, when microtubules were visualized in living plant cells by microinjecting fluorescent tubulin, the lifetime of individual microtubules was found to be extremely short (Yuan et al. 1994; Wymer and Lloyd 1996, Himmelspach et al. 1999). The injected tubulin was incorporated extremely rapidly into the preexisting cortical network. Upon bleaching the fluorescence by a laser beam, the fluorescence of the bleached spot recovered within a few minutes, indicating an extremely high turnover of tubulin dimers. This dynamics of tubulin assembly and disassembly contrasts with the concept of a mechanically coupled microtubular helix.
5. By using lines of *Arabidopsis thaliana* expressing a fusion of an α-tubulin with GFP it became possible to analyse and quantify the dynamic behaviour of individual microtubules in living epidermal cells (Shaw et al. 2003). Microtubules were found to move through the cortex by a treadmilling mechanism. Interestingly, both ends of the microtubule contributed to a net motility in the direction of the plus-end. When parts of these microtubules were bleached in fluorescence-recovery after photobleaching (FRAP) experiments, the bleached region did not move, suggesting that translocation of assembled microtubules did not occur. Thus, it was the assembly and disassembly of microtubules that was responsible for the net movement of microtubules. This conclusion is supported by experiments where the be-

haviour of the plus-end marker EB1 was followed by means of a GFP-tag. Here, a conspicuous, bidirectional movement of the plus-ends was observed in interphase arrays of microtubules (Dhonukshe et al. 2003; Chan et al. 2003). In contrast to the situation in animal cells, where catastrophic disassembly is fast but relatively rare, treadmilling in *Arabidopsis thaliana* was found to be quite common but moderate, possibly through tight regulation of the minus-ends.

6. When microtubule reorientation was followed in vivo upon microinjection of fluorescent neurotubulin (Yuan et al. 1994; Wymer and Lloyd 1996, Himmelspach et al. 1999), the reorientation was observed to proceed in two distinct stages. The first stage local phase transitions from transverse to longitudinal arrays lead to individual discordant microtubules that herald the prospective orientation of the array. These discordant "exploratory" microtubules subsequently become more frequent, leading to a patchy situation where longitudinal and transverse microtubules coexist in the very same cell. Only during a second stage are microtubules coaligned into a new parallel array whose direction is defined by the original, "exploratory" microtubules. This coalignment is characterized by a distinct group behaviour of cortical microtubules, as demonstrated quite recently (Chan et al. 2007): By using spinning-disc microscopy in seedlings that expressed fluorescently tagged versions of tubulin or the tubulin plus-end marker EB1, it was possible to follow microtubule reorientation over longer timescales at high temporal resolution. This approach uncovered patches of microtubules that act in concert and are of equal polarity. These domains move around the cell until they collide with other patches. New microtubules are preferentially generated along tracks where other microtubules had been before.

These observations led to a revision of the original dynamic-spring model (for review see Lloyd 1994). The actual *reorientation* involves direction-dependent changes of microtubule lifetime. For instance, in an array that undergoes reorientation from a transverse into a longitudinal orientation, the "exploratory" longitudinal microtubules will acquire increased stability, whereas the transverse microtubules will be more labile. This reorientation in sensu strictu will then be followed by a phase of *coalignment* where the reoriented, but still disordered, microtubules are coupled into patches of identical polarity. These patches subsequently move around the cell and progressively align into a new parallel array. From the mechanistic point of view, the first phase would require changes in the activity of structural microtubule-associated proteins (MAPs) that control the lifetime of a given microtubule, whereas the second phase would be driven by microtubule motors.

A central prerequisite for the first phase (*reorientation*) would be differences in the lifetime of microtubules that are dependent on some kind of vector, i.e. orientation with respect to the cell axis. The direction of this

unknown vector would then change in response to the signal that triggers microtubular reorientation.

Stable microtubules have been observed in both animals and plants to consist of tubulin that is post-translationally modified (for review, see MacRae 1997). All α-tubulins, with the exception of one species (the slime mould *Physarum polycephalum*; Watts et al. 1987), carry a carboxy terminal tyrosine, which can be post-translationally cleaved off by a tubulin tyrosine carboxypeptidase. The carboxy terminal tyrosine can be restored by a tubulin tyrosine ligase. The biological role of this detyrosination process is not really understood but, in mammalian cells, microtubules consisting of detyrosinylated tubulin are less dynamic (Kreis 1987). The initial model assumed that the detyrosinylated tubulin was the cause of the increased stability. However, it turned out later that the tubulin tyrosine carboxypeptidase, responsible for this modification, preferentially binds to tubulin that is assembled in microtubules, whereas it shows less affinity for dimeric tubulin. Conversely, the tubulin tyrosine ligase acts predominantly on dimeric tubulin (Kumar and Flavin 1981). This would favour an alternative scenario where tubulin tyrosination would primarily depend on microtubule dynamics (Khawaja et al. 1988). In fact, the dynamics of microtubules assembled in vitro from tyrosinylated or detyrosinylated tubulin is indistinguishable (Skoufias and Wilson 1998). Detyrosination has been described for plant tubulin as well and can be triggered by signals that control growth (Duckett and Lloyd, 1994). Although it is not known whether detyrosination is cause or consequence of microtubule stability, it can be used as a marker for microtubules with increased lifetime. There exist a couple of well-characterized monoclonal antibodies that detect tyrosinylated tubulin (Kilmartin et al. 1982; Kreis 1987). Using such antibodies it should be possible to test whether signal-triggered microtubule re-orientation really involves direction-dependent differences of microtubule lifetime. Using maize coleoptiles as model, it could be shown that detyrosination can be controlled via auxin (Wiesler et al. 2002). In fact, the longitudinal microtubules produced in response to auxin depletion predominantly contained the detyrosinylated form of α-tubulin, indicating direction-dependent differences in microtubule stability.

The stability of microtubules is generally believed to depend on the activity of structural MAPs that decrease the frequency of microtubule catastrophe (Bin-Bing and Kirschner 1999; Caudron et al. 2000). In vitro, the assembly of microtubules is promoted by warm temperatures, by GTP, and by magnesium. In vivo, the nucleation of new microtubules occurs on the surface of specialized organelles, the centrosomes. Higher plants are unique in this respect because they do not possess centrosomes, and an entire chapter of the present volume (see Chapter "Microtubules and the Evolution of Mitosis" of this book) is dedicated to this topic. Higher plants organize microtubules on functional analogues, so-called microtubule-organizing centres (MTOCs). The major MTOC in dividing cells seems to be the nuclear sur-

face (Lambert 1993). In fact, it is possible to induce microtubule asters by addition of purified nuclei (Stoppin et al. 1994). In differentiated interphase cells, the activity of the nuclear envelope is masked by cortical MTOCs that become manifest during the recovery of microtubules from disassembly induced by drugs, low temperature or high pressure (Marc and Palevitz 1990; Cleary and Hardham 1993). These MTOCs contain microtubule-nucleating factors such as γ-tubulin (Liu et al. 1994; Pastuglia et al. 2006) or elements of the tubulin-chaperoning complex CCT (Himmelspach et al. 1997; Nick et al. 2000). A couple of so-called structural plant MAPs have been identified in the meantime that can regulate different aspects of microtubule dynamics such as nucleation, severing or bundling (for review see Lloyd et al. 2004).

However, the central problem of reorientation remains to be solved: how the stability of the discordant microtubules can be regulated differently from that of the bulk microtubules that are still oriented in the original orientation. It seems that there must be interactions with a vectorial field or lattice that are regulated. Membrane-bound MAPs that are regulated by signal-transduction chains might be the key players in this context. For instance, certain isoforms of phospholipase D have been isolated as microtubule-binding proteins (Marc et al. 1996) and participate in the interaction of cortical microtubules with the plasma membrane (Gardiner et al. 2001).

It is obvious that the direction-dependent stability of a given microtubule cannot be intrinsic to the microtubule itself, but is produced by the interaction of this microtubule with a directional lattice or vectorial field. This lattice or field could be a different component of the cytoskeleton, such as actin filaments (the interaction of microtubules and actin filaments is explored in the chapter "Crossed-wires: Interactions and cross-talk between the microtubule and microfilament networks in plants" of this book). It could be a physical field, such as mechanical strain or bound dipoles, or it could be an apoplastic lattice, such as the cellulose microfibrils that have been found to feed back on microtubules (see above). The MTOCs could be transported and organized along such a lattice (Fig. 9a). This would cause a dynamic shift in the nucleation activity. It would not explain, however, why microtubules are more stable when they grow into a certain, the "right", direction as compared to their fellow microtubules that grow into the "wrong" direction. It could be the cross-linking with proteins that stabilizes microtubules (e.g. through bundling or capping). If the distance between such microtubule stabilizers were dependent upon direction, it would provide a mechanism to favour certain microtubule orientations. A simple possibility for such a mechanism is depicted in Fig. 9b, where the hypothetical microtubule stabilizers are aligned on a longitudinal lattice that can change between a disperse and a bundled configuration. Experiments in which the orientation of microtubules changed after pharmacological manipulation of actin (Kobayashi et al. 1988; Seagull 1990; Nick et al. 1997) suggest that this lattice is related to actin microfilaments. In the disperse configuration of this lattice, the average minimal

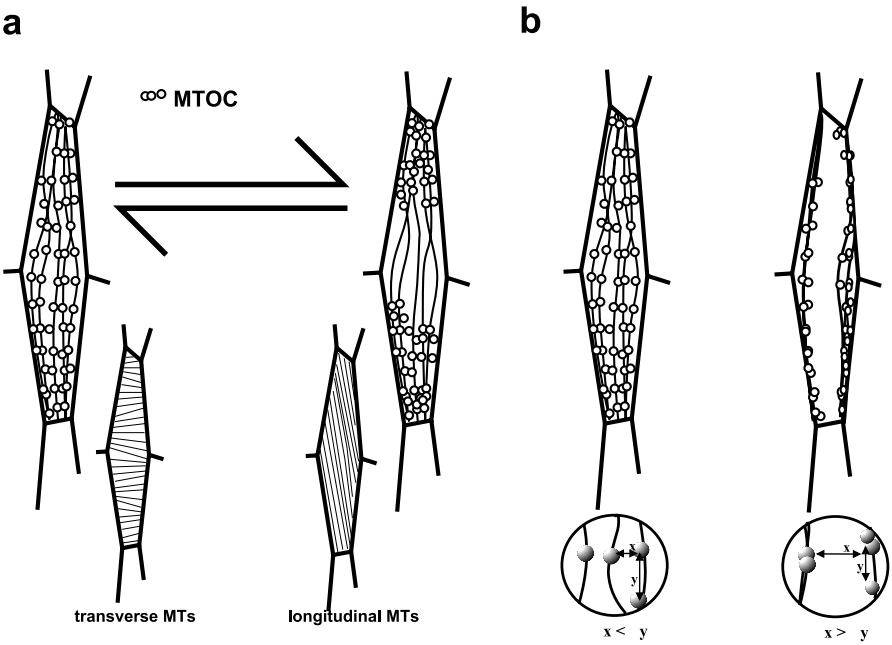


Fig. 9 Model for the directional reassembly of microtubules. **a** Microtubule-organizing centres moving along a directional matrix/lattice resulting in a redistribution of microtubule-nucleating sites. **b** Microtubule-organizing centres are tethered to a directional matrix/lattice. Upon bundling of this lattice, longitudinal microtubules will be favoured over transverse microtubules due to a lower minimal distance (Δy) in the longitudinal direction as compared to the minimal distance in the transverse direction (Δx)

distance between the microtubule stabilizers would be smaller in the transverse direction, favouring a transverse orientation of microtubules. In the bundled configuration, the average distance in the transverse direction would increase such that a longitudinal microtubule would become more stable than its transverse counterpart.

6 Mechanisms for the Control of Cell Axis

Plants can basically use two mechanisms of adjusting their axis in congruence with their environment. To respond rapidly, they can change the axis of cell expansion. To obtain more persistent changes, for instance in a situation where the environmental change is not transient, they can alter the axis of cell division. Microtubules are central players in both adaptive processes and, in addition, seem to be targets of numerous signalling cascades that control their organization and dynamics. In a nutshell, their function is twofold: they

act as devices that collect and process some signals, and they act as devices that transform the outcome of this signal processing into persistent changes of shape. In fact, they represent a versatile tool for linking signalling with morphogenesis.

The versatility of this tool is illustrated by the ample opportunities to control cell expansion. The control of cell expansion by environmental stimuli such as light or gravity includes: stimulus perception; signal-transduction cascades that are often organized as complex networks; response of the (still unknown) directional matrix that defines direction-dependent microtubule assembly and disassembly leading to a net reorientation of cortical microtubules; coalignment of a new, parallel array and reorientation of cellulose deposition, resulting in a directional switch in the anisotropy of the cell wall; and, eventually, a switch in the axis of preferential cell expansion. Each of these steps is regulated and can be used to control cell axis (both by the plant itself and by biotechnological manipulation):

1. Manipulation of stimulus perception. The dense canopies characteristic of industrial agriculture lead to a pronounced shade-avoidance response that is triggered by the plant photoreceptor phytochrome (Smith 1981). When stem elongation is stimulated in consequence of shade avoidance, this will render the plant prone to lodging and thus will reduce yield (Oda et al. 1966). The agronomical impact of this phenomenon was demonstrated by experiments in which phytochrome was overexpressed, resulting in reduced shade avoidance. This approach allowed higher yields (Robson et al. 1996). However, the consequences of this strategy are not confined to cell expansion, but affect all responses that are dependent on phytochrome including the composition of photosystems, branching, tropistic responses, hormonal balance and induction of flowering. This pleiotropy will cause, depending on species and crop type, undesired side effects that are difficult to foretell. The same type of argumentation is valid not only for shade avoidance, but in principle for any other signalling pathway that affects cell elongation, for instance, by temperature, nutrient uptake or abiotic or biotic factors.
2. Manipulation of signalling cascades. The classic approach for the suppression of lodging through dwarfing genes (Peng et al. 1999) or growth regulators such as CCC or ethephone belong to this approach (Andersen 1979). Since most signal cascades are not linear, but split and interwoven into complex networks, again a high degree of pleiotropy is expected. The experience with undesired side effects of ethephone such as incomplete grain filling (Makela et al. 1996) or reduced root development (Luib and Schott 1990) illustrate the drawbacks of this approach. In plants, where coordination is not brought about by a neuronal network, but basically by chemical signalling, it will be difficult to define signalling events that are confined to one specific target. Thus, a strategy aimed at events down-

stream of the various branching points of signal transduction is expected to be more localized.

3. Manipulation of the directional matrix. This would represent the most specific target because it is expected to affect cortical microtubules in the first place and thus mainly the axis of cell expansion. The nature of this matrix is to be identified, but experiments in which actin drugs have been observed to affect the organization of microtubules (Kobayashi et al. 1988; Seagull 1990; Nick et al. 1997) suggest that the interaction between microfilaments and microtubules (as covered in detail in the chapter "Crossed-wires: Interactions and cross-talk between the microtubule and microfilament networks in plants" of this book) might be crucial in this context.
4. Manipulation of microtubule disassembly and reassembly. At present, this is the target easiest to access. Disassembly of microtubules can be blocked by taxol (Parness and Horwitz 1981) or it can be enhanced by a broad panel of compounds including the alkaloids colchicine, vinblastine and colcemid, or by herbicides such as dinitroanilines or phenylcarbamates (for review see Morejohn 1991; Vaughn 2000). Disassembly of microtubules can also be triggered through the calcium–calmodulin pathway (Fisher et al. 1998) or through low temperature (see the chapter "Microtubules as Sensors for Abiotic Stimuli" of this book). In the meantime, a few microtubule-associated proteins have been identified that participate in the regulation of microtubule dynamics. These include the microtubule-severing katanins, bundling proteins such as EF-1α (Durso and Cyr 1994), MAP65 or MOR1. It should be mentioned that microtubule stability can also be regulated through signals that might act through altering the activity of such microtubule-associated proteins (for reviews see Nick 1998, 1999). These include hormones such as abscisic acid (Sakiyama and Shibaoka 1990; Wang and Nick 2001), auxin (Wiesler et al. 2002), but also exogenous factors such as blue light (Nick and Schäfer 1994) or temperature (Abdrakhamanova et al. 2003). These signals and molecules could be used as tools to control cell axis, especially if they are targeted to the still-unknown lattice that decides over the orientation-dependent differences in microtubule turnover.
5. Manipulation of post-translational modification. Tubulins are subject to elaborate post-translational modifications (for review see Ludueña 1998) that correlate with enhanced stability of microtubules. Whereas this increased stability was originally thought to be the consequence of the modification, it is now thought to be the cause (for review see Bulinski and Gundersen 1991; Erck et al. 2000): The modifying enzymes seem to prefer microtubules as substrates rather than soluble tubulin heterodimers (Kumar and Flavin 1981). Thus, a microtubule with an increased lifetime will be modified to a larger degree than a rapidly cycling microtubule. These modifications include phosphorylation of β-tubulin, acety-

lation, polyglutamylation and detyrosination/retyrosination of α-tubulin. Recently, even a nitration of α-tubulin has been discovered (Cappelletti et al. 2003) in neural cells. From these modifications, acetylation (Vaughn and Renzaglia 2006) and detyrosination (Duckett and Lloyd 1994; Wiesler et al. 2002) have been shown to occur in plants as well. The responsible enzymes have to be identified. However, the enzyme responsible for the retyrosination of α-tubulin has been cloned from pig brain (Ersfeld et al. 1993), and homologues of this enzyme are present in plants (Nick et al., unpublished results). A homologue of the retyrosination enzyme has been recently shown to be responsible for polyglutamylation in *Tetrahymena* and mouse (Janke et al. 2005). The post-translational modifications are thought to act as signals that regulate the interaction of microtubules with organelles or microtubule motors (Gurland und Gundersen 1995; Kreitzer et al. 1999) and thus differentiate different populations of microtubules that differ in function. It could be shown for auxin-dependent reorientation of cortical microtubules that the discordant microtubules that herald the new orientation of the array are detyrosinated, whereas the microtubules that still maintain the original orientation are not (Wiesler et al. 2002). The increased detyrosination of the discordant microtubules might enhance their interaction with kinesins (Kreitzer et al. 1999) such that they are preferentially moved, leading in the coalignment of the new, longitudinal array. By modification of the retyrosination enzyme it might be possible to suppress or to enhance the coalignment of microtubules and thus to control cell axis in a specific manner.

6. Manipulation of cellulose synthesis. As pointed out above, cortical microtubules and cellulose microfibrils are linked through a self-referring feedback control. Pharmacological or molecular interference with cellulose synthesis could be used to generate even non-intuitive consequences. However, a constitutive manipulation would simply result in a thinning of cell walls and a reduced mechanical stability (Edelmann et al. 1989). Conversely, the cell wall could be irreversibly stiffened by induction of peroxidase-triggered lignification (Fry 1979; Liszkay et al. 2004). If the interaction of microtubules and cellulose microfibrils were altered in a switchable way, this might provide an elegant tool for achieving specific alterations of cell axis.

Since length control represents a central topic of agriculture, microtubule-based rationales designed to regulate the axis of growth are expected to be a meaningful biotechnological strategy. The basic idea is to utilize the morphogenetic responses that occur naturally in a plant and to target these responses either to cells, where they usually do not occur, or to modify them minimally to obtain changes in the response amplitude. In the ideal case, a manipulation of plant axis and shape could be produced without the help

of heterologous genes and promotors, if the endogenous genes and promotors are recombined in an intelligent way. Even relatively subtle changes in assembly dynamics, microtubule bundling or post-translational modification should produce conspicuous and specific effects on plant shape.

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Crossed-Wires: Interactions and Cross-Talk Between the Microtubule and Microfilament Networks in Plants

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Abstract In plant cells, the cytoskeleton comprises distinct and highly dynamic arrays of microtubules and actin microfilaments. The basic structures and proteins of both the microtubules (~25 nm-diameter polymers of α- and β-tubulin heterodimers), and the microfilaments (~7 nm-diameter polymers of 42 kDa actin monomers) are conserved in all eukaryotic organisms, and occur in all cell types. The third cytoskeletal array present in animal cells, intermediate filaments, are of a more varied composition and their presence has not (yet) been demonstrated in plant cells.

The basic organization of microtubules and microfilaments in various plant cells was determined over several decades from static images of fixed material. These images often demonstrated that microfilaments co-align with microtubules. As functional and molecular studies have become more prevalent, it has become apparent that co-ordination of dynamic microtubules and microfilaments is necessary for many facets of growth and development, and that cross-talk exists between them. Numerous studies have shown such interactions in animal cells (Gavin 1997; Goode et al. 2000; Dehmelt and Halpain 2003), and it is the diversity of these processes in plants that forms the subject of this review. As such, this review takes a broad approach to the topic. Defining microtubule–microfilament cross-talk (or microfilament–microtubule cross-talk for those of an actin persuasion) as any type of relationship between microtubules and microfilaments, the review commences with a reassessment of early work into colocalization between microtubules and microfilaments (Sect. 1), which leads to information about microtubule–microfilament interactions (Sect. 2). In this review, the term "interactions" implies a direct, physical relationship between the two components of the cytoskeleton, whereas "cross-talk" is used in a more encompassing way that includes indirect interactions. Section 3 considers proteins that might mediate direct microtubule–microfilament interactions. However, taking the broad view of microtubule–microfilament cross-talk leads to discussion of systems where both microtubules and microfilaments play a role, but without any direct involvement with one another. Such microtubule–microfilament co-ordination seemingly occurs in organelle movement and shaping (Sect. 4). A further component of microtubule–microfilament cross-talk involves indirect, but specific interplay between the networks via the Rop-signalling pathway (Sect. 5).

The cytoskeleton performs numerous fundamental roles within plant cells, and plant biologists have demonstrated that the microtubules and microfilaments function independently in many of these. However, as this review documents, on the occasions when these two networks come together, and there is interplay between them, dissecting the tangled cross-wires of the microtubules and microfilaments can become difficult.

1
Microfilament Organization, Function, and Colocalization with Microtubules

As a basis for understanding direct microtubule–microfilament interactions, and for also understanding indirect cross-talk, microfilament localization studies are essential. Despite microfilaments being more "delicate" than microtubules, and thus comparatively more difficult to observe in fixed tissues, many studies over the last thirty years have demonstrated that microfilaments can colocalize with microtubules in plant cells. This section looks at evidence for colocalization in both interphase and dividing cells, and the various techniques used to collect this evidence.

1.1
Colocalization During Cell Division

During cell division, when the microtubule arrays of the preprophase band, mitotic spindle, and phragmoplast define the various phases of mitosis and cytokinesis, microfilaments also undergo systematic reorganizations. Colocalization of microtubules and microfilaments occurs in the preprophase band (Traas et al. 1987) where the majority of polymerized actin is present as single microfilaments, rather than as bundles, and is thus organized differently to the bundled microfilaments present in interphase cells (Ding et al. 1991b), and while colocalization does not occur in the mitotic spindle, it is re-established in the phragmoplast (Gunning and Wick 1985). This colocalization is not complete, with slight differences in the organization of phragmoplast microtubules and microfilaments detectable by electron microscopy (Kakimoto and Shibaoka 1988), immunofluorescence (Collings et al. 2003; Collings and Wasteneys 2005) or upon microinjection (Zhang et al. 1993). To date, neither the mechanisms by which microfilaments and microtubules colocalize during cell division nor the cause for the subtle differences between the two patterns, have been addressed.

1.2
Microtubule-Associated Filaments Observed by Electron Microscopy

Electron micrographs of plant cells demonstrate that 7-nm-wide filaments often run along microtubules (Fig. 1A). Even from their earliest observation in *Lilium* pollen tubes, it was suggested that (based on their diameter) these filaments were composed of actin (Franke et al. 1972). This was later confirmed in *Lilium* pollen tubes with actin antibodies (Lancelle and Hepler 1991; Fig. 1B), and in *Hydrocharis* root hairs with heavy meromyosin labelling (Tominaga et al. 1997). These microtubule-associated microfilaments are separated by 10 to 15 nm from the microtubules (Ding et al. 1991a), but are linked by extensive cross-bridging (Lancelle et al. 1987; Ding et al. 1991a), and although they can be

preserved by conventional aldehyde fixation, their preservation is improved by the use of freeze fixation and substitution methods (Tiwari et al. 1984). These microfilaments seem to be ubiquitous in plants, being present in tip-growing pollen tubes (Franke et al. 1972; Lancelle et al. 1987; Lancelle and Hepler 1991), and root hairs (Seagull and Heath 1979; Tominaga et al. 1997). Significantly, they also occur in various cell types undergoing diffuse growth (Hardham et al. 1980; Tiwari et al. 1984; Ding et al. 1991a; Fig. 1A).

Despite numerous reports on microtubule-associated microfilaments during the 1970s and 1980s, research into these structures has been neglected during the subsequent years. If these structures are indeed microfilaments, then it might be expected that they should be sensitive to disruption with

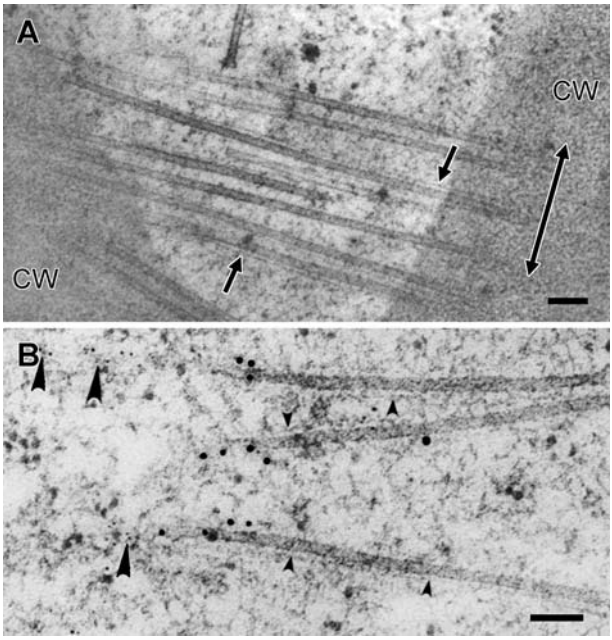

Fig. 1 Freeze substitution demonstrates that cortical microtubules can be accompanied by microfilaments. **A** A glancing section through a diffusely expanding wheat root-tip cell shows cortical microtubules that align transverse to the long axis of the cell (indicated by a *double-ended arrow*). Numerous transversely oriented microtubule-associated microfilaments are also present (*arrows*). *CW* = cell wall. **B** In tobacco pollen tubes, double labelling with anti-actin and anti-tubulin antibodies was visualized with 5- and 10-nm gold particles, respectively. Single microfilaments associated with the cortical microtubules, although they labelled poorly with the actin antibodies (*small arrowheads*), while actin localization continued as an extension of a microtubule as it disappeared out of the section (*large arrowheads*). Image in **A** by Suresh Tiwari (formerly Australian National University) and provided by Brian Gunning. Image in **B** is modified from Lancelle and Hepler (1991), and is reproduced with permission from Springer. *Bars* in **A** and **B** equal 100 nm

drugs targeted to microfilaments such as latrunculin or cytochalasin. These experiments seem not to have been conducted. Furthermore, it has not yet been established how these microtubule-associated microfilaments are stabilized, which proteins form the cross-bridges between them, and what roles they might play.

1.3 Cortical Microfilaments Observed by Fluorescence Microscopy in Fixed Tissue

Is there evidence from fluorescence microscopy that cells contain microtubule-associated microfilaments? Virtually all fluorescent methods for visualization of plant microfilaments demonstrate a fine cortical array that runs parallel to the transverse cortical microtubules. These methods include rhodamine-phalloidin labelling (Traas et al. 1987; Sonobe and Shibaoka 1989; Blancaflor 2000; Collings et al. 2001), immunofluorescence (McCurdy and Gunning 1990; Collings and Wasteneys 2005), immuno- or rhodamine-labelling of membrane ghosts (Collings et al. 1998), microinjections of rhodamine-phalloidin into living cells (Cleary 1995), microinjections of fluorescently tagged recombinant *Arabidopsis* fimbrin (Kovar et al. 2001), and visualization with fusions of green fluorescent protein (GFP) and the actin-binding domains of talin (Kost et al. 1998) and fimbrin (Sano et al. 2005). However, although immuno-double labelling shows extensive overlap between the two patterns, such colocalization is not conclusive proof that microtubules and microfilaments do interact.

Cortical microfilaments are often referred to as "fine" and "delicate", although care needs to be taken with these terms. For example, "fine" is sometimes used to imply that the cortical array comprises single microfilaments because it appears dimmer than the subcortical bundles, even though it has not yet been demonstrated whether these cortical microfilaments are bundled or not. Describing cortical microfilaments as "delicate" is appropriate, for they are more difficult to preserve than the subcortical bundles. In general, their preservation in fixed material is aided by pre-treatments with maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Sonobe and Shibaoka 1989) prior to aldehyde fixation. MBS, sometimes described as a prefixative, is a protein cross-linker and should be considered as a fixative in its own right. It stabilizes microfilaments by covalently cross-linking adjacent actin monomers within the microfilament (Sutoh 1984; Sonobe and Shibaoka 1989), and MBS treatments of purified plant actin result in a ladder of dimers and larger oligomers on SDS-PAGE gels. Thus, although MBS is not needed to see transverse microfilaments (see for example Traas et al. 1987; Wernicke and Jung 1992; Collings et al. 1998), MBS pre-treatment increases their preservation. Nevertheless, some caution is warranted in using MBS because it may lead to an over-representation of cortical microfilaments in artifactual arrays (Collings and Wasteneys 2005).

1.4 Cortical Microfilaments Viewed in Living Cells

Transverse cortical microfilaments have been seen in fluorescence microscopy with a variety of methods including microinjection of labelled phalloidin (Cleary 1995) or actin-binding proteins (Kovar et al. 2001), and through the expression of GFP-tagged reporters (Kost et al. 1998; Sano et al. 2005). Labelling with GFP-ABD2, the current probe-of-choice in *Arabidopsis* (Sheahan et al. 2004; Wang et al. 2004), confirms immunolabelling studies (Sano et al. 2005). Elongating root epidermal cells contain dynamic arrays of longitudinally oriented microfilament bundles and a dimmer transverse network (Fig. 2A). Some limitations currently exist with reporter genes for microfilaments: a recent study showed that GFP-fimbrin, GFP-talin and GFP-ADF1 all reported the fine cortical microfilaments near the tips of growing pollen tubes in different ways, with GFP-fimbrin most closely approaching the "gold-standard" of electron microscopy following rapid freezing (Wilsen et al. 2006). Understanding of microfilament dynamics and cross-talk with microtubules will only be possible through the development of a new generation of fluorescent reporter fusions, including GFP-actin, and the development of stably transformed *Arabidopsis* plants expressing both microtubule- and

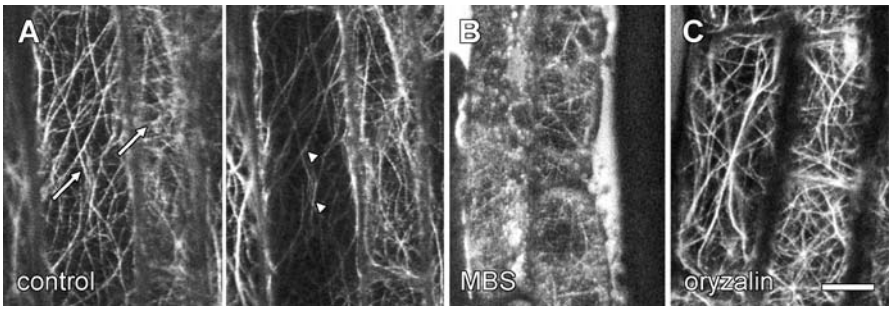

Fig. 2 Transverse microfilaments occur in the cortex of elongating *Arabidopsis* root epidermal cells where they lie parallel to cortical microtubules. Confocal imaging of epidermal cells of *Arabidopsis* roots stably expressing GFP-ABD2 confirms that the transverse organization of cortical microfilaments depends on microtubules. **A** In control roots, a single optical section taken through the outer cortex (*left*) showed a network of transverse microfilaments (*arrows*) whereas an optical section taken 1 μm further into the cell (*right*) showed predominantly longitudinal microfilament bundles (*arrowheads*). The transverse microfilaments were not as prominent as found with immunolabelling. **B** Treatment with MBS (400 μM, 10 min) marginally increased the amount of transverse microfilaments but led to a long-term reduction in microfilament labelling. Attempts to fix GFP-ABD2 with aldehydes, and hence complete double labelling assays, failed as the GFP marker dissociated from the microfilaments during the fixation process. **C** Microtubule depolymerization with oryzalin (20 μM, 30 min) caused a complete loss of transverse microfilaments. *Bar* in **C** = 10 μm for all images

microfilament-reporting constructs. To date, the use of dual-labelling plants has been limited (Saedler et al. 2004; Timmers et al. 2007).

1.5 Microfilaments and Cell Expansion

Traditionally, while microfilaments have been thought to regulate the expansion of tip-growing cells, microtubules have been thought to control the expansion of diffusely growing cells (Kropf et al. 1998). However, experimentation demonstrates that this proposed and simple relationship does not exist. Thus, while active elongation and tip-growth is associated with regions of diffuse and dynamic microfilaments in pollen tubes and root hairs, (Fu et al. 2001; Ketelaar et al. 2003), microtubules are also active in controlling the growth of these cells (Bibikova et al. 1999; Anderhag et al. 2000; Ketelaar et al. 2003).

In cells showing complex expansion patterns, such as trichomes and leaf pavement cells, volume increases occur through growth in specific regions of the cell wall that coexist with areas in which the cell does not expand. Not only do the cells showing this type of growth require both microtubules and microfilaments to regulate their final shape (Panteris and Galatis 2005; Smith and Oppenheimer 2005), regions of diffuse and dynamic microfilaments are associated with the regions of these cells that are actually protruding (Fu et al. 2002; Frank et al. 2003). The polymerization of microfilaments in these cells is controlled by the ARP2/3 complex, and mutations affecting this complex (or signalling to it) modify microfilament polymerization and thus cell growth and shape (reviewed in Kotzer and Wasteneys 2006).

The proposed relationship between microtubules controlling diffuse growth, and microfilaments controlling tip-growth, also fails in diffusely expanding cells. In these cells, where microtubules delineate the control of cellulose deposition and thus the direction of cell expansion (Paredez et al. 2006), dynamic microfilaments do not accumulate at sites of cell expansion. Instead, microfilaments have been reported to accumulate at the non-expanding ends of the cells (Baluška et al. 2003). Even so, microfilaments contribute to cell elongation in diffusely growing cells. In elongating roots, microfilament disruption with microfilament antagonists reduces cell elongation and promotes lateral cell expansion. This results in a shorter root with a swollen tip (Baskin and Bivens 1995; Blancaflor 2000; Collings et al. 2006). Genetic manipulations in *Arabidopsis* also suggest that microfilaments contribute to the controlled expansion (reviewed in Blancaflor et al. 2006). The *act2-2D* and *act7* mutants, in which actin genes are disrupted, show reduced cell elongation and radial expansion defects (Nishimura et al. 2003; Gilliland et al. 2003). Furthermore, the over- or under-expression of five different actin-binding proteins [profilin (Ramachandran et al. 2000); actin-depolymerization factor (ADF) (Dong et al. 2001b); class XI myosin (Holweg

and Nick 2004); actin interacting protein (AIP) (Ketelaar et al. 2004); and, cyclase-associated protein (CAP) (Barrero et al. 2002)] all modify cell elongation in *Arabidopsis*. Additionally, some GFP tags for microfilaments also result in decreased cell and tissue elongation (Sheahan et al. 2004).

The mechanism(s) through which microfilaments contribute to diffuse expansion remain undetermined. In tip-growing cells, and cells showing complex expansion patterns, diffuse and dynamic microfilaments generated by the ARP2/3 complex are associated with the sites of active outgrowth. However, in diffusely expanding cells, this array of microfilaments is not present at all, and, although ARP2/3 proteins are expressed throughout the plant (Li et al. 2003), these cells lack phenotypes in ARP2/3-related mutants (Li et al. 2003; Mathur et al. 2003). If microfilaments are important in diffuse cell expansion, then it follows that other pathways regulate their polymerization.

Microfilaments are essential for basic processes associated with overall cell growth, including cytoplasmic streaming and the delivery of Golgi vesicles to sites of active exocytosis (Boevink et al. 1998; Nebenführ et al. 1999). However, additional microfilament-dependent processes exist. As microfilaments appear to be involved in exocytosis and endocytosis (Grebe et al. 2003), they are in part responsible for the cellular distribution of important proteins in cell patterning, such as auxin influx and efflux carriers, which undergo cycling to and from the plasma membrane (Muday and Murphy 2002). However, microtubules remain important for the control of cell expansion (see Chap. 1 of this book), and as discussed in the following sections, it is also possible that cross-talk between microtubules and microfilaments may be a further means through which microfilaments regulate cell expansion.

2 Evidence for Microtubule–Microfilament Cross-Talk

2.1 Pharmacology Provides Evidence for Cross-Talk and Direct Interactions

Pharmacological evidence demonstrates that in certain cells, and at certain times, microtubules and microfilaments can show direct interactions. In these experiments, a range of specific cytoskeletal antagonists have been used: should microfilament disruption modify microtubule organization, or should microtubule de- or over-stabilization modify microfilaments, then this provides evidence for direct interactions, especially in cases where microtubules and microfilaments co-align. It must be emphasized, however, that because these experiments have investigated a range of different tissues in a range of different species with a variety of drugs, the results are often inconsistent.

The pharmacological approaches to the cross-talk between microtubules and microfilaments can be summarized in several ways. First, treatments that

stabilize microtubules and microfilaments promote microtubule-associated microfilaments whereas treatments that disrupt the cytoskeleton reduce the number of these microfilaments. Second, microfilament disruption over long periods can cause microtubule rearrangements, although the evidence for this is equivocal. And third, microfilament disruption often (but not always) prevents microtubule reorganizations from occurring normally. Thus, microfilaments rely on cortical microtubules for their cortical organization and stability, but microtubules may require microfilaments for their (re)organization.

Stabilization of cortical arrays promotes the presence of transverse cortical microfilaments, as shown through at least five different experimental approaches:

- Microtubule depolymerization causes the loss of transverse cortical microfilaments, as viewed by phalloidin labelling in cultured carrot cells (Traas et al. 1987), by immunofluorescence in *Arabidopsis* root epidermal cells (Collings and Wasteneys 2005) and in *Arabidopsis* roots expressing GFP-ABD2 (Fig. 2C).
- Immunolabelling shows that microtubule disruption in the *mor1* mutant renders the cortical microfilaments hypersensitive to microfilament disruption (Collings et al. 2006).
- Taxol that promotes microtubule stability enhances the presence of microtubule-associated microfilaments in maize roots labelled with phalloidin (Chu et al. 1993; Blancaflor 2000).
- In tobacco BY-2 suspension cells, taxol stabilization of microtubules prior to and during protoplasting promotes retention of aligned microtubules on plasma membrane ghosts, and also the co-alignment of cortical microfilaments (Collings et al. 1998) (Fig. 3A,B).
- And whilst not an experiment involving applied cytoskeletal antagonists per se, the microinjection of recombinant ADF (actin depolymerization factor) into *Tradescantia* stamen hairs causes a breakdown of the longitudinal subcortical and transvascuolar microfilament bundles that generate cytoplasmic streaming, and their reorganization into transverse bundles. While double-labelling of microfilaments along with microtubules was not possible, it was suggested that the orientation of the newly formed microfilaments was controlled by microtubules (Hussey et al. 1998). It seems that ADF can generate stabilized microfilaments, for the transient expression of GFP-ADF fusions in *Allium* epidermal cells suggests that the ADF might promote microfilament bundling (Dong et al. 2001a).

Numerous studies have investigated the long-term effects of microfilament disruption on microtubule organization, but the conclusions from these studies are somewhat variable. While some studies suggest that microtubules remain unaffected by microfilament disruption (for example, Hush and Overall 1992; Collings et al. 2006), others have reported changes, notably an in-

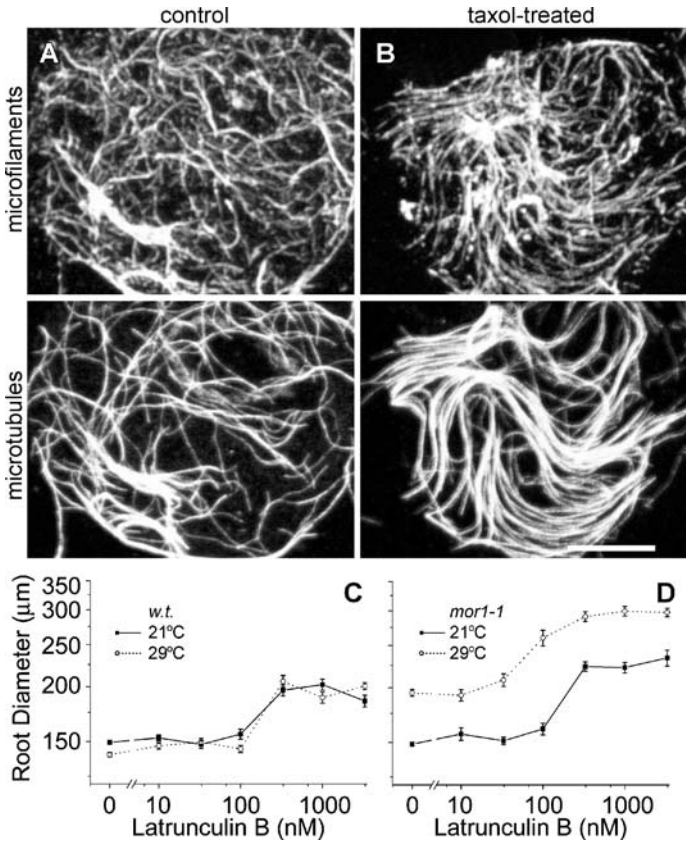

Fig. 3 Modulation of the microtubule cytoskeleton can cause changes to microfilaments and/or to microfilament-related responses. **A**,**B** The membrane-associated cytoskeleton of tobacco BY-2 cells found on membrane ghosts responded to stabilization of microtubules with taxol (10 μM, 3 h). Compared to control ghosts that had random microfilaments and microtubules (**A**), ghosts from taxol-treated cells retained highly aligned microtubules that were parallel to ordered microfilaments (**B**). *Bars* in **A** and **B** = 10 μm. **C**,**D** Average *Arabidopsis* root diameters were recorded for wild-type (**C**) and *mor1* mutant plants (**D**) incubated on different concentrations of latrunculin B for 48 h at either 21 °C or 29 °C, with points representing the means and standard errors for 30 or more roots. While wild-type plants showed a similar latrunculin response at both temperatures, the *mor1* plants were hypersensitive to latrunculin, undergoing root swelling at much lower drug concentrations, but only at the restrictive temperature. Images **A** and **B** are modified from Collings et al. (1998) and reprinted with permission of the American Society of Plant Biologists, while images **C** and **D** are modified from Collings et al. (2006) and reprinted with permission of New Phytologist

creased randomization of transverse microtubules (Seagull 1990; Blancaflor 2000; Ueda and Matsuyama 2000). However, reports of impaired or abnormal microtubule reorganization in response to microfilament disruption seem to

be more common (first reviewed in Collings and Allen 2000) with at least nine independent reports in varying systems:

- In the developing tracheary elements of cultured *Zinnia* cells, microfilament disruption prevents microtubule reorganizations that normally occur during wall reinforcement and suggests that microfilaments "play an important role in the change in the orientation of arrays of microtubules from longitudinal to transverse" (Kobayashi et al. 1988).
- During cotton fiber development, microtubules (Seagull 1992; Andersland et al. 1998), and microfilaments (Andersland et al. 1998) undergo a sequential and extensive series of reorganizations necessary for controlling cell wall deposition. Microfilament disruption prevents microtubule rearrangements, and results in the eventual formation of disorganized microtubule arrays (Seagull 1990). Significantly, although microtubule-associated microfilaments have not been detected by electron microscopy in cotton fibers (Tiwari and Wilkins 1995), a putative microtubule–microfilament cross-linking protein has been isolated from these cells (Preuss et al. 2004) (Sect. 3.1).
- Long-term treatments with low levels of cytochalasin disrupt transverse cortical microfilaments and prevent re-organization of microtubules into the normally present transverse bands in developing wheat mesophyll cells (Wernicke and Jung 1992).
- In the elongating epidermal cells of adzuki bean epicotyls, microtubules undergo a cyclic re-orientation from transverse to longitudinal. Microfilament disruption promotes longitudinal microtubules at the expense of transverse microtubules (Takesue and Shibaoka 1998).
- In living epidermal cells of *Allium* undergoing the cessation of elongation, Jan Marc's group has shown that microfilament disruption slows reorientation of microtubules from transverse to oblique and longitudinal (Sainsbury et al. in preparation).
- In dividing epidermal and root-tip cells of *Allium*, the microtubule preprophase band becomes progressively narrower as preprophase progresses. This microtubule remodelling cannot proceed when microfilaments are disrupted, as demonstrated in immunolabelled cells (Mineyuki and Palevitz 1990, Eleftheriou and Palevitz 1992), and in living tissue expressing GFP-MBD (Granger and Cyr 2001).
- In tobacco BY-2 cells, the recovery of interphase microtubules after cytokinesis is blocked by microfilament disruption even though these treatments do not modify interphase cortical microtubules. This observation was first made by immunofluorescence (Hasezawa et al. 1998) and has been confirmed in living cells expressing GFP-tagged tubulin (Yoneda et al. 2004).
- Asymmetric mitosis in developing stomatal complexes of *Zea* features a microtubule "half-spindle", spatially separated from microfilaments,

that links the dividing nucleus to the plasma membrane. Microfilament disruption, either with drugs or through the mutant *brick1* where Rop signalling is modified (see Sect. 5 of this chapter), results in microtubule reorganization and realignment of the mitotic spindle (Panteris et al. 2006).

One again, however, systems exist in which microtubules continue to undergo reorganizations even after microfilament disruption (Hush and Overall 1992; Ueda and Matsuyama 2000).

In conclusion, pharmacological experiments demonstrate that microtubule-microfilament cross-talk can occur in the form of apparently direct interactions between the two components of the cytoskeleton. Changes in microtubule organization can be dependent on the presence of microfilaments but the stability of microtubule-associated microfilaments can also be dependent on microtubules.

2.2 Molecular Genetics Provides Further Evidence for Microtubule–Microfilament Cross-Talk

A range of cytoskeletal mutants in *Arabidopsis* and other plants have implications for studies of microtubule–microfilament cross-talk. In *Arabidopsis*, the combination of pharmacological and mutational approaches demonstrates cross-talk between microtubules and microfilaments, although not whether there is a direct interaction. In plants in which microtubules are disrupted, including the *mor1* mutant at its restrictive temperature and the constitutive mutants *botero* and *spiral1*, inhibition of root growth and radial swelling occur at lower latrunculin concentrations than in wild-type plants (Fig. 3C,D). This hypersensitivity effect can be mimicked in wild-type plants by low concentrations of oryzalin (Collings et al. 2006). We have used this hypersensitivity to latrunculin to isolated further temperature-sensitive mutants. To date, however, it is not clear, whether this hypersensitivity results from some direct interaction between transverse cortical microtubules and microfilaments, or through indirect cross-talk.

One interesting mutant has also been isolated from mutagenized rice plants screened for resistance to microtubule depolymerization. The rice mutant *Yin-Yang* was initially isolated on the basis of its resistance to the microtubule–depolymerizing herbicide ethyl-*N*-phenyl carbamate. However, auxin-dependent cell elongation in *Yin-Yang* is significantly more sensitive to microfilament disruption with cytochalasin D than wild-type plants (Wang and Nick 1998; Waller et al. 2000). Unfortunately, the identity of the *Yin-Yang* mutation is not yet known.

Mutants have been notably informative in dissecting the control of microtubule dynamics by microfilaments. In root hairs of the *distorted2* (*dis2*)

mutant of *Arabidopsis*, in which the ARP2/3 complex that regulates microfilament polymerization is disrupted, cortical microtubules are organized normally (Schwab et al. 2003). In the *dis2* mutant, however, endoplasmic microtubules are overly clustered and bundled at sites where microfilaments polymerize and aggregate abnormally. These microtubules are also hyperstable to microtubule disruption, suggesting that they are not as dynamic as in wild-type plants (Saedler et al. 2004). Significantly, experiments using low latrunculin concentrations that did not inhibit cytoplasmic streaming, but which were likely to reduce rates of microfilament polymerization, also generated bundled and less dynamic microtubules and thus phenocopied the *dis2* mutation (Saedler et al. 2004). A similar situation exists in the elongating root hairs of *Medicago* where microfilament disruption with latrunculin reduces the rate of endoplasmic microtubule assembly by 40%, as measured with confocal microscopy. As dual expression of GFP-ABD2 and dsRed-MBD demonstrated little physical contact between the microtubules and microfilaments, an as yet unknown and indirect form of cross-talk between the microtubules and microfilaments was suggested (Timmers et al. 2007).

The temperature-sensitive *Arabidopsis rsw6* mutant also demonstrates control of microtubule dynamics by microfilaments. At the permissive temperature, root epidermal cells maintain normal microtubule arrays but when seedlings are transferred to the restrictive temperature, the ordering of microtubules across the entire root breaks down even though individual cells retain parallel microtubules. Microfilament-disruption with latrunculin minimizes this microtubule reorientation, and it was speculated that microfilaments are "important in the early stages of reorientation but not for directly positioning microtubules" (Bannigan et al. 2006). Furthermore, unlike most other microtubule-related mutants, the microtubules in *rsw6* are hyperstable to microtubule disruption, as found in *dis2* (Bannigan et al. 2006).

Mutants also suggest that microfilaments can control microtubule organization. In expanding trichomes, mutations that modify microfilament organization through disruption of the ARP2/3 complex, or signalling to this complex, result in reorganization of microtubules (Schwab et al. 2003; Zhang et al. 2005), and similar changes in microtubule organization are caused by microfilament disruption with cytochalasin (Schwab et al. 2003).

3 Proteins That Might Mediate Direct Microtubule–Microfilament Interactions

If microtubules and microfilaments can interact directly in plant cells, what proteins mediate these interactions? In animal cells, various proteins have been implicated. These include the microtubule actin cross-linking factor (Leung et al. 1999), two microtubule-associated proteins (MAPs), MAP1 and MAP2 (Dehmelt and Halpain 2003), formins, the microtubule motor dynein

and related dynactin complexes, coronin, various complexes involving the microfilament-dependent motor protein myosin, and a class of actin-binding proteins referred to as ERM proteins (Goode et al. 2000). Plants, however, lack MAPs1 and 2, coronin, ERM proteins and dynein.

Plants do have representatives of two classes of protein, myosins and formins, which in animals can interact with both microtubules and microfilaments, but there is no evidence for this ability for the plant homologues of these proteins. Plant myosins belong to classes VIII and XI (Riesen and Hanson 2007) and do not contain the 110 amino acid microtubule-binding tail domain which is sometimes found in myosin classes VI and VII and which is referred to as the myosin tail homology domain (MyTH) (Oliver et al. 1999). Although the *Arabidopsis* calmodulin-binding kinesin contains this MyTH domain (Fig. 4A), biochemical evidence suggests that this myosin-like motif binds microtubules rather than microfilaments, and that this microtubule binding occurs independently of the kinesin motor domain (Narasimhulu and Reddy 1998; Oliver et al. 1999). Animal formins and formin homologues constitute a diverse family of proteins that regulate the cytoskeleton through a formin homology 2 (FH) domain. Although generally considered to be actin-binding proteins, formins can also regulate microtubule dynamics (Faix and Grosse 2006). While formin homologues exist in plants (Deeks et al. 2002), their putative microtubule-binding activities have not yet been tested.

Nevertheless, several candidate proteins for linkers between microtubules and microfilaments have been described in plants, with biochemical evidence for two in particular, calponin homology domain-containing kinesins (Preuss et al. 2004), and MAP190 (Igarashi et al. 2000). However, even for these two proteins, the evidence for a role as linkers between microtubules and microfilaments has remained equivocal.

The continued investigation of the plant cytoskeleton through a variety of approaches, including such novel strategies as identifying cytoskeletal interacting proteins through their effects in mammalian cells (Abu-Abied et al. 2006) will undoubtedly identify many further proteins, and the next few years will show which of these can act as linkers between microtubules and microfilaments.

3.1 Class-14 Kinesin Containing Calponin-Homology Domains

Kinesins are microtubule-based motor proteins that can be divided into 14 subfamilies based on variations in a conserved motor domain, although outside this motor there is little or no sequence conservation. Plants are unusual in their complement of kinesins. Not only does *Arabidopsis* have the most kinesins (61) of any sequenced organism (Reddy and Day 2001), but their distribution into 10 of the 14 described kinesin subfamilies is uncommon, be-

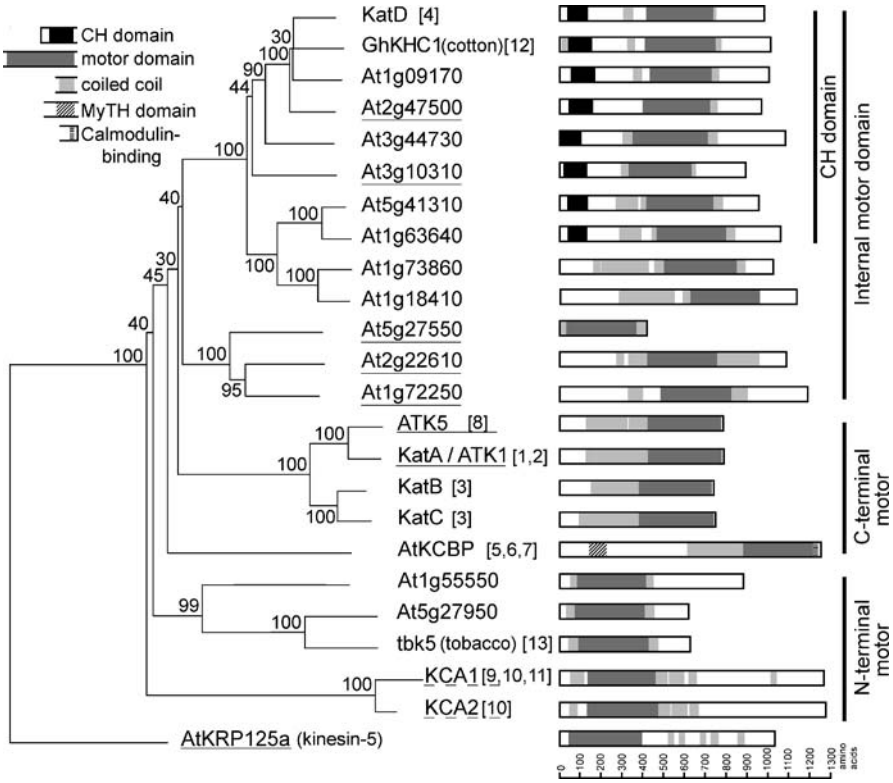

Fig. 4 Calponin homology domain-containing class-14 kinesins (CHK14s) are potential linking proteins between microtubules and microfilaments. Seven *Arabidopsis* kinesins (and a cotton protein GhKHC1) that group together based on motor domain homologies have a conserved structure with an N-terminal calponin homology domain, and an internal motor domain surrounded by two coiled-coil domains. Motor domains of the 21 *Arabidopsis* class-14 kinesins, along with one each from cotton and tobacco, were aligned in ClustalW. A neighbor-joining phylogeny was constructed using AsaturA and visualized in JTreeView using the *Arabidopsis* kinesin-5 AtKRP125a as an outgroup. Schematic diagrams show motor and calponin homology domains determined in HMMSmart, coiled-coil regions found by Parcoil2, a myosin tail homology domain (MyTH), and an experimentally determined calmodulin-binding domain (Reddy et al. 1996). The names of class-14 kinesins that are upregulated during cell division are underlined (Vanstraelen et al. 2006a), and two further class-14 kinesins, not part of this group but known to function in division (Vanstraelen et al. 2004, 2006b) are underlined with dashes. Class-14 kinesins are named either by their gene loci, or, where characterized, by their published names. References (in *square brackets*): *1* = Mitsui et al. 1993; *2* = Chen et al. 2002; *3* = Mitsui et al. 1994; *4* = Tamura et al. 1999; *5* = Reddy et al. 1996; *6* = Oppenheimer et al. 1997; *7* = Song et al. 1997; *8* = Ambrose et al. 2005; *9* = Kong and Hanley-Bowdoin 2002; *10* = Vanstraelen et al. 2004; *11* = Vanstraelen et al. 2006b; *12* = Preuss et al. 2004; *13* = Matsui et al. 2001

cause two of these groups, kinesins of the classes 7 and 14, contribute more than half the proteins (15 and 21 respectively). The completed genomes for rice and poplar also show similarly large numbers of kinesins with skewed distributions, a highly atypical pattern compared to non-plant organisms (Richardson et al. 2006).

Compared to other kinesins, class-14 kinesins are unusual with respect to their structure and biochemistry. While the motor domain is N-terminal in the other kinesin classes, the motor domains of class-14 kinesins can be located either at the N- or C-terminal or even in the center of the protein. And while all other kinesins drive motion towards the plus end of microtubules, class-14 kinesins are minus-end directed motors. This reversal of direction has been attributed to a specific neck domain (Endow and Waligora 1998) that is found in all plant kinesin of class 14 (Reddy and Day 2001). Numerous class-14 kinesins have been functionally characterized in plants including at least eight from *Arabidopsis*, and one each from cotton and tobacco (Fig. 4).

Phylogenetic analysis of the class-14 kinesis in *Arabidopsis* shows that they cluster into different sub-groups. One of these subgroups, of interest to studies of microtubule–microfilament interactions, contains an internal motor surrounded by two regions of coiled coil, and an N-terminal calponin homology (CH) domain (Fig. 4). This clade of CH-domain containing class-14 kinesins (CHK14s) is unique to higher plants and not found in other organisms. *Arabidopsis* contains 7 of these CHK14s that range in size from 100 to 128 kDa. Expression analysis of these proteins through the Genevestigator database demonstrates that all are expressed at varying levels through the plant.

These CHK14s are significant for microtubule–microfilament cross-talk because the CH domain is a known actin-binding motif, making these the only plant proteins known to contain *bona fide* microtubule-interacting and microfilament-binding motifs. Although KatD, the only characterized *Arabidopsis* CHK14 was not investigated for its microfilament-binding activity (Tamura et al. 1999), a cotton homologue of this protein, GhKHC1, has been studied in this context. GhKHC1 bundles microfilaments in vitro, and antibodies raised against GhKHC1 decorate not only cortical microtubules but possibly also cortical microfilaments (Preuss et al. 2004). However, the interactions between CHK14s such as GhKHC1 and microfilaments are not well understood, as CHK14s contain only a single CH domain (Fig. 4). Typically, two CH domains are required to mediate actin binding in CH domain-containing proteins such as α-actinin or spectrin, or even four CH domains in the cases of animal and plant fimbrins, whereas proteins with only a single CH domain are thought to be involved in signal transduction (Gimona et al. 2002). How then does GhKHC1 bind to microfilaments in vitro? Preuss et al. (2004) cite unpublished data in which they tested whether the CH domain alone is sufficient to bind microfilaments and they

found this not to be the case. This does not preclude a role for the CH domain in signalling to the cytoskeleton because the protein calponin, which itself only contains a single CH domain, is an actin-binding protein with the site of actin-binding mapped to a region between the CH domain and the C-terminus of the protein (Gimona and Mital 1998). Thus, the status of CHK14s as linking proteins between microtubules and microfilaments remains unclear.

3.2 MAP190

Cortical microfilaments and microtubules co-align in tobacco BY-2 cells (Sonobe and Shibaoka 1989; Collings et al. 1998). Detailed biochemical analyses of these cells have been possible through the generation of cytoplasm- and cytoskeleton-rich vacuole-free miniprotoplasts (Sonobe 1996). From such miniprotoplasts, fractions with microtubule-associated proteins were enriched leading to the isolation of a 190-kDa protein (Igarashi et al. 2000). Antibodies against this protein demonstrated that it could co-sediment with purified microtubules or microfilaments. However, as this assay was conducted with the crude protein fractions, it was not determined whether MAP190 itself was responsible for these activities directly. Immunolocalizations also showed that MAP190 localized to the interphase nucleus and only colocalized to the cytoskeleton during cell division (Igarashi et al. 2000). Analysis of the cloned gene and its *Arabidopsis* homologue (At2g03150) revealed the presence of only EF-hands and nuclear localization sequences, and no canonical domains or motives predicted to mediate cytoskeletal binding (Igarashi et al. 2000; Hussey et al. 2002). Thus it is still not clear, whether ABP190 qualifies as a true microtubule- and microfilament-binding protein.

3.3 Other Proteins That Can Bind Microtubules and Microfilaments

Various other proteins have now been identified in plants that might be able to bind to either microtubules or microfilaments, but not necessarily at the same time.

Using *Arabidopsis* cell cultures as a starting material, Chuong et al. (2004) conducted a proteomic examination of microtubule-binding proteins. While this approach did not recover several of the well characterized MAPs such as MAP65, MOR1, MAP190, and kinesins, other established MAPs were present. Several proteins of relevance to microtubule–microfilament interactions were also identified including EF1α, and actin-11, an actin isoform generally associated with reproductive tissues (Huang et al. 1997).

The protein synthesis factor EF1α might contribute to microtubule–microfilament cross-talk. EF1α is a well characterized actin-binding protein

in other organisms (Yang et al. 1990), while in plants, EF1α colocalizes with microtubules in cultured carrot (Durso et al. 1996) and tobacco BY2 cells (Hasezawa and Nagata 1993), and with microfilament bundles in maize endosperm (Clore et al. 1996). Purified carrot EF1α co-sediments with microfilaments in vitro (Yang et al. 1993), and recombinant maize EF1α acts as a microfilament-bundling protein and harbors multiple actin-binding sites (Gungabissoon et al. 2001). Purified carrot EF1α also acts a microtubule-bundling and stabilizing protein (Moore et al. 1998), and contains multiple microtubule-binding sites (Moore and Cyr 2000). As with many other proteins that can bind to microtubules and microfilaments, there is no clear evidence that EF1α can bind to both microtubules and microfilaments simultaneously, thus mediating direct interactions. Some evidence comes from *Physarum* EF1α which does bind microtubules to microfilaments (Itano and Hatano 1991).

Two other plant proteins bind both microtubules and microfilaments, although it has not been shown whether this happens concurrently or under the same conditions. The phospholipase D (PLD) family of signalling proteins has, among many things, been implicated in cytoskeletal responses. An *Arabidopsis* PLDδ was isolated in a microtubule-affinity screen as a microtubule-binding protein, and decorates both microtubules and the plasma membrane (Gardiner et al. 2001). However, an *Arabidopsis* PLDβ was also identified that binds both monomeric actin and microfilaments, typical of different PLD isoforms from animal and even bacterial sources (Kusner et al. 2003). As PLD inhibitors modify both microtubules and microfilaments in *Arabidopsis* (Motes et al. 2005), it is possible that PLDs could act as linkers between them.

Dynamin and dynamin-related proteins are GTPases that cause fission and deformation of membranes, and function in a large range of different cellular processes including endo- and exocytosis, protein sorting and vesicle trafficking. Plant dynamin-related proteins are grouped into six families (DRP1 through DRP6), and DRP3 was recently isolated as a microtubule binding protein from tobacco BY-2 cells. This protein co-sediments in vitro with both microtubules and microfilaments (Hamada et al. 2006), but evidence for functional interactions with either microtubules or microfilaments was not provided.

Further candidates for proteins mediating interactions between plant microtubules and microfilaments seemed, until recently, to be the LIM proteins. While these proteins are known to associate with the actin cytoskeleton in animal cells, immunofluorescence suggested that, in plant cells, they colocalize with microtubules (Brière et al. 2003). However, subsequent in vitro analysis showed that recombinant tobacco LIM is a microfilament bundling protein, and that GFP-tagged LIM localized to microfilaments in vivo (Thomas et al. 2006). The initially reported association of LIM to microtubules visualized by immunofluorescence was therefore interpreted as “an artefact resulting from the use of detergents”.

4
Microtubules and Microfilament-Based Cytoplasmic Streaming

Cytoplasmic streaming in higher plants depends exclusively on microfilaments, for streaming ceases when microfilaments are disrupted whereas streaming continues, seemingly unaffected, when microtubules are depolymerized. Nevertheless, evidence exists for cross-talk between microtubules and microfilaments in the control of organelle movement and positioning even though this does not seem to involve direct interactions. Furthermore, there is experimental evidence for even closer forms of cross-talk with microtubules potentially undergoing re-alignment due to the forces placed on them by streaming, and for effects of microtubule stability on the stability of microfilaments.

4.1
Organelle Positioning and Shaping

Until recently, it has been thought that organelle positioning in interphase cells of higher plants depended exclusively on microfilaments. By labelling mitochondria, plastids, peroxisomes, and the components of the secretory pathway with fluorescent dyes or GFP, their motion has been shown to be inhibited by microfilament disruption rather than by depolymerization of microtubules. Further, myosin associates with all these organelles (Wada and Suetsugu 2004; Riesen and Hanson 2007). Reports on microtubule-based motility in interphase higher plant cells have been rare, although kinesin motors may associate with vesicles during trichome development (Lu et al. 2005), and during cell division (Lee et al. 2001; Vanstraelen et al. 2006a). If organelle motility is microfilament-dependent, why consider organelle motion in a review of microtubule–microfilament interactions? Careful observations have demonstrated that while organelle motility is microfilament-based, positioning of stationary organelles is microtubule-based. Thus, microtubule–microfilament cross-talk in the control of organelle positioning and shaping is indirect and antagonistic.

Considerable evidence suggests that microtubules interact with mitochondria. Not only do kinesins associate with the mitochondria in *Arabidopsis* (Ni et al. 2005) and tobacco (Romagnoli et al. 2007), but GFP-tagged mitochondria, which normally move along microfilaments, align along microtubules when streaming is inhibited (van Gestel et al. 2002). This is consistent with observations in pollen tubes which suggest that the mitochondrial kinesins act to decrease mitochondrial motility (Romagnoli et al. 2007). Similarly, mitochondrial fusion during cell de-differentiation is also microtubule dependent and can be impaired by microtubule depolymerization and a kinesin inhibitor (Sheahan et al. 2005). Microtubule-based positioning of mitochondria has also been suggested in *Arabidopsis* based on analysis

of the *fmt* mutant (Logan et al. 2003; Logan 2006), and mitochondria in the alga *Chara* also appear to have the ability to move along microtubules (Foissner 2004).

Plastids also show microtubule-based localization. In tobacco, plastid motility is microfilament-based but microtubule disruption reduces stromule formation and changes plastid shape. Moreover, microtubule disruption increases the rate of plastid streaming (Kwok and Hanson 2003). In certain C4 plants, the aggregation of specialized chloroplasts is also microtubule-dependent (Chuong et al. 2006), and in the moss *Physcomitrella*, plastid movements in response to blue (but not red) light are mediated by both microfilaments and microtubules (Sato et al. 2001). Thus, the interactions of mitochondria and plastids with microtubules act to confine their movement whereas interactions with microfilaments generate motility.

Interactions with microtubules are poorly documented for the organelles of the endomembrane and secretory system. Microtubule depolymerization significantly increases streaming in cells with actively streaming Golgi where it was concluded that "microtubules do not appear to have an effect on movement, except for a subset of cells in which they seem to limit streaming" (Nebenführ et al. 1999). With peroxisomes, the evidence for interactions with microtubules is indirect. Several peroxisomal matrix proteins have been isolated from microtubule affinity studies (Chuong et al. 2002; Harper et al. 2002), and proteomic analysis of microtubule-associated proteins in *Arabidopsis* revealed numerous peroxisomal-targeted proteins (Chuong et al. 2004). This led to the suggestion that peroxisomal proteins might be "stored" on microtubules awaiting import into the peroxisomes (Muench and Mullen 2003). In conclusion, all organelles of plant cells undergo microfilament-based cytoplasmic streaming, but in addition depend on microtubules that act antagonistically on streaming and seem to play a role in organelle positioning.

4.2 Microtubule Organization and Cytoplasmic Streaming

As the diffuse elongation of plant cells ceases, microtubules re-orient from transverse to oblique through processes that are still only partially understood (see Chap. 1 of this book). It is possible that this re-orientation is a passive process dependent on microfilament-based cytoplasmic streaming. In the alga *Chara*, cortical microtubules will reorient passively to the direction of microfilament-based streaming upon wounding of the cell (Foissner and Wasteneys 1999). This process has also been investigated in higher plants. In *Allium* epidermal cells, where cytoplasmic streaming is predominantly longitudinal like other elongate plant cells, microtubule reorient slowly from transverse to oblique in the course of development. Agents that disrupt microfilaments slow this change in microtubule re-orientation. And as

re-orientation is similarly inhibited by treatments that inhibit cytoplasmic streaming, including myosin inhibitors or blocking ATP synthesis with dinitrophenol (DNP), it is possible that microtubules reorganize passively with cytoplasmic flow (Sainsbury et al., in preparation).

4.3 Hypersensitivity to Microfilament Disruption when Microtubules are Depolymerized—A Special Case of Cross-Talk

Microtubule–microfilament cross-talk in cytoplasmic streaming can also occur in a more apparent but still indirect manner. Microtubule depolymerization hypersensitizes streaming to microfilament disruption in certain algal cells (*Chara*, *Nitella* and *Spirogyra*) so that, while microtubule depolymerization does not modify streaming, it makes streaming more sensitive to microfilament disruption (Wasteneys and Williamson 1991; Collings et al. 1996; Foissner and Wasteneys 2000). Although cytoplasmic streaming in *Allium*, *Vallisneria* and *Tradescantia* do not show this hypersensitization response (Collings et al. 1996), it has been reported for the root hairs of two aquatic higher plants. In *Limnobium*, microtubule depolymerization hypersensitized microfilaments to non-inhibitory concentrations of cytochalasin and latrunculin (Nakasuka and Shimmen 2006), while in the related species *Hydrocharis*, microtubule depolymerization did not block streaming, but it did prevent the recovery of streaming after cytochalasin removal, apparently because microfilaments themselves did not recover their normal, bundled, array (Tominaga et al. 1997).

The mechanisms underlying this hypersensitization remain unknown. However, in *Nitella*, the subcortical microfilament bundles that drive cytoplasmic streaming are separated from the cortical microtubules by a layer of chloroplasts. Thus, a direct interaction between microtubules and microfilaments seems unlikely. Instead, this spatial separation led to an indirect model to explain microtubule–microfilament cross-talk. "A more plausible hypothesis is that the [microfilaments] are sensitized to cytochalasin by tubulin dimers or microtubule-associated proteins, large amounts of which are released when microtubules are depolymerized. Microtubule components, as opposed to assembled microtubules, could act on [microfilaments] at any point in the cell to which diffusion takes them." (Collings et al. 1996). Although the identity of the factor(s) involved remains unknown, this model has recently been revived to explain Rop-based signalling between microtubules and microfilaments.

5
Rop-Based Signalling to Both Microtubules and Microfilaments

5.1
Rops in Epidermal Cells

Cross-talk between microtubules and microfilaments has been explored in detail for the development of the interdigitated epidermal or pavement cells of *Arabidopsis* and other plants. During leaf development, epidermal cells with simple shapes undergo co-ordinated and selective, localized expansion to form highly complex and interlocking patterns (Fig. 5A). Outgrowths (lobes) of one cell must be matched with reduced or inhibited growth (necks) of the adjoining cell. This requires both intracellular and as yet unknown intercellular signalling to coordinate growth between adjacent cells (reviewed in Panteris and Galatis 2005; Smith and Oppenheimer 2005).

These selective growth processes involve the cytoskeleton. Microtubules are associated with the development of the necks and the inhibition of growth there (Panteris and Galatis 2005). In *Vigna*, microtubule bundles are found associated with the non-growing neck regions of the expanding cells (Panteris et al. 1993) and as similar events occur in *Arabidopsis* (Wasteneys et al. 1997; Fu et al. 2002), this suggests that the cell-wall thickening present in the lobe necks might be dependent on the presence of microtubules. This is analogous to microtubule-based wall deposition in other diffusely growing plant cells. Chemical disruption of microtubules in *Vigna* (Panteris et al. 1993) prevents interdigitation occurring. Microtubule mutants can also show reduced interdigitation (Whittington et al. 2001; reviewed in Kotzer and Wasteneys 2006).

While microtubules are associated with the neck regions where growth is inhibited, diffuse cortical microfilaments are found in the actively growing cell outgrowths of *Arabidopsis* (Fu et al. 2002) and maize (Frank et al. 2003). Furthermore, microfilament disruption with cytochalasin in *Vigna* also reduces the waviness of pavement cells (Panteris and Galatis 2005), and various microfilament-related mutants, including those that affect the ARP2/3 complex in *Arabidopsis* or the *brick1* mutant in *Zea*, also show reduced interdigitation (Fu et al. 2002; Frank et al. 2003; Li et al. 2003; reviewed in Kotzer and Wasteneys 2006). As both microtubules and microfilaments seem to be required for the developmental processes associated with interdigitation, it is perhaps not surprising that evidence exists in *Arabidopsis* for cross-talk via the Rop-signalling pathway (Fu et al. 2005; Bannigan and Baskin 2005; Kotzer and Wasteneys 2006).

Rops (Rho of plants) are a plant-specific family of Rho GTPases that act as master switches controlling multiple downstream pathways (Gu et al. 2004). Rops are activated by binding GTP and turn on effector proteins, RICs, that contain CRIB (Cdc42/Rac-interactive binding) motifs (Wu et al. 2001; Fu

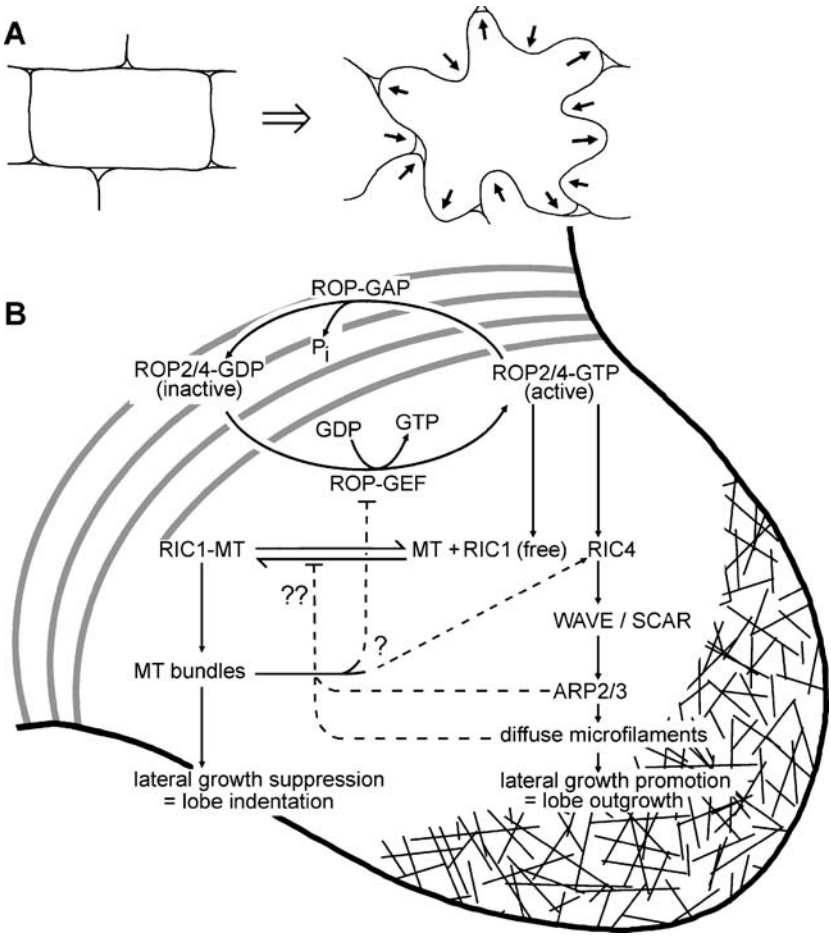

Fig. 5 The development of pavement cells in *Arabidopsis* requires Rop GTPase-dependent signalling to the cytoskeleton, and a feedback loop between microtubules and microfilaments (Fu et al. 2005). **A** In the leaf primordia, pavement cells have a regular shape but this is modified by co-ordinated cell expansion to form lobes between adjacent cells and an interdigitated morphology. **B** Microtubule bands (*in grey*), found connecting the non-growing neck regions alternate with regions of diffuse microfilaments in the tips of growing lobes. This patterning is controlled by Rop-based signalling. Activated Rops promote microfilament through the RIC4 and ARP2/3 pathway and cause the microtubule banding protein RIC1 to dissociate from microtubules leading to their destabilization. Cross-talk between microtubules and microfilament is evident with feedback from the microtubule bundles inhibiting RIC4 activity and microfilament polymerization (Fu et al. 2005) in an unknown way, indicated by a *question mark*. Feedback from diffuse microfilaments to decrease microtubule stability is evident in root hairs (Saedler et al. 2004, Timmers et al. 2007) and suggested for *Zea* stomatal development (Panteris et al. 2006). Were these systems also active in pavement cells, a self-reinforcing cycle would result, although it remains unclear whether signalling would come from microfilaments themselves or elements that signal to them (indicated by *paired question marks*)

et al. 2002). In *Arabidopsis* epidermal cells, two closely related Rops (Rop2 and Rop4) interact with RIC1 and RIC4 to regulate the cytoskeleton and the formation of pavement cells (Fig. 5B). Rops positively control microfilament formation through activating RIC4 which in turn promotes microfilament polymerization through the WAVE/SCAR and ARP2/3 pathways (Fu et al. 2002). This pathway explains why mutations affecting microfilaments through these complexes reduce pavement cell formation. Rops also negatively control microtubule polymerization because Rop-dependent de-activation of RIC1, a microtubule-associated protein, prevents it from binding to and bundling microtubules (Fu et al. 2002). Thus, the formation of lobed pavement cells is Rop/RIC-dependent and requires signalling to both microfilaments and microtubules (Fig. 5B).

Although microtubules and microfilaments are co-ordinated by the same signalling molecules in this model, this by itself does not imply cross-talk. This has, however, been demonstrated. The status of microtubule polymerization controls RIC4 activity, with microtubule bundling inhibiting Rop/RIC interactions and locally preventing microfilament polymerization. This feedback has been demonstrated by direct, FRET (fluorescence resonance energy transfer)-based measurements of the interactions between Rop2 and RIC4, which increase following both microtubule destabilization or depolymerization (Fu et al. 2005). How this cross-talk occurs exactly is not yet clear, although in a mechanism analogous to that suggested in *Nitella* (Collings et al. 1996, Sect. 4.3), it is possible that Rop activity is controlled through the release of some microtubule-associated element (Fu et al. 2005). This factor might directly inactivate RIC4, thus directly blocking microfilament polymerization, or it might prevent Rop activation and indirectly block microfilament polymerization (Fig. 5B, question mark).

In cells showing complex expansion patterns such as pavement cells, microtubules and microfilaments show complementary patterns with microfilament patches being present in sites of active growth. A similar pattern of complementary microtubules and microfilaments, also occurs during the asymmetric divisions of *Zea* stomatal mother cells, and can be disrupted by latrunculin or mutation of *brick1*. This patterning "favors the idea that some [MF] nucleating factor(s), but not the [MFs] themselves, is (are) implicated in the depletion of the microtubules" (Panteris et al. 2006).

5.2 A Rop-Based Feedback Cycle Between Microtubules and Microfilaments?

The microtubule-based control over microfilament polymerization present in expanding pavement cells is not sufficient to demonstrate a self-reinforcing feedback cycle, for no evidence yet suggests that microfilaments control microtubule organization in pavement cells. Yet evidence for such a control does exist in root hairs, trichomes and during cell division. In root hairs,

microfilament disruption, either in the *dis2* mutant (Saedler et al. 2004) or following treatment with low levels of latrunculin (Saedler et al. 2004; Timmers et al. 2007), results in hyperstabilized microtubules through an unknown mechanism, while in developing trichomes, disruption of microfilaments leads to changes in microtubule organization (Schwab et al. 2003; Zhang et al. 2005) (see Sect. 2.2). In stomatal mother cells, microfilament disruption changes microtubule organization (Panteris et al. 2006). If similar mechanisms were active in pavement cells, local activation of Rops and RIC4 would generate dynamic microfilaments that would sustain microtubules dynamics and suppress microtubule bundling such that a self-reinforcing cross-talk feedback cycle would emerge. It remains unclear, however, whether the microfilaments themselves, or factors signalling to the microfilaments, would be involved in such a feedback (Fig. 5B, paired question marks).

6 Conclusions

The advent of molecular genetics has allowed investigations of the plant cytoskeleton to move beyond simple characterizations of the different structures that occur in cells, and beyond the not-so-simple identifications of cytoskeletal proteins as bands on gels. This approach has identified an elegant feedback system through which Rop-based signalling to both microtubules and microfilaments is reinforced with signalling from microtubules to microfilaments. The possibility of a complete feedback loop is also suggested by evidence for signalling from microfilament to microtubules. Yet, as documented in this review (I hope), interactions and cross-talk between microtubules and microfilaments in plants are not limited to this Rop-based system. It would seem probable that in the next several years, advances in proteomics and molecular genetics, coupled with developments in live-cell imaging, and even a return (to prominence) of electron microscopy, will also identify which proteins allow microtubules to interact directly with microfilaments, and, more importantly, why they do this.

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Microtubules and the Control of Wood Formation

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Abstract Cell walls are reinforced by cellulose microfibrils, which resist cell expansion in response to turgor pressure. The orientation of cellulose microfibrils in the primary walls of cambial derivatives determines the direction of cell expansion, thereby controlling the shape and size of secondary xylem cells in trees. In addition, the texture of the secondary wall, in particular the orientation of cellulose microfibrils in the middle layer of the secondary wall (S_2 layer), is closely related to the physical properties of secondary xylem cells. Thus, the orientation of cellulose microfibrils in the secondary walls determines the mechanical properties of wood. In addition, the secondary xylem cells form modifications of the cell wall, such as pits and perforations, by the localized deposition of cellulose microfibrils. These pits and perforations provide a pathway for liquid flow between secondary xylem cells. Thus, the ability to control the orientation and localization of cellulose microfibrils in the secondary wall might allow us to change the quality of wood and its products. There is considerable evidence that the dynamics of cortical microtubules are closely related to the orientation and localization of newly deposited cellulose microfibrils in the differentiating secondary xylem cells. Thus, manipulation of cortical microtubules would allow control of the texture of cell wall, with a consequent improvement of wood quality.

1 Significance of Cell-wall Texture for Wood Quality

Wood has been used for thousands of years as a raw material for timber, furniture, pulp and paper, chemicals, and fuels. In addition, since wood is a major carbon sink, it is expected to play an important role in removing the excess of atmospheric CO_2 that is generated by the burning of fossil fuels (IPCC 2007). Therefore, there is still great demand for wood as a renewable bio-material and source of bio-energy.

Wood is produced by the vascular cambium (cambium) of living organisms, namely trees. Therefore, wood quality can vary significantly even within a single species, depending on environmental conditions, such as climate, soil, and spacing of growing trees. In addition, wood quality varies among species and within individual trees according to cambial age, stem position, and distance from the crown. Tree-to-tree variability within members of a species under identical growth conditions shows that genetic factors also influence wood quality. These evidences indicate the strong possibility that wood qual-

ity might be improved not only by silvicultural treatments, such as pruning, thinning, and fertilization, but also by breeding to select genetically desirable trees (Panshin and de Zeeuw 1980; Zobel and van Buijtenen 1989; Zobel and Jett 1995). Developed molecular biological approaches also have demonstrated their potential to improve wood quality (Higuchi 1997; Mellerowicz et al. 2001). Differences of wood quality, in particular of mechanical properties, are largely due to differences in wood structure. Thus, wood structure is one of the most important targets in attempts to control wood quality.

The orientation of cellulose microfibrils, referred to as the microfibril angle (the angle between cellulose microfibrils and the main cell axis) in the secondary wall is one of the most important ultrastuctural characteristics that determine the properties of wood and its products (Cave and Walker 1994). In particular, the angles of the middle layer of the secondary wall (S_2 layer), which is the thickest layer in the secondary wall, are of major importance. The microfibril angles of the S_2 layer are negatively correlated with the modulus of elasticity (MOE) of wood (Wardop 1951, Cave 1968). Thus, wood with large microfibril angles has low strength. In addition, microfibril angles in the S_2 layer affect the shrinkage and swelling of wood in response to changes in moisture content (Barber and Meylan 1964; Harris and Meylan 1965). Wood exhibits limited longitudinal shrinkage, because of the steep orientation of cellulose microfibrils in the S_2 layer. However, in wood where the S_2 layer has large microfibril angles, such as juvenile wood, a greater longitudinal shrinkage up to 10% is exhibited (Tsoumis 1991). Large changes in dimensions caused by the shrinkage result in changes of shape, warping, and collapse in wood and its products. Therefore, the control of microfibril angles in the secondary wall, in particular in the S_2 layer, might provide a powerful biological tool for changing the properties of wood. This chapter describes the involvement of cortical microtubules in the control of the size and shape of secondary xylem cells and the texture of the cell wall.

2 Cellular Mechanisms of Wood Formation

Although wood is of great economical importance, the precise process of its formation (xylogenesis) is not yet fully understood (see Higuchi 1997; Lachaud et al. 1999; Sundberg et al. 2000; Mellerowicz et al. 2001; Plomion et al. 2001; Chaffey 2002a; Samuels et al. 2006). Therefore, in order to create "new woods" with more desirable qualities by biotechnological manipulation, more detailed information is needed on the cellular and molecular aspects of wood formation.

Increases in the diameter of tree stems are due to the activity of cambium (Catesson 1994; Larson 1994; Funada 2000; Kitin et al. 2000, 2001). The cambium is defined as the actively dividing layer of cells that lies between and

gives rise to the secondary xylem and phloem (Fig. 1; IAWA Committee 1964). The cambium consists of fusiform cambial cells and ray cambial cells. The mean lengths of fusiform cambial cells range from 1100 μm to 4000 μm in conifers (gymnosperm trees) and from 170 μm to 940 μm in hardwoods (angiosperm trees) (Larson 1994). The lengths of fusiform cambial cells vary depending on the species and the age of the cambium. The higher resolution in the third dimension of confocal scanning electron microscopy showed that the long fusiform cambial cells were mononucleate in all cases despite their length (Kitin et al. 2002).

The periclinal division of cambial cells leads to an increase in stem diameter. The division of cambial cells produces the secondary phloem towards the outer face and the secondary xylem towards the inner face. Usually, many more secondary xylem cells are produced as compared to secondary phloem cells (Larson 1994). Thus, it is the matured xylem cells that are generally used as wood.

Prior to cell division, the preprophase band (PPB) of microtubules is formed in most plants cells (see chapter "Control of Cell Axis" in this book). The location of the PPB generally predicts the plane of the future cell plate (see Wick 1991). The PPB, spindle microtubules, and phragmoplast microtubules have been observed in dividing ray cambial cells of *Abies sachalinensis* (Oribe et al. 2001). However, there is no clear evidence for the existence of a PPB in fusiform cambial cells, which form long axially oriented plane of cell plate, in woody plants (Evert and Deshpande 1970; Farrar and Evert 1997; Oribe et al. 2001; Chaffey and Barlow 2002; Rensing et al. 2002). Thus, cell division without formation of a PPB might occur in fusiform cambial cells.

The cambial activity of trees exhibits annual periodicity in temperate zones (Fig. 1). Cambial activity ceases in the autumn or winter seasons. Cambial dormancy in winter can be considered to consist of two stages, namely, rest and quiescence (Catesson 1994; Larson 1994). The resting stage is controlled by endogenous signals and it is followed by the quiescent stage, which is controlled

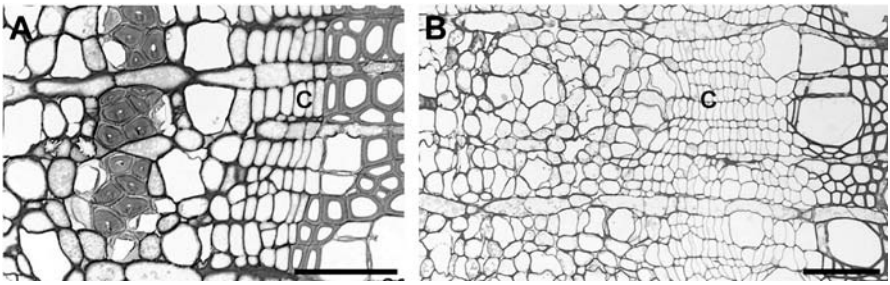

Fig. 1 Light micrographs of transverse sections of dormant cambium (**A**) and active cambium induced by localized heating (**B**) of the main stem in *Populus sieboldii* x *P. grandidentata* (Courtesy of Mrs. S. Begum). (*C*) cambium. *Bars* = 50 μm

by environmental conditions. The resting stage of dormancy is a physiological state wherein the cambium cannot divide, even under favourable growth conditions. By contrast, during the quiescent stage of cambial dormancy, the cambium is able to divide when exposed to appropriate environmental conditions. Cambial activity generally resumes in the spring, with a change from the quiescent dormant state to the active state (cambial reactivation). The localized heating of stems in winter induces localized cambial reactivation in evergreen conifers (Barnett and Miller 1994; Oribe and Kubo 1997; Oribe et al. 2001, 2003; Grièar et al. 2006) and a deciduous hardwood (Begum et al. 2007). Therefore, increase in temperature might be a limiting factor in the onset of cambial reactivation during the quiescent dormant state.

The rapid increase in cambial activity from spring to early summer is associated with an increase in the total amount of the auxin indole-3-acetic acid (IAA) in the cambial region (Sundberg et al. 1991; Funada et al. 2001a, 2002). Precise studies by cryo-sectioning in combination with microscale gas chromatography-mass spectrometry have demonstrated a steep radial gradient in the level of IAA across the cambial region (Uggla et al. 1996; Hellgren et al. 2004). The level of endogenous IAA is maximal in the zone of cambial cells. Thus, auxin appears to regulate the number of dividing cambial cells, playing an important role in positional signaling (Uggla et al. 1996; Sundberg et al. 2000).

As soon as a cambial cell looses the ability to divide, it will start to differentiate into secondary phloem or xylem cells. The stages in the development of secondary xylem cells can be categorized as follows: cambial cell division, cell enlargement, cell wall thickening, cell wall sculpturing (formation of modified structure), lignification, and cell death (Panshin and de Zeeuw 1980; Thomas 1991; Funada 2000). Fusiform cambial cells differentiate into longitudinal tracheids (tracheids), vessel elements, wood fibers, and axial parenchyma cells, while ray cambial cells differentiate into ray parenchyma cells. In some conifers, such as *Pinus*, ray cambial cells differentiate into ray parenchyma cells and ray tracheids. Cells derived from fusiform cambial cells increase in length and in diameter as they approach their final shape during differentiation. For example, tracheids in conifers increase only slightly in length but they do increase considerably in radial diameter. Vessel elements do not increase significantly in length, whereas their increases in radial and tangential diameter is conspicuous (Kitin et al. 1999, 2003).

The very thin (often less than 0.1 μm-thick) and plastic cell wall, which is characteristic for the stage of cell enlargement, is termed primary wall. Cellulose is the major component of the cell wall. Cellulose is highly crystalline and has a very high tensile strength. Thus, cellulose microfibrils form a framework in the cell wall. The primary wall consists of loose aggregates of cellulose microfibrils (Harada 1965; Harada and Côté 1985; Abe and Funada 2005). This structure allows relatively unimpeded expansion of the cells deriving from the cambium. In addition, the primary wall of cambial cells has

a characteristic histochemical composition that allows for considerable extensibility (Catesson 1990, 1994; Catesson et al. 1994; Guglielmino et al. 1997). Localized loosening of the cell wall, in particular at cell junctions, also occurs during cell expansion (Funada and Catesson 1991; Catesson et al. 1994). Such partial lysis of the cell wall is closely associated with a localized decrease in the level of calcium ions that are bound to the cell wall.

When cell expansion is almost complete, well-ordered cellulose microfibrils are deposited, establishing the so-called secondary wall (Harada 1965). Once the formation of the secondary wall has begun, no further expansion of cells occurs. The secondary xylem cells of woody plants, such as tracheids and wood fibers, have cell walls with a highly organized structure. Continuous deposition of the secondary wall increases the thickness of the cell wall. The thickness of the cell wall varies depending on cell function, cambial age, and the season at which the cell is formed (earlywood or latewood). In general, cells that function to support the tree, such as tracheids and wood fibers, form thick secondary walls. Thus, the ultrastructure of tracheids and wood fibers is of great importance to define the mechanical properties of wood. The cell wall supports the heavy weight of the tree itself and functions in the transport of water from roots to leaves, which can sometimes bridge distances of more than 100 m (Utsumi et al. 2003). In addition, the cell wall prevents microbial and insect attack, thereby protecting the tree during its very long life, which, in some cases, can exceed several thousands years.

With the onset of secondary wall deposition, the lignification begins at the intercellular layer, progressing to the primary wall and, eventually, to the secondary wall (Takabe et al. 1981b). When lignification has been completed, cell death (cell autolysis) occurs immediately in tracheary elements, such as tracheids and vessel elements. Secondary xylem cells, which contribute to the mechanical support of the tree or to the conduction of water, pass through the developmental stages mentioned above. This process of cell death might be expected to resemble the programmed cell death, which occurs in differentiating tracheary elements derived from single cells isolated from the mesophyll of *Zinnia elegans* (Fukuda 1996, 2004; Fukuda and Komamine 1980; Kuriyama and Fukuda 2002).

By contrast, ray parenchyma cells remain alive for several years or more without immediate autolysis of cell organelles (Miyazaki et al. 2002; Nakaba et al. 2006). Even after maturation, namely, secondary wall formation and lignification, they retain their organelles and remain viable (Funada 2000). As a result, ray parenchyma cells play an important role in the storage and radial transport of materials, mediating radial continuity in the stem (Catesson 1990; Sauter 2000; Chaffey and Barlow 2001). In addition, these cells contribute to the formation of heartwood (Bamber and Fukazawa 1985). The process of differentiation, and in particular, the death of long-lived ray parenchyma cells, differ from those of short-lived tracheids or vessel elements (Nakaba et al. 2006).

Since tracheids or ray parenchyma cells derived from fusiform cambial cell or ray cambial cells are aligned in a radial direction, successive aspects of xylogenesis can be observed in a radial file within a single specimen. Thus, cambial derivatives are a suitable system to follow the process of differentiation of secondary xylem cells in situ.

3 The Interaction of Cortical Microtubules and Cellulose Microfibrils in the Primary Wall

The intracellular pressure of the protoplast against the cell wall (turgor pressure) originates from the vacuole and provides the driving force for the enlargement of plant cells. The increase in the volume of the vacuole is derived from a gradient in the water potential between cytoplasm and vacuole and the apoplast (Kutschera 1991). When the turgor pressure in the cell exceeds the yield point of the cell wall, the cell can expand. As the cell expands, the cell wall becomes stiffer and, consequently, its yield point increases. Then, the rate at which the cell expands decreases gradually and finally cell expansion ceases.

The turgor pressure is exerted equally in all directions within a cell. If there are no reinforcement mechanisms, cells are expected to expand spherically. However, the cellulose microfibrils, with their considerable tensile strength, reinforce the cell wall and resist expansion in response to the turgor pressure. The predominant orientation of cellulose microfibrils in the cell wall is usually perpendicular to the direction of cell expansion (Green 1980; Taiz 1984). Cellulose microfibrils in elongating cells are oriented transversely (Green and Kings 1966). The change in the direction of growth is related to the reorientation of cellulose microfibrils (Ridge 1973; Lang et al. 1982). The orientation of newly deposited cellulose microfibrils on the inner surface of the primary wall determines the direction and extent of cell expansion, thereby determining the final shape and size of the cell.

When transverse sections of secondary xylem cells are examined by polarization microscopy, the primary wall is only slightly birefringent as a result of the differences in orientation of the cellulose microfibrils in the primary wall. Electron microscopic observations with replica methods have shown that the cellulose microfibrils in the primary wall are not well ordered and are separated from each other by relatively large interspaces (Harada and Côté 1985). Randomly oriented cellulose microfibrils are observed during cell expansion of tracheids, wood fibers, and ray parenchyma cells (Imamura et al. 1972; Fujii et al. 1977; Thomas 1991; Abe et al. 1995b). The loose and random network of cellulose microfibrils in the primary wall allows for easy expansion of secondary xylem cells.

In macerated tracheids of *Pinus radiata*, cellulose microfibrils are oriented approximately axial relative to the cell axis on the outer surface, whereas they

are transverse on the inner surface (Wardrop 1958). Observations by conventional microscopy, X-ray diffraction, and electron microscopy, have led to diagrammatic representations for the organization of the primary wall of tracheids or wood fibers (Wardrop 1964; Wardrop and Harada 1965; Harada and Côté 1985). In such models, the thin primary wall is composed of two layers with differently oriented cellulose microfibrils. Wardrop (1958) interpreted the differences in the orientation of cellulose microfibrils between the inner and outer surfaces of the primary wall of tracheids in terms of the multi-net growth hypothesis that was originally proposed by Roelofsen and Houwink (1953). According to this hypothesis, cellulose microfibrils are originally deposited in a direction transverse to the cell axis and their orientation shifts passively in the longitudinal direction during cell enlargement.

Cellulose microfibrils have been observed by field emission-scanning electron microscopy (FE-SEM) in differentiating tracheids or wood fibers (Fig. 2; Abe and Funada 2005; Abe et al. 1991, 1992, 1994, 1995a,b, 1997; Prodhan et al. 1995a,b; Awano et al. 2000; Yoshida et al. 2000; Hosoo et al. 2002). FE-SEM provides images of relatively large areas at high resolution. Thus, it is a particularly useful tool to follow changes in the orientation of newly deposited cellulose microfibrils during xylem differentiation.

The orientation of cellulose microfibrils of the radial walls in differentiating tracheids of *Abies sachalinensis* changes during cell expansion (Abe et al. 1995b). The cellulose microfibrils on the innermost surface of the primary wall are not well ordered. Most of cellulose microfibrils in the tracheids at the

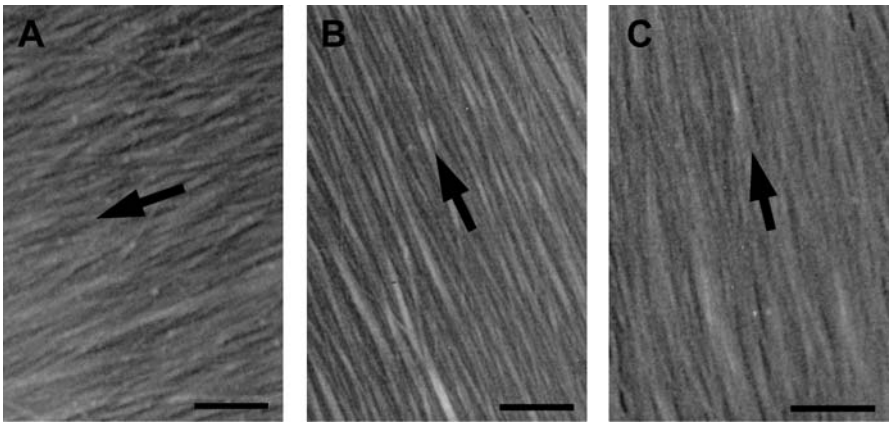

Fig. 2 Scanning electron micrographs (FE-SEM), showing newly deposited cellulose microfibrils (viewed from the lumen side) on the inner surface of the secondary wall of tracheids of *Abies sachalinensis*. The axes of tracheids in the photographs are vertical. The orientation of cellulose microfibrils (*arrows*) changes from a flat S-helix (**A**) over a flat Z-helix (**B**), to a steep Z-helix (**C**). The helical direction, as observed from the outer face of the cells, is designated an S-helix or a Z-helix relative to the longitudinal axis of the cell. *Bars* = 0.25 μm

early stage of cell expansion are predominantly oriented longitudinally. Longitudinally oriented cellulose microfibrils would restrain the turgor-driven longitudinal expansion. As the cell expands, the predominant orientation of cellulose microfibrils on the innermost surface changes from longitudinal to transverse. At the final stage of cell expansion, cellulose microfibrils are oriented transversely to the cell axis. These observations suggest that it is not necessary to adopt the multi-net growth hypothesis to explain the difference in orientation of cellulose microfibrils between the outer and inner parts of the primary wall in tracheids.

Longitudinally oriented cellulose microfibrils in the primary wall of the fusiform cambial cells serve first to facilitate lateral expansion. In addition, transversely oriented cellulose microfibrils at the final stage of cell expansion probably prevent further lateral expansion. Therefore, the mechanism that controls the orientation of cellulose microfibrils in the primary wall determines the shape and the size of secondary xylem cells.

Observations in a wide variety of plant cells for over four decades have revealed that cortical microtubules play an important role in the orientation of newly deposited cellulose microfibrils (Ledbetter and Porter 1963; Hepler and Palevitz 1974; Gunning and Hardham 1982; Robinson and Quader 1982; Giddings and Staehelin 1991; Seagull 1991; Shibaoka 1994; Nick 2000; Baskin 2001). Cellulose is synthesized by enzyme complexes that are often referred to as terminal complexes. The terminal complexes are localized in the plasma membrane (Herth 1985; Schneider and Herth 1986; Kimura et al. 1999). The only components of cellulose synthase complex that have been identified in higher plants are the cellulose synthase (CesA) proteins (Richmond and Somerville 2000). At least three different CesA proteins, named IRREGULAR XYLEM (IRX) proteins, are required for cellulose synthesis during secondary wall formation in *Arabidopsis thaliana* (Taylor et al. 2003). The IRX proteins are localized at the bands of secondary wall and co-localize with cortical microtubules (Gardiner et al. 2003).

It has been postulated that cortical microtubules, which are closely associated with the plasma membrane, guide the movement of these complexes, because co-alignment of cortical microtubules and newly deposited cellulose microfibrils has been often observed in cells of both lower and higher plants (Ledbetter and Porter 1963; Giddings and Staehelin 1991; Baskin 2001). In addition, microtubule-depolymerizing agents, such as colchicine, usually disrupt the orientation of cellulose microfibrils (Robinson et al. 1976; Srivastava et al. 1977; Hogetsu and Shibaoka 1978). Moreover, the disruption of the array of transverse cortical microtubules in mutants of a katanin-like protein of *Arabidopsis thaliana* is associated with radial cell expansion and a concomitant disruption of orientation of cellulose microfibrils (Burk and Ye 2002). Recent direct visualization of CesA in living cells of transgenic *Arabidopsis thaliana* plants revealed that the CesA complexes moved in plasma membranes at constant rates (Paredez et al. 2006). In addition, the movement of

CesA complexes in linear tracks was coincident with cortical microtubules. These observations support the hypothesis that cortical microtubules control the movement of cellulose synthase complexes.

Two models have been proposed for the mechanism by which cortical microtubules control the orientation of newly deposited cellulose microfibrils (Heath and Seagull 1982; Gidding and Staehelin 1991, see chapter "Control of Cell Axis" of this book). In model 1, cellulose-synthase complexes are guided directly on cortical microtubules, with putative physical links between microtubules and the cellulose-synthase complexes (Heath 1974). The linked molecules might be pulled by kinesin-like motor proteins. In model 2, cellulose-synthase complexes move in membrane channels delimited by cortical microtubules. The complexes might be propelled forward by forces that result from the polymerization and crystallization of cellulose microfibrils. Gidding and Staehelin (1988) favored model 2, because, using transmission electron microscopy (TEM), they found that cellulose-synthase complexes existed between or adjacent to cortical microtubules, rather than directly on top of cortical microtubules. In contrast, using high-resolution SEM, Vesk et al. (1996) observed that individual cortical microtubules were positioned directly adjacent to individual cellulose microfibrils in the cell wall. Their observations suggest the possibility that the orientation of each cellulose microfibril might be controlled directly by cortical microtubules.

The average diameter of microtubules is very small, about 24 nm. Thus, the cortical microtubules have been mainly observed using TEM since the first observations by Ledbetter and Porter (1963). TEM reveals the fine structure of microtubules and the interactions between their components. However, it is difficult to examine the microtubules in relatively large areas of plant material by TEM, because this technique requires very small slightly oblique ultrathin sections. Thus, successive changes in the orientation of cortical microtubules during xylem differentiation cannot be easily followed.

With the successful introduction of indirect immuno-fluorescence microscopy, it became possible to visualize microtubules over large areas within plant tissues (Fig. 3a; Lloyd 1979, 1987; Wick et al. 1981). Cortical microtubules in the cambial cells and their derivatives of woody plants have been observed by a similar technique (Uehara and Hogetsu 1993; Abe et al. 1995b; Prodhan et al. 1995a; Chaffey 2002b; Chaffey et al. 1997a,b,c, 1999). In addition, confocal laser scanning microscopy in combination with immunofluorescence staining made it possible to construct three-dimensional images of microtubules in cambium and differentiating secondary xylem or phloem cells of several woody plants (Abe et al. 1995a; Funada 2002; Funada et al. 1997, 2000, 2001b; Furusawa et al. 1998; Chaffey et al. 1997a, 1999, 2000a, 2002; Nakaba et al. 2006). This method provides a powerful tool to follow dynamic changes in microtubule organization.

FE-SEM has also been used to observe the fine structure of microtubules in wide regions of plant tissues (Fig. 3b; Hirakawa 1984; Abe et al. 1994;

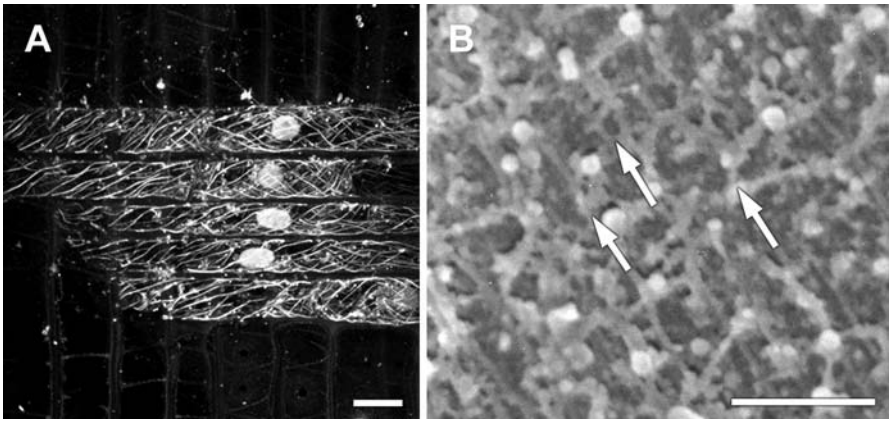

Fig. 3 Visualization of cortical microtubules. **A** Projection of a z-stack of immunolabeled cortical microtubules in differentiating ray parenchyma cells of *Taxus cuspidata* obtained by confocal laser scanning microscopy. *Bar* = 25 μm. **B** Scanning electron micrograph (FE-SEM) of cortical microtubules (*arrows*), viewed from the lumen side, in differentiating wood fibers of *Fraxinus mandshurica* var. *japonica*. *Bar* = 0.5 μm

Vesk et al. 1994, 1996; Prodhan et al. 1995a). In addition, recent advances of four-dimensional analysis (3-D analysis over time) using stable expression of genes for tubulin or microtubule-associated proteins (MAP) in fusion with green fluorescent protein (GFP) in living plant cells allow for the dynamics of microtubules to be followed (Marc et al. 1998; Paredez et al. 2006; Yoneda et al. 2007). Stable expression of GFP-tubulin fusion protein in transgenic *Arabidopsis thaliana* cell suspensions enables time-lapse observations of microtubules during the differentiation of tracheary elements (Oda et al. 2005; Oda and Hasezawa 2006). The use of GFP-fusion proteins will provide valuable new information about the roles of microtubules in cambial cells and differentiating secondary xylem cells of woody plants.

The presence of cortical microtubules in cambial cells and their derivatives in woody plants has been confirmed by TEM (Cronshaw 1965; Srivastava 1966; Srivastava and O'Brien 1966; Robards and Humpherson 1967; Itoh 1971; Nobuchi and Fujita 1972; Fujita et al. 1974, 1978; Tsuda 1975; Barnett 1981; Inomata et al. 1992; Prodhan et al. 1995a; Chaffey et al. 1997a,b,d, 1999, 2002a). In fusiform cambial cells, microtubules in the peripheral cytoplasm are oriented both longitudinally and horizontally with respect to the long axis of the cell (Srivastava 1966). Cortical microtubules appear to be linked to the plasmalemma by bridges (Fujita et al. 1974; Barnett 1981). The cortical microtubules are found both in active and in dormant fusiform cambial cells, but they are more abundant in active cambial cells, compared to dormant cambial cells (Itoh 1971; Tsuda 1975). Random arrays of cortical microtubules have been demonstrated in fusiform cambial cells by immunofluorescence (Abe et al. 1995a,b; Chaffey et al. 1997a,b,c, 1999, 2002a; Funada et al. 1997), and by

TEM in ultrathin sections (Chaffey et al. 1997a,d). The random arrays of cortical microtubules in fusiform cambial cells might be a general phenomenon in woody plants, regardless of whether they are conifers or hardwoods (Chaffey et al. 1997c). A careful study of cortical microtubules in differentiating tracheids has revealed that the predominant orientation of cortical microtubules is longitudinal with respect to the axis of the cell at the early stage of cell expansion (Fig. 4A; Abe et al. 1995a,b; Funada et al. 1997). The predominant orientation changes progressively from longitudinal to transverse during the radial expansion of cells. Finally, ordered and transversely oriented cortical microtubules are observed in tracheids at subsequent stages of differentiation, during which radial expansion ceases. Chaffey et al. (1997c, 2002) also found that the orientation of cortical microtubules changed from random within active fusiform cambial cells to helical within developing wood fibers. These observations indicate that the orientation and organization of cortical microtubules in differentiating tracheids and wood fibers changes successively during formation of the primary wall.

The progressive changes in the orientation of cortical microtubules resemble the reorientation of newly deposited cellulose microfibrils in tracheids during formation of the primary wall, suggesting a close relationship between the orientation of the cortical microtubules and the cellulose microfibrils. It appears that the cortical microtubules are involved in determining the orien-

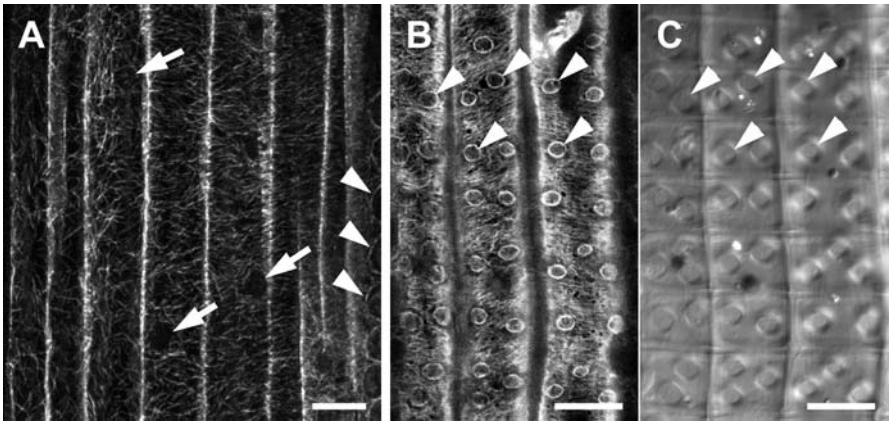

Fig. 4 Immunofluorescence images (**A**,**B**) and Nomarski differential interference contrast image (**C**)showing the arrangement of cortical microtubules obtained by confocal laser scanning microscopy. **A** Cortical microtubules during formation of the primary wall in differentiating tracheids of *Abies sachalinensis*. Cortical microtubules disappear locally (*arrows*) at sites of future intertracheal bordered pits, and circular bands of cortical microtubules (*arrowheads*) are visible around the edges of developing bordered pits. **B**,**C** Circular bands of cortical microtubules (*arrowheads* in **B**) are superimposed on the edges of the pit borders of differentiating tracheids in the cross-field (*arrowheads* in **C**) in *Taxus cuspidata*. *Bars* = 25 μm

tation of newly deposited cellulose microfibrils by controlling the movement of the cellulose-synthase complexes in expanding tracheids. Cortical microtubules are arranged perpendicularly to the direction of elongation in elongating cells, whereas cortical microtubules are oriented multidirectionally in isotropically expanding cells (Hogetsu and Oshima 1986; Hogetsu 1989). Thus, the orientation of cortical microtubules during the formation of primary walls reflects the direction of cell expansion. In the case of derivatives of fusiform cambial cells, lateral expansion might be the consequence of the predominantly longitudinal orientation of cellulose microfibrils in the primary wall. Thus, the fusiform cambial cells are able to expand radially, because cortical microtubules and cellulose microfibrils are arranged longitudinally. In contrast, the ordered and transversely oriented cellulose microfibrils on the innermost surface of the primary wall impede the lateral expansion of these cells. Their orientation might be controlled by cortical microtubules that are transverse as well.

4 The Interaction of Cortical Microtubules and Cellulose Microfibrils in the Secondary Wall

The structure of the secondary wall is not homogeneous. The secondary wall consists of three main layers: the outermost layer (S_1 layer); the middle layer (S_2 layer); and the innermost layer (S_3 layer) facing the lumen side. Each of these layers can be identified by polarization microscopy, as originally described by Kerr and Bailey (1934) and Bailey and Vestal (1937). The identification of the three layers is facilitated by differences in orientation of the cellulose microfibrils in the various layers. The cellulose microfibrils in the S_1 and S_3 layers form a flat helix relative to the cell axis, whereas those in the S_2 layer form a steep helix. Detailed models for the structure of cell walls in tracheids and wood fibers have been proposed from the results of electron microscopy (Wardrop 1964; Harada and Côté 1985; Abe and Funada 2005). Electron microscopic observations of cross sections reveal that the S_2 layer dominates. In tracheids and wood fibers, about 80% of the cell wall (in terms of thickness) are generally occupied by the S_2 layer. In addition, there are intermediate layers interspersed between the S_1 and the S_2 layers, and between the S_2 and the S_3 layers, respectively (Harada and Côté 1985). These layers are correlated to progressive changes in the angles of cellulose microfibrils relative to the cell axis. Thus, these layers cannot be easily discriminated by clear borders (Abe and Funada 2005; Abe et al. 1992).

When cell expansion ceases, well-ordered and transversely oriented cellulose microfibrils are observed on the innermost surfaces of the cell walls in differentiating tracheids of *Abies sachalinensis* (Abe et al. 1995a,b, 1997). These cellulose microfibrils are considered to belong to the secondary wall

because of their texture. They are deposited just before birefringence becomes detectable by polarization microscopy and the cessation of radial tracheid expansion (Abe et al. 1997). Thus, the radial expansion of tracheids might be restricted by the deposition of well-ordered secondary wall.

Several authors have proposed that the S_1 layer has a "crossed fibrillar texture" due to alternation between an S-helix and a Z-helix (Wardrop 1964; Wardrop and Harada 1965; Harada and Côté 1985). The helical direction, as observed from the outer face of the cells, is designated as S-helix (left-handed helix) or a Z-helix (right-hand helix) relative to the longitudinal axis of the cell. In contrast, Dunning (1968, 1969) proposed that the "crossed fibrillar texture" was merely due to a progressive change in the orientation of cellulose microfibrils within the S_1 layer. Further observations indicate that the first-deposited, well-ordered cellulose microfibrils are oriented in a flat S-helix and then the orientation of newly deposited cellulose microfibrils changes from an S-helix to a flat Z-helix from the outer toward the inner part in the S_1 layer, in a clockwise direction, as viewed from the lumen side (Abe et al. 1991, 1997; Kataoka et al. 1992; Prodhan et al. 1995a; Abe and Funada 2005). Brändström et al. (2003) also found evidence for the absence of a "crossed fibrillar structure texture" with cellulose microfibrils in alternating S- and Z-helices in tracheids of *Picea abies*.

During the formation of the secondary wall in tracheids or wood fibers, the cellulose microfibrils change their orientation progressively from a flat helix to a steep Z-helix in a clockwise rotation when viewed from the lumen side (Fig. 2). They are oriented at about 5–20° with respect to the cell axis. No cellulose microfibrils with an S-helix are observed during formation of the S_2 layer. Meylan and Butterfield (1978) observed the direction of cellulose microfibrils using SEM in the S_2 layer in tracheids, wood fibers, and vessel elements of over 250 woody plants and concluded that the pattern was always a Z-helix.

This shift in the angles of cellulose microfibrils is considered to generate a semi-helicoidal structure (Abe et al. 1991, 1995a; Prodhan et al. 1995a). The concept of a helicoidal pattern has been proposed for the cell walls of numerous plants (Roland and Vian 1979; Neville and Levy 1984; Roland et al. 1987). The pattern consists of a series of planes, in which the direction of cellulose microfibrils changes progressively. The arc-shaped or bow-shaped patterns observed using TEM in oblique ultrathin sections of tracheids or wood fibers are consistent with a helicoidal structure (Parameswaran and Liese 1982; Roland and Mosiniak 1983; Prodhan et al. 1995b; Donaldson and Xu 2005). Roland and Mosiniak (1983) demonstrated that a rotational change in the orientation of cellulose microfibrils from the S_1 to the S_2 layer produced an intermediate twisted appearance in wood fibers of *Tilia platyphyllos*.

The cellulose microfibrils of the S_2 layer are closely aligned with a high degree of parallelism. The continuous deposition of cellulose microfibrils in one direction produces a thick cell-wall layer with a consistent texture. When

the rotational change in the orientation of cellulose microfibrils is arrested, a thick cell-wall layer is formed as a result of the repeated deposition of cellulose microfibrils (Roland and Mosiniak 1983; Roland et al. 1987; Abe et al. 1991; Prodhan et al. 1995a). The thickness of the secondary wall is important in terms of the properties of wood, because it is closely related to the specific gravity of wood (Panshin and de Zeeuw 1980; Zobel and van Buijitenen 1989). The duration of the arrest in the orientation of cellulose microfibrils seems to determine the thickness of the S_2 layer and, thus, the thickness of the secondary wall. In contrast, Kataoka et al. (1992) proposed an alternative model where the repetition of alternating changes in the direction of cellulose microfibrils results in a thick cell wall in tracheids. However, a repeated alternation in the direction of cellulose microfibrils has not been confirmed by other investigators.

The average microfibril angles in the S_2 layer differ among species, and between the radial and tangential wall, and they depend also on the time of cell formation (Saiki 1970; Harada and Côté 1985; Saiki et al. 1989; Donaldson and Xu 2005). For example, the angle in differentiating earlywood tracheids is 3°–14° relative to the cell axis in *Abies sachalinensis*, but 9°–21° relative to the cell axis in *Larix kaempferi*, and 17°–32° relative to the cell axis in *Picea jezoensis* (Abe and Funada 2005). In addition, the microfibril angles in the S_2 layer can vary within the stem. The angles are usually large in the growth ring near the pith, namely, in the juvenile wood, and they decrease towards the bark side with increasing age of the cambium (Watanabe et al. 1963; Donaldson 1996; Donaldson and Burdon 1995; Hirakawa and Fujisawa 1995; Hirakawa et al. 1997). Moreover, tracheids of compression wood, which are formed on the lower side of inclined stems in conifers, have large microfibril angles of about 45° with respect to the cell axis (Timell 1986; Yoshizawa 1987). These variations in microfibril angles are due to differences in the pattern of orientation of cellulose microfibrils. For example, while the direction of cellulose microfibrils in normal wood tracheids rearranges into a steep Z-helix that is oriented at about 5–20° from the tracheid axis, the orientation of cellulose microfibrils in compression wood tracheids becomes oblique until the cellulose microfibrils are oriented in a Z-helix with an angle of about 45° from the tracheid axis. Such a rotation of cellulose microfibrils might control the microfibril angle in the S_2 layer, and, thus, represents one of the most important factors that determines wood properties.

At the final stage of the formation of secondary walls, the orientation of newly deposited cellulose microfibrils changes from a steep Z-helix to a flat helix, with counterclockwise rotation when viewed from the lumen side, from the outer towards the inner part of the layer. This corresponds to a directional switch in the orientation of the cellulose microfibrils from clockwise to counterclockwise (when viewed from the lumen side) during formation of the secondary wall. The deposition of cellulose microfibrils in a flat helix results in the S_3 layer. The orientation of cellulose microfibrils deposited on the in-

nermost S_3 layer varies among tracheids from an angle of 40° to the cell axis in a Z-helix to an angle of 20° in an S-helix (Abe et al. 1992). The most frequently observed orientation of cellulose microfibrils in the innermost surface of tracheids ranges from 70° to 80°, from 60° to 70°, and from 40° to 50° in the S-helices of *Larix kaempferi, Picea jezoensis* and *Picea abies*, respectively (Abe etal. 1992; Abe and Funada 2005). However, the cellulose microfibrils in Z-helices are occasionally observed on the innermost surfaces in tracheids in the late part of the annual ring. The cellulose microfibrils in Z-helices on the innermost surfaces might be the result of the incomplete rotation of cellulose microfibrils within the S_3 layer. The cellulose microfibrils in the S_3 layer are deposited in bundles and a complete lamella without any gaps between cellulose microfibrils is not formed (Abe et al. 1991, 1994). This texture differs from that of the S_2 layer, where the cellulose microfibrils have a high degree of parallelism. When transverse sections are observed by polarization microscopy, the S_3 layer exhibits birefringence, as does the S_1 layer. The shift in angles of cellulose microfibrils is more abrupt during the transition from the S_2 to the S_3 layer, as compared to the transition from the S_1 to the S_2 layer (Harada and Côté 1985; Abe et al. 1991). The rate of change in the orientation of cellulose microfibrils determines the structure of the cell wall layer.

The S_3 layer is generally thinner than the S_1 and S_2 layers. The thickness differs among tracheids (Singh et al. 2002). For instance, in earlywood, the thickness of radial walls of tracheids is 0.14–0.35 μm for the S_1, 0.98–1.93 μm for the S_2, and 0.07–0.15 μm for the S_3 layer. In latewood, it is 0.37–0.62 μm for the S_1, 2.13–6.94 μm for the S_2, and 0.08–0.14 μm for the S_3 layer (Saiki 1970). In addition, the thickness of the S_3 layer varies considerably among species. Liese (1963) reported that the thickness of the S_3 layer ranged between 0.07 and 0.08 μm in *Pinus sylvestris* and *Picea abies*. Saiki (1970) reported that the thickness of the S_3 layer ranged between 0.04 and 0.22 μm in *Pinus densiflora, Cryptomeria japonica, Chamaechyparis obtusa, Larix kaempferi*, and *Thuja orientalis*.

Wardrop (1964) reported that the S_3 layer was absent in some of the normal tracheids in *Picea*. Prodhan et al. (1995b) reported that cellulose microfibrils in juvenile wood were sparsely distributed on the innermost surfaces of wood fibers and, thus, no S_3 layer could be observed by polarization microscopy. It is also well known that the tracheids of the compression wood of conifers lack an S_3 layer (Timell 1986; Yoshizawa 1987). Some gelatinous fibers of tension wood (the so-called S_1 + S_2 + gelatinous layers or S_1 + gelatinous layers types) that are formed on the upper side of inclined stems in hardwoods also lack an S_3 layer (Prodhan et al. 1995a,b). In these tracheids or wood fibers, the rotation of cellulose microfibrils might be limited to a single semi-helicoidal pattern, as proposed by Roland et al. (1987). The difference in the rotation of cellulose microfibrils leads to variations of microfibrillar angles on the innermost surface of the cell wall in the tracheids or wood fibers.

A schematic model of the orientation of newly deposited cellulose microfibrils in a tracheid is shown in Fig. 5. As mentioned above, the direction of orientation of cellulose microfibrils changes progressively with changing speed of rotation during the formation of the secondary wall (Funada 2000). Thus, if cortical microtubules control the orientation of cellulose microfibrils in the secondary wall in tracheids or wood fibers, cortical microtubules are expected to be parallel to the newly deposited cellulose microfibrils and they are expected to change their orientation progressively during formation of the secondary wall.

Cortical microtubules are abundant throughout the formation of the secondary wall in differentiating xylem cells (Barnett 1981), and numerous parallel cortical microtubules, with a steep angle relative to the cell axis, are observed during formation of the secondary wall in wood fibers as well (Chaffey et al. 1997a). Cortical microtubules and cellulose microfibrils have been observed to be parallel in differentiating tracheids and during the formation of the secondary wall in several woody plants (Cronshaw 1965; Nobuchi and Fujita 1972; Robards and Kidwai 1972; Fujita et al. 1974; Barnett 1981; Hirakawa 1984; Inomata et al. 1992; Abe et al. 1994, 1995a; Prodhan et al. 1995a; Chaffey et al. 2002). Cronshaw (1965) observed a large number of cortical microtubules in developing wood fibers of *Acer rubrum*. When the S_2 layer is developing, parallel cortical microtubules are oriented in a helical direction. In oblique sections, cortical microtubules are seen to be oriented parallel to the orientation of cellulose microfibrils, as seen upon negative staining of the secondary wall in developing tracheids or wood fibers (Barnett 1981; Inomata

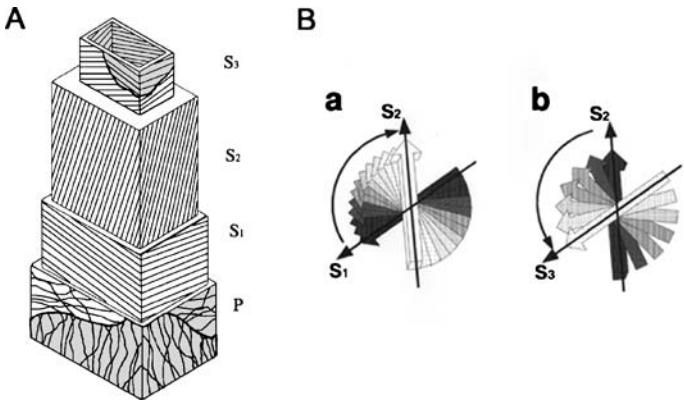

Fig. 5 A schematic model of cell wall texture in tracheids. **A** The orientation of newly deposited cellulose microfibrils. **B** Progressive changes in the orientation of cellulose microfibrils (*arrows*) viewed from the lumen side during the formation of secondary wall from S_1 layer to S_2 layer (*a*) and from S_2 layer to S_3 layer (*b*). P is primary wall; S_1 is outer layer of secondary wall; S_2 is middle layer of secondary wall; S_3 is inner layer of secondary wall

et al. 1992). The cellulose microfibrils seem to be packed towards the inner face of the cell wall in almost the same direction as cortical microtubules (Inomata et al. 1992).

During the formation of the secondary wall, following the cessation of cell expansion, the cortical microtubules are aligned in well-ordered arrays (Fig. 6; Uehara and Hogetsu 1993; Abe et al. 1994, 1995a,b; Prodhan et al. 1995a; Chaffey et al. 1997a, 1999, 2002; Funada et al. 1997; Furusawa et al. 1998; Samuels et al. 2002). Thus, two distinct arrangements of cortical microtubules are clearly detectable in differentiating tracheids or wood fibers: random arrays that are visible during the formation of primary walls and well-ordered arrays that appear during the formation of secondary walls. The shift from random to well-ordered arrays seems to be a gradual process. During the successive steps of xylem differentiation, no tracheids or wood fibers with disassembled cortical microtubules can be seen in any radial files. The absence of disassembled microtubules indicates that the orientation of cortical microtubules might change progressively, without complete depolymerization. Robert et al. (1985) first observed, in ethylene-treated pea epicotyls and mung-bean hypocotyls, that the reorientation of cortical microtubules occurred from transverse to oblique to longitudinal without complete depolymerization. Observations by micro-injection of fluorescently labelled tubulin into living plant cells demonstrated that cortical microtubules reorient in a continuous and complex manner (Yuan et al. 1994, 1995; Himmelspach et al. 1999). It has been proposed that the reorientation is initiated by the

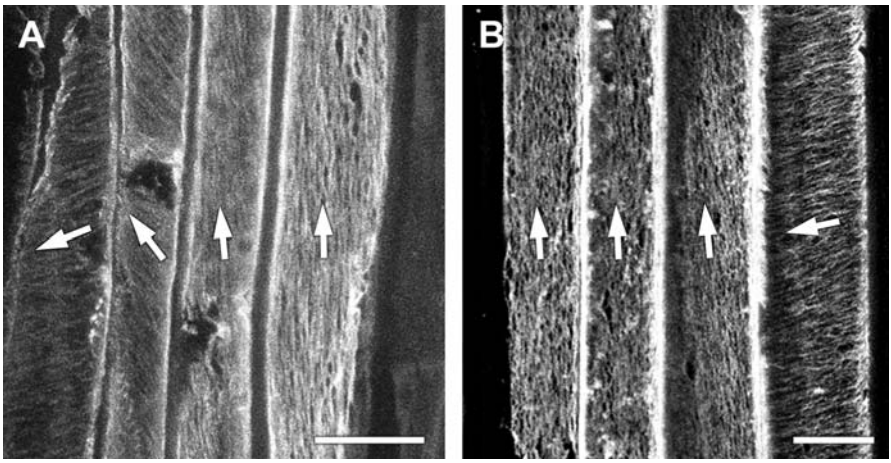

Fig. 6 Immunofluorescence images, obtained by confocal laser scanning microscopy, showing the successive changes in the orientation of cortical microtubules, viewed from the lumen side, in differentiating tracheids of *Abies sachalinensis*. Changes in the orientation of cortical microtubules (*arrows*) from a flat S-helix to a steep Z-helix (**A**) and from a steep Z-helix to a flat S-helix (**B**) during formation of the secondary wall. *Bars* = 25 μm

appearance of discordant cortical microtubules that do not share the existing alignment, but anticipate the new direction. As the existing microtubules destabilize, the old alignment is gradually replaced by a new alignment. Time-lapse observations using tobacco Bright Yellow-2 (BY-2) cells expressing MBD-DsRed (the microtubule binding domain of the MAP4 fused to the *Discosoma* red fluorescent protein) have also revealed that the cortical array organization does not require complete depolymerization of the microtubules (Dixit and Cry 2004). Although the process is not fully understood, modifications of MAPs, such as the 65-kDa MAP (MAP65; Chan et al. 1999), might be related to the stability of cortical microtubules (Nick 2000; Oda and Hasezawa 2006). More detailed observations of these proteins are needed if we are to clarify the mechanisms related to the stability of cortical microtubules and, subsequently, the molecular basis of their reorientation in secondary xylem cells. Recently, Mao et al. (2006) observed that the MAP65 was involved in the induced bundling of cortical microtubules in differentiating tracheary elements of *Zinnia elegans* cultured cells.

Successive changes in the orientation of cortical microtubules can be observed in differentiating tracheids or wood fibers during the formation of secondary walls (Abe et al. 1994, 1995a; Prodhan et al. 1995a; Furusawa et al. 1998; Chaffey et al. 2002). The orientation of cortical microtubules changed by clockwise rotation from a flat S-helix to a steep Z-helix when viewed from the lumen side (Fig. 6A). This shift in the direction of cortical microtubules is completed within three or four tracheids or wood fibers in a radial file. Then, the cortical microtubules are oriented in a steep Z-helix at almost the same angle over the next ten to fifteen tracheids or wood fibers of the radial file. After further differentiation, the orientation of cortical microtubules returns from the steep Z-helix to a flat S-helix in tracheids or wood fibers (Fig. 6B). This shift is completed within one or two tracheids or wood fibers in a radial file.

These observations provide strong evidence for the hypothesis that the orientation of cortical microtubules changes progressively and in a similar manner to the changes in the orientation of newly deposited cellulose microfibrils during the formation of the secondary wall. Thus, there is a very close relationship between cortical microtubules and newly deposited cellulose microfibrils. The cortical microtubules might control the ordered orientation of cellulose microfibrils in the semi-helicoidal cell walls of tracheids or wood fibers in woody plants.

During the formation of the secondary wall in tracheids or wood fibers, the orientation of cortical microtubules changes abruptly from a steep Z-helix to a flat S-helix, in contrast to the gradual change from a flat S-helix to a steep Z-helix. The shift in angles of newly deposited cellulose microfibrils is more abrupt during the transition from a Z-helix to a flat S-helix (from the S_2 to the S_3 layer), compared to the transition from a flat S-helix to a steep Z-helix (from the S_1 to the S_2 layer). The velocity of reorientation of microtubule might be related to the reorientation of newly deposited cellulose microfibrils. Therefore, the ro-

tational motion of cortical microtubules reflects the thickness of intermediate layers and the semi-helicoidal pattern of the secondary wall.

A similar parallelism between the orientation of cortical microtubules and cellulose microfibrils has been observed in the tension wood fibers of hardwoods. Tension wood is produced on the upper sides of inclined stems in response to gravitational stimulation. Tension wood fibers are usually characterized by the presence of gelatinous fibers, which form a gelatinous layer on the inner part of the cell wall. Cellulose microfibrils in the gelatinous layer are oriented parallel or nearly parallel to the longitudinal axis of the fibers. In gelatinous fibers of the so-called S_1 + gelatinous layers type of hardwoods, such as *Fraxinus mandshurica* var. *japonica*, the cellulose microfibrils change their orientation from their original S-helix until they are oriented approximately parallel to the cell axis (Prodhan et al. 1995a,b). In parallel, the cortical microtubules in these fibers change their orientation from a flat helix to a direction parallel or nearly parallel to the cell axis during the formation of the secondary wall, similar to the rotation in the orientation of the cellulose microfibrils. Parallel or nearly parallel cortical microtubules are observed in differentiating gelatinous fibers of *Populus euroamericana* (Nobuchi and Fujita 1972; Fujita et al. 1974), *Salix fragilis* (Robards and Kidwai 1972) and *Populus tremula* x *Populus tremuloides* (Chaffey et al. 2002). During the formation of the gelatinous layer, the spacing of the cortical microtubules is close and their parallelism is pronounced. In contrast to normal wood fibers, cortical microtubules in gelatinous fibers do not change their orientation from parallel or nearly parallel to a flat helix at the final stage of secondary wall formation. This absence of microtubule reorientation is mirrored in the orientation of cellulose microfibrils, which remain parallel or nearly parallel to the cell axis in the inner layer of gelatinous fibers.

Isolated single cells from the mesophyll of *Zinnia elegans* that differentiate to tracheary elements have been used as excellent experimental system to study wall formation in vitro (Fukuda 1994, 1996, 2004; Fukuda and Kobayashi 1989). In this system, an increase in the number of microtubules accompanies the reorganization of microtubules and the formation of the secondary wall. Similarly, the number of cortical microtubules increases four-fold early in the synthesis of the secondary wall in cotton fibers, as compared with the number during synthesis of the primary wall (Seagull 1992). This increase in the number of microtubules depends on the synthesis of tubulin de novo, which has been shown in the *Zinnia* system to be regulated at the transcriptional level (Fukuda 1994, 1996). DNA microarray analysis using *Populus tremula* x *Populus tremuloides* revealed that ten tubulin genes were strongly upregulated during the formation of the secondary wall (Hertberg et al. 2001). In tracheids, the density of cortical microtubules changes during formation of the secondary wall (Abe et al. 1994). In developing tracheids of *Abies sachalinensis*, the average densities of cortical microtubules are 5.4 microtubules per μm of cell wall for the S_{12} layer (between the S_1 and the S_2 layer), 9.3 for the S_2 layer, and 5.7 for

the S_3 layer, respectively. The deposition of cellulose microfibrils is more abundant during the formation of the S_2 layer than during the formation of S_1 or S_3 layers (Takabe et al. 1981a). The high density of cortical microtubules might be synchronized in relation to the active synthesis of cellulose.

During the formation of the gelatinous layer in developing gelatinous fibers in hardwoods, the average densities of cortical microtubules range around 20 per μm (Fujita et al. 1974) and 17–18 per μm of cell wall (Prodhan et al. 1995a). These cortical microtubules are mutually close with strong parallelism between them. The high density of cortical microtubules during formation of the S_2 and gelatinous layers might be related to the deposition of closely packed cellulose microfibrils in these layers. Kimura and Mizuta (1994) proposed the hypothesis that cortical microtubules at high density are involved in the orientation of cellulose microfibrils, while those at low density are not. Thus, the density distribution of microtubules might regulate orientation and texture of cellulose microfibrils in the cell wall, and, therefore, the orientation of microtubules might be controlled by factors that regulate the synthesis of tubulin (discussed in the chapter "Plant tubulin genes: regulatory and evolutionary aspects" of this book).

5 The Influence of Cortical Microtubules on Heterogeneous Cell Wall Structure

Heterogeneous thickenings of the secondary wall are observed in many plant cells. These secondary thickenings are visible as annular, spiral, reticulate, scalariform, or pitted patterns. They are due to the localized deposition of components of the cell wall, in particular cellulose microfibrils. The localized deposition of cellulose microfibrils might be related to a heterogenous distribution of cellulose-synthase complexes (Herth 1985; Schneider and Herth 1986). Groups of cortical microtubules can be observed under ridges of secondary wall. They are oriented parallel to the cellulose microfibrils and parallel to the bands of the cell wall in developing tracheary elements (Hepler and Newcomb 1964; Pickett-Heaps 1974). Hardham and Gunning (1979) observed that groups of cortical microtubules appeared prior to the localized deposition of the secondary wall. Thus, the cortical microtubules might determine the pattern of the cell wall by defining the localization of cellulose microfibrils in the cell wall. Recent time-lapse imaging of microtubules in living tracheary elements of *Arabidopsis thaliana* cell suspension showed that bundles of microtubules appeared at prospective sites of secondary-wall deposition (Oda et al. 2005; Oda and Hasezawa 2006). Subsequently, a single bundle of microtubules beneath the narrow secondary wall was separately arranged into a pair of bundles that are located along both sides of the developing secondary wall. These observations confirmed the crucial role of cortical microtubules in the regulation of deposition of secondary wall.

Modifications in structure are normal features of the cell wall in the secondary xylem cells of woody plants (Panshin and de Zeeuw 1980; Ohtani 2000). These modifications, such as helical thickenings, pits, and perforations, are formed by localized deposition of cell wall material. Their size, structure, and number are characteristic anatomical features for individual species and, thus, they are frequently used for the identification of woods.

The secondary xylem cells develop localized ridges made of parallel bundles of cellulose microfibrils on the innermost surface of the secondary wall. The cellulose microfibrils are oriented helically with respect to the cell axis. Such a thickening of the cell wall is known as helical thickening or spiral thickening. Helical thickenings are observed in tracheids or wood fibers of only a few species, but they are relatively common in vessel elements (Ohtani 2000). Helical thickenings vary considerably among species in terms of helix direction, width and height of ridges, frequency of thickenings, and the interval between ridges.

At the final stage of formation of the secondary wall, bands of obliquely oriented cortical microtubules appear in tracheids of conifers, such as *Taxus cuspidata*, in which helical thickenings are generally formed (Fig. 7A, Uehara and Hogetsu 1993; Furusawa et al. 1998). These bands of cortical microtubules are first approximately 3–4 μm wide. Then the bands become narrow and rope-like. Slightly disordered cortical microtubules are observed between the bands. These rope-like bands of cortical microtubules are oriented helically beneath the cell wall around tracheids. Different arrangements of cortical microtubules are observed within the same tracheid (Furusawa

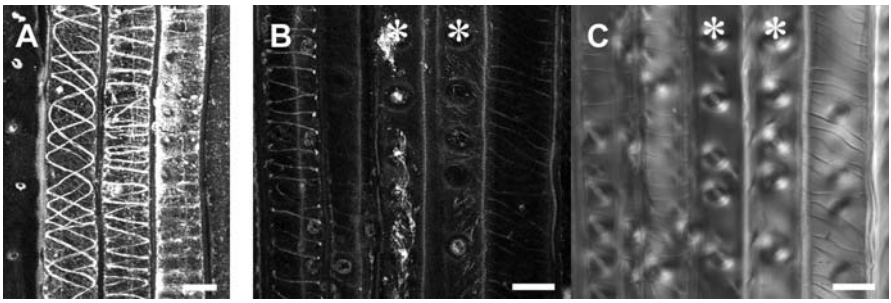

Fig. 7 Nomarski differential interference contrast image and immunofluorescence images, obtained by confocal laser scanning microscopy, showing the localization of cortical microtubules, which can be seen at the final stage of formation of the secondary wall in differentiating tracheids of *Taxus cuspidata*. **A** Bands of helically oriented cortical microtubules are visible. **B** Disruption of cortical microtubules in some differentiating tracheids by the application of microtubule-assembly inhibitor colchicine. Two tracheids lack helically oriented cortical microtubules (*asterisks*). **C** Nomarski differential interference contrast image of the same section as in (**B**). No helical thickenings are developed in the tracheids, which lack cortical microtubules (*asterisks*). *Bars* = 25 μm

et al. 1998). In one part of the tracheid, cortical microtubules are localized in a band-like pattern with disordered microtubules between bands, whereas in another part of the tracheid, rope-like bands of cortical microtubules with few microtubules between the bands are observed. Such a tracheid might represent a transitional stage during the dynamic relocalization of cortical microtubules. The changes in the arrangement of cortical microtubules can occur nonuniformly within a single tracheid. As the tracheids differentiate, the cortical microtubules eventually disappear.

When rope-like bands of cortical microtubules are observed, localized ridges of cell wall materials are clearly visible by bright-field microscopy (Uehara and Hogetsu 1993; Furusawa et al. 1998). The rope-like bands are superimposed on the localized ridges. This spatial congruence suggests that these bands of cortical microtubules might be involved in the formation of the helical thickenings. Thus, cortical microtubules control the localized deposition of cellulose microfibrils.

In differentiating vessel elements of hardwoods, such as *Aesculus hippocastanum*, in which helical thickenings are generally formed, similar bands of cortical microtubules are observed (Chaffey et al. 1997c, 1999). At the final stage of formation of the secondary wall, wide and nearly transverse bands of cortical microtubules are present. Later, the bands are replaced by narrower bands to form pairs of parallel bundles. The formation of bands of cortical microtubules appears to take place before helical thickenings are detectable. These results suggest that cortical microtubules drive the formation of the helical thickenings in vessel elements. Each pair of parallel bundles of cortical microtubules is often connected to a neighboring bundle by finer threads of bridging-cortical microtubules. These bridging-cortical microtubules, spanning the region between cortical microtubules, may act as "spacers" (Chaffey et al. 1999).

The direct application of the microtubule-depolymerizing agent colchicine to main stems disrupts some cortical microtubules in differentiating tracheids of *Taxus cuspidata*. Certain tracheids lack cortical microtubules at the final stage of formation of the secondary wall (Funada et al. 2001b; Fig. 7B,C). Such tracheids have no helical thickenings. In contrast, other tracheids that are endowed with helically oriented cortical microtubules form typical helical thickenings. These results confirm a role for localized cortical microtubules in the formation of the helical thickenings, which control the localized deposition of cellulose microfibrils.

The pits of secondary xylem cells, gaps in the secondary wall, are important areas that ensure cell-to-cell movement of water, nutrients, micromolecules, macromolecules, and ions. Thus, the minute structure of pits has been well documented mainly by electron microscopy (see Liese 1965; Harada and Côté 1985; Ohtani 2000).

During early stages of primary wall-formation in differentiating tracheids, cortical microtubules disappear locally from areas of prospective intertracheal bordered pits (Fig. 4A; Abe et al. 1995b, Funada et al. 1997), consistent

with the finding that the development of bordered pits begins long before the formation of the secondary wall in tracheids (Barnett and Harris 1975; Barnett 1981) and that bordered pits are delineated in the primary wall (Bauch et al. 1968; Fengel 1972; Imamura and Harada 1973). The accumulation of cell-wall material, which delineates the prospective sites of bordered pits, could be detected in the primary wall of differentiating vessel elements of *Kalopax pictus* using resin-cast replicas (Kitin et al. 2001). Thus, localized disappearance of cortical microtubules in the early stage of formation of the primary wall might be closely related to the determination of pit sites (Funada 2000; Funada et al. 2000, 2001b).

The tracheid forms the bordered pits and the ray parenchyma cell forms the simple pits. Half-bordered pit pairs are formed in a cross-field, which is the rectangle formed by the contacting walls of a tracheid and a ray parenchyma cell in conifers. Some pine trees, such as *Pinus densiflora*, have large window-like cross-field pits with a broad aperture and an overhanging border on the tracheid side (Panshin and de Zeeuw 1980; Ohtani 2000). Confocal microscopy has revealed the detailed process by which cross-field pits form in a radial file (Funada et al. 2001b). Cortical microtubules are oriented at random in expanding tracheids. Microtubule-free regions within randomly oriented cortical microtubules are visible in differentiating tracheids at the early stage of cell expansion. As tracheids expand, these regions gradually enlarge. The large "holes" within cortical microtubules in tracheids might be related to the formation of window-like cross-field pits. Later, localized cortical microtubules appear at the periphery of the microtubule-free regions. These localized cortical microtubules are superimposed on the pit borders of tracheids in the cross-field. In contrast, no microtubule-free regions are found in adjacent ray parenchyma cells. Therefore, the local disappearance of cortical microtubules occurs only in differentiating tracheids. The difference in behavior of cortical microtubules between tracheids and ray parenchyma cells is closely related to the fact that the localized deposition of cell wall materials in the cross-field occurs in tracheids, but not in adjacent ray parenchyma cells.

Upon advanced differentiation of tracheids, well-ordered cortical microtubules are visible in pit-free regions. Such cortical microtubules are distorted around the aperture of bordered pits in a streamline pattern (Fig. 8A,B). In contrast, obliquely oriented cortical microtubules are observed contiguously in adjacent ray parenchyma cells, including the areas of cross-field pits (Fig. 8B,C).

Enlarged perforations within the reticulum of cortical microtubules are also detected at prospective pit sites in differentiating vessel elements of *Aesculus hippocastanum* and *Populus tremula* x *Populus tremuloides* (Chaffey et al. 1997b, 1999, 2002). The appearance of microtubule-free regions might be regarded as a diagnostic indicator for the sites of bordered pits. In addition, contact pits, which develop between vessel elements and adjacent ray parenchyma cells (Murakami et al. 1999), are first detected as microtubule-free

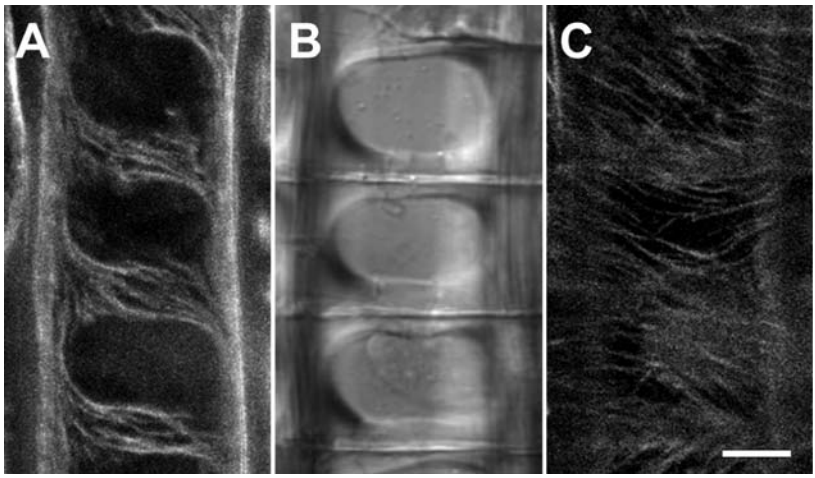

Fig. 8 Immunofluorescence images (**A**,**C**) and Nomarski differential interference contrast image (**B**), obtained by confocal laser scanning microscopy, showing the localization of cortical microtubules, which can be seen during formation of the half-bordered pits of *Pinus densiflora*. **A** Distortion of well-ordered cortical microtubules in a differentiating tracheid around the aperture of window-like cross-field pits. **B** Nomarski differential interference contrast image of the same section as in (**A**) and (**C**). **C** Cortical microtubules in adjacent ray parenchyma cells. Obliquely oriented cortical microtubules are visible throughout the areas of cross-field pits. *Bars* = 10 μm

regions within a random array of cortical microtubules (Chaffey et al. 1999, 2002).

Therefore, the localized disappearance of microtubules might be involved in the determination of prospective pit regions. Pit-free and pit-forming regions are delineated by localized cortical microtubules at early stages of primary-wall formation. It is unknown how this disappearance of cortical microtubules is determined, but local differences in the properties of the plasma membrane/cell wall might be related to the development of microtubule-free regions (Chaffey et al. 1997b), such that the plasma membrane is separated into two domains.

Bordered pits are characterized by a membrane that is overarched by the secondary wall. Concentrical rings of cellulose microfibrils on the inner surface of pit borders produce the border thickening (Liese 1965; Harada and Côté 1985). In differentiating tracheids at the final stage of cell expansion, circular bands of cortical microtubules are observed at the periphery of the microtubule-free regions (Hirakawa and Ishida 1981; Uehara and Hogetsu 1993; Abe et al. 1995b, Chaffey et al. 1997c; Funada et al. 1997, 2001b). Similar circular bands of cortical microtubules can be seen around the aperture of bordered pits in differentiating vessel elements of *Salix fragilis* (Robards and Humpherson 1967), *Aesculus hippocastanum* (Chaffey et al. 1997b, 1999), and

Populus tremula x *Populus tremuloides* (Chaffey et al. 2002). When the circular bands of cortical microtubules are observed, the pit borders are clearly visible by brightfield microscopy (Uehara and Hogetsu 1993; Funada et al. 1997, 2001b; Chaffey et al. 2002). The circular bands of cortical microtubules are superimposed on the edges of developing bordered pits (Fig. 4B,C). Thus, the circular bands of cortical microtubules are likely involved in the deposition of concentrically oriented cellulose microfibrils at the pit borders. In contrast, microtubule-free regions that are surrounded by bands of cortical microtubules become pit membranes. Hogetsu (1991) and Uehara and Hogetsu (1993) have proposed the hypothesis that the circular bands of cortical microtubules determine and maintain a boundary between the pit and non-pit regions that respectively activate and inactivate the deposition of the secondary wall. In consequence, the pit membrane is not committed for the deposition of a secondary wall.

As tracheids differentiate, the circular bands of cortical microtubules narrow centripetally. The reduction in diameter of circular bands of cortical microtubules is associated with the development of the over-arching border of bordered pits. Similarly, developing contact cells form a ring of cortical microtubules at the periphery of pits (Chaffey et al. 1999). However, unlike bordered pits, they do not decrease in diameter during differentiation. These differences in the behavior of cortical microtubules thus determine the minute structure of each pit type.

The results mentioned above indicate that the localized appearance or disappearance of cortical microtubules controls the localized deposition of cellulose microfibrils, resulting in modifications of the cell wall. However, the mechanisms for the reorientation and localization of cortical microtubules are not fully understood. Kobayashi et al. (1987, 1988) and Fukuda and Kobayashi (1989) proposed that actin filaments might play an important role in shifting the orientation and localization of microtubules in differentiating tracheary elements in cultured cells of *Zinnia elegans*. They observed that reticulate bundles of microtubules and aggregates of actin filaments between microtubules simultaneously emerged from sites that are adjacent to the plasma membrane just prior to the formation of the secondary wall. Subsequently, aggregates of actin filaments extended transversely to the cell axis and microtubules became transversely aligned between actin filaments. Transverse ridges of secondary wall were formed above the transverse bundles of microtubules in tracheary elements (Falconer and Seagull 1985). The disruption of actin filaments by cytochalasin B prevents the reorientation of microtubules into transverse arrays. These results suggest that actin filaments might regulate the orientation and localization of microtubules in tracheary elements of *Zinnia elegans* in vitro.

The presence of actin microfilament bundles has been reported for cambial cells of woody plants (Srivastava 1966; Srivastava and O'Brien 1966; Tsuda 1975; Goosen-de Roo et al. 1983; Chaffey et al. 1997a, 2000b, 2002; Rensing

and Samuels 2004). The microfilaments are generally oriented longitudinally. The lengths of microfilament bundles range from 7 μm to 17 μm (Catesson 1990). Chaffey et al. (1997a, 2000b, 2002) observed in *Aesculus hippocastanum* and *Populus tremula* x *Populus tremuloides* that bundles of axially oriented microfilaments were present in fusiform cambial cells and the longitudinal orientation of microfilaments was retained in cambial derivatives during xylem differentiation, even though the orientation of cortical microtubules had changed. Similar bundles of actin filaments were oriented axially in fusiform cambial cells in *Pinus densiflora* (Funada 2000, 2002; Funada et al. 2000). Their orientation in differentiating tracheids does not change during formation of the primary and secondary walls, even though the orientation of cortical microtubules changes. In some tracheids, transversely or obliquely oriented actin filaments are observed at the final stage of xylem differentiation, but in other tracheids the axial orientation is maintained. These observations suggest that there is no clear relationship, in terms of orientation, between cortical microtubules and actin filaments in cambial derivatives gaps in the secondary wall. Actin filaments might not play a major role in the reorientation of cortical microtubules in the differentiating secondary xylem cells that are derived from cambial cells. Chaffey et al. (1997a) pointed out that the behavior of cortical microtubules and actin filaments in a natural system, such as the differentiation of cambial derivatives, might differ from that in cell culture, such as the *Zinnia* system.

In contrast, a colocalization of cortical microtubules and actin filaments could be observed at the periphery of the bordered pits in differentiating vessel elements (Chaffey et al. 2000b, 2002). The reduction in diameter of localized rings consisting of microtubules and actin filaments was associated with the reduction in the diameter of aperture of the bordered pits. These observations suggest a role of the two cytoskeletal components in the development of the bordered pits. In addition, myosin was localized at the periphery of the bordered pits, suggesting that an acto-myosin system in analogy to a plant muscle is present at the apertures of bordered pits during their development (Chaffey and Barlow 2002). We need further work to elucidate the role of actin filaments and myosins in controlling the orientation and localization of cortical microtubules in the natural system of secondary xylem differentiation (discussed in more detail in the chapter "Crossed-wires: Interactions and cross-talk between the microtubule and microfilament networks in plants" of this book).

6
Potential Approaches to the Control of Cell-wall Texture

The secondary xylem cells of woody plants, such as tracheids, vessel elements, and wood fibers, are characterized by cell walls with a highly organized

structure. The orientation of cellulose microfibrils in the primary wall determines the direction of cell elongation and expansion, thereby controlling the shape and size of xylem cells. In addition, the orientation of cellulose microfibrils is one of the most important characteristics that determine the physical properties of wood. In particular, microfibril angles of the S_2 layer have a significant influence on the strength of wood. It has been suggested that the microfibril angles might be genetically controlled (Donaldson and Burdon 1995; Hirakawa and Fujisawa 1995). The microfibril angles differed significantly among clones of different origin. Thus, detailed screens to select plus trees with low microfibril angles should be included in future breeding programs.

As mentioned above, with respect to control of cellulose microfibril orientation and deposition in secondary xylem cells, cortical microtubules are considered to play important roles both in normal wood and reaction wood. Cortical microtubules might control the movement of cellulose synthesizing complexes (terminal complexes) in the plasma membrane (Baskin 2001; Paradez et al. 2006). Therefore, to control the microfibril angles, it is important to identify the genes that determine the angles of the S_2 layer. Although the mechanism of microtubule reorientation is not yet fully understood, valuable information has accumulated from recent molecular approaches. For example, the change of one amino acid in the tubulin protein resulted in altered orientation of cortical microtubules in epidermal cells of *Arabidopsis thaliana* (Thitamadee et al. 2002). In addition, microfibril angles in the secondary wall are correlated with the expression of a specific β-tubulin gene (*EgrTUB1*) in wood fibers of *Eucalyptus grandis* (Spokevicius et al. 2007). Therefore, molecular changes in the proportion of specific β-tubulin monomers in microtubules may influence the positioning of cellulose microfibrils in secondary walls of secondary xylem cells. Directional factors that control the orientation of cortical microtubules during the formation of secondary walls will be characterized in the near future. Such approaches might be important to create "new designer wood", where desired properties have been engineered through the control of the cell wall texture. Biotechnological control of the cell wall texture (such as microfibrillar angles) and thickness of the secondary wall in tracheids or wood fibers by manipulation of cortical microtubules could provide new tools, which would permit control of wood quality.

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Microtubules and Pathogen Defence

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Abstract The cytoskeletal network of plant cells represents a dynamic structure that responds to external stimuli by changes of organization. An attack of pathogenic microbes represents an external stress that seriously threatens plant survival. Growing evidence from recent research indicates that cytoskeletal elements, such as microtubules and microfilaments, are central players in plant defence responses. Tubulin and actin inhibitors suppress the polarization of cellular events related to plant defence, such as massive cytoplasmic aggregation, deposition of papillae and the accumulation of autofluorescent compounds at the sites of fungal penetration. Simultaneously, these inhibitors allow non-pathogenic fungi to penetrate successfully into non-host plants. Thus, microtubules and microfilaments, through the temporal and spatial regulation of molecules and/or organelles in the host cell, seem to control responses conferring resistance to attempted fungal penetration. In addition, elements of the plant cytoskeleton seem to play a critical role in hypersensitive cell death. On the other hand, several plant pathogens produce anti-cytoskeletal compounds during invasion, suggesting that the plant cytoskeleton represents an advantageous target for plant pathogens and symbionts. The possibility of enhancing plant resistance to pathogens via artificial manipulation of cytoskeletal elements will be discussed.

1 Dynamic Reorganization of the Cytoskeleton During Pathogen Attack

The cytoskeleton can respond by adaptive reorganization to a variety of external stimuli. In addition to comprehensive reviews of the plant cytoskeleton (Volkmann and Baluška 1999; Staiger 2000; Breviario and Nick 2000; Wasteneys 2004; Wasteneys and Yang 2004), a number of specific reviews focused on adaptive reorganization of the cytoskeleton in response to abiotic stresses (see chapter "Microtubules as sensors for abiotic stimuli" in this volume), such as gravitropic stimulation (Perbal and Driss-Ecole 2003; Wasteneys 2000), low temperature (Browse and Xin 2001), hormone treatment (Ishida and Katsumi 1991), and wounding (La Claire 1989) have been published. Increasing evidence strongly suggests that the plant cytoskeleton is a key player in the defence against pathogens, including viruses (see chapter "Microtubules and viral movement" in this volume), fungi, oomycete species and, possibly, bacteria. The first part of this chapter will therefore de-

scribe the previous work on dynamic rearrangement and disruption of the cytoskeleton during pathogen attack.

Rearrangements of the cytoskeleton during attempted infection have been reported for several plant–fungus systems. The first report of cytoskeletal responses in plant cells during fungal attack was made by our group. In the interaction between barley coleoptiles and the non-pathogenic powdery mildew, *Erysiphe pisi*, microfilaments and microtubules are rearranged in the coleoptile during attempted penetration by the fungus (Kobayashi et al. 1991, 1992). When appressoria of the non-pathogenic fungus initiated penetration attempts, microfilaments and microtubules reorganized into a radial array focused towards the site of penetration (Fig. 1). Kobayashi et al. (1992, 1996) reported that such cytoskeletal rearrangements were more prominent in host–pathogen interactions that were incompatible. Similar, major rearrangements of cytoskeletal components during interaction with pathogenic fungi or oomycetes have been observed in other host–pathogen systems as well, for instance for cowpea and *Uromyces vignae* (Škalamera and Heath 1998), onion and *Magnaporthe grisea* (Xu et al. 1998) or *Botrytis allii* (McLusky et al. 1999), *Arabidopsis* and *Phytophthora sojae* (Cahill et al. 2002; Takemoto et al. 2003), or *Arabidopsis* and *Peronospora parasitica* (Takemoto et al. 2003) or *Colletotrichum* (Shimada et al. 2006). This sug-

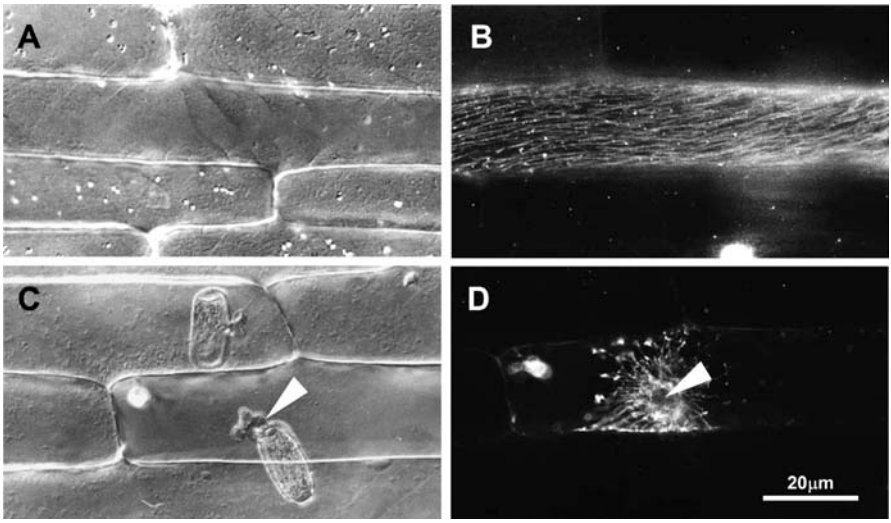

Fig. 1 Rearrangement of microtubules in barley coleoptile cells inoculated with the non-pathogenic *E. pisi*. *A*, *C* Differential interference contrast images. *D* Epifluorescence images of immunostained microtubules. *A*, *B* Oblique orientation of microtubules in epidermal cells of uninoculated coleoptiles. *C*, *D* Microtubules 24 h after inoculation with numerous short and condensed microtubule bundles forming a radial array at the site of attempted penetration (*arrowheads*)

gests that the cytoskeleton is commonly involved in a broad range of host or non-host interactions. During the interaction between flax and the flax rust fungus, *Melampsora lini*, radial arrays of microfilaments and microtubules at the encounter site were limited to incompatible interactions, suggesting that the cytoskeleton is involved in gene-for-gene-dependent host resistance as well as in non-host resistance (Kobayashi et al. 1994). Moreover, even in tobacco cell cultures (i.e., isolated from a tissue context), the cytoskeleton plays an important role in defence mechanisms against fungal penetration (Kobayashi et al. 2003). In this cultured cell system, radial rearrangements of the actin cytoskeleton at fungal penetration sites was observed, similar to the situation in tissue. Altogether, rearrangement of the cytoskeleton against fungal penetration attempts might be a basic and original response of plant cells.

In several plant–pathogen interactions, the dynamic rearrangement of the cytoskeleton is replaced by a disruption. In cultured parsley cells challenged by the non-pathogenic *Phytophthora infestans*, a localized disruption of microtubules occurred simultaneously with a rearrangement of microfilaments at the penetration site (Gross et al. 1993). Disruption of the cytoskeleton was also observed in host cells undergoing a hypersensitive response (HR). Cahill et al. (2002) showed that the usual "focusing" of microtubules towards the penetration site did not occur in soybean during compatible or incompatible interactions with *Phytophthora sojae*. Instead, there was rapid disruption of microtubules during an early stage of incompatibility in association with HR. A similar disruption of microtubules and actin filaments during HR was observed in flax cells suffering penetration by the incompatible *Melampsora lini* (Kobayashi et al. 1994). The possible role of dynamic cytoskeletal reorganization for penetration resistance and HR will be discussed in the next section.

2
Possible Roles of the Cytoskeleton in Pathogen Defence

Although evidence supporting the importance of the cytoskeleton in plant defence has accumulated over recent years, the actual role of the cytoskeleton during the manifestation of pathogen defence has not been elucidated yet. Generally, the cytoskeleton controls cell shape, cell motility, cell migration, and cell polarity through the spatial and temporal regulation of molecules and organelles in the cell. In plants, the cytoskeleton participates in diverse cellular processes such as cytoplasmic streaming (Kamiya 1981), motility of organelles (Williamson 1986), cell wall synthesis (Hardham et al. 1980), and cell division (Katsuta and Shibaoka 1992). In this context, the following possibilities can be envisaged with respect to the potential role of the cytoskeleton in plant defence responses:

1. Penetration resistance through polarization of defence-related reactions
2. Hypersensitive cell death
3. Intracellular and intercellular signal perception, triggering defence responses

In this section, we will discuss these possible roles of the cytoskeleton in plant defence.

2.1 Role of the Cytoskeleton in Penetration Resistance to Fungal Pathogens

A number of pathogenic fungi form infection structures, such as germ tubes and appressoria, when they attack and then directly penetrate into plant cells. When plant cells sense fungal penetration attempts, they express various morphological and physiological responses contributing to penetration resistance. Penetration resistance is the first line of defence against pathogenic fungi, because direst penetration is essential for, at least some, fungal pathogens (Tsuji et al. 2003; Xu et al. 1998). The plant cytoskeleton seems to be involved in the expression of penetration resistance. Major changes of intracellular organization that are well-detectable by light microscopy are known as common features of penetration resistance. These include a movement of the nucleus towards the encounter site (Pappelis et al. 1974; Gross et al. 1993), cytoplasmic aggregation (Bushnell and Bergquist 1975; Kunoh et al. 1985a), alterations in the arrangement of cytoplasmic strands (Kitazawa et al. 1973; Kobayashi et al. 1993), and changes in the velocity of cytoplasmic streaming (Tomiyama 1956; Kobayashi et al. 1990). Takemoto et al. (2003) investigated cytoplasmic aggregation during the interaction of *Arabidopsis* with oomycete pathogens using a panel of transgenic *Arabidopsis* plants expressing subcellular structures tagged with the green fluorescent protein (GFP). They observed that cytoplasmic aggregation included the accumulation of the endoplasmic reticulum and the enrichment of Golgi bodies around the penetration site, suggesting that cytoplasmic aggregation represents a defence mechanism based on the localized production and secretion of plant materials (Takemoto et al. 2003). As motive force and guiding track for the movement of organelles and cytoplasm, the plant cytoskeleton is expected to play a role in these defence-related responses (Kamiya 1981; Williamson 1986; Seagull 1989). In fact, in barley cells, treatment with cytochalasins (i.e., actin inhibitors) inhibited cytoplasmic aggregation in response to penetration attempts of the non-pathogen *Erysiphe pisi* (Kobayashi et al. 1997b). The result strongly suggests that the actin cytoskeleton is involved in cytoplasmic aggregation accompanying fungal attack.

In addition to the accumulation of organelles, it is observed that various defence-related compounds become localized around fungal penetration sites. The papilla, a cell wall apposition at the penetration site, which is formed in the center of cytoplasmic aggregates, has been thought to repre-

sent an important defence reaction (Aist 1976; Heath and Heath 1971). In onion cells that have been inoculated with *Botrytis allii*, fluorescent phenolic compounds, with potential anti-fungal activity, accumulated at sites of attempted penetration and were associated with a reorganization of the actin cytoskeleton (McLusky et al. 1999). When barley cells were treated with cytochalasins, this strongly inhibited the accumulation of callose, a major component of papilla (Kobayashi et al. 1997b). A similar polar accumulation of proteins, polysaccharides, and fluorescent substances could be observed at fungal penetration sites in barley cells, and this polar accumulation was completely blocked by treatment with inhibitors of cytoskeletal polymerization and depolymerization (Fig. 2, Kobayashi et al. 1997b). Treatment with actin inhibitors effectively increased the penetration efficiency of non-pathogenic fungi on non-host plants (Kobayashi et al. 1997c). *Erysiphe pisi*, a pathogen of pea, normally fails to penetrate into non-host plants such as barley, wheat, cucumber, and tobacco. However, when tissues of these non-host plants were treated with cytochalasins, this fungus became able to penetrate and formed haustoria in epidermal cells of these plants. Moreover, treatment of these plants with various species of cytochalasins allowed several other non-pathogenic biotrophic, hemibiotrophic, and necrotorophic fungi to penetrate the cells of these non-host plants in a concentration-dependent manner. These results strongly suggest that actin microfilaments play an important role in the polarization of defence-related compounds at the attacked site and thus participate in the expression of penetration resistance (Schmelzer 2002).

During the past few years, genetic and molecular biology approaches have advanced our understanding of the molecular basis of penetration resistance. *Arabidopsis* penetration (*pen*) mutants are partially compromised in penetration resistance (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). PEN1 is a syntaxin that acts in a pre-invasion non-host resistance pathway that seems to be different from the mechanism described above. PEN1 is likely to be part of a SNARE complex that secretes vesicle cargoes to the site of attempted fungal invasion, contributing to formation of cell wall appositions (Collins et al. 2003). PEN2 is a peroxisomal glucosyl hydrolase (Lipka et al. 2005), and PEN3 encodes a plasma membrane ABC transporter (Stein et al. 2006). Both PEN2 and PEN3 are also recruited to attempted fungal penetration sites. Recruitment of PEN1 to fungal penetration sites was not dependent on the actin cytoskeleton (Bhat et al. 2005). Shimada et al. (2006) found that GFP-tagged PEN1 did not accumulate at sites of attempted penetration by either adapted or non-adapted *Colletotrichum* species, whereas pronounced focal accumulations of PEN1 were observed upon inoculation with powdery mildews. In contrast, inhibition of actin filaments by cytochalasin treatment indicated a functional contribution of the actin cytoskeleton for penetration resistance to *Colletotrichum* species. Altogether, the function of PEN1 seems to be specific for powdery mildews and independent of actin, whereas PEN2 and PEN3 contribute to a more general penetration resistance. Consis-

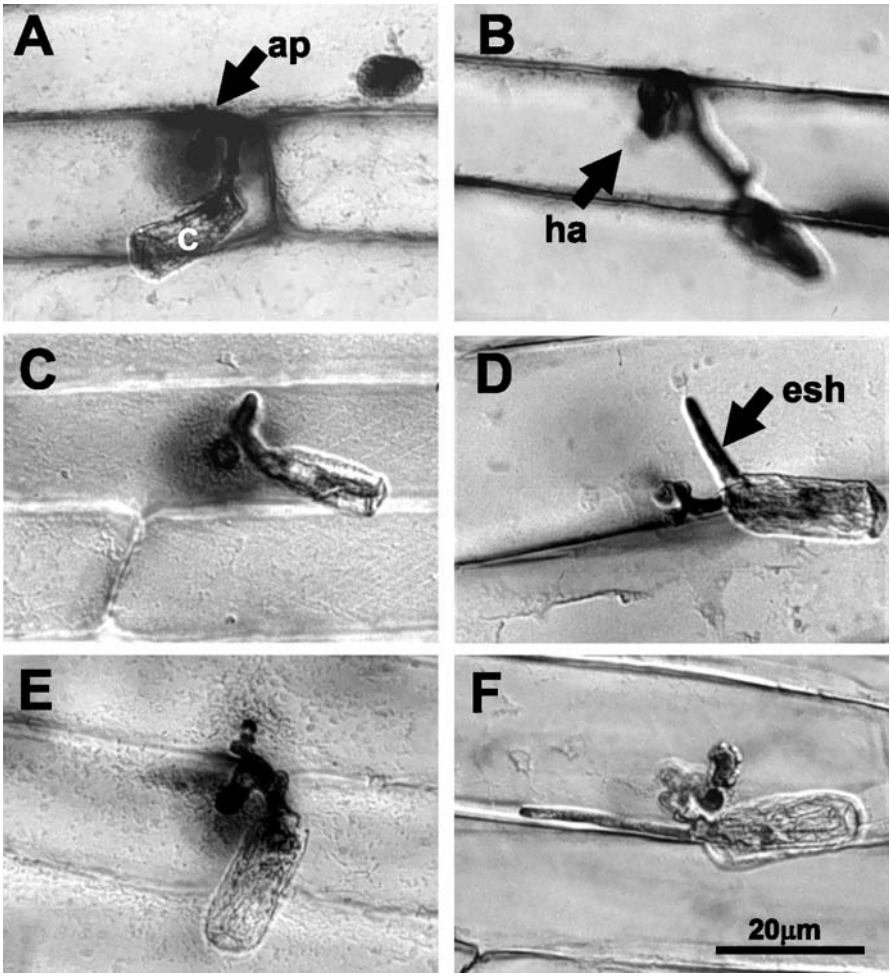

Fig. 2 Bright-field micrographs showing the effects of the cytochalasin A on localized accumulation of defence-related materials in barley coleoptile cells 24 h after inoculation with *E. pisi*. *A*, *C*, *E* Untreated controls. *B*, *D*, *F* Treatment with 1 $\mu g\,mL^{-1}$ cytochalasin A. *A*, *B* Signal after staining with amido black to visualize protein accumulation. *C*, *D* Signal after acidic Schiff reaction to visualize carbohydrate accumulation. *E*, *F* Signal after staining with lesorcinol blue to visualize callose. Note the absence of signals and the successful penetration of *E. pisi* and formation of haustoria in the cytochalasin-treated cells shown in *B*, *D* and *F*. *ap* Appressorium, *ha* haustorium, *esh* elongating secondary hypha

tent with this global contribution to penetration resistance, *pen2* and *pen3* mutants display similar phenotypes that are not observed in *pen1* mutants. Moreover, mutations in *PEN2* partially suppressed the phenotype of *pen3* mutants, suggesting that the functions of PEN2 and PEN3 might be interdependent, whereas PEN1 acts through an independent mechanism (Stein et al.

2006). Since the global penetration resistance (mediated by the PEN2/PEN3 pathway) can be suppressed by cytochalasins, actin might act as a track for PEN2-containing peroxisomes and/or vesicles (Stein et al. 2006). Thus, the actin cytoskeleton may act cooperatively with PEN2 and PEN3 in penetration resistance against a broad range of pathogenic fungi.

2.2 Role of the Cytoskeleton in the Hypersensitive Reaction

As described in Sect. 2, the plant cytoskeleton is dynamically reorganized in cells that undergo a hypersensitive reaction. The involvement of the plant cytoskeleton in the hypersensitive reaction is supported by several findings. The hypersensitive reaction of barley coleoptile cells that had been challenged by an incompatible strain of *Blumeria graminis hordei* was partially inhibited by cytochalasin B, a blocker of actin polymerization (Hazen and Bushnell 1983). Similarily, Škalamera and Heath (1998) reported that hypersensitive cell death in cowpea cells upon infection with the cowpea rust fungus (*Uromyces vignae*) was inhibited by cytochalasin E. In the flax and flax rust fungus (*Melampsora lini*) system, a rapid hypersensitive response, developing about 24 h after inoculation, normally inhibits fungal development and invasion by an incompatible interaction. However, in the presence of oryzalin,, a blocker of microtubule polymerization, the occurrence of hypersensitive cell death was delayed and reduced in frequency (Kobayashi et al. 1997a). Microtubules were not disrupted in flax cells at the first contact with the fungal structure but disappeared as soon as haustoria formed, even in a very early stage of infection. In incompatible interactions between soybean and *P. sojae*, treatment of hypocotyls with oryzalin prior to inoculation led to a partial breakdown of the incompatible response (Cahill et al. 2002). In tobacco cells, treatment with cryptogein, a protein secreted by *Phytophthora cryptogea* that triggers a hypersensitive-like response of tobacco cells, caused a rapid and severe disruption of the microtubular network (Binet et al. 2001).

These results suggest that the both actin and the microtubule cytoskeleton are involved in the expression of hypersensitive reactions. There is an interesting analogy with cell death in self-incompatibility. Geitmann et al. (2000) reported that treatment with S-protein, a determinant of self-incompatibility of pollen, causes a rapid reorganization and depolymerization of endoplasmic actin filament bundles in incompatible pollen of *Papaver rhoeas*. In addition to the rapid arrest of pollen-tube growth, treatment with S-protein can also trigger programmed cell death of self-pollen (Thomas and Franklin-Tong 2004), suggesting that the actin cytoskeleton participates in the induction or manifestation of programmed cell death. Although the exact function of the cytoskeleton in the expression of the hypersensitive response remains to be elucidated, there is growing evidence that the plant cytoskeleton participates in cellular signaling cascades.

2.3 Role of the Cytoskeleton in Intra- and Intercellular Signal Transmission of Pathogen Defence

Although there is not much direct evidence connecting the cytoskeleton to defence-related signal transduction, circumstantial evidence is accumulating. In potato tissues treated with elicitors prepared from *Phytophthora infestans*, cytochalasin D and some inhibitors of the signal transduction cascade (including staurosporine, ophiobolin, and quinacrine) inhibited the accumulation of rishitin, a potato phytoalexin that is produced in potato tissues in response to this elicitor (Furuse et al. 1999). Similarly, cytochalasin A blocked the accumulation of phenylalanine ammonia-lyase, and thus blocked phytoalexin formation in pea tissues treated with a fungal elicitor (Sugimoto et al. 2000). These results indicate that the factors involved in elicitor-induced signal transduction are linked with the actin cytoskeleton. Alternatively, the cytoskeleton might ensure rapid transport of these signals.

During recent years, many authors have reported that components of signal-transduction chains, such as mitogen-activated protein kinase cascades (MAPK), or PI-3 kinase and phospholipase D (PLD), are directly or indirectly bound to the cytoskeleton in the context of hormonal responses, cell cycle regulation, signaling of abiotic stresses, and defence mechanisms (Bögre et al. 2000; Gardiner et al. 2001; Nishihama et al. 2002; Soyano et al. 2003; Xu et al. 1992). In eukaryotic cells, external signals are received by receptors in or at the surface of cells, and the signals are transmitted to an intracellular target through modulation of a cascade of second-messengers. In plants, it is the extracellular matrix (ECM) that is involved in cell–cell communication in a wide range of developmental, reproductive, and pathogenic processes (Liu et al. 2001). In animal cells, the integrins, talin, spectrin, α-actinin, vitronectin or vinculin form direct links between the cytoskeleton, the plasma membrane, and the ECM. However, these components seem to be absent from the *Arabidopsis* genome (Brownlee 2002). Recently, Gardiner et al. (2001) demonstrated that the enzymatically active phospholipase D binds both microtubules and the plasma membrane in tobacco BY-2 cells. Dhonukshe et al. (2003) found that treatment of tobacco BY-2 cells with 1-butanol, a potent activator of PLD, caused partial depolymerization and release of cortical microtubules from the plasma membrane, suggesting that the activation of PLD triggers microtubular reorganization. Moreover, it was suggested that both PLD and phosphatidic acid, which is produced by hydrolysis of membrane lipids, are involved in plant defence responses against bacterial (Andersson et al. 2006) and fungal pathogens (de Jong et al. 2004; Profotova et al. 2006), and that microtubules may transmit the signals from ECM to cytoplasm.

A second line of evidence is based on the MAPK cascade, which is very important for the disease resistance of plants (Asai et al. 2002; He et al. 2006;

Pedley and Martin 2005; Zhang and Klessig 2001). Recent work showed that NPK1, one of the tobacco MAPKs, might be associated and regulated by microtubule motor proteins (Bögre et al. 2000 Nishihama et al. 2002; Soyano et al. 2003). Phosphorylation of cytoskeletal proteins and/or binding proteins by MAPKs resulted in the rearrangement of cytoskeletal arrays leading to morphological changes and cell polarization (Šamaj et al. 2004).

Although signal transmission is thought to be conveyed mainly by intracellular diffusion, the cytoskeleton provides tracks that might allow for rapid transmission of the modulated signals. Transfer of resistance from a cell that had been actually attacked by *E. pisi* to unchallenged adjacent cells was observed in barley (Kunoh et al. 1988). Microfilaments have been detected as components of plasmodesmata in several plant species (White et al. 1994; Blackman and Overall 1998; Baluška et al. 2004). Recently, both actin and myosin localized to plasmodesmata have been demonstrated to play a role in gating plasmodesmata (see chapter "Microtubules and viral movement" in this volume; Heinlein 2002), raising the possibility that the cytoskeleton contributes to intercellular signal transmission.

3
The Cytoskeleton as a Possible Target of Pathogenicity and Symbiosis

As described above, plant actin is conceived as a key player in penetration resistance, hypersensitivity, and signal transduction during pathogenesis. During pharmacological studies, cytoskeletal inhibitors have been found to affect the expression of various defence responses, indicating that the plant cytoskeleton might be an advantageous molecular target for plant pathogenic microbes. In addition to pathogenic interactions, symbiotic interactions would appear to require cytoskeletal reorganization during the establishment of symbioses. In this section, we therefore will discuss the plant cytoskeleton as a potential target for pathogen invasion and symbioses.

3.1
Disorganization of the Cytoskeleton by Pathogenic Factors

As described above, a disruption of the cytoskeletal network was observed during pathogen attack for a number of plant–pathogen interactions. In mammalian cells, the cytoskeleton of the host cell is a major virulence target of effector proteins from pathogenic bacteria (Büttner and Bonas 2003; Cornelis 2002). For example, YopE, YpkA, and YopT from *Yersinia* spp. directly influence the activity of Rho GTPases (Aepfelbacher and Heesemann 2001), which are key regulators of the actin cytoskeleton. Plant pathogenic bacteria share some homologs of these type-III effectors from animal bacteria, although the target of these plant type-III effectors are not Rho GTPases (Shao

et al. 2002). When *Arabidopsis* leaves were inoculated with *Pseudomonas syringae*, the actin cytoskeleton became disrupted during an early stage of infection (Fig. 3). This result indicates that plant bacteria are also endowed with type-III effectors that target to the plant actin cytoskeleton, although such effectors have not been identified yet. Recent studies have revealed that some plant pathogenic fungi produce compounds that are directed against elements of the plant cytoskeleton and contribute to the pathogenicity of these fungi. The virulence of the blast fungus *Pyricularia*, attacking *Digi-*

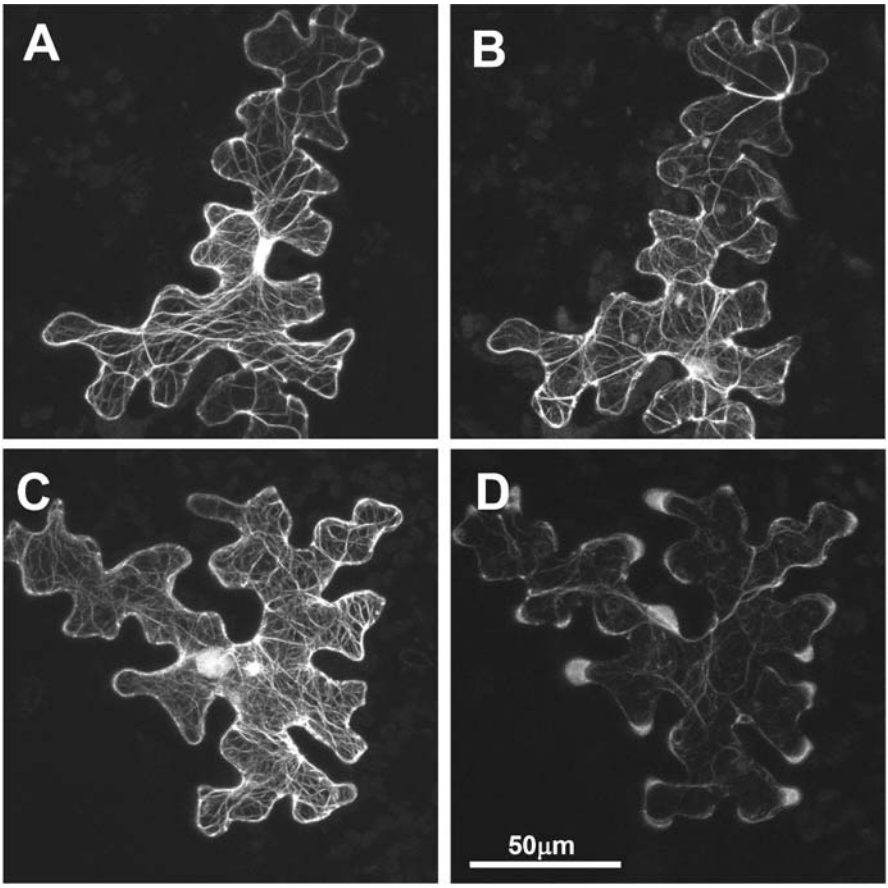

Fig. 3 Confocal laser scanning micrograph of *Arabidopsis* leaf pavement cells that were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. The *Arabidopsis* epidermal cells expressed transiently GFP-mouse talin upon biolistic bombardment. *A*, *B* Infiltration with 10 mM $MgCl_2$ (mock treatment). *C*, *D* Infiltration with *Pst* DC3000 cells. *A*, *C* 0 h after treatment. *B*, *D* 15 h after treatment. The actin cytoskeleton had mostly vanished in the *Arabidopsis* cells inoculated with bacteria, whereas intact microfilaments were observed in the mock-treated cell

taria plants correlates with the production of pyrichalasin H, a cytochalasin species (Tsurushima et al. 2005). Pretreatment of leaf sheaths of crabgrass with pyrichalasin H led to the penetration and colonization by non-host isolates. These results indicate that production of pyrichalasin H is a determinant for the pathogenicity of different *Digitaria* isolates of this blast fungus. Yuan et al. (2006) reported that actin microfilaments and microtubules were severely disrupted in *Arabidopsis* suspension-culture cells when these cells were treated with high concentrations of *Verticillium dahliae* toxin (VD toxin). It is highly suggestive that the most well-known class of actin inhibitors, the cytochalasins, are specifically produced by various genera of fungi as a tool for overcoming plant defence (Thomas 1978).

3.2 Role of the Cytoskeleton in Symbiosis

Plant cells engage in mutualistic endosymbioses with microorganisms including Gram-negative bacteria (*Rhizobium*) and fungi of the *Zygomycetes* (arbuscular mycorrhiza). Evidence has accumulated suggesting that the arbuscular mycorrhiza and the root-nodule symbioses of legumes are based on some common core components of plant cells, including the cytoskeleton (Parniske 2000).

The early stages of root-nodule development are mediated by an exchange of signals between plant and rhizobia that controls the altered gene expression on the bacterial part, and the cell growth, division, and differentiation on the part of the host (Gage and Margolin 2000). Cytoskeletal reorganizations occur during the various stages of symbiotic interactions or in response to Nod factors, i.e., the signal molecules produced by rhizobia during the initiation of nodule formation. A transient fragmentation of actin (Cárdenas et al. 1998) and a reorganization of microtubules (Timmers et al. 1998, 1999) were observed during all the early symbiotic steps in legumes. Treatment with Nod factors such as Nod RM-IV (Sieberer et al. 2005; Weerasinghe et al. 2003) or lipochitin oligosaccharide (Miller et al. 1999; Vassileva et al. 2005) caused dynamic redistributions and reorganizations of the cytoskeleton in legume root hairs. It is presumed that these modifications of the cytoskeleton contribute to the modification of plant defence (Parniske 2000) or to the commitment of the host tissues towards the differentiation of structures adapted for the endosymbiosis of rhizobia (Timmers et al. 1998). Similarly, microfilaments and microtubules reorganized drastically upon infection of arbuscular mycorrhizal fungi (Genre and Bonfante 1998, 2002).

The influence of the mycorrhizal fungus *Gigaspora margarita* on cytoskeletal organization in the root-epidermal cells of *Lotus japonicus* was compared between wild-type and the host mutant *ljsym4*, in which the fungus is confined to the epidermal cells (Genre and Bonfante 2002). Epidermal cells of the mutant responded by a disorganization of microtubules and microfilaments

before and during attempted fungal penetration, whereas the cytoskeleton of the wild-type remained highly organized and, in a late stage, enveloped the growing hyphae of the fungus. The close relationship between the organization of the host cytoskeleton and the compatibility with the fungus suggests that a correct reorganization of the cytoskeleton in the epidermis is necessary for fungal development and the suppression of plant defence. The recent advances in genomics have made it possible to obtain global images of gene expression and protein synthesis. Using this approach, Lohar et al. (2006) reported that some genes that were regulated differentially during an early stage of nodulation in *Medicago truncatula* belong to families encoding proteins involved in the cytoskeleton, calcium transport and binding, reactive oxygen metabolism, as well as in cell wall functions. This was confirmed by Amiour et al. (2006), who investigated modifications of the *M. truncatula* root proteome during the early stage of arbuscular mycorrhizal symbiosis. The response to the formation of fungal appressoria included alterations of proteins related to cytoskeletal reorganization, such as actin depolymerization factor.

Endosymbiosis is characterized by the "symbiosome", a compartment within host cells in which the symbiotic microorganisms are either partially or completely enclosed by a host-derived membrane (Parniske 2000). Although structural similarities in fungal symbiotic interfaces have been recognized for a long time, the genetic and molecular overlap of root symbioses with endosymbiotic pathogenic interactions (including powdery mildews and rusts) have remained unclear. Insight into the genetic and molecular basis of the cytoskeletal modifications that occur during endosymbiosis may contribute to a better understanding of plant defence in general and the similarity between mutualistic and pathogenic endosymbioses in particular.

4 Prospects – Potential of the Cytoskeleton for Molecular Breeding of Pathogen-Resistant Plants

During the past few years, enormous progress has been made in the understanding of the molecular basis of both non-specific general resistance and specific R-gene mediated resistance (Jones and Dangl 2006). Two classes of defence mechanisms are triggered, either through the pathogen-associated molecular patterns (PAMPs) receptor (Gómez-Gómez and Boller 2000; Zipfel et al. 2006) or through nucleotide-binding (NB) leucine-rich repeat (LRR) proteins, which are encoded by R-genes (Hammond-Kosack and Jones 1997; van der Biezen an Jones 1998). Insight into the molecular basis of disease resistance will provide novel strategies for the development of disease-resistant crops with the help of molecular breeding. As described above, the plant cytoskeleton plays an important role in both plant defence and microbial symbiosis. As

a highly conserved subcellular structure of higher plant cells, the cytoskeleton will also provide new targets for molecular breeding of disease-resistant crops.

Early cytological studies indicated that penetration resistance to fungal pathogens was not only due to passive mechanisms, but that it is also highly inducible (Kunoh et al. 1985b; Snyder and Nicholson 1990), although the molecular basis of the respective signal perception and defence machinery have remained unclear. Little time is required for fungal pathogens to penetrate plant cell walls and to subsequently invade the cells. Successful suppression of fungal attack therefore relies upon rapid responses to microbial attack. In this context, the organization of the cytoskeleton that is regulated by a number of binding proteins (Deeks et al. 2002; Steiger et al. 1997; McCurdy et al. 2001; Vantard et al. 2002; Wasteneys and Galway 2003) has attracted attention. Recently, small GTPases of the Rho family, which appear to control coordinately different cellular activities through interaction with multiple target proteins (Gu et al. 2004; Hall 1998; Nibau et al. 2006), have emerged as key regulators of the actin cytoskeleton. Recent work indicates that a unique member of plant Rho small GTPases, Rac, is a pivotal component of plant defence responses (Agrawal et al. 2003; Fujiwara et al. 2005; Jung et al. 2006; Moeder et al. 2005; Ono et al. 2001; Schultheiss et al. 2002). The first report of a role of Rac in plant defence by Kawasaki et al. (1999) reports that overexpression of a constitutively active (i.e., GTP-bound) form of a Rac results in enhanced hypersensitive responses and in resistance to the rice blast disease. Overexpression of a constitutively activated barley Rac (RACB) in single cells partially suppressed the actin reorganization usually observed upon attack by the barley powdery mildew, *B. graminis*, whereas a knockdown of RACB promoted actin focusing (Opalski et al. 2005). These results indicate that RACB is involved in the modulation of actin reorganization and cell polarity during the interaction of barley with this powdery mildew. Thus, evidence for Rac GTPases as good candidates for enhanced disease resistance through the manipulation of cytoskeletal reorganization is increasing.

Plants had to evolve a network of complex and diverse defence mechanisms designed to combat a wide variety of pathogens. As described above, the plant cytoskeleton represents a prominent target for pathogenic fungi because it plays a central role in penetration resistance, conveying a major general resistance to fungi. So far, only a few examples have been reported where pathogenicity has been related to an anti-actin activity of fungi (Tsurushima et al. 2005; Yuan et al. 2006). However, disruption of the actin cytoskeleton by anti-actin reagents that could suppress actin-dependent penetration resistance caused a prominent induction of defence responses in tobacco cells (Kobayashi et al. 2007). In R-gene-mediated specific resistance, effectors that enable pathogens to overcome general resistance have been recently shown to be recognized, directly or indirectly, by specific NB-LRR proteins encoded by R-genes (Jones and Dangl 2006). According to the "guard hypothesis" (Dangle and Jones 2001), perturbation of a host target by a pathogenic effec-

tor generates a "pathogen-induced modified self" molecular pattern, which in turn activates the corresponding NB-LRR protein. It is likely that disintegration of important components of general resistance, such as the plant cytoskeleton, may be "guarded" by unknown monitoring proteins. It is highly suggestive that deficiencies in the function of genes related to general resistance, such as the callose synthase GSL5 (Nishimura et al. 2003) or the ABC transporter PEN3/PDR8 (Stein et al. 2006), result in activation of salicylic acid-dependent resistance. A better understanding of these mechanisms may help us to develop a novel type of disease-resistant plants.

The cytoskeleton controls a variety of cellular activities and thus appears to play an important role in defence-related responses. Pathogens often take advantage of the genetic conservation and functional plasticity of the plant cytoskeleton. However, this also means that the cytoskeleton is a good target for approaches with the aim of protecting plants from various biotic stresses.

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Microtubules and Viral Movement

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Abstract The spread of plant virus infection depends on specialized virus-encoded movement proteins (MP) that target plasmodesmata (PD) to facilitate viral movement from cell to cell. Cell biological studies have shown that the MP of tobacco mosaic virus (TMV) accumulates in PD and also associates with membranes and the cytoskeleton during infection. Whereas the targeting of the protein to PD involves the endoplasmic reticulum–actin network, additional contacts with elements of the microtubule (MT) cytoskeleton are implicated in the transport of viral RNA. This article provides an overview of recent and current findings that describe the interactions between MP and MT during early and late stages of TMV infection.

1 Introduction

The spread of tobacco mosaic virus (TMV) within infected plants depends on the replication of the viral RNA (vRNA) genome in each infected cell and on the successful transport of vRNA into the adjacent cells. Both processes depend on intricate interactions between the virus and its host. The vRNA encodes four major protein products: the 126- and 183-kD replicase proteins, the 30-kD movement protein (MP), and the 17.5-kD coat protein (CP). The two virus-encoded replicase proteins are found in replication complexes isolated from infected plants (Osman and Buck 1996; Watanabe et al. 1999). Although they do interact (Goregaoker et al. 2001), the 183-kD protein alone is sufficient for replication in protoplasts, whereas the 126-kD protein has complementary functions that increase the efficiency of replication (Ishikawa et al. 1986; Lewandowski and Dawson 2000). The MP and CP are dispensable for replication (Lewandowski and Dawson 2000; Meshi et al. 1987; Takamatsu et al. 1987), but are required for cell-to-cell and long-distance movement (Dawson et al. 1988; Deom et al. 1987). Whereas the MP provides the basic trans-acting functions required for both cell-to-cell and long-distance movement, the CP is required for long-distance transport but not for local cell-to-cell movement (Dawson et al. 1988; Hilf and Dawson 1993; Saito et al. 1990; Siegel et al. 1978), indicating that TMV moves between cells in a nonencapsidated form. The MP binds single-stranded nucleic acids in vitro (Citovsky et al.

1990), and since complexes of MP and vRNA were isolated from TMV-infected plants (Dorokhov et al. 1983, 1984), it appears probable that MP binds vRNA and, thus, forms the core of the particle that spreads between cells. However, whether such vRNA particles are indeed formed in vivo remains to be shown. A number of observations suggest that, in addition to the MP, the 126-kD replicase is also involved in cell-to-cell movement (Derrick et al. 1997; Hirashima and Watanabe 2001; Holt et al. 1990; Knapp et al. 2001, 2005). However, the mechanistic role of the 126-kD replicase in movement is not known. In contrast, for the MP, it has been established that this protein localizes to plasmodesmata (PD) and modifies the size-exclusion limit (SEL) of the pores (Atkins et al. 1991; Ding et al. 1992a; Moore et al. 1992; Oparka et al. 1997; Tomenius et al. 1987; Waigmann et al. 1994; Wolf et al. 1989), thus enabling the movement of macromolecules including vRNA. Moreover, during infection as well as in transient expression assays, the protein associates with endoplasmic reticulum (ER) membranes and the cytoskeleton, suggesting a role of these cellular components in vRNA movement (Heinlein et al. 1995, 1998a; McLean et al. 1995). Microtubules (MT) seem to represent an important player, although recent years have seen some debate about their role. This article aims to give an overview of the interactions between MP and host cell components, and will pay particular attention to a discussion of MT and their function in viral pathogenesis. For further reading about PD, other MP-interacting factors, and plant virus infection in general, the reader is referred to other recent review articles (Heinlein and Epel 2004; Oparka 2005; Waigmann et al. 2007; Waigmann and Heinlein 2007).

2 Studies to Localize the MP in Infected Cells

One approach to gain insight into the mechanisms by which MP facilitates the spread of vRNA has been to localize MP in infected cells. First attempts to identify intercellular targets of the protein employed immunoelectron microscopy (Atkins et al. 1991; Meshi et al. 1992; Moore et al. 1992; Tomenius et al. 1987) and biochemical fractionation using virus-infected tissues and transgenic plants overexpressing MP (Deom et al. 1990; Moore et al. 1992; Moser et al. 1988). According to these studies, the MP was present in cell wall- and plasma membrane-rich fractions and was localized to branched PD. Other biochemical analyses indicated that the MP is associated with the microsomal fraction as an integral membrane protein (Reichel and Beachy 1998). Consistently, biochemical studies using recombinant proteins expressed in *Escherichia coli* led to the suggestion that MP contains two protease-insensitive α-helical transmembrane domains (Brill et al. 2000).

With the introduction of the *Aequorea victorea* green fluorescent protein (GFP) into plant biology (Baulcombe et al. 1995; Haseloff and Amos 1995;

Niedz et al. 1995), TMV derivatives were developed that express the MP as a functional MP:GFP fusion protein (TMV-MP:GFP) (Epel et al. 1996; Heinlein et al. 1995) and thus allow the analysis of infection sites as well as the subcellular localization and function of MP during vRNA replication and movement in living plant leaf tissues (Heinlein et al. 1995, 1998a; Padgett et al. 1996). Infection in leaves of susceptible *Nicotiana* species, such as *Nicotiana benthamiana*, by TMV-MP:GFP produced radially expanding fluorescent infection sites (Fig. 1A). The leading edge of these sites reflects the leading front of the spreading infection, as was shown by experiments involving manual incisions to the leaf lamina. When these incisions were made just in front of

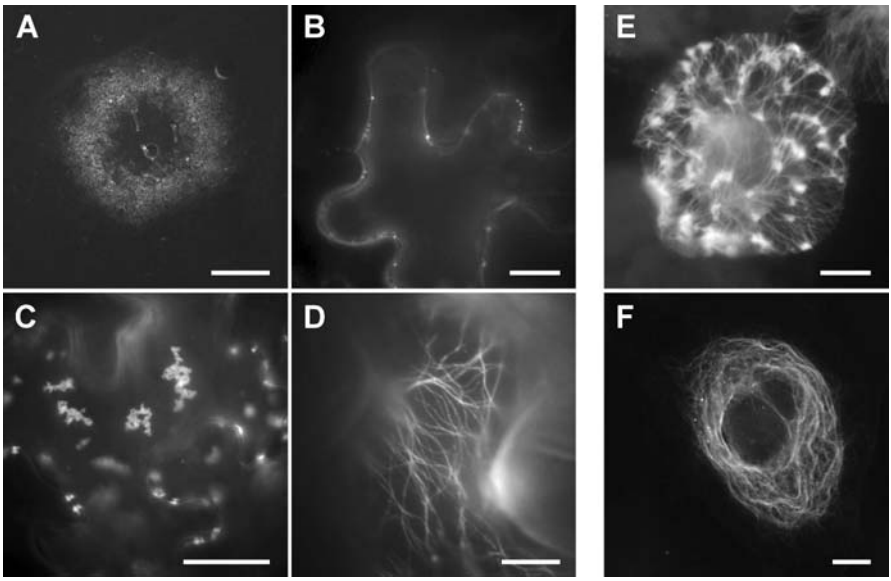

Fig. 1 The MP of TMV localizes to MT and other subcellular components (Heinlein et al. 1998a). The distribution of MP between inclusion bodies (IB) and MT is temperature-dependent (Boyko et al. 2000b). The pictures show the distribution at room temperature (22 °C). **A** Infection site of TMV-MP:GFP in *N. benthamiana* (5 days post infection (dpi), 22 °C). *Scale bar*: 500 μm. **B** Enlargement of an epidermal cell located at the front of the infection site. The MP is localized to PD in the lateral cell walls. Only one cell is focussed in the illumination beam. Adjacent cells are also infected but not shown here. *Scale bar*: 10 μm. **C** Enlargement of an epidermal cell located in the highly fluorescent area of the infection site. The MP is localized to large IB that are formed by transient aggregation of the ER. *Scale bar*: 10 μm. **D** Enlargement of an epidermal cell located at the trailing rim of the highly fluorescent area. The MP:GFP is localized to MT. *Scale bar*: 5 μm. **E** Distribution of MP:GFP in a TMV-MP:GFP-infected BY-2 protoplast at 16 hours post infection. The protein is localized to MT and MT-associated IB. *Scale bar*: 10 μm. **F** Mammalian COS7 cell transiently expressing wild-type MP and stained with MP antibody. The MP is localized to MT. MT in MP-expressing cells are detached from the centrosome and form abnormal arrays that encircle the nucleus. *Scale bar*: 10 μm

the leading edge of fluorescence, further spread of infection was interrupted. However, when the incision was made just behind the leading fluorescent cells, the infection continued to spread (Oparka et al. 1997). Since the CP is not required for cell-to-cell movement of the virus, the TMV-MP:GFP derivatives were constructed with and without CP. A CP-containing construct that was analyzed in detail produced less MP:GFP during infection as compared to the construct lacking the CP (Heinlein et al. 1998a). However, the efficiency by which the virus spreads between cells was not affected (Heinlein et al. 1998a; Szécsi et al. 1999). This observation indicates that the abundance of MP:GFP produced during TMV-MP:GFP infection is higher than the amount needed for the spread of infection. Yet, the amount of MP:GFP produced by TMV-MP:GFP is lower than the amount of MP produced by wild-type TMV (Szécsi et al. 1999). Interestingly, TMV is able to move from cell to cell even if the level of MP produced during TMV infection is reduced to 2% (Arce-Johnson et al. 1995). Although at first glance this may appear surprising, this finding is rather consistent with the fact that infection of a new cell starts from zero and spreads forward into yet another cell within a short time, before any higher amount of MP can accumulate. Therefore the cells at the front of the spreading infection site contain very low amounts of the protein.

Of course, it needs to be asked why at later stages of infection the cells accumulate MP to levels much higher than to the level required for the spread of infection. It may be possible that high amounts of MP are required for movement in certain hosts of TMV. On the other hand, the high amounts of MP that accumulate in cells behind the infection front may provide auxiliary functions, for example, in the protection of the replicating virus against plant defense responses or against competing viruses. Nevertheless, as described below (Sect. 2.1), the strong expression of MP:GFP in infected cells made it possible to visualize binding targets of the protein by fluorescence microscopy. Whether the MP:GFP-labeled structures are involved in the function of MP in vRNA movement, or whether the observed interactions only occur due to excessive MP:GFP, has been determined by functional tests, as is also described further below (Sects. 2.2 and 2.3).

2.1
MP:GFP Associates with MT and Other Specific Targets in Infected Cells

When leaves of *N. benthamiana* are infected by TMV-MP:GFP or TMV-MP:GFP-CP and then observed by fluorescence microscopy, the accumulation of MP:GFP at specific subcellular sites can be followed over time. Cells were sequentially analyzed starting with cells at the leading front of the infection site, representing the earliest stages of the infection, and progressing toward the center of the infection site, representing progressively later stages of the infection (Heinlein et al. 1995, 1998a; Oparka et al. 1997). Thus, during early stages of the infection, MP:GFP accumulates in PD (Fig. 1B) and also asso-

ciates with the cortical ER. Later on, the protein accumulates in ER-associated inclusion bodies (IB) as well as on MT (Fig. 1C,D). Finally, MP:GFP fluorescence disappears from all locations except from PD (Heinlein et al. 1998a). Since CP-expressing constructs express lower amounts of MP:GFP than the constructs lacking the CP, they accumulate less detectable MP:GFP in IB and on MT (Heinlein et al. 1998a). Associations of MP:GFP with PD, IB, and MT were also observed by Padgett et al. (1996) who investigated infection sites of constructs derived from tomato mosaic tobamovirus Ob (ToMV-Ob). The accumulation pattern of the MP:GFP of this virus was compared between two different hosts, *N. benthamiana* and *N. tabacum*. Although the overall expression level of MP:GFP was lower in *N. tabacum* than in *N. benthamiana*, the protein accumulated on MT throughout the fluorescent area of the infection site. Thus, the observation of MT-associated MP:GFP cannot be simply ascribed to unspecific association due to excess MP:GFP, but rather reflects a specific interaction of MP with the host. The presence of MP-associated MT in cells near the leading edge of infection sites in *N. tabacum* is consistent with a role of MT in targeting the MP and/or vRNA to PD. However, since in *N. benthamiana* MP:GFP-associated MT were visible only in cells behind the leading edge of infection, i.e., rather adjacent to cells in which the MP:GFP disappeared except from PD, Padgett et al. (1996) suggested that MT may have a role in MP degradation. Thus, whether MT function in the targeting of MP and/or vRNA to PD or whether they rather play a role in the degradation of MP has been subject to intense investigations during recent years (Ashby et al. 2006; Boyko et al. 2007; Gillespie et al. 2002) (see also Sect. 2.9).

2.2 Localization of MP to IB and Role of ER

Immunostaining of infected cells with antibodies against luminal ER proteins led to the conclusion that the IB are derived from cortical ER to which MP localizes very early during infection (Heinlein et al. 1998a; Más and Beachy 1999). In addition to MP, they contain replicase (Heinlein et al. 1998a), vRNA (Más and Beachy 1999), and also accumulate CP (Asurmendi et al. 2004), indicating that they are associated with virus-replication complexes (VRC) (Asurmendi et al. 2004; Kawakami et al. 2004; Liu et al. 2005).

Recent studies on mechanically inoculated epidermal cells indicate that VRC-containing IB may undergo cell-to-cell movement from the initially inoculated cells to adjacent cells (Kawakami et al. 2004). However, while it seems plausible that the spread of infection is associated with the cell-to-cell movement of membrane-associated VRC, it seems rather unlikely that TMV replication and vRNA movement depend on the formation of IB. For example, although there is evidence that the formation of IB involves MP (Más and Beachy 1999; Reichel and Beachy 1998), TMV mutants that lack MP replicate normally (Meshi et al. 1987). Moreover, a TMV derivative encoding a func-

tional MP:GFP mutant that lacks 55 amino acid from the C terminus of MP allowed the spreading of infection in *N. benthamiana* leaves, although cells within infection sites did not exhibit any IB that contain MP:GFP (Boyko et al. 2000c). Finally, the amount of MP:GFP in IB is strongly reduced upon increasing the temperature by 10 degrees from 22 to 32 °C, although the higher temperature is three times more permissive for movement than the lower temperature. Apparently, this observation may indicate that IB are formed as a consequence of local overaccumulation of MP at ER-associated replication sites under restrictive temperature conditions (Boyko et al. 2000b). This notion is further supported by evidence indicating that infection associates with the ER through an intrinsic property of vRNA and/or replicase (Más and Beachy 1999). Thus, it appears likely that the association of MP with the native cortical ER network early in infection and its subsequent presence in the ER-derived IB during later stages of infection (Heinlein et al. 1998a) occurs as a consequence of ER-associated viral replication. However, irrespective of whether or not IB are formed, the underlying ER is contiguous between adjacent cells through PD (Ding et al. 1992b; Epel 1994; Overall and Blackman 1996) and thus is likely to play a central role in guiding MP and vRNA from replication sites to the channel. This hypothesis is consistent with the tight association of ER membranes with actin filaments, which provide structural stability and motor force to the network (Allan and Brown 1988; Karchar and Reese 1988; Lichtscheidl and Baluska 2000; Quader et al. 1989; Staehelin 1997).

2.3
Localization of MP to MT Correlates with Function in vRNA Transport

The observation that MP decorates MT (Heinlein et al. 1995, 1998a; McLean et al. 1995; Padgett et al. 1996) and also actin microfilaments (McLean et al. 1995) suggested the involvement of cytoskeletal elements in PD targeting and cell-to-cell movement of vRNA (Carrington et al. 1996; Heinlein et al. 1995; Zambryski 1995). Obviously, this hypothesis is consistent with observations in many different biological systems that the coordinated activities of cytoskeletal components are responsible for the specific transport of RNAs, as well as the anchoring of RNAs at their destinations (reviewed by Palacios and St. Johnston 2001; St. Johnston 2005). However, unlike the situation in animal systems, where the role of MT in RNA transport is well documented, there are only very few examples for such a role of MT in the plant kingdom (e.g., Becht et al. 2006).

Nevertheless, in vivo studies using TMV derivatives encoding functional, dysfunctional, and temperature-sensitive mutants of MP fused to GFP demonstrated a correlation between the association of MP:GFP with MT and the function of MP:GFP in the intercellular spread of infection, thus indicating a role of MT in vRNA movement during early infection (Boyko et al. 2000a,b,c, 2002, 2007; Kotlizky et al. 2001; Fig. 2).

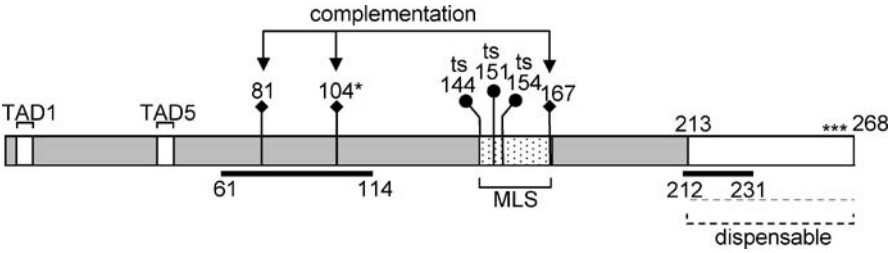

Fig. 2 Mutations in MP that have been used to characterize the functional significance of MT association of the protein. The analysis of progressive C- and N-terminal deletion mutations demonstrated that the function of MP in TMV movement as well as MT association depend on amino acids 1–213, and that the 55 C-terminal amino acids of the protein are dispensable (Boyko et al. 2000c). TAD1 and TAD5 are three amino acid deletions in MP. TAD1 (also referred to as MPΔaa3–5, NT1, dMP) inactivates MP and causes constitutive MT association of the MP. It also interferes with normal MT association of wild-type MP in trans (Kotlizky et al. 2001). TAD5 inactivates MP and interferes with MT association of MP in plant cells (Kahn et al. 1998). Exchange of the proline residue for serine at position 81 of the MP inactivates the protein and interferes with MT association. This defect is alleviated by additional threonine for isoleucine and arginine for lysine exchange mutations at positions 104 and 167, respectively. Functional intramolecular complementation between residues 81, 104, and 167 indicates that these residues are critical for the central fold of the protein (Boyko et al. 2002). Temperature-sensitive amino acid exchange mutations are clustered in a domain of MP showing similarity with the M-loop of tubulin (M-loop similarity, MLS). Exchange of arginine for glycine, glycine for valine, or proline for serine at positions 144, 151, and 154, respectively, causes reversible inactivation of MP with respect to function and MT association at the restrictive temperature (Boyko et al. 2000a, 2007). MT association of the MP may be regulated by phosphorylation. Domains of MP to which phosphorylation has been mapped are highlighted by *black bars*. Phosphorylation of specific amino acids that have been addressed experimentally is indicated by *asterisks*. Phosphorylation of the threonine at position 104 as well as phosphorylation events at amino acid positions 258, 261, and 265 in the C terminus of MP appear to restrict the function of MP in TMV movement (Karger et al. 2003b; Trutnyeva et al. 2005)

1. The analysis of infection by TMV-MP:GFP demonstrated that the increased efficiency by which TMV spreads in infected plants at an increased temperature of 32 °C (Boyko et al. 2000b; Lebeurier and Hirth 1966; Matthews 1991) is correlated with an increased association of MP with MT (Boyko et al. 2000b).
2. A series of TMV-MP:GFP derivatives carrying progressive N-terminal and C-terminal deletion mutations in MP was used to demonstrate that amino acids 1–213 required for the function of MP in virus movement are also required for MT association of the protein. Any amino acid deletion at the N terminus or truncation from the C terminus toward the N terminus beyond amino acid 213 led to functional inactivation of MP and abolished the ability of the protein to associate with MT (Boyko et al. 2000c).

3. An internal three amino acid deletion mutation spanning amino acids 49–51 (TAD5) abolished both function and MT association of the protein (Kahn et al. 1998), whereas the deletion of amino acids 3–5 (MP^{NT-1}, also referred to as TAD1, dMP, MPΔaa3–5), caused a loss of function due to constitutive MT association of the protein (Kotlizky et al. 2001). Moreover, expression of MP^{NT-1} interfered with MT association of virus-encoded MP during infection (Kotlizky et al. 2001), thus providing a possible explanation for virus resistance in plants expressing the mutant protein (Cooper et al. 1995; Lapidot et al. 1993).
4. The role of MT gained support also by the analysis of a TMV mutant carrying a proline-to-serine exchange mutation at position 81 of the MP (Pro81Ser), which abolishes the function of the protein in TMV movement. Interestingly, the activity of this mutant protein is restored if the Pro81Ser mutation is complemented by additional mutations at two other amino acid positions (Thr104Ile and Arg167Lys) of the protein (Deom and He 1997). Functional restoration of the Pro81Ser mutation by the Thr104Ile and Arg167Lys mutations was conserved when these mutations were tested in the context of the MP encoded by TMV-MP:GFP. Importantly, functional recovery correlated with recovery of MT association of the protein, thus supporting a role of MP:MT interactions during TMV movement (Boyko et al. 2002). Moreover, the fact that the function of MP in vRNA transport was restored by intramolecular complementation of the dysfunctional Pro81Ser amino acid exchange mutation by distant Thr104Ile and Arg167Lys exchange mutations indicates that the core region of MP folds into a functionally required tertiary structure, which allows distant primary sequence and secondary structure elements to interact (Boyko et al. 2002; Fig. 2).
5. Perhaps the strongest line of evidence for a functional role of MT in TMV movement was provided by the analysis of temperature-sensitive mutations in MP (Fig. 2), which confer a movement-deficient phenotype at an increased temperature of 32 °C (Jockusch 1968; Nishiguchi et al. 1978; Ohno et al. 1983). The amino acid exchange mutations responsible for the temperature-sensitive movement phenotype were introduced into TMV-MP:GFP and did indeed confer temperature sensitivity to this virus. This was first tested by the formation and size of lesions in hypersensitive tobacco (*N. tabacum* cv Xanthi NN). The plants carry the N-resistance gene that upon infection triggers a hypersensitive reaction that leads to the formation of necrotic lesions. To address the movement of the virus, a temperature-shift protocol was applied (Boyko et al. 2000a, 2007; Jockusch 1968) which uses the fact that the N-gene mediated hypersensitive response is temperature sensitive (Kassanis 1952). Thus, plants kept at high temperature (32 °C) allow a virus with an intact movement function to spread efficiently from cell to cell. Subsequently, when the plants are placed at a temperature permissive for N (22 °C), the newly infected

cells are revealed by necrosis triggered by the hypersensitive reaction. However, while TMV-MP:GFP lesions showed a brownish halo of newly infected necrotizing cells, and thus revealed that the MP:GFP is active at high temperature, no newly infected cells were observed by this reaction in the case of the mutant derivatives, thus confirming that these mutants carry a temperature-sensitive movement function, which is inactivated at high temperature. The same mutants were then used for inoculation of *N. benthamiana*, which does not carry the N gene and is a susceptible host for TMV. As for the parental virus TMV-MP:GFP (Heinlein et al. 1998a), the mutant viruses also formed infection sites that appeared as radially expanding green-fluorescent rings. However, unlike in the case of TMV-MP:GFP, the infection sites caused by the mutant viruses ceased to expand at high temperature and thus confirmed the temperature sensitivity caused by the mutations. Importantly, the subcellular analysis of the infection sites revealed conspicuous changes in the localization of MP:GFP in response to temperature. Whereas the MP:GFP of the parental virus showed reduced accumulation in IB and increased association with MT at high temperature, which previously was shown to be correlated with strongly increased cell-to-cell spread of the virus (Boyko et al. 2000a,b), the temperature-sensitive MP:GFP proteins accumulated in IB but failed to show any association with MT under these conditions (Boyko et al. 2000a, 2007). The temperature-induced changes in function and subcellular localization patterns are fully reversible. Thus, when the temperature is decreased from restrictive temperature (32 °C) to permissive temperature (22 °C), the infection sites caused by the mutant viruses resume expansion and the mutant MP:GFP proteins again exhibit MT association (Boyko et al. 2000a, 2007).

Collectively, these functional studies revealed a strict correlation between MT association and function of MP in viral movement. A role of MT was also indicated by studies using infected protoplasts and a combination of antibody labeling and in situ hybridization procedures, which showed that vRNA was localized to MT in a manner dependent on MP (Más and Beachy 1999). Moreover, the localization of vRNA on MT depended on the competence of MP to bind MT, since vRNA was mislocalized in cells expressing a mutant MP (TAD5, Kahn et al. 1998) that binds vRNA but fails to associate with MT (Más and Beachy 2000).

The mutations employed in functional analysis revealed the importance of specific domains of MP that appear to be involved in MT association. It appears highly intriguing that amino acid mutations that affect MP function and MT association in a temperature-sensitive manner cluster in a small domain of MP that bears structural similarity to the M-loop of α-, β-, and γ-tubulin (Boyko et al. 2000a; Fig. 2). The tubulin M-loop interacts with the N-loop of tubulin molecules in the adjacent MT protofilaments and thus plays an im-

portant role in the assembly and stability of MT (Nogales et al. 1999). The similarity with the tubulin M-loop may allow the MP to directly bind to free or assembled tubulin of either isoform, including γ-tubulin, and may also identify the MP as a binding target for tubulin cofactors.

2.4 In Cells Engaged in vRNA Transport the MP is Associated with Mobile, MT-Proximal Particles

Although the analysis of virus mutants indicated a correlation between a MP function in viral movement and the association of the protein with MT, the particular role of MT during the movement process remains unclear and requires further study. Despite the observation of MP:GFP accumulation on MT in cells near the infection front (Boyko et al. 2000b; Padgett et al. 1996) or in cells infected with temperature-sensitive viruses undergoing functional recovery (Boyko et al. 2000a, 2007), it remains unclear whether MP binds MT in parallel, during, or after MP has executed its function in vRNA movement. To determine whether or not there is a direct role of MT in TMV movement, the movement process should be visualized directly. Unfortunately, direct visualization is hampered by the generally very low abundance of MP in cells at the front of the spreading infection site. Thus, although cells at the leading front of spreading infection sites of TMV-MP:GFP or TMV-MP:GFP-CP showed detectable MP:GFP fluorescence in PD, other associations with cellular components that might be involved in the targeting of MP and/or vRNA to PD could not be detected (Heinlein et al. 1998a).

One way out of this dilemma is again the use of TMV derivatives carrying temperature-sensitive mutations. As described above (see Sect. 2.3), these mutations allow specific interference with the cell-to-cell progression of infection at the restrictive temperature. Because, despite these mutations, the virus continues to replicate and produce MP:GFP under these conditions, the leading front cells become loaded with the protein. Having now accumulated a high amount of MP in the leading front cell, the participation of this protein in the movement process can be studied upon restoration of function at the permissive temperature. Thus, as described above (see Sect. 2.3), previous studies have shown that the protein that accumulates in IB in cells of the leading front at the restrictive temperature redistributes onto MT when the restricting temperature is replaced by the permissive temperature, and this redistribution is correlated with the restoration of the vRNA cell-to-cell movement function of MP (Boyko et al. 2000a). However, in these experiments, the cells were observed one day after the onset of the application of the permissive temperature. As a result of this timing, the observed reassociation of MP:GFP with MT may again represent a rather late event, i.e., an event occurring after the vRNA has already been transported into noninfected cells.

To overcome this problem, in recent studies the cells were monitored directly after the transfer of the infected plants from the restrictive to the permissive temperature (Boyko et al. 2007). Interestingly, under these conditions, small, mobile, MP:GFP-associated particles were observed that apparently moved away from the IB toward the cell periphery. This finding suggests a role of mobile, MP:GFP-associated particles in vRNA movement. The reinvestigation of infection sites caused by functional TMV derivatives under normal conditions demonstrated the presence of similar MP:GFP-carrying particles in the front cells of infection, thus excluding the possibility that the particles occur as an artifact of temperature-sensitive mutations or the applied temperature conditions. However, due to the very low fluorescence of particles in infection sites of TMV-MP:GFP, only rare images could be taken by long exposure of a highly sensitive CCD and any analysis of their dynamic behavior was impossible. Nevertheless, cells in the leading front containing mobile particles could be observed in plants infected with a chimeric virus in which the MP of TMV-MP:GFP was replaced by the MP of ToMV-Ob (Padgett and Beachy 1993). Inoculation of plants in which MT were labeled by expression of GFP-tagged TUA6 from *Arabidopsis thaliana* (*tua-GFP*; Gillespie et al. 2002) revealed that at least some of the mobile particles translocated in apparent proximity to MT (Boyko et al. 2007). Importantly, in cells in which the MP:GFP started to accumulate on the MT themselves, the particles ceased to be mobile. While it remains unclear whether the accumulation of MP:GFP on MT directly inhibited the trafficking of MP-containing particles, this observation may indicate that TMV effectively limits intracellular transport processes upon transition from the early to mid stages of infection. Thus, it needs to be noted that there are two different associations of MP with MT that need to be distinguished. At early infection stages, during which vRNA transport is likely to occur, MP is observed in mobile MT-proximal particles. Later during infection, when particle movements are abolished, MP is clearly seen to accumulate on MT (Boyko et al. 2007).

It may be possible that such sequential activities are regulated at the level of phosphorylation, since MP is known to be phosphorylated in vivo and in vitro (Citovsky et al. 1993; Haley et al. 1995; Karpova et al. 1999; Waigmann et al. 2000; Watanabe et al. 1992). Interestingly, amino acid residue Thr-104, which apparently interacts with Pro-81 and Arg-167 and is involved in MP function and MT interaction (Boyko et al. 2002; Deom and He 1997), was also identified as a phosphorylated residue (Karger et al. 2003; Fig. 2). Phosphorylation at this site may cause a change in the interaction with Arg-167, which is located within the M-loop similarity domain of MP and thus may affect the role of this domain in target binding. Phosphorylation of Thr-104 is not essential for MP function, since replacement of Thr-104 by the non-phosphorylatable Ala does not affect viral movement. However, substituting Thr-104 with negatively charged phosphorylation mimicking amino acid residue Asp strongly inhibits cell-to-cell spread

of the mutant virus in *N. tabacum* plants. Thus, phosphorylation at Thr-104 may serve as an inactivation mechanism. The phosphorylated and inactivated form of MP may be MT-associated, as suggested by the observation that MT-associated MP occurs in the form of potentially phosphorylated isoforms that, at the resolution of two-dimensional electrophoresis, differ by ionic charge (Ashby J., Brandner K., Boutant E., Heinlein M., unpublished results). Whether MP phosphorylated at Thr-104 is indeed MT associated has not been investigated. However, the possibility that phosphorylation turns MP into an inactive, MT-associated form would be consistent with the occurrence of functionally different isoforms of MP during early and late stages of infection.

The observation of mobile, proximal particles containing MP:GFP might be reminiscent of mobile, MT-associated RNA particles observed in animal systems (Hirokawa 2006; Sossin and DesGroseillers 2006) and suggests that MT play a role during early infection, potentially in the transport of the infectious particle that spreads between cells. Additional studies will be required to determine whether the particles do indeed contain vRNA and whether they are targeted to PD for intercellular transport. Moreover, their potential association with membranes must be investigated, since recent observations in initially infected cells suggest that TMV-MP:GFP infection spreads both intracellularly and from cell to cell in the form of membrane-derived, MP:GFP-associated replication complexes (Kawakami et al. 2004). However, although the particles may be associated with membranes and replication complexes, they probably do not represent secretory vesicles since neither TMV movement nor the targeting of MP to PD is affected by treatment of plants with brefeldin A, a known secretory pathway inhibitor (Tagami and Watanabe 2007).

In the context of the hypothesis that the particles may contain replication complexes, it is important to note another observation, namely that the mobile particles that emerged from large IB upon functional recovery of temperature-sensitive MP:GFP preceded the formation of new IB in the periphery of the cells. Indeed, although peripheral IB occur in cells infected under normal conditions, especially at the sites near PD (Padgett et al. 1996), they were absent in cells infected by temperature-sensitive virus at the restrictive temperature and only occurred at later time points following recovery at the permissive temperature. Given the evidence suggesting that vRNA moves in association with subunits of replication complexes (Kawakami et al. 2004), and the observations indicating that MP:GFP-containing replication sites occur near MT (Heinlein et al. 1998a), it appears conceivable that the MT-associated particles represent subunits of replication complexes that move to or along the cellular periphery not only to target PD and to release vRNA and associated factors for intercellular movement, but also for laying down the foundation for new peripheral infection sites.

2.5
TMV Movement is Not Affected by MT-Disrupting Agents

The observation that the spread of infection correlates with mobile MP:GFP-associated particles that are translocated along MT in cells at the leading front of infection provides strong evidence for a direct role of MT in TMV RNA transport. To investigate the role of MT in TMV movement, several laboratories, including ours, tested the spread of infection in the presence of MT-disrupting agents. Surprisingly, it was found that the process of viral cell-to-cell spread could not be efficiently inhibited (Ashby et al. 2006; Gillespie et al. 2002; Kawakami et al. 2004). There are two possibilities to explain this finding. The first and most straightforward explanation would be that TMV movement is MT-independent. However, in this case it would be important to ask why the interaction of MP with MT is conserved in many cell types and why it correlates with MP function. The other possible explanation, which is also very simple, is that the treatment of leaves with MT polymerization inhibitors does not disrupt MT completely. Since the spread of infection requires the intercellular transport of only very few virus particles (Li and Roossinck 2004; Sacristan et al. 2003), a complete disruption of MT activities is expected to be necessary to affect TMV movement. Given also that local events at only one of the many PD that connect a cell with adjacent cells may suffice to infect the adjacent cell, and that both the MP and the viral genome are expressed to high levels during infection (Arce-Johnson et al. 1995; Heinlein et al. 1998a; Padgett et al. 1996; Szécsi et al. 1999), the inhibition of virus movement should be extremely difficult to achieve unless a full disruption of the cellular transport mechanism can be established. Studies to investigate the role of MT by applying MT-disrupting agents used GFP fused to Arabidopsis α-tubulin TUA6 (tua-GFP) as a visible MT marker to demonstrate successful MT disruption in parallel assays (Gillespie et al. 2002). However, although these assays demonstrated the accumulation of nonlocalized tua-GFP and, thus, provided evidence for inhibited polymerization of new MT, these assays did not provide sufficient resolution to demonstrate the disruption of existing, perhaps stabilized, MT. Indeed, by staining cells treated by antimicrotubular drugs with a tubulin antibody, Seemanpillai et al. (2006) could demonstrate that the treated cells, in which tua-GFP-labeled MT have disappeared, can still contain fragments of polymerized endogenous tubulin or even intact MT. Moreover, inoculation of treated plants with a TMV derivative encoding a mutant but functional MP:GFP that exhibits enhanced MT association in cells at the leading front of infection revealed the presence of MP:GFP-labeled MT in newly infected cells into which the virus had just spread. Thus, although TMV infection may not require MT to be intact completely, a role of localized MT activities during the vRNA movement process cannot be ruled out (Seemanpillai et al. 2006). The lack of inhibition of the vRNA movement by MT-disrupting agents may in part also be due to the ability of MP to stabilize MT (see be-

low, Sect. 2.6). In fact, since the MP can move between cells by itself (Kotlizky et al. 2001; Waigmann et al. 1994), it may be possible that the protein is able to stabilize MT in cells ahead of the spreading virus.

2.6 Characterization of MT-Associated MP

The ability of MP to interact with MT is preserved upon transient expression in both plant cells or mammalian cells (Boyko et al. 2000a; Ferralli et al. 2006; Heinlein et al. 1998a), indicating that the ability to bind MT is independent of other viral products or specific plant factors. Studies in MP-transfected mammalian cells indicate that the association of MP with MT persists upon disruption of the ER and actin networks, as well as upon inactivation of the MT motor dynein (Ferralli et al. 2006). The protein may bind to MT even without the aid of any accessory protein, since recombinant MP isolated from *E. coli* was shown to bind to both tubulin dimers and assembled MT in vitro (Ashby et al. 2006; Ferralli et al. 2006). Cosedimentation assays, in which a given amount of preassembled MT were incubated with increasing amounts of MP, indicate that MP binds MT in a saturable manner, with a K_d value of 71.6 ± 14.5 nM and a binding stoichiometry of 1.4 ± 0.12 MP/tubulin monomer (Ashby et al. 2006). It is unclear whether a single equivalent binding site for MP resides on the MT surface or whether MP binds MT in a dimeric form (Brill et al. 2004). Nevertheless, the binding affinity of MP for MT is within the known range for high-affinity microtubule-associated proteins (MAP) (Ackmann et al. 2000). Moreover, similar to other MAPs, the MP stabilizes MT upon binding. MP:MT complexes isolated from TMV-infected plant cells as well as MP:MT complexes formed in vitro were shown to resist treatments with cold, calcium, and high concentrations of NaCl (Ashby et al. 2006; Boyko et al. 2000a). Moreover, MP-associated MT complexes formed in vivo during infection in *N. benthamiana* leaves, in MP-transgenic BY-2 cell lines, or in transfected mammalian cells, are stable against MT-disrupting agents (Ashby et al. 2006; Ferralli et al. 2006).

The stability of MT generated by MP association appears to be higher than the stability conferred by other tested plant and mammalian MAPs (Ashby et al. 2006; Ferralli et al. 2006). For example, MT stabilized by MP in transfected mammalian cells resist the treatment with cold or with 100 μM nocodazole, whereas MT stabilized by ectopically expressed MAP2 do not (Ferralli et al. 2006). This enhanced stability of MP-associated MT may be related to the increased salt stability of the MP:MT interaction (Ashby et al. 2006; Boyko et al. 2000a). This feature may also indicate that MP interacts with tubulin through a rather specific mechanism. The existence of such a specialized mode of binding is supported by the fact that all temperature-sensitive mutations in MP that affect the function of the protein in vRNA in a temperature-sensitive manner cluster in the small domain in MP showing structural similarity to

the M-loop of tubulin (Boyko et al. 2000a, see Sect. 2.3). This loop mediates tubulin–tubulin interactions between adjacent MT protofilaments. It has been speculated that in mimicking this important tubulin–tubulin interaction domain, the MP may be able to intercalate into the MT lattice after or during assembly (Boyko et al. 2000a; Heinlein 2002). However, results of experiments involving fluorescence recovery after photobleaching (FRAP) argue against this possibility. In infected cells as well as in cells expressing MP:GFP from a transgene, fluorescence recovery of MT-associated MP was uniform within the bleached area of the MT, that is, in no cases did MP:GFP appear to move directionally along the MT from nonbleached areas into bleached areas. In this respect, the MP:GFP fluorescence recovery pattern was the same as that observed for GFP:MAP65.5, thus indicating that MP binds to the external surface of MT, just as a conventional MT-stabilizing MAP (Ashby et al. 2006). On the other hand, one has to take note of the fact that MT in plants are bundled and may even occur in opposite directions within the bundles. Thus, directional movements of MP within the lattice of dynamic, treadmilling MT may not be revealed by FRAP experiments.

2.7 MT Might Not Represent the Initial Target of MP

While the similarity to the tubulin M-loop may allow the MP to directly interact with tubulin, it may also pinpoint the protein as a target for interactions with MT-assembly cofactors or other MAP. Thus, although MP accumulates on MT, the initial target of MP may be a different factor. A first indication to support this possibility is provided by the observation that MP associates with mobile particles, situated proximally to MT, before it accumulates on the MT themselves (Boyko et al. 2007). Additional evidence comes from experiments using MP-transfected mammalian cells, which showed that MP expression interferes with the centrosomal attachment of MT as well as with the ability of centrosomes to renucleate MT upon depolymerization (Ferralli et al. 2006). The apparent ability of MP to interfere with centrosomal MT-nucleation activity was conserved even when the ability of MP to bind MT was abolished by an amino acid exchange mutation. This finding may indicate that MP interacts with factors involved in MT nucleation, polymerization, or anchorage before it eventually accumulates on the MT themselves. Thus, further research has to address the exciting potential ability of MP to manipulate the dynamics of the MT cytoskeleton rather than just binding it. Given the observation that MP overexpression in mammalian cells interferes with MT nucleation (Ferralli et al. 2006), it may be possible that MP has the ability to interact with MT-assembly factors and to reorient or newly establish specific MT-nucleating sites. Since in cells at the leading front of infection the MP is expressed to only very low levels and accumulates in or near PD (Heinlein et al. 1998a), such activity of the protein may play a role in the vicinity of the channel.

Interestingly, it was shown that transgenic tobacco plants overexpressing MP contain fibrous MP-associated material in the cavity of their PD (Ding et al. 1992a; Lapidot et al. 1993; Moore et al. 1992), which may be consistent with an ability of MP to cause the formation of a cytoskeletal intercellular transport structure within PD. This hypothesis may be supported by the analysis of multicellular cyanobacteria overexpressing MP, where cytoskeletal, likely FtsZ-associated, filaments across the intercellular septa have been observed (Heinlein 2006; Heinlein et al. 1998b; Boutant and Heinlein, unpublished results). Potentially related to a cytoskeleton-manipulating ability of MP may also be the observation that infected plant protoplasts, or protoplasts transiently expressing the MP, form plasma membrane protrusions into the medium (Heinlein 2002; Heinlein et al. 1998a), a behavior that may reflect a process by which MP causes the formation of a transport structure in the PD of walled cells. Evidence that the protrusions of protoplasts or PD contain tubulin is lacking. However, the hypothesis that MP may form a MT-based transport structure in PD may not be too far-fetched. A precedent for a viral manipulation of MT-organizing complexes in the formation of an intercellular transport structure is provided by the cell-to-cell transmission of human T-lymphotropic virus (HTLV-1) (Derse and Heidecker 2003; Igakura et al. 2003), which involves a reorganization of MT and a relocation of the MT-organizing center to cell–cell contacts and the formation of a "virological synapse" which allows the transfer of viral material between cells. Moreover, given that so-called tubule-forming plant viruses cause the assembly of a large tubelike transport structure in PD (Huang et al. 2000; Kasteel et al. 1996, 1997; Ritzenthaler and Hofmann 2007; Ritzenthaler et al. 1995; Satoh et al. 2000; van Lent et al. 1991; van Lent and Schmitt-Keichinger 2006), the ability of plant viruses to induce the formation of specific transport structures and gross structural changes in PD is well documented. Although the tubular transport structures of tubule-forming viruses are assembled from viral MP, it may nevertheless be possible that the TMV MP has specificially adapted to interact with the MT system to induce a novel transport structure through manipulation of tubulin and MT-assembly factors. Alternatively, the MP may target MT for anchorage, i.e., to sites at which movement-competent vRNA particles or replication-associated Ibs/VRC are formed. During early infection, however, an interaction with MT-assembly factors may be needed to induce tubulin assembly in order to interfere with constitutive anchorage and to release vRNA particles (VRC) for movement.

2.8 MPB2C, a MP-Binding Factor Involved in the Accumulation of MP on MT Late in Infection

The notion that MT-associated MP, which accumulates during late stages of infection, may be inactive in TMV movement is supported by results obtained

by the functional characterization of MPB2C, a MT-associated protein involved in accumulating MP on MT (Curin et al. 2007; Kragler et al. 2003). MPB2C has been isolated using MP as a bait in a membrane-based yeast screening system (Kragler et al. 2003). Transient expression of this protein mediated increased accumulation of MP on MT and reduced the ability of MP to move from cell to cell. When the MPB2C gene was silenced in *Nicotiana* plants (Curin et al. 2007), cell-to-cell movement of transiently expressed MP and spread of TMV were unimpaired, indicating that MPB2C has no essential role in TMV movement. However, a nearly complete loss of accumulation of transiently expressed MP on MT was observed in silenced plants, indicating that MPB2C is involved in targeting MP to MT. These findings support the concept that the accumulation of high levels of MP on MT in late stages of infection is dispensable for movement. However, although it has been shown that MPB2C expression and, thus, MPB2C-mediated accumulation of MP on MT interferes with MP movement, it is unknown whether MPB2C-mediated accumulation of MP on MT also interferes with TMV movement. The demonstration of such an inhibition of viral spread would support a role of MPB2C in controlling vRNA movement through sequestration of MP.

2.9 Possible Functions for MP-Associated MT

Upon accumulation of MP on MT, the MT-associated dynamic movements of MP-associated particles are arrested (Boyko et al. 2007), and the ability of MP to diffuse cell-to-cell is inhibited (Kragler et al. 2003). This suggests that the MT-associated MP is functionally inactive. Indeed, this hypothesis is consistent with the observations that (a) the accumulation of MP occurs in cells behind the leading front of infection (Heinlein et al. 1998a); (b) vRNA movement already occurs in cells, where the abundance of MP is low (Arce-Johnson et al. 1995; Heinlein et al. 1998a); thus, vRNA movement does not require the high amounts of MP that accumulate on MT; and (c) silencing of MPB2C, a factor apparently involved in MT accumulation of MP, does not interfere with TMV movement (Curin et al. 2007). Therefore, why does the MP accumulate on MT? Does the accumulation of MP on MT occur as a consequence of MP overaccumulation or could MT-associated MP exert specific functions during late infection? At this time, at least three scenarios might be considered: (1) MT-associated MP inhibits molecular motors, (2) MT-associated MP is destined for degradation, and (3) MT-associated MP supports vRNA replication.

1. The possibility that the accumulation of MP on MT interferes with the activity of molecular motors is supported by the results of in vitro assays (Ashby et al. 2006). Here, rhodamine-labeled MT were absorbed onto the surface of a glass perfusion chamber precoated with human kinesin-1. Subsequently, following the addition of purified MP, but not following

the addition of control proteins, kinesin-mediated MT translocations were strongly inhibited. No inhibition was observed when MP was added to the perfusion chamber prior to introducing the fluorescent MT, indicating that MP does not directly bind kinesin or causes nonspecifically anchorage of the MT to the glass surface. Staining of the MT with antibodies confirmed the association of the MP with the length of the filaments. These data are consistent with the known inhibition of motor activity by other MAPs, such as MAP2 and Tau (Ebneth et al. 1998; Hagiwara et al. 1994; Lopez and Sheetz 1993; Seitz et al. 2002; Stamer et al. 2002; Trinczek et al. 1999; von Massow et al. 1989), and support the idea that during late infection, the accumulation of MP on MT could negatively regulate MT-dependent trafficking of endogenous plant and/or viral factors. This could be of interest to the virus in various ways. For example, this activity of MP could help to restrict the backward spread of vRNA into already infected cells and thus ensure that infection spreads efficiently into noninfected cells. It is also conceivable that this activity interferes with the transport of competing viruses or of defense-related signals, such as the virus-induced silencing signal (Dunoyer and Voinnet 2005; Heinlein 2005).

2. A role of MP-accumulating MT in the degradation of MP is suggested by the observation that the accumulation of MP on MT during late stages of infection coincides with subsequent disappearance of the protein (Padgett et al. 1996). Moreover, specific proteasome inhibitors led to increased levels of stable, high molecular weight forms of MP in protoplasts (Reichel and Beachy 2000), and since inhibitor treatments also led to the apparent redistribution of MP from MT onto cortical ER in planta (Gillespie et al. 2002), it has been suggested that targeting of MP for degradation by the 26S proteasome is MT-dependent (Gillespie et al. 2002). However, given that stable poly-ubiquitinated proteins are likely to accumulate in the proteasome-targeting pathway under these conditions, and a precedent exists for an ER-associated degradation system in plants (Kirst et al. 2005; Muller et al. 2005), it appears likely that, independent of MT, the ER mediates the proteasome-dependent degradation of MP. And, indeed, a MP mutant that does not accumulate in association with ER-derived IB has been reported to exhibit prolonged stability in association with MT (Kotlizky et al. 2001). Nevertheless, despite these considerations, a role for MT in the degradation pathway was investigated by testing whether MT-associated MP is ubiquitinylated. Poly-ubiquitinated MP was indeed present in crude extracts from protoplasts infected with TMV derivatives. However, the corresponding high molecular weight forms of MP were totally absent from MT-enriched fractions, indicating that the association of MP with MT is unlikely to represent the ubiquitin-mediated degradation pathway for MP. This conclusion was supported by the observation that MP disappears during late infection even in the presence of MT-disrupting agents and, hence, is MT-independent (Ashby et al. 2006). Thus, although

MT as well as other MP-accumulating sites can be described as reservoirs for MP that eventually become degraded, it seems unlikely that MT have any specific role in this respect. It is conceivable that MP is continuously degraded in the cytoplasm of infected cells. Given that the protein is transiently expressed within a short time window during infection (Heinlein et al. 1998a; Padgett et al. 1996; Watanabe et al. 1984), the disappearance from all dynamic associations, including MT, is expected once the protein has passed its expression peak.

3. A function of MT-associated MP in vRNA replication and, thus, the productivity of the virus is suggested by the occurrence of the complex during late infection stages, when infection concentrates cellular activities on the production of virions. Consistent with such a function of MT-associated MP, the ER-derived IB that are associated with viral replication (Heinlein et al. 1998a; Más and Beachy 1999) are found near MT (Heinlein et al. 1998a), and various observations suggest that MT participate in their formation. The formation of the bodies during early infection and the subsequent reconstitution of normal, preinfection ER during late infection has been correlated with the occurrence and subsequent disappearance of MT-associated MP, respectively (Reichel and Beachy 1998). Moreover, recent observations in transfected mammalian cells indicate that ER membranes are recruited to MP-associated MT (Ferralli et al. 2006). Thus, it appears conceivable that MT-associated MP causes changes in ER architecture, for example through interference with MT motors (Ashby et al. 2006). Moreover, since MP has transmembrane domains and may act in association with membranes (Brill et al. 2000, 2004), MP may also change the ER architecture through MT association per se. Recent preliminary results obtained in our laboratory by immunolabeling of infected protoplasts indicate that the viral replicase colocalizes with MP on MT, whereas in the absence of MT-associated MP the replicase occurs in the vicinity, but not in such proximity, of the filaments (Groner, Ashby, Heinlein, unpublished results). Since replicase is associated with the ER, this finding is in agreement with the possible recruitment of ER to the MT via ER-associated MP. The recruitment of ER and replicase to the MT by MP may have a role in supporting the production of virions during late infection. On the other hand, this behavior may also reflect a process during early infection by which movement-competent replication complexes (Kawakami et al. 2004) and perhaps also the mobile, MT-proximal MP particles (Boyko et al. 2007) are formed.

2.10 The Targeting of MP to PD May be MT-Independent

Although the intercellular transport of vRNA appears to involve MT, there is evidence that at least the accumulation of MP to PD may be MT-independent.

First hints came from the observation that a movement-defective mutant MP (TAD5), lacking amino acids 46–51, localizes to PD and IB, but not to MT (Kahn et al. 1998). Moreover, temperature-sensitive mutations in MP that interfere with function and MT association of the protein still allow the MP to accumulate in PD at the restrictive temperature (Boyko et al. 2000a, 2007). A recent study applying FRAP on PD in tissues treated with specific inhibitors indicated that the accumulation of MP in PD involves the actin/ER network (Wright et al. 2007). Thus, it was shown that that the accumulation of MP in PD is inhibited in the presence of high concentrations of brefeldin A, which also resulted in the disruption of the ER. In contrast, PD targeting of MP was not inhibited in the presence of lower concentrations of the inhibitor, which nevertheless interfered with the integrity of Golgi complexes. These observations indicate that the targeting of MP to PD involves an intact ER network but does not rely on the secretory pathway. Moreover, consistent with the close association of the ER with actin filaments, the accumulation of the protein in PD was also inhibited by inhibitors of the actin cytoskeleton rather than by inhibitors of the MT cytoskeleton. It should be noted, however, that disruption of the ER–actin network only led to a reduction but not to a full inhibition of the targeting of MP to PD. Indeed, Prokhnevsky and colleagues (2005) found no evidence for inhibited PD targeting by MP upon prolonged treatment with actin-disrupting agents. This seems to indicate that either the inhibitor treatments were not efficient or that actin filaments are dispensable for the PD targeting of at least a fraction of the MP. Since MP is associated with the ER, the protein may target PD via diffusion in the ER even if the ER-associated microfilaments are disrupted (Wright et al. 2007). Moreover, since MT-disrupting agents may not disrupt all MT in treated tissues (Seemanpillai et al. 2006), the conclusion that MT are not involved in the PD targeting by MP may be premature.

3 Role of MT in Other RNA and Viral Transport Systems

Since TMV RNA moves between cells in the form of a nonencapsidated protein:RNA complex, the observation of MP-proximal particles that move along MT in leading front cells of TMV infection sites is reminiscent of RNA-containing particles reported for many systems, such as neurons (Hirokawa 2006; Knowles et al. 1996; Kohrmann et al. 1999; Muslinov et al. 2002; Smith 2004; Sossin and DesGroseillers 2006; Tiruchinapalli et al. 2003), oligodendrocytes (Ainger et al. 1993), fibroblasts (Sundell and Singer 1990), *Xenopus* oocytes (Forristal et al. 1995; Kloc and Etkin 1995), and *Drosophila* embryos (Ferrandon et al. 1994; Januschke et al. 2002; MacDougall et al. 2003). MT-dependent transport of RNA granules in these systems appears to depend on the activity of MT motor proteins (Hirokawa 2006). A role of MT and motor proteins

is also involved in the cytoplasmic transport of animal viruses (Dohner et al. 2005; Greber and Way 2006; Leopold and Pfister 2006; Radtke et al. 2006) and other cases of mRNA transport (Bullock et al. 2006; Czaplinski and Singer 2006; Palacios and St. Johnston 2001; St. Johnston 2005; Weil et al. 2006).

However, in contrast to animal cells, for plants the list of examples supporting a role for MT and motor proteins in the transport of mRNA macromolecules and viruses is rather short. In fact, research to unravel the mechanisms underlying the ability of plants to support the inter- and intracellular transport of RNA molecules (Haywood et al. 2002; Lucas et al. 2001; Okita and Choi 2002; Yoo et al. 2004) is still in its infancy and relies to some extent on RNA viruses as a model, as presented here for the RNA of TMV. The best-studied nonviral system is probably the localization of storage protein mRNA in cereal endosperm. In fact, a role of the cytoskeleton has been implicated in the localization of prolamine storage protein mRNA in rice. However, when an adapted GFP-based monitoring system (Bertrand et al. 1998) was applied, and prolamine and glutelin RNAs were detected as large, mobile particles, their movements were found to depend on microfilaments rather than on MT (Hamada et al. 2003). A second potential system to investigate the role of the plant cytoskeleton in RNA transport is represented by the MT-dependent transport of an RNA binding protein required for the establishment of hyphal cell polarity in *Ustilago maydis* (Becht et al. 2006).

With respect to plant viruses, TMV is probably the most thoroughly studied viral system implying a role of MT for transport. Since the amino acids affected by point mutations causing temperature sensitivity in MT association and function are conserved within tobamoviruses (except the arginine affected by Ni2519, Boyko et al. 2007, which is not conserved in some tobamoviruses, such as TVCV, CrTMV, YoMV, and RGMV), it will be interesting to know whether other MPs from this group, in addition to those of TMV and ToMV-Ob, also interact with MT. Another virus for which a role of MT has been documented is grapevine fanleaf virus (GFLV). Unlike TMV, this virus moves in the form of virions through tubules assembled by its MP within PD. The targeting of the GFLV-MP to PD has been studied in BY-2 cells (Laporte et al. 2003) and was shown to be inhibited in the presence of brefeldin A, suggesting a role of the secretory pathway. Consistent with this finding, the protein cofractionates with membranes also associated with KNOLLE, a syntaxin involved in cytokinesis (Lauber et al. 1997). Interestingly, GFLV-MP was mistargeted in the presence of a MT-disrupting inhibitor, whereas an actin inhibitor had no effect. These results imply a role of MT in providing the correct subcellular address for the targeting of the GFLV-MP to PD. However, further studies are needed to determine the exact function of MT during PD targeting. Moreover, targeting of the MP of cowpea mosaic virus (CPMV), another tubule-forming virus, to peripheral foci was not dependent on the secretory pathway or on the cytoskeleton (Pouwels et al. 2002), suggesting that tubule-forming viruses are fundamentally different with respect to their mechanisms of spread.

There is evidence that MT may play a certain role during infection by potato virus X (PVX) and beet yellows virus (BYV). Movement of PVX depends on three "triple-gene-block (TGB)" MP and CP, each of which is essential, but not sufficient, for virus translocation (Chapman et al. 1992; Morozov and Solovyev 2003). Although there is strong evidence for the association of the TGB MP of PVX and of related viruses with the endomembrane system (Cowan et al. 2002; Gorshkova et al. 2003; Haupt et al. 2005; Ju et al. 2005; Krishnamurthy et al. 2003; Mitra et al. 2003; Morozov and Solovyev 2003; Solovyev et al. 2000; Zamyatnin et al. 2002), the PVX CP and whole PVX virions were reported to bind to taxol-stabilized MT and to compete with MAP2 binding in vitro (Serazev et al. 2003). An additional viral protein that has been reported to bind MT in vitro is the 65-kDa Hsp70 homolog (hsp70h) of BYV, one of the five viral proteins required for movement of this virus (Karasev et al. 1992). However, a role of MT in BYV movement as suggested by this finding will need further support, since when transiently expressed in fusion with GFP or mRFP in agroinfiltrated cells, the 65-kDa protein is targeted to PD independently of MT but rather depending on actin (Prokhnevsky et al. 2005). Finally, the cauliflower mosaic virus (CaMV) aphid transmission factor (ATF) binds MT in vitro and in vivo (Blanc et al. 1996), indicating a role of MT in the uptake and plant-to-plant transmission of this virus by aphids.

4 Concluding Remarks

The finding that the MP of TMV interacts with MT and that this activity of MP correlates with the function of this protein in TMV RNA movement indicates a role of MT in the transport of the vRNA. This hypothesis has recently been further confirmed by the observation of MT-proximal, MP-containing particles in cells at the leading front of infection (Boyko et al. 2007). However, further studies are needed to determine whether these particles contain vRNA and whether they are indeed targeted to PD. Research on TMV has also provided evidence that the MP is targeted to PD independently of MT association (Boyko et al. 2000a, 2007; Kahn et al. 1998; Wright et al. 2007). This pathway involves the actin-ER network that connects viral replication sites with PD (Wright et al. 2007). Results of MT inhibitor treatments have suggested that this pathway may be sufficient for spreading infection into adjacent cells (Gillespie et al. 2002). However, since the inhibitors may not be 100% effective, conclusions based on the absence of effects of MT inhibitor are difficult to make (Seemanpillai et al. 2006). On the contrary, the analysis of functionally switchable, temperature-sensitive mutations in MP indicates that the trafficking of vRNA does not occur if the ability of MP to associate with MT is inhibited (Boyko et al. 2000a, 2007). Nevertheless, although the direct correlation between MT association of MP and its function in TMV RNA

movement has been made, it remains to be seen whether the ability of MP to interact with MT has indeed a direct role in TMV movement or whether other functions of MP that may also be affected by the temperature-sensitive mutations may play this role.

In vivo observations have suggested that vRNA moves between cells in the form of larger, membrane-associated replication complexes (Kawakami et al. 2004). However, direct in vivo evidence demonstrating that the movement process involves the formation of a MP:vRNA complex is still lacking. Recent advances in the development of quantitative fluorescence microscopy technologies to label RNA and proteins in vivo and to demonstrate their molecular interactions (Fricker et al. 2006) may allow one to detect vRNA in vivo and to directly address the interactions of vRNA with MP, MT, and the ER.

However, unlike TMV movement, which does not require encapsidation and may thus have allowed specific adaptation to RNA transport mechanisms, other viruses, for example viruses that require CP or even encapsidation for movement, are likely to have evolved other ways to interact with the host cell and to spread infection. Indeed, several viruses were shown to interact with endomembranes and not with MT and thus indicate that diverse pathways do exist. Further insight is needed to better describe these pathways, to understand the molecular virus–host interactions involved, and to determine whether they may be used in parallel or rather by specific viruses.

It will also be important to address the role of these pathways in the intercellular transport of endogenous proteins and RNA macromolecules. Further analysis of the mechanism by which TMV and other related tobamoviruses move from cell to cell also has the potential to reveal new important insights into the dynamics and function of plant MT. Tobamoviruses that infect *A. thaliana*, such as oilseed rape mosaic virus (ORMV) (Aguilar et al. 1996), turnip vein clearing virus (TVCV) (Lartey et al. 1997), or TMV-Cg (Diaz-Griffero et al. 2006), already pave the way to the application of *Arabidopsis* functional genomics (Carr and Whitham 2007; Huang et al. 2005; Whitham et al. 2003), and new biochemical and genetic screening approaches in this system will enhance our ability to identify novel genes and proteins involved in replication and movement, i.e., the pathway that guides these viruses and potentially other macromolecules to PD.

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Microtubules as Sensors for Abiotic Stimuli

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Abstract Microtubules are generally perceived as structural, static elements that basically function either as supporting scaffolds or barriers. This view has been increasingly challenged during the last decade of the last century, when in-vivo imaging of microtubules revealed that they are endowed with complex and highly nonlinear dynamics. This indicates that, in addition to their traditional structural functions, microtubules must play a role in more volatile events that have to be organized in space and time. It has become clear that microtubules are subject to numerous signalling chains and that this is especially important in plants, where morphogenesis is under tight control of a broad panel of environmental cues. However, it has remained a bit more implicit that microtubules are not only targets for signalling, but participate very actively in signal transduction itself. This work ventures to review and to emphasize this aspect. It begins with a survey of the physiological and molecular evidence of microtubules as targets for signalling, but then changes perspective focussing on the mechanosensory properties of microtubules. It is proposed that the nonlinear dynamics of microtubule assembly provide the strong and sensitive signal amplifier necessary for the sensing of minute mechanic stimuli. Using gravi- and cold-sensing as examples, it is shown, how this mechanism can be used very efficiently to detect abiotic stimuli and to adapt to even harsh environments.

1 Microtubules as Sensors: Physiological Mechanisms

The perception of abiotic stimuli other than light poses demanding challenges to signalling: a physical stimulus has to be transformed into a biochemical output. 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 has to be efficiently amplified.

This problem is even accentuated in plants, because plant cells are subject to continuous pressure from inside (produced by the expanding protoplast) and outside (produced by the expanding neighbouring cells). These pressures are in the range of several bars and are responsible for large background forces against which the minute changes of mechanical energy have to be discriminated. A second challenge is the lack of specialized sensory organs—sensing is diffusely spread over a large number of cells. Thus, each individual cell has to produce a sufficient sensory output by itself. The maximally possible input energies are therefore limited and hardly exceed thermal noise.

Mechanochemical sensors in higher plants are involved in numerous responses to physical stimuli including sensing of gravity, touch, wind, (for review see Telewski 2006), but also cold and salt. Experiments with aequorin-transformed plants have shown that these stimuli trigger the release of calcium in specific time signatures (Knight et al. 1991). Therefore, mechanosensitive calcium channels have been proposed to transduce these physical stimuli into calcium influx as chemical signals. In fact, when touch-insensitive mutants were isolated in *Arabidopsis*, three of the four genes identified in this approach turned out to be calmodulins (Braam and Davis 1990), and touch-responses can be suppressed by inhibitors of calmodulin (Jones and Mitchell 1989). However, the touch-sensitive calcium channels that are postulated to trigger this signal chain have remained elusive, at least in plants. This might be related to the highly artificial conditions required to identify stretch-activated ion fluxes by patch-clamp techniques. Removal of the cell wall, isotonic conditions, and suction by the holding electrode create conditions, where most ion channels would be defined as mechanosensitive (Gustin et al. 1991).

The transition to terrestrial life forms required very efficient systems to efficiently sense and respond to gravity and mechanical tension. It is even possible to understand plant evolution in terms of adaptation to this task (Niklas 1997). Despite this impact, mechanosensing has remained obscure so far. A simple stretch-activated ion-channel system is certainly not sufficient to cope with the challenge to detect a minute input (deformation of a membrane) against a background of fairly large turgor pressures. Thus, efficient systems of input amplification are required. It is likely that similar systems operate in other organisms as well, however, in plants they have to be particularly effective.

What are the requirements for such input amplifiers? (1) They should be able to collect small and diffuse mechanic energies (for instance from changes in membrane fluidity, Los and Murata 2004) and to concentrate them into a local, stronger stimulus (stress-focussation). (2) They should be anisotropic to efficiently transfer mechanical translocations. (3) They should be endowed with a certain rigidity. (4) They should be endowed with positive autoregulation to efficiently amplify small inputs.

These four preconditions are met by microtubules that therefore represent good candidates for such input-amplifiers. Their bending modulus corresponds to that of glass (Gittes et al. 1993)—unlike actin, for instance. They are long, hollow cylinders and their growth and shrinkage is not a continuous process, but subject to catastrophic phase transitions. A recent publication (Grishuk et al. 2005) could show that disassembly of microtubules can generate substantial forces that are about tenfold higher than even those caused by microtubule motors.

A range of observations from different organisms suggests that microtubules are involved in the perception of abiotic stimuli:

1. *Perception of touch in Caenorhabditis*: A screen for mutants that are insensitive to touch, Chalfie and coworkers identified transmembrane proteins that possibly represent elements of a mechanosensitive channel (Chalfie and Au 1989). However, they also recovered a couple of mutants in tubulins that participated in specialized microtubule bundles characteristic for the touch-sensitive cells (e.g. Fukushige et al. 1999). These observations led to a working model, where the primary input—a deformation of the membrane—was amplified by a microtubule-based lever system ("toilet-flush system") into a focussed mechanical force that is large enough to open the putative ion channel (Chalfie 1993).
2. *Gravitropism*: The trigger (susception in sensu Björkman 1988) is the sedimentation of statoliths. The force exerted by these statoliths is believed to be sensed by mechanosensitive ion channels. This gravitropic sensing can be blocked by antimicrotubular drugs in the rhizoid of *Chara* (Friedrich and Hertel 1973) as well as in moss protonemata (Schwuchow et al. 1990; Walker and Sack 1990) or in coleoptiles of maize (Nick et al. 1991) and 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 is reduced either as a consequence of a mutation (Nick et al. 1994) or treatment with taxol, this results in a strong inhibition of gravitropic responses (Nick et al. 1997; Godbolé et al. 2000; Gutjahr and Nick 2006).
3. *Mechanic stimuli affect microtubule orientation*: The application of mechanical fields (Hush and Overall 1991), high pressure (Cleary and Hardham 1993) or artificial bending of coleoptiles (Zandomeni and Schopfer 1994) can induce a reorientation of cortical microtubules. Centrifugation experiments in regenerating tobacco protoplasts (Wymer et al. 1996) suggest that microtubules are aligned in parallel to the administered centrifugal force. Although the stimuli used in these studies were several orders of magnitude above those that typically occur in a physiological context, there are indications that microtubules are aligned by mechanical strain during development as well. For instance, when new leaf primordia are laid down, sharp transitions in microtubule orientation arise at the boundary of the incipient primordium. These sharp transitions are subsequently smoothened by realignments of microtubules such that the pitch of cortical microtubules changes gradually over several tiers of cells in parallel to the stress-strain pattern predicted for the environment of a protruding primordium (Hardham et al. 1980).
4. *Volume regulation*: The ability to regulate cell volume depending on the osmolarity of the environment represents an evolutionary ancient achievement and can be observed already in bacteria, where osmoregulation involves mechanosensitive channels of large conductance (MscL) that can open and allow mass transport of osmotically active ions parallel to the gradient of chemical potential for those particles (Chang et al. 1998). Again,

the primary stimulus is a deformation of the membrane. In the case of MscL, this has to be quite drastic nearly in the range of membrane breakage to trigger opening of the channel. In eukaryotes, more subtle inputs are sufficient to trigger volume regulation. Recent studies on the role of the cytoskeleton in the volume regulation of mammalian spermatozoa (Petrunkina et al. 2004) or plant protoplasts (Komis et al. 2002) indicate that microtubules participate in the earliest events of volume regulation.

5. *Temperature sensing*: Since microtubules disassemble in the cold, there exist a couple of studies, where their behaviour was followed in the context of low-temperature responses. When microtubules were manipulated pharmacologically, this was accompanied by changes in cold hardiness. For instance, a treatment with taxol was reported to reduce freezing tolerance in rye roots (Kerr and Carter 1990) or spinach mesophyll (Bartolo and Carter 1991b). Conversely, freezing tolerance could be induced by a mild treatment with pronamide (a herbicide that affects microtubule assembly) in a way similar to cold acclimation (Abdrakhamanova et al. 2003) indicating that a sensory microtubule population acts as a "thermometer" that triggers or modulates adaptive responses to low temperature.

These examples may suffice to illustrate the importance of microtubules for the sensing of abiotic stimuli. The primary sensors of these responses have remained obscure so far, but it seems that microtubules act as amplifiers in concert with these primary sensors. For several of the responses described above, the removal of microtubules cannot completely interrupt sensing, but results in a decreased sensitivity and thus in a delay of the response. For instance, treatment with microtubule assembly-blockers delays the onset of gravitropic bending in coleoptiles (Nick et al., unpublished results), but eventually gravitropism initiates suggesting that microtubules are not the conditio sine qua non, but rather act as positive modulators of the primary sensing response.

2 Microtubules as Sensors: Molecular Mechanisms

Although a sensory function of microtubules in the sensing of abiotic stimuli is supported by a large number of observations from different organisms, the molecular base of this sensory function has remained enigmatic so far. Principally, there are two possible routes and at the present (Fig. 1), limited, state of knowledge it is not possible to rule out any of those. And this may not be necessary, because these routes are not mutually exclusive:

1. Microtubules as Susceptors for Mechanosensitive Ion Channels.
   In the first model, the actual perception of the mechanic stimulus occurs through mechanosensitive ion channels (Fig. 1A). The primary input are minute deformations of the membrane with energies that are, in

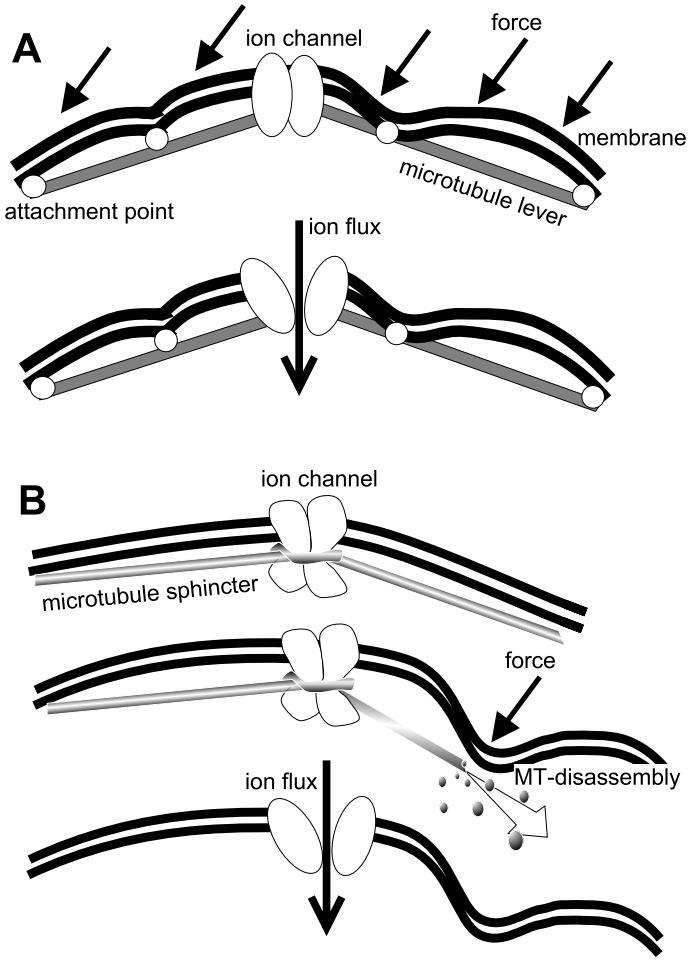


Fig. 1 Models for the role of microtubules in mechanosensing. **A** Microtubules acting as mechanosusceptors in sensu (Björkman 1988). Membrane deformations are collected and focussed by a microtubule lever system towards a mechanosensitive ion channel such that the input energy exceeds thermal noise. **B** Microtubules acting as mechanoreceptors in sensu strictu. Microtubules constrict the opening of ion channels and disassemble upon mechanic load. Note that, in this model, the ion channel acts as a transducer, not as a receptor [in contrast to the model depicted in (**A**)]

most cases, below the fluctuations due to thermal noise. In order to obtain a sensible signal, these primary deformations have to be focussed by a lever system. The role of microtubules in this model would be that of a (mechanic) susceptor in sensu (Björkman 1988).

Elimination of microtubules has been repeatedly found to activate calcium channels. Antimicrotubular compounds such as oryzalin, ethyl-*N*-

phenylcarbamate, or colchicine induce a six- to tenfold increase in the activity of calcium channels (Ding and Pickard 1993; Thion et al. 1996, 1998). Moreover, cold-induced calcium fluxes are amplified conspicuously by these drugs in tobacco cells (Mazars et al. 1997). However, the characterization of a channel activity as mechanosensitive is usually based on patch-clamp experiments and does not prove any physiological function in mechanosensing.

These pharmacological findings from plant cells are supported by the results from genetic screens for touch-sensitive ion channels in *Caenorhabditis elegans*. Using a system, where the phobic response to a specific touch stimulus was screened, so-called mechanosensation defective (*mec*) mutants could be recovered (Chalfie and Au 1989; for review see Chalfie 1993). Some of the mutated genes encoded a novel class of transmembrane proteins, the so-called degerins, that might represent components of a touch-sensitive ion channel. However, two of these mutants, *mec7* and *mec12* were affected in a unique set of microtubules consisting of 15 protofilaments that were confined to the axons of the touch-sensitive neurons responsible for the phobic response (Chalfie and Thomson 1982). MEC12 and MEC7 were later shown to encode specific isotypes of α-tubulin (Savage et al. 1989) and β-tubulin (Fukushige et al. 1999), respectively. In both mutants, the loss of mechanosensation was correlated by specific changes in the organization of these 15-protofilament microtubules. This led to a model, where the microtubules act through specific linker proteins as a kind of lever system that amplifies minute deformations of the perceptive membrane into a strong aperture of the putative channels (Chalfie 1993). A similar set-up, where specialized microtubules are able, via an intermediate protein to induce a functional spatial arrangement of receptors or ion channels has been proposed for the clustering of glycine receptors in rat spinal cord synapses, where the microtubule-associated protein gephyrin plays the role of the intermediate linker (Kirsch et al. 1993).

2. Microtubules as Primary Deformation Sensors.

   In the model sketched above, microtubules act as signal amplifiers for mechanosensitive ion channels. However, microtubules might be the sensors themselves (Fig. 1B). Even in vitro the assembly of microtubules from soluble tubulin heterodimers could be shown to depend on vectorial forces. The preferential direction of the assembled microtubules could be modulated by centrifugal force (Tabony and Job 1992) although one should note that the forces acting here are orders of magnitude above the minute inputs that trigger the perception of abiotic stimuli in a physiological context. The same argument is true for experiments, where the microtubules could be reoriented by bending of maize coleoptiles by application of defined weights (Zandomeni and Schopfer 1994). The forces acting in those experiments approached the limits of membrane integrity

and thus are far beyond the physiologically relevant range. This emphasizes the necessity of efficient signal amplification in deformation sensing. As will be explained in more detail later, due to their nonlinear dynamics microtubules themselves should be able to amplify small mechanic stimuli into clear net outputs that can be processed by downstream signalling cascades. It should also be kept in mind that microtubules can generate force not only through microtubule-motors such as kinesins or dyneins. In addition, microtubule disassembly could be recently shown to generate a force that is quite considerable and even exceeds the forces produced by motor proteins (Grishchuk et al. 2005).

Summarizing, both models for the sensory role of microtubules are compatible with our (admittedly still limited) knowledge on the molecular base of abiotic sensing. Both models rely on positive feedback circuits that are able to amplify the minute inputs (small deformations of the perceptive membranes in the first model or changes in the dynamic equilibrium between assembly and disassembly of microtubules themselves in the second model) into clear and nearly qualitative outputs that can then be processed by downstream signalling cascades. The distinction between the two models described above was introduced for the sake of conceptual clarity, it might be not as pronounced in the biological context of a cell, where both mechanisms could act in a complementary fashion. It will be a challenge for the next years not only to identify the molecular elements acting in the perception and modulation of abiotic stimuli, but to understand their interaction and systemic properties. This will require integration of molecular data with cell biological and physiological analysis and even mathematical modelling.

3 Plant Microtubules as Mechanosensors

Plant growth and development responds very sensitively to mechanical stimulation, a phenomenon that has been termed thigmomorphogenesis (Jaffe 1973). For instance, cell expansion is redistributed from elongation towards lateral thickening, when a shoot is repeatedly touched or bent. Later, it was possible to demonstrate thigmomorphogenesis on the cellular level as well. For instance, when a protonema of the fern *Adiantum* was squeezed by a needle, chloroplasts "fled" from the contact site (Sato et al. 1999), whereas in parsley cells, the nucleus, probably as an element of a defence response to pathogen invasion (see Chapter "Microtubules and pathogen defence", in this volume), approached the needle (Gus-Mayer et al. 1998). Alternatively, cells (protoplasts) were embedded in agar and mildly centrifuged or squeezed, resulting in a respective alignment of cell division or cell expansion with the force vector (Wymer et al. 1996; Zhou et al. 2007).

It has long been speculated that calcium fluxes are involved in the signal transduction that culminates in thigmomorphogenesis. By generating transgenic tobacco plants that expressed the luminescent calcium reporter aequorin (Knight et al. 1991), it became possible to observe these fluxes directly and to demonstrate that the signature triggered by a touch stimulus was specifically different from those induced by other stimulation qualities such as cold. Since these changes of intracellular calcium levels occur rapidly after stimulation (Legue et al. 1997), mechanosensitive calcium channels have been postulated as the primary element of mechanosignalling.

Recently, homologues of the bacterial MscS (for mechanosensitive channel of small conductance) have been identified in *Arabidopsis thaliana*. One of these homologues, MSL3, could functionally complement a bacterial mutant affected in the function of mechanosensitive channels suggesting that MSL3 is indeed a mechanosensitive ion channel. GFP fusions of MSL3 and a second homologue, MSL2, were demonstrated, by fluorescence microscopy, and by subcellular fractionation, to be localized in discrete patches in the plastid envelope. Moreover, they colocalized with the plastid division factor MinE (see Chapter "Microtubules and the Evolution of Mitosis", in this volume) indicating an interaction of MSL2 and MSL3 with plastid division. In fact, mutants in these bona-fide channels harboured chloroplasts that were irregular in size, shape and partially number. Thus, these channels regulate morphogenesis and development of plastids. In other words: during endosymbiosis of the prokaryotic plastid ancestors, these channels underwent a shift in function from osmoregulation (that has been taken over by the eukaryotic "host" cell) towards regulation of plastid morphogenesis. An attractive model assumes that MSL2 and MSL3 sense membrane tension in the plastid envelope and feed this information through interactions with MinE (and, indirectly, MinD) into the machinery that defines the location of the plastid division ring (see Chapter "Microtubules and the Evolution of Mitosis", in this volume).

Thus, homologues of prokaryotic mechanosensitive channels seem to exist in plants. The putative channels that are responsible for thigmomorphogenesis, have remained elusive, though. By patch-clamp analysis, it was possible to detect mechanosensitive calcium fluxes in membrane preparations (Ding and Pickard 1993). These fluxes could be inhibited by lanthanoid ions and were capacitated by antimicrotubular agents indicating that microtubules control the permeability of these channels for calcium.

In the context of an expanding tissue (that is characterized by considerable tension), microtubules seem to be directly involved in osmoadaptation. By application of osmotic stress to root tips of *Triticum turgidum* microtubules could be induced to disassemble and to reorganize in massive bundles, the so-called macrotubules (Komis et al. 2002). When this response was suppressed by treatment with oryzalin, the protoplasts were not any longer able to adapt by controlled swelling to the osmotic stress and perished. A pharmacolog-

ical study (Komis et al. 2006) revealed that inhibitors of phospholipase D, such as butanol-1 or *N*-acetylethanolamine, suppressed osmotic adaptation as well as the formation of the macrotubules. In contrast, phosphatidic acid, a product of phospholipase D, enhanced osmoadaptation and macrotubule formation and was able to overcome the inhibitory effect of butanol 1. These observations demonstrate that the microtubule response (formation of macrotubules) is essential for osmoadaptation, and that signalling through phospholipase D acts upstream of microtubules in this response.

A recent study (Zhou et al. 2007) highlights the interaction of microtubules with the cell-wall-cytoplasmic continuum during mechanosensing. Agarose-embedded suspension cells of chrysanthemum were subjected to compression force. Under these conditions, the axis of cell expansion could be aligned in a direction perpendicular to the vector of force. When microtubules were removed by oryzalin prior to the treatment or when the cell-wall cytoplasmic continuum was impaired by treatment with RGD-peptides (that, in animal cells, interfere with adhesion sites), this alignment response was interrupted. Elimination of actin filaments by cytochalasin B did not produce this effect. Thus, microtubules, probably in conjunction with the cell wall, are essential for the cellular response to mechanic stimulation. However, as in the macrotubule system, it is not clear, whether microtubules act as transducers or even effectors of the mechanic stimulus or whether they convey a true sensory function.

Using tension-free protoplasts, Wymer et al. (1996) were able to align microtubules by a short centrifugation and thus to orient the axis of cell expansion in a direction perpendicular to the force vector (Fig. 2). They used this system to dissect a possible sensory role of microtubules. Since microtubules are necessary for the directional synthesis of cellulose (see Chapter "Control of cell axis", in this volume), a transient elimination of microtubules using the herbicide amiprophosmethyl was used. After washing out the herbicide, microtubules recovered such that the directionality of cellulose synthesis and thus the cell axis could become manifest. Using this approach, microtubules were eliminated for the short interval corresponding to the time of centrifugation and then allowed to recover without any significant effect on viability or regeneration of the protoplasts. This transient microtubule elimination was then administered either immediately before or immediately after the centrifugation stimulus (Fig. 2). When microtubules were eliminated subsequent to the centrifugation, the alignment of cell axis by the stimulus was not impaired. However, when microtubules were eliminated just prior to the centrifugation and allowed to recover immediately after the end of stimulation, the alignment disappeared completely. This demonstrated clearly that microtubules are essential for the sensing of this mechanic stimulus.

Thus, there is clear evidence for a microtubule function in the mechanosensing of plants, and experimental systems have been developed, where this question can be addressed. It should now become possible to discriminate

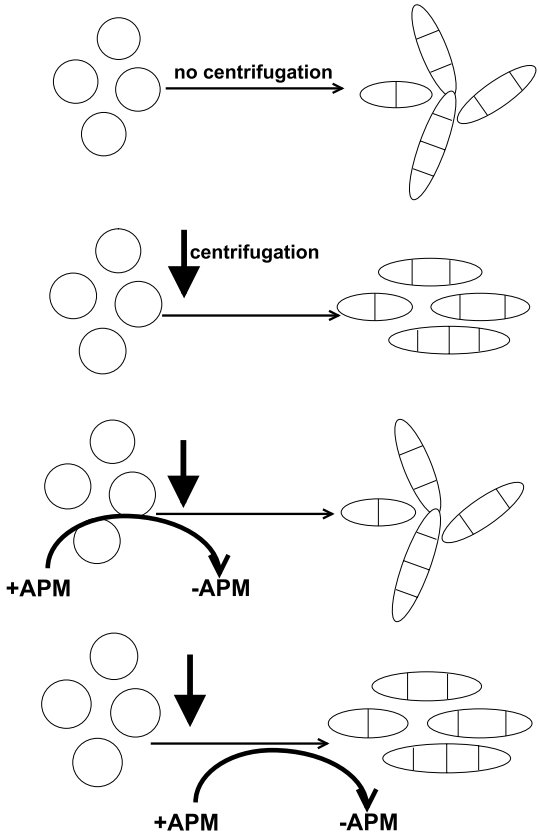


Fig. 2 Demonstration of a true sensory role for microtubules in plant mechanoperception. Tobacco protoplasts were embedded in agarose and then subjected to a short mechanic stimulation (mild centrifugation). Cell expansion and cell division were subsequently aligned in a direction perpendicular to the vector of force. When microtubules were eliminated by amiprophosmethyl (APM) prior to centrifugation and allowed to recover afterwards, cells elongated and divided normally, but without alignment. APM treatment of identical duration but administered following the centrifugation was not effective

between mechanosusception by microtubules (Fig. 1A) and a microtubular mechanoreceptor function in *sensu strictu* (Fig. 1B).

4 Plant Microtubules as Gravisensors

Mechanosensation is not confined to the perception of touch, but includes a range of abiotic stimuli with gravity certainly being of central importance. Upon the transition to terrestrial life, plants had to cope with the loss of

buoyancy as an important supportive element. This required the development of mechanical support, the vascular bundle as the central element of the telomes that represent the modular elements of all land plants (Zimmermann 1965). However, it is not sufficient to generate a force-bearing element, what matters, is the spatial arrangement of these elements in a manner such that they provide optimal mechanical support, but simultaneously consume minimal biomass and are as light as possible. This optimization task can only be achieved, when the arrangement of supportive structures is guided by the pattern of mechanical strain. The ultimate source of these strains is gravity. Thus, gravity has to be perceived very efficiently and it has, in addition, to be linked to morphogenesis.

This link becomes manifest in two basic phenomena:

1. When the orientation of a plant is changed with respect to gravity, it will respond by bending that will restore the original orientation and thus will minimize mechanical stress (gravitropism).
2. When new organs are laid down and oriented, these processes are often adjusted with respect to gravity (gravimorphosis).

Microtubules and gravitropism: For the rhizoid of *Chara* the classical experiments of Johannes Buder (1961) have shown that barium-sulfate containing vesicles, the *Glanzkörperchen*, are necessary and sufficient for gravisusception. For higher plants the classical starch-statolith theory (Nemec 1900; Haberland 1900) postulated that amyloplasts in the perceptive tissues (e.g. root cap or bundle sheath cells) are responsible for the susception of the gravitropic stimulus. A long tradition of experimentation demonstrated that amyloplasts are necessary for efficient gravitropism. For instance, gravitropic sensitivity was reduced in starch-deficient mutants. However, it took almost a century until it could be shown that amyloplast translocation is sufficient to trigger a tropistic growth response. By using high-gradient magnetic fields, Kuznetsov and Hasenstein (1996) succeeded to induce bending in vertically oriented roots and thus were able to proof the starch-statolith concept for gravisusception. It represents an irony of science history that this breakthrough was not achieved by the elaborate and expensive microgravity experiments in the context of space research, but instead through a very cheap, but well-designed ground experiment. Thus, in higher plants as well, the primary stimulus is produced by statolithic particles (the amyloplasts), but the actual perception event remains to be revealed. It had been postulated for the rhizoid of *Chara* that a sedimentation of the *Glanzkörperchen* to the lower flank of the rhizoid would divert vesicle flow towards the upper side such that more material is intussuscepted into the upper flank resulting in a growth differential driving downward bending (Sievers and Schröter 1971). This hypothesis was later extended to negative gravitropism by combining sedimentation with a different mode of growth (Hodick 1994). According to this model, the actual perception of gravity would rely upon a proximity

mechanism. It is to be doubted that proximity is used for graviperception in higher plants, since already classical studies (Rawitscher 1932) using intermittent stimulation could show that perception can occur in the absence of amyloplast sedimentation. Moreover, dose-response studies employing centrifugation show that the output (gravitropic curvature) is dose-dependent even for stimuli that completely saturate amyloplast sedimentation. Even for the rhizoid of *Chara*, for which the proximity mechanism had been postulated originally, it could be demonstrated that stimuli that produce a complete sedimentation of the *Glanzkörperchen* can nevertheless be discriminated (Hertel and Friedrich 1973). This suggests that the actual perception of gravity is not based on proximity, but on pressure exerted by the statoliths to a mechanosensitive receptor.

If gravity is not perceived by proximity, but by pressure, this poses a big challenge to the sensing mechanism. Since gravity is sensed by individual cells (in contrast to the direction of light in phototropism—Buder 1920; Nick and Furuya 1995), the maximal energy available for stimulation is the potential energy of the sensing cell. This energy barely exceeds thermal noise, if it is not focussed upon small areas. 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 coleoptiles of maize (Nick et al. 1991) and 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 is reduced either as a consequence of a mutation (Nick et al. 1994) or treatment with taxol, this results in a strong inhibition of gravitropic responses (Nick et al. 1997; Godbolé et al. 2000; Gutjahr and Nick 2006).

The gravitropically induced reorientation of cortical microtubules has been observed for both shoot (Nick et al. 1991) and root gravitropism (Blancaflor and Hasenstein 1993). In maize coleoptiles, the microtubules in the epidermal cells of the upper flank of the stimulated organ assumed a longitudinal orientation, whereas the microtubules in the lower flank remained transverse. By microinjection of fluorescent tubulin into epidermal cells of intact maize coleoptiles it was later even possible to demonstrate the gravitropic microtubule reorientation in vivo (Himmelspach et al. 1999). The time course of this response was consistent with a model where gravitropic stimulation induced a lateral shift of auxin transport towards the lower organ flank and, as a consequence, a depletion of auxin in the upper flank. The microtubular response was thought to be primarily by this decrease in auxin concentration rather than by gravity itself. In maize roots, however, where a similar reorientation could be observed in the cortex (Blancaflor and Hasenstein 1993), the time course of reorientation was found to be slower than the changes in growth rate induced by gravity.

This leads to the question, whether the gravitropic response of microtubules is direct or whether microtubules merely respond to changes in growth rate. In fact, it is possible to induce microtubule reorientation by bending coleoptiles with manual force (Zandomeni and Schopfer 1994)—microtubules will then become longitudinal in the concave flank, but remain transverse in the convex flank. To dissect the gravitropic response and a potential response to changed growth rate, microtubule behaviour was followed in coleoptiles that were prevented by a surgical adhesive from elongation and either kept in horizontal orientation (such that a gravitropic stimulation occurred) or in vertical orientation (such that growth was inhibited in the absence of a gravitropic stimulus). In this setup, a microtubule reorientation from transverse to longitudinal could be observed only in the horizontal orientation (Himmelspach and Nick 2001) demonstrating unequivocally that microtubules, at least in this system, responded to gravity rather than to the inhibition of growth.

Microtubules and gravimorphosis: The impact of gravimorphosis is already illustrated by the simple observation that roots form at the lower pole of a plant. Although a considerable amount of phenomenological work has been dedicated to this problem at the turn of the century (Vöchting 1878; Sachs 1880; Goebel 1908), the underlying mechanisms have remained obscure so far. One reason for this problem has been certainly the use of adult organs, where polarity has already been fixed and is hard to invert. In the meantime, new systems have been introduced that may be more suited to study gravimorphosis. Germinating fern spores, for instance, initiate their development with a first asymmetric division that separates a larger, vacuolated rhizoid precursor from a smaller and denser thallus precursor. 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. The axis of the first division is strictly aligned with gravity. When the spore is tilted after the axis of the first division has been determined, the rhizoid will grow in the wrong direction and cannot adjust this error (Edwards and Roux 1994). Prior to division, at the time when the spore is competent to the aligning 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 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. It should be mentioned that a similar mechanism of gravimorphosis has been described for the determination of the grey crescent in frog eggs (Gerhart et al. 1981), where the dorsiventral axis is determined by an interplay of 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).

Microtubules versus mechanosensitive channels in gravisensing: Since microtubules guide the anisotropic deposition of cellulose in the cell wall (see Chapter "Control of cell axis", in this volume), it is not trivial to discriminate their function in gravity-sensing from their participation in the control of axial cell expansion. When gravitropic bending is inhibited by antimicrotubular agents, this might be caused by a block of the sensory or of the effector function of microtubules. To discern these microtubular functions, lateral transport of auxin induced by gravitropic stimulation was analyzed as an event situated upstream of differential growth using radioactively labelled auxin in rice coleoptiles (Godbolé et al. 2000). Lateral auxin transport could be blocked by ethyl-*N*-phenylcarbamate (EPC), a herbicide that binds to the carboxyterminus of α-tubulin and inhibits assembly of tubulin heterodimers to the growing ends of microtubules (Wiesler et al. 2002). Interestingly, taxol inhibited lateral transport partially without any inhibition of longitudinal transport of auxin. This indicates that the presence of sensory microtubules is not sufficient for gravity sensing—they have to be endowed with turnover to fulfil their function. The high dynamics of this sensory microtubule population might also explain the extreme sensitivity of gravisensing to low temperature that would be otherwise difficult to explain (Taylor and Leopold 1992). These observations favour a model, where microtubules are actively sensing gravity (Fig. 1B) rather than merely acting as gravisusceptors (Fig. 1A).

The gravisensory function of microtubules can be specifically blocked by acrylamide (Gutjahr and Nick 2006), a widely used inhibitor of intermediate-filament function in mammalian cells (Eckert and Yeagle 1988). Similar to EPC, acrylamide interrupts a very early step in the gravitropic response chain, clearly upstream of auxin redistribution and differential growth. There are no clear homologues of intermediate-filament proteins known in the plant kingdom, but acrylamide treatment specifically disrupts microtubules, leaving, for instance, actin filaments, untouched (Gutjahr and Nick 2006). The immediate target of acrylamide in mammalian cells seems to be a kinase that phosphorylates keratin (Eckert and Yeagle 1988). Since kinases and phosphatases have been shown to regulate the organization of plant microtubules (Baskin and Wilson 1997), the inhibition of gravitropism by acrylamide might be caused by interference with the regulatory circuits active in the highly dynamic microtubule population responsible for gravisensing.

By application of artificial bending stress in antagonism to a gravitropic stimulus, it is possible to separate the response of gravity from the secondary mechanic stimulus that is induced by the differential growth during gravitropic bending (Ikushima and Shimmen 2005). When, under these conditions, the activity of mechanosensitive channels was suppressed by gadolinium in hypocotyls of adzuki beans, this suppressed the (mechanically induced) reorientation of microtubules in the effector tissue, whereas gravitropic curvature proceeded unaltered (indicating that the microtubule

population resident in the inner tissues of the apical hook that is responsible for gravisensing remained functional). Thus, at least in this system, mechanosensing is sensitive to gadolinium, gravisensing is not.

Although our knowledge on the primary events of mechano- and gravisensing in plants is extremely limited, it is clear already at this stage that the role of microtubules might differ qualitatively. In mechanosensing, microtubules seem to act as susceptor structures that focus deformation stress towards ion channels (Fig. 1A). In contrast, in gravisensing, the necessity for high dynamics and dimer turnover favours a direct sensory role of microtubules (Fig. 1B). Thus, nature might utilize both mechanisms simultaneously to sense (and possibly to discriminate) different stimuli. The challenge for future research in this field will be to design experimental approaches with clear outputs based on clear concepts on the sensing mechanism. Only in a second step it will become possible to define and test molecular and cellular candidates.

5 Microtubules as Thermometers

In temperate regions, temperature poses major constraints to crop yield. Attempts to increase photosynthetic rates by conventional breeding programs, 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 be reached only, 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 limited by the cold sensitivity of leaf growth (Watson 1952; Monteith and Elston 1971). This conclusion is supported by the finding that in cool climates the production of biomass is not source-, but sink-limited (Warren-Wilson 1966). The major target seems to be the root—it is thus the cold response of roots that defines the velocity of shoot development (Atkin et al. 1973).

However, the issue of cold sensitivity in agriculture is not confined to the temperate regions. Many tropical and subtropical plants suffer severely when they are exposed to cool temperatures that are even still far above the freezing point. This poses extreme problems when fruits have to be harvested and cooled for transport and processing, because these fruits rot rapidly as soon as they return to warmer temperatures. This phenomenon has been known for a long time and was originally termed *Erkältung* (chilling damage) by Molisch (1897) to distinguish it from the damage that is caused by actually freezing the tissue. In extreme cases, even very moderate cooling can produce irreversible damage, when it hits a very sensitive period of development. For instance, the fertility of rice is extremely and irreversibly reduced when temperature drops below 18 °C during flower development. The economical consequences of this phenomenon can be drastic—for instance, during the

cool summer of 1993, the rice yield was reduced by around 25% according to estimates of the Japanese Ministry of Agriculture, Forestry and Fishery.

The extent of cold sensitivity varies between different species and even between different cultivars of the same crop. Those plants that can cope with cool, but non-freezing temperatures below 10 °C, are termed chilling-resistant, whereas the term freezing-resistant is used for plants that can survive temperatures below zero, such as winter wheat or rye (Lyons 1973). It should be kept in mind that the degree of cold sensitivity can change depending on development and environment. For instance, the freezing resistance of many species can be increased by pretreatment with cool, but non-freezing temperature. This so-called cold acclimation or cold hardening during the autumn determines the survival during the winter (Stair et al. 1998). The problem of cold tolerance is not only important for agronomy, but represents an interesting scientific issue as well, because similar to mechanic and gravity-stimulation, a physical signal has to be transformed into a cascade of biochemical signalling events.

In contrast to chilling damage, the cellular consequences of freezing injury are well understood. Especially during rapid freezing, ice crystals form and disrupt internal and external membranes which will kill the cell instantaneously (Burke et al. 1976). As long as freezing occurs at a slow pace and does not exceed a certain limit, the ice will form outside the cell and will remain on the surface of the cell walls, in vessel elements and on the external surface. This does not kill the cell as long as the ice crystals do not penetrate the plasma membrane. However, the plant will dry out in the long term because the access of water to the roots is impaired (Mazur 1963). Therefore, reduced dehydration by reduced transpiration is a strategy to cope with freezing stress. This is a major reason for the dominance of xeromorphic species (such as the conifers) in subarctic or subalpine forests. A second strategy against freezing injury seems to be the expression of specific hydrophilic proteins (Hughes and Dunn 1996). Some of these proteins seem to correspond to the antifreezing proteins found in Antarctic fishes (Kurkela and Frank 1990). These proteins are thought to reduce the threshold temperature for the phase transition into the solid state in membranes and cytoplasm. For instance, one of these proteins, COR15a stabilizes the lamellar phase of chloroplasts in low temperatures (Steponkus et al. 1998).

The actual reason for chilling injury is less evident and has not received the same degree of attention as freezing injury. An apparently trivial consequence of low temperature are reduced rates of biochemical processes. This should result in a reduced metabolic activity, but cannot explain irreversible damages. However, the temperature dependence of different enzymes varies considerably (Guy 1990): whereas maize starch synthetase is inhibited already at 12 °C, and the rice tonoplast proton-ATPase at 10 °C, the maize PEP carboxylase is still active at 4 °C. Thus, when the temperature drops below 10 °C, the enzymes of the respiratory chain will be more affected (possibly because

they are bound to membranes), whereas some of the glycolytic enzymes are still active. This will result in metabolic imbalance and the accumulation of ethanol and acetaldehyde (Lyons 1973).

Membrane-bound enzymes are more susceptible to chilling injury as compared to soluble enzymes (Lyons 1973). The reason has to be sought in the fluidity of the membranes which is tightly coupled to the abundance in unsaturated fatty acids. A membrane that is composed exclusively from fully saturated lipids should exhibit phase separation at around 30 °C, whereas the introduction of one *cis*-double bond in the centre of the molecule would decrease this phase transition down to 0 °C (Ishizaki-Nishizawa et al. 1996). The degree of lipid saturation can be manipulated by overexpression of desaturases in plants and this has been shown repeatedly to modify chilling sensitivity in plants (Murata et al. 1992; Wolter et al. 1992; Kodama et al. 1994; Ishizaki-Nishizawa et al. 1996).

One of the most pronounced and rapid cellular responses to chilling is the cessation of cytoplasmic streaming (Kamiya 1959; Woods et al. 1984; Tucker and Allen 1986) which occurs within a few minutes after a drop to 10 °C in chilling-sensitive species such as cucumber or tomato (Sachs 1865), whereas it can proceed in chilling-resistant plants down to 0 °C (Lyons 1973). When the period of chilling exceeds a few hours, cytoplasmic streaming fails to recover. Kinetic studies in subtropical species such as maize, lima bean and cotton (Lyons 1973) showed developmental differences with high sensitivity during periods of elevated cell growth. Additionally, high chilling sensitivity is characteristic for morphogenetic responses to light such as axis formation in lower plants (Haupt 1958) or phototropism in maize (Nick and Schäfer 1991).

Microtubules of both plants and animals disassemble in response to low temperature, but the degree of cold sensitivity depends on the type of organism. Whereas mammalian microtubules disassemble already at temperatures below +20 °C, the microtubules from poikilothermic animals remain intact below that temperature (Modig et al. 1994). In plants, the cold stability of microtubules is more pronounced as compared to animals (Juniper and Lawton 1979) reflecting the higher developmental plasticity. However, the critical temperature where microtubule disassembly occurs varies between different plant species (Jian et al. 1989; Chu et al. 1992; Pihakaski-Maunsbach and Puhakainen 1995): In chilling-sensitive plants such as maize, tomato or cucumber, microtubules disassemble already at temperatures above 4 °C, whereas they can withstand 0 °C in moderately resistant plants such as spinach and beet. In cold-resistant species such as winter wheat or winter rye even temperatures as low as –5 °C will not eliminate microtubules. Even within a given species, the cold sensitivity of microtubules can vary considerably (Abdrakhamanova et al. 2003).

The close correlation between microtubular cold sensitivity and the chilling sensitivity of cell growth is supported by the observation that abscisic

acid, a hormonal inducer of cold hardiness (Holubowicz and Boe 1969; Irving 1969; Rikin et al. 1975; Rikin and Richmond 1976) can stabilize cortical microtubules against low temperature (Sakiyama and Shibaoka 1990; Wang and Nick 2001). Tobacco mutants, where microtubules are more cold stable due to expression of an activation tag, are endowed with cold resistant leaf expansion (Ahad et al. 2003). Conversely, destabilization of microtubules by assembly blockers such as colchicine or podophyllotoxin increased the chilling sensitivity of cotton seedlings, and this effect could be rescued by addition of abscisic acid (Rikin et al. 1980). Gibberellin, a hormone that has been shown in several species to reduce cold hardiness (Rikin et al. 1975; Irving and Lanphear 1986), renders cortical microtubules more cold-susceptible (Akashi and Shibaoka 1987).

It is possible to increase the cold resistance of an otherwise chilling-sensitive species by precultivation at moderately cool temperature. The genes that are activated during this so-called cold hardening are partially identical to those that respond to abscisic acid (for a review see Hughes and Dunn 1996), and the tissue content of abscisic acid increases during cold hardening (Lalk and Dörffling 1985; Lång et al. 1994). On the other hand, mutants that are not able to sense abscisic acid are nevertheless capable of cold hardening (Gilmour and Thomashow 1991) indicating the coexistence of at least two parallel pathways that differ with respect to their dependency on abscisic acid.

Cold hardening can be detected on the level of microtubules as well. Microtubules of cold-acclimated spinach mesophyll cells coped better with the consequences of a freeze-thaw cycle (Bartola and Carter 1991a). Although abscisic acid can increase the cold resistance of microtubules (Sakiyama and Shibaoka 1990), it seems not to be the only trigger. When the microtubular response to abscisic acid was compared to the response to cold hardening, it differed in both orientation and degree of bundling (Wand and Nick 2001), again supporting the existence of a pathway that is independent of abscisic acid.

Microtubules are not only the target of cold stress, they seem, in addition, to participate in cold sensing itself, triggering a chain of events that culminates in increased cold hardiness. When microtubule disassembly is suppressed by taxol, this is reported to suppress cold hardening (Kerr and Carter 1990; Bartolo and Carter 1991b). This indicates that microtubules have to disassemble to a certain degree in order to trigger cold hardening. To test this hypothesis, cold hardening was followed in three cultivars of winter wheat that differed in freezing tolerance (Abdrakhamanova et al. 2003). During cultivation at 4 °C, the growth rate of roots recovered progressively as a manifestation of cold hardening. In parallel, the roots acquired progressive resistance to a challenging freezing shock which would impair growth irreversibly in nonacclimated 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, when a transient disassembly was artificially

generated by a pulse treatment with the antimicrotubular herbicide pronamide in the sensitive cultivar, this could induce freezing tolerance. This demonstrates that a transient, partial disassembly of microtubules is necessary and sufficient to trigger cold hardening suggesting that microtubules act as "thermometers".

Similar to mechano- and gravity-sensing this leads to the question, whether microtubules act as susceptors (Fig. 1A) or as true receptors (Fig. 1B) for low temperature. The primary signal for cold perception is thought to consist of increased membrane rigidity (Los and Murata 2004; Sangwan et al. 2001). For instance, the input of low temperature can be mimicked by chemical compounds that increase rigidity, such as demethylsulfoxide, whereas benzyl alcohol, a compound that increases membrane fluidity, can block cold signalling (Sangwan et al. 2001). Using aequorin as a reporter in transgenic plants, rapid and transient increases of intracellular calcium levels in response to a cold shock could be demonstrated monitoring changes of bioluminescence (Knight et al. 1991). Pharmacological data (Monroy et al. 1993) confirmed that this calcium peak is not only a byproduct of the cold response, but necessary to trigger cold acclimation. This peak is generated through calcium channels in conjunction with calmodulin. Calcium/calmodulin in turn are intimately linked to microtubule dynamics. Immunocytochemical data show that microtubules are decorated with calmodulin depending on the concentration of calcium (Fisher and Cyr 1993). It was further suggested that the dynamics of microtubules is regulated via a calmodulin-sensitive interaction between microtubules and microtubule-associated proteins such as the bundling protein EF-1α (Durso and Cyr 1994). However, the interaction could be even more direct, because cleavage of the C-terminus of maize tubulin was shown to render microtubules resistant to both low temperature and calcium (Bokros et al. 1996). If the release of calcium from intracellular pools was blocked by treatment with lithium, an inhibitor of polyphosphoinositide turnover (Berridge and Irvine 1984), this resulted in increased cold stability of microtubules in spinach mesophyll (Bartolo and Carter 1992). Using a cold-responsive reporter system it could be demonstrated that disassembly of microtubules by oryzalin could mimick the effect of low temperature, whereas suppression of microtubule disassembly by taxol suppressed the activation of this promotor by low temperature (Sangwan et al. 2001). In the same system, treatment with the calcium ionophore A23187 was observed to be inductive, whereas the Ca-channel blocker gadolinium suppressed cold induction of the reporter. These data favour a model, where microtubules act as receptors that limit the permeability of calcium channels that are triggered by membrane rigidification (Fig. 3).

When microtubules function as modulators of calcium-channel activity and when microtubule integrity is regulated through calcium/calmodulin this would set up a regulatory circuit capable of self-amplification: Stable microtubules that limit the activity of cold-induced voltage-dependent calcium

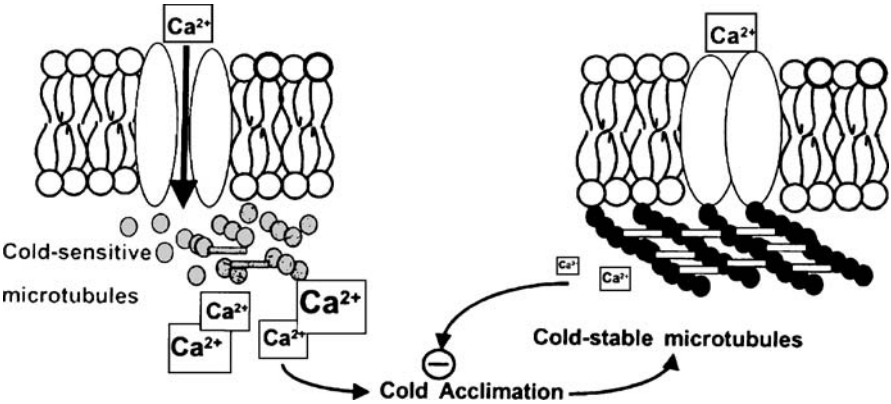


Fig. 3 Perceptive role of microtubules in cold sensing. Cold induced disassembly of specific microtubules that control the permeability of mechanosensitive calcium channels amplifies the influx of calcium, triggering further disassembly of microtubules in a positive feedback loop. The calcium-triggered transduction cascade culminates in changes of gene expression that will produce cold-hardening, including the formation of cold-stable microtubules such that the microtubule-dependent perception of cold will be alleviated (sensory adaptation)

channels, would, upon disassembly, release this constraint and this would elevate the activity of the channels resulting in an increased influx of calcium. This calcium influx, in turn would result in further disintegration of the microtubular cytoskeleton and thus trigger by this positive feedback the influx of additional calcium. A very small initial calcium influx might thus be amplified into a strong signal that can be easily processed by the activation of calcium-dependent signalling cascades. The resulting signal cascade will activate cold-hardening as an adaptive response to cold stress. Interestingly, microtubules will be rendered cold stable as a consequence of this cold-hardening (Pihakaski-Maunsbach and Puhakainen 1995; Abdrakhamanova et al. 2003), which in turn, should result in a reduced activity of the calcium channels that respond to membrane rigidification. Thus, microtubules would not only endow cold sensing with high sensitivity, but, in addition, with the ability to downregulate sensitivity upon prolonged stimulation, a key requirement for any biological sensory process.

6 Outlook

Comparison of the Three Sensory Mechanisms

This work summarizes evidence for a microtubule function in the sensing of touch, gravity and low temperature. Although we are still far from under-

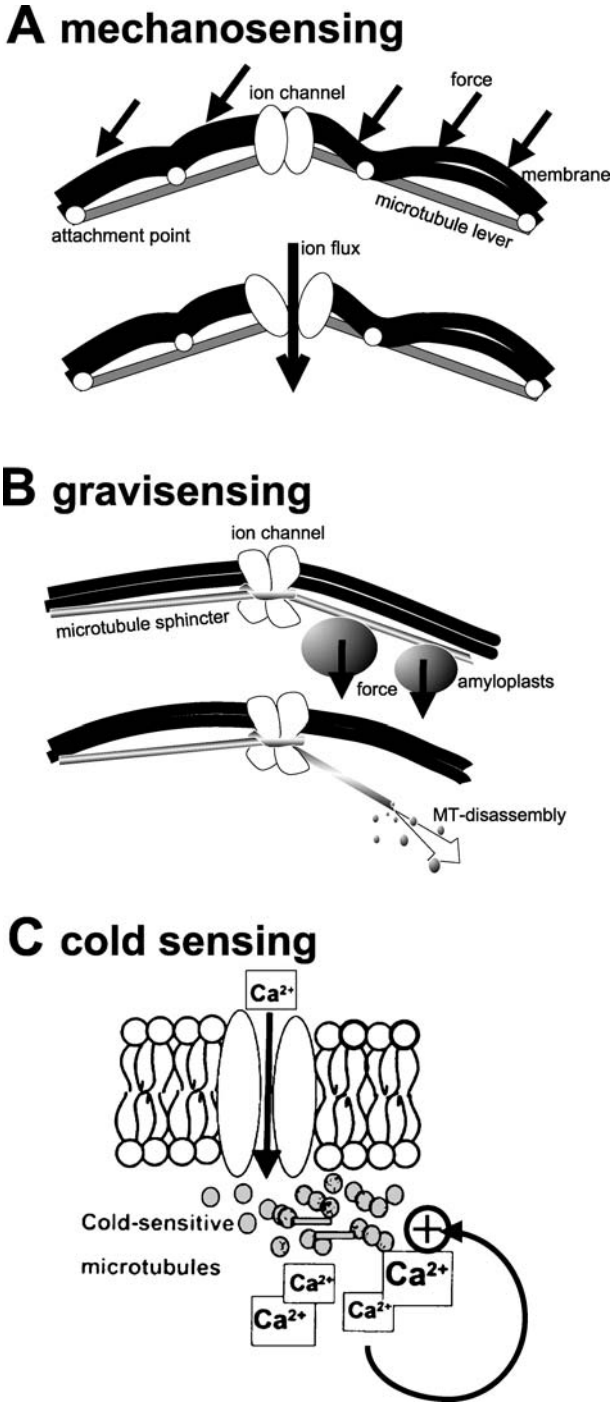
standing the actual setup of the sensory machinery, already at this stage first differences between the different stimulus qualities emerge (Fig. 4):

For mechanoperception, microtubules seem to interact with stretch-activated ion channels. They might act as mechanosusceptors (Fig. 4A) focussing diffuse membrane deformations upon specific membrane areas. Since the definition of a stretch-activated channel is experimentally very problematic and prone to artifacts (Gustin et al. 1991), the possibility should be tested seriously that there are no a priori stretch-activated channels in plants. It might be the combination of a channel with a microtubule lever system that generates the mechanogating of the channel. The same channel would not be defined as mechanosensitive, if the membrane would be stripped of microtubules.

The situation might be different for the sensing of gravity (Fig. 4B). Here microtubules themselves could act as primary sensors. The few experiments, where the involvement of ion channels has been addressed experimentally (Ikushima and Shimmen 2005) suggest that these channels might be dispensable for gravity sensing. The necessity of microtubule turnover in the sensing of gravity indicates a true perceptive function rather than stimulus susception.

The sensing of cold (Fig. 4C) seems to represent a third mechanism, where the gating of cold-sensitive channels (that probably respond to membrane rigidity as an input) is limited by microtubules. When these microtubules disassemble in response to cold, this will release the constraints upon the activity of the ion channels such that calcium can enter which will facilitate, through interaction with calmodulin, further disassembly of microtubules and thus trigger a positive feedback loop. In this system microtubules would play a dual function—in the first phase perception in sensu strictu and susception in the subsequent phase.

It is their innate molecular properties that render microtubules ideal for the sensing of minute and noisy inputs. Microtubule dynamics are nonlinear and endowed with autocatalytic properties: (A) Under cellular conditions, microtubules of different orientation compete for the incorporation of free heterodimers. In all organisms investigated so far tubulin synthesis is tightly regulated by an elaborate system of transcriptional and post-transcriptional controls, probably to avoid the accumulation of (highly toxic) supernumerous free heterodimers (for details refer to Chapter "Plant tubulin genes: regulatory and evolutionary aspects", in this volume). (B) Although the term "cytoskeleton" evokes the idea of a rigid framework that stabilizes the structure of a cell, such associations are far from reality. The half-time of a plant microtubule, for instance, has been calculated to be in the range of 30–60 s (Hush et al. 1994). Therefore, it is more appropriate to conceive microtubules as states of dynamic equilibrium between assembly and disassembly of tubulin heterodimers. It is this dynamic equilibrium that provides the major source for the characteristic nonlinearity of microtubule dynamics.


A mechanosensing
ion channel
force
membrane
microtubule lever
attachment point
ion flux
B gravisensing
ion channel
microtubule sphincter
force
amyloplasts
MT-disassembly
C cold sensing
Ca2+
Cold-sensitive
microtubules
Ca2+
Ca2+
Ca2+
Ca2+

◀ **Fig. 4** Potential differences in microtubule function during the sensing of touch, gravity and cold. **A** Mechanosusceptor function of microtubules during stretch-activation of mechanosensitive ion channels. **B** Microtubules as primary gravity sensors, possibly in interaction with microtubule-gated ion channels. **C** Dual function of microtubules as primary sensors and amplifiers (susceptors) during cold sensing

Interestingly, the relation between assembly and disassembly is practically never balanced—there is always one dominating over its antagonist. This statement is valid in both space and time. In space, because dimer addition and dispersal define a distinct polarity of each individual microtubule with dimer addition dominating at the plus end, dimer dissociation at the minus end. In time, because each microtubule can switch between a growing state, when dimer addition at the plus end predominates over dimer dissociation at the minus end, and a shrinking state, when dimer dissociation at the minus end exceeds dimer addition at the plus end. The switch between both states is swift and dramatic, so dramatic that it has been termed microtubule catastrophe. These conversions depend on associated proteins that can increase or decrease the frequency of transition between growth and shrinkage.

Because of their nonlinear growth microtubules are often involved in developmental patterning. For instance, the induction of the grey crescent in the developing frog egg is produced in an epigenetic process, where a gravity-dependent gradient of developmental determinants in the central yolk interacts with a second, displaced, gradient in the egg cortex (Gerhart et al. 1981). The displacement of the egg cortex is driven by microtubules and triggered by the penetration of the sperm. The sperm induces the nucleation of microtubules that act as tracks for a kinesin-driven movement (Elinson and Rowning 1988). The movement, in turn, triggers shear forces that align the nucleation of additional microtubules in a direction parallel to the movement, whereas deviant microtubules more frequently undergo catastrophic transitions. The resulting net alignment of tracks increases the efficiency of movement and thus the aligning force. This culminates in a rapid rotation of the cortical plasma in a direction from the sperm towards the more remote equator of the egg. This movement will then cause an overlap of upper cortex with a small region of the lower core and eventually trigger inductive events that define the Spemann organisator.

The combination of nonlinear, autocatalytic dynamic states of individual microtubules with the tight control of free heterodimers accentuating mutual competition between individual microtubules generates system properties that are highly relevant for sensory processes. Microtubules fulfil all formal criteria of a reaction-diffusion system *in sensu* Turing (1952). This means that they can be understood as ideal pattern-generators that are able to produce qualitatively clear, neat outputs from minute, and highly noise-contaminated inputs. It can be modelled how due to their innate dynamic

properties microtubules will spontaneously self-organize in response to even weak external factors such as gravity or mechanic fields (Tabony et al. 2004). It thus seems that nature has made ample use of these unique molecular properties to build sensory systems that are both sensitive and robust against stochastic noise.

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Plant Tubulin Genes: Regulatory and Evolutionary Aspects

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Abstract Once transcribed at its own DNA locus, a tubulin monomer knows that it will be eventually incorporated within microtubules (MTs). What it does not know, though, is how it will ultimately reach its final destination, how complex the path will be, and at which regulatory step it will have to stop. In fact, this will depend upon the status of the cell, the genetic background and the external stimuli. In this article, we will try to guide the reader through the many places of this fascinating journey. In the post-genomic era it may appear anachronistic to keep studying plant tubulin genes. We all know what they do: they encode tubulin, one of the major cytoskeletal proteins, the building block of microtubules (MTs). However, accumulation of the transcripts of the various tubulin genes of plants, tubulin synthesis and post-translational modification, tubulin folding and assembly into MTs, and control of MT dynamics and positioning remain as fascinating, vast and still unresolved fields of investigation, full of novelties and peculiarities. We may as well call this arena of research microTUBULOMICS, recognizing the very many aspects that impinge on tubulin expression and subunit turn-over, accumulation, and interaction with proteins and drugs (Fig. 1).

1
All Aboard – Tubulin Genes at the Nuclear Station

Tubulin synthesis and availability is firstly controlled at the level of nuclear transcription. The plant cell has the possibility to choose which of the different members of the two α- and β-tubulin gene families should be expressed and this may depend on the cell cycle, the stage of development, the tissue and organ as well as different internal and external stimuli. In the classical model, nuclear mRNA transcription is controlled by promotors and 5′ and 3′ sequences that flank the coding region. Typical experiments involve the isolation of tubulin sequences 5′ of the ATG initiation codon, the construction of transformation vectors in which these putative regulatory sequences are fused to a reporter gene (GUS or GFP), and the production of transgenic plants in which the expression profile driven by that specific promotor activity can be followed (examples in Chu et al. 1998; Stotz and Long 1999; Abe et al. 2004). In this way, a role for any given tubulin isotype can be proposed that is based on the specific pattern of expression that its own promotor/regulatory sequence is able to sustain. Using this approach, several laboratories have recorded the specificity of expression of particular tubulin genes in roots, flowers, and pollen or in response to cold, fungal colonization

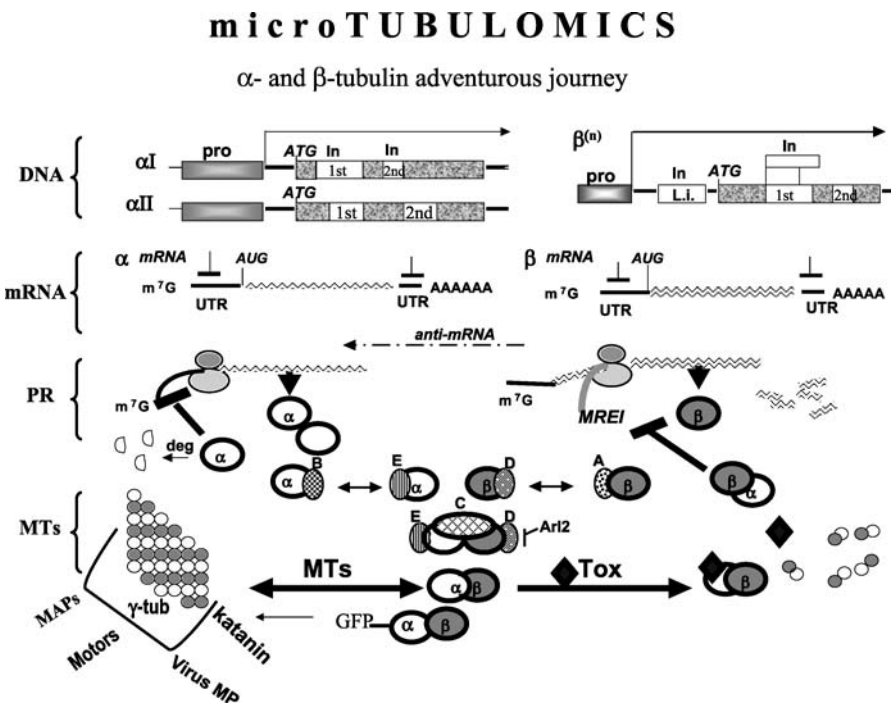

Fig. 1 microTUBULOMICS: plant tubulin regulatory issues. Different control mechanisms operating at DNA, mRNA, protein synthesis (PR), folding and assembly into MTs levels are depicted. This scheme is mainly tailored on what has been reported for the rice tubulin genes. The situation in other plant species may be partially different (i.e., there are three gene subfamilies for the alpha tubulin of maize) (Villemur et al. 1992, 1994; Qin et al. 1997). *DNA level*: The genomic organization of generic plant α- and β-tubulin genes is shown. α-tubulin genes are divided into their corresponding two gene subfamilies (I and II). Promotor (pro) and direction of transcription are shown. Intron sequences are indicated by white boxes and their different sizes and location are also represented. *mRNA level*: 5′–3′ UTR sequences as possible site for translational repression are shown as well as naturally occurring antisense tubulin mRNA molecules (Dolfini et al. 1993; Breviario, unpublished). *Protein level*: Translational control for α-tubulin and MREI-mediated mechanism for β-tubulin mRNA degradation are shown. Tubulin folding brought about by proteins of the chaperone complex TFC is shown. Arl2 is a regulatory G-protein. *Microtubule level*: incorporation of tubulin dimers into the MTs is shown with lateral contacts involving α–α and β–β monomer interactions. The depolymerizing effect of a generic anti-tubulin drug (Tox) is also represented. Further interactions of MTs with generic microtubule-associated proteins (MAPs), motor proteins, severing proteins (katanin), virus movement proteins (MP), and γ-tubulin for microtubule-dependent microtubule nucleation are shown. The incorporation of a chimeric GFP-tubulin into the Mts is represented. The scheme is principally tailored for the cortical MT array

or light (reviewed in Breviario and Nick 2000). Sometimes, tubulin transcriptional downregulation depends on a combination of regulatory elements present at both the 5′ and the 3′ flanking sequence as it was the case for light-regulated TUB1 gene of soybean (Doyle and Han 2001).

However, none of these studies had ever included tubulin introns as putative regulatory elements. The need to consider this issue has been uncovered by two different laboratories working on rice tubulins. Both laboratories documented, in transient or stable transgenic approaches, that full transcriptional expression of a reporter gene was achieved only when the tubulin regulatory sequences driving GUS expression contained a tubulin intron sequence. The *OsTubB16* (now *TUB4* according to the recent nomenclature by Yoshikawa et al. 2003) promotor activity was strongly enhanced by the presence of an intron located in the leader sequence of the same gene (Morello et al. 2002), whereas the *OsTubA1* promotor activity was enhanced by the presence of the first intron from its coding sequence (Jeon et al. 2000). This intron could also enhance GUS expression if juxtaposed to the 35S-CaMV promotor. Experiments performed with the rice constructs carrying TUA1 regulatory elements showed that the intron was not only enhancing the level of GUS expression, but was responsible for a specific pattern of expression that was restricted to meristematic tissues (Jeon et al. 2000). Experiments performed on the regulatory regions of the two other rice α tubulin genes (TUA2 and TUA3) confirmed these findings and indicated that the presence of the first intron is strictly required for high-level expression of all rice α-tubulin genes (Fiume et al. 2004). Thus, intron-mediated enhancement of expression (IME), participates in the control of tubulin expression in plants. These experiments have also shown a certain level of sequence dependency since the regulatory effect exerted by any of the rice α-tubulin introns cannot be replaced by the regulatory intron of TUB4 (Fiume et al. 2004).

However, the molecular mechanisms of IME remain largely unknown and could not be attributed to specificities of the nucleotide sequences (Rose and Beliakoff 2000; Rose 2002). Introns act in concert with other regulatory sequences and may exhibit gene-dependent requirements (Rethmeier 1997; Fiume et al. 2004). A very exciting aspect of intron and intron-derived sequences, such as spliced non-coding RNA (ncRNA), relies on a novel theory of eukaryotic evolution that calls for multiple interactions between ncRNAs, proteins, and DNA (Mattick 2004). According to this theory, ncRNAs have evolved to function as endogenous network control molecules that enable direct gene–gene communication and multitasking of eukaryotic genomes (Mattick and Gagen 2001). The evidence for the presence of such intron-derived microRNAs has been steadily accumulating (Li et al. 2007). To study the function of ncRNA, tubulin would be a good model since it is ubiquitous in all eukaryotes and was subject to long and well-documented evolution (Moriya et al. 2001; Jost et al. 2004). In fact, tubulin introns have been con-

served through evolution and are found, from yeasts to plants, in well-defined positional blocks within their respective genomic sequences (Dibb and Newman 1989; Liaud et al. 1992). This feature has been exploited to estimate the sizes of plant tubulin gene families as well as their polymorphic content (Montoliu et al. 1992; Bardini et al. 2004; Breviario et al. 2007).

Experiments currently under way also show that the removal of regulatory introns of rice TUB4 and TUA2 genes alters the patterns of tissue specific expression rather than, in the case of TUB4, reducing the expression of the GUS reporter (Breviario, unpublished). IME has been definitively shown for the rice tubulins. It remains to be elucidated to what extent it is present in other plant tubulin genes.

The functionally variable tubulin introns provide sequence polymorphisms that are also useful for the genetic characterization of plant species and varieties and the investigation of eukaryotic evolution (Lynch et al. 2002), whereby the early stages are yet to be resolved (Moriya et al. 2001). Differentiation of the microtubule system may have been a landmark of early eukaryotic evolution with a divergence between a plant protist-oxymonad and an animal parabasolid clade (Moriya et al. 2001). On this premise, a plant species- and variety-specific assay (TBP for Tubulin Based Polymorphism) based on length polymorphism of the introns present in the β-tubulin gene family has been developed and optimized (Bardini et al. 2004; Breviario et al. 2007). Based on the selective PCR-mediated amplification of the two introns present in the coding sequences of plant β-tubulin genes, the TBP assay has been successfully applied to a large number of plant species and varieties. As further advantage this method does not require a priori knowledge of the beta-tubulin sequences for the species of interest.

Tubulin introns are unique by their simplicity of detection, genomic multilocus distribution, and length polymorphisms. These specific features may be consequences of their potential role in gene regulation (Jeon et al. 2000; Fiume et al. 2004). Compared to coding sequences, the phylogeny of intron sequences is not easily discernable due to their rapid evolution. In contrast, intron position is stable to such an extent that non-conserved positions are considered to have arisen most recently by specialized mechanisms such as intron-sliding (Rogozin et al. 2000; Edvardsen et al. 2004). Remarkably, intron position is biased among species. Most species are characterized by a well-defined number of introns, the positions of which are strongly conserved (Dibb and Newman 1989). This means that intron positioning has not occurred randomly. Moreover, intron location is biased toward the 5′ end of a gene. Again, this bias may be explained by possible regulatory roles that may involve, in a cooperative way, introns and untranslated 5′ sequences (Sakurai et al. 2002). Accordingly, intron position is conserved among tubulin genes of a given species. The potential of TBP for the analysis of alternative intron functions is still to be exploited, given the fact that alternative splicing resulting in diverse plant tubulins has not been reported, yet.

There are several important reasons to use plant tubulin genes to investigate the role of introns under the two aspects of evolution and gene expression:

1. The tubulin gene family is widely used as a phylogenetic marker especially to investigate early eukaryotic evolution. α- an β-tubulin genes have the widest taxonomic representation and have been identified in the putatively early diverging eukaryotic clades (Edgcom et al. 2001; Hampl et al. 2005; Moriya et al. 2001; Keeling and Doolittle 1996). More specifically, tubulin introns have been used successfully to establish evolutionary relationships in fungi, protozoa, urochordates, flagellates, endophytes and within the tuber genus (Hampl et al. 2005; Wang et al. 2006; Gentile et al. 2005; Schroeder et al. 2002; Widmer et al. 1998; Edvardsen et al. 2004; Begerow et al. 2004).
2. Alternative splicing of tubulin introns, leading to the production of different tubulin proteins, has not been reported so far.
3. Tubulin introns, in the vast majority of eukaryotic species, are not spread randomly within the genome but are present in 16 well-defined clusters. Cluster combinations change among the different eukaryotic species. Reported exceptions are *Oikopleura* and *Caenorhabditis* that show short introns at non-conserved positions within the α-tubulin genes (Edvardsen et al. 2004).
4. Higher plant beta-tubulin introns are located at conserved positions within their respective genes.
5. Intron position is biased toward the 5′ end of a tubulin gene, i.e., the region mainly involved in the control of gene expression.
6. Plant tubulin genes are more numerous than their animal counterparts.
7. Accumulating evidence shows a role of plant tubulin introns in the control of gene expression.
8. The development of microtubules represents a landmark in eukaryotic evolution that can be compared with changes in the genomic organization and sequence of tubulin genes. Such changes have mainly occurred in the non-coding parts because of the obviously strict functional requirements of the coding regions. This is also the reason why evolution has worked differently on coding as compared to intron sequences.

The structural and functional analysis of tubulin introns may help to identify the direction of intron evolution: gain, loss, or both? A matter that has not been clarified yet. Of lately, introns have gained new credit in the scientific community. DNA data released from several genome projects have provided evidence that 12 different vertebrate species share up to 99% of identity in protein encoding DNA sequences, while 2/3 of their genomic differences are found in introns (Gibbs 2003). Moreover, species complexity is not related to genome sizes, as more gene-encoded products may be found in organisms with relatively smaller genomes (Mattick 2004). Thus, we are left with the be-

lief that what was once called junk DNA may actually have an important role both in modulation of gene expression (Sinibaldi and Mettler 1992) and in eukaryotic evolution, as stated by Mattick and Gagen (2001) and extensively discussed by Rodriguez-Trelles et al. (2006).

2 Next: Post-Transcriptional and Post-Translational Check Points

Availability of α-tubulin and β-tubulin monomers can also depend on control mechanisms exerted at the level of mRNA stability and protein synthesis. Two separate mechanisms that down-regulate protein synthesis in the presence of increasing amounts of unpolymerized tubulin dimers and monomers have been described for animal cells. One is operating on the nascent chain of the newly synthesized β-tubulin polypeptide: unpolymerized tubulin dimers bind to the MREI motif of the N-terminal end, common to all β tubulins, and cause the activation of a ribosome-based RNAse that specifically degrades tubulin mRNA. So far, no one has demonstrated an MREI-based regulatory feedback mechanism in plants, but the possibility exists, as almost all plant β-tubulins contain MREI as the initial tetrapeptide. Indirect evidence that a similar mechanism may also occur in plants comes from experiments performed on cultured soybean cells transfected with apoaequorin and challenged with the addition of elicitors (Ebel et al. 2001). Glucan elicitors induce differential degradation of mRNA for two β-tubulins, with down-regulation of tubB1 mRNA but an unaltered level of tubB2 mRNA. In explanation, the authors point out that while the N-terminus of β1-tubulin contains the canonical MREI autoregulatory sequence, the N-terminal sequence of β2-tubulin, MRES, diverges. On the other hand, in rice cells in which extensive MT depolymerization was caused by treatments with oryzalin, the amount of tubulin monomers was drastically lowered whereas tubulin mRNA levels were almost unaltered, suggesting the presence of different regulatory mechanisms (Gianì et al. 1998). Thus, the question of whether plant cells may control the amount of tubulin through a mechanism that operates at the level of mRNA stability remains open. In this context, the finding that oryzalin causes a rapid decrease in de-novo synthesis of rice α-tubulin and β-tubulin, but does not change the level of tubulin mRNA, suggests that a mechanism operating at the level of mRNA translation rather than mRNA stability may be active under these conditions (Gianì et al. 2002). Translational control exerted at the level of tubulin 5′-UTR sequences may also explain the data obtained from the transformation of maize and tobacco with exogenous tubulin (see below). More substantial evidence for such a translational control exists for the synthesis and accumulation of α-tubulin in mammalian cells (Gonzales and Cabral 1996).

Tubulin folding is another key step that regulates the amount of tubulin subunits available for MT polymerization in concert with translational and

transcriptional controls. Tubulin folding is carried out by a cytosolic chaperone complex consisting of several proteins. Some are specific for monomeric α-tubulin (TFC B and E) or β-tubulin (TFC A and D), whereas others (TFC C) are specific for heterodimeric tubulin (Steinborn et al. 2002). At the dimeric stage, folding is also under regulation of the small G-protein Arl2. Hydrolysis of GTP by β-tubulin in the complex acts as a switch for the release of the native tubulin heterodimer (Tian et al. 1999). Therefore, tubulin folding is an important process that modulates MT dynamics. In fact, it has been recently shown that *A. thaliana* cells that over-expressed TFC B have fewer MTs compared to control cells suggesting that the sequestering of α-tubulin, determined by high amount of TFC B, reduces the actual number of microtubules (Dhonukshe et al. 2006). This is likely to influence the amount of the monomeric β-tubulin that is actually incorporated into the MTs. The cytosolic chaperone complex TFC may be involved in maintaining the correct balance in intracellular tubulin α and β monomer pools as shown in *Arabidopsis*, where a mutation in *Kis*, the Arabidopsis ortholog of tubulin folding cofactor (TFC) A, could be rescued by overexpression of α-tubulin (Kirik et al. 2002). This is consistent with the idea that the overexpression of α-tubulin in *kis* mutants counterbalances the excess of free monomeric β-tubulin resulting from a reduced activity of the chaperone protein TFC A.

The future characterization of the tubulin-binding sites on folding proteins will help in the development of chemicals that interfere with tubulin folding and thus MT dynamics. This is actually a very promising new field of investigation with potential for medical application, because some human diseases have been shown recently to result from mutations in tubulin-folding factors that affect tubulin dimer formation and MT dynamics (Kortazar et al. 2006).

An important aspect of the tubulin chaperone complex is related to the kinetics of the equilibrium between the monomeric α- and β-tubulins that are recruited for dimer assembly and those monomers that remain sequestered by TFC B (α-tubulin) and A (β-tubulin) (Fig. 1). These sequestered monomers form a storage pool of tubulin moieties ready for assembly and are expected to be needed during phases of intense MT polymerization and growth (Lopez-Fanarraga et al. 2001). On the contrary, shrinkage or depolymerization of MTs, due to physiological constraints or drug treatments, could result in an excess of unincorporated tubulin that could then undergo protein degradation, as shown for rice cells treated with oryzalin (Gianì et al. 1998, 2002).

Post-translational modification of α- or β-tubulins may also influence the stability of microtubules as well as their interactions with proteins and ligands such as drugs, while contributing to tubulin diversity. These modifications typically include phosphorylation, polyglycylation and acetylation, for which no precise location on tubulin proteins has been reported, while polyglutamylation and tyrosination/detyrosination are restricted to the highly variable acidic carboxy-terminal region (Smertenko et al. 1997; Erck et al. 2000). In most cases, the modifying enzymes preferentially act on

the tubulin subunits that are part of assembled microtubules, whereas the enzymes that catalyze the reverse reactions prefer the α–β dimer of non-polymerized tubulin (Laurent and Fleury 1996). This is certainly true for tyrosination/detyrosination (see below). Tubulin post-translational modifications are evolutionary conserved suggesting that they play important roles in vivo such as the binding of molecular motors to the external surface of MTs (Rosenbaum 2000). Despite their undisputable importance, their occurrence and role in plants has been the subject of few, not yet conclusive and sometimes controversial reports. Tubulin phosphorylation, for instance, remains still elusive (MacRae 1997). Only indirect evidences has been provided by studies with inhibitors of protein kinases and phosphatases (Baskin and Wilson 1997) or by analysis of two-dimensional phosphoprotein pattern resulting by treatment of *Nicotiana tabaccum* cells with lipopolysaccharides (Gerber et al. 2006). A systematic study on α-tubulin post-translational modifications based on specific antibodies against acetylated, polyglutamylated, and tyrosinated maize α-tubulin in different tissues of maize has been published recently (Wang et al. 2004). Tyrosination of α-tubulin was by far the predominant modification, especially in leaves.

Although more extensively studied in animals than in plants (Duckett and Lloyd 1994; Smertenko et al. 1997; Gilmer et al. 1999), tubulin tyrosination deserves a few additional lines for its predominance and importance. Tyrosination of α-tubulin is a signature for rapid growth. It is ubiquitous for the simple reason that all α-tubulin genes in any given plant (or animal) contain a C-terminal tyrosine. This tyrosine is cleaved by a (yet unidentified) tyrosine carboxypeptidase (TCP) as first step of a cycle, and can be recycled due to a specific tubulin-tyrosine ligase (TTL) (Erck et al. 2000). This tyrosination cycle seems to be evolutionary conserved because detyrosinated tubulin has been reported in many eukaryotes (Preston et al. 1979). While TCP can bind only microtubules, TTL acts on unpolymerized tubulin dimers through a specific binding with the β-tubulin moiety (Wehland and Weber 1987). Because of this, microtubules with a long lifetime are rich in detyrosinated α-tubulin, whereas microtubules with a fast turn-over contain more tyrosinated α-tubulin. Therefore detyrosination is the consequence rather than the cause of microtubule stability (Khawaja et al. 1988). The detyrosinated tubulin is likely to be endowed with enhanced affinity for kinesins (Liao and Gundersen 1998). Both the status of tubulin tyrosination and the activity of TTL have been reported as important markers for tumor progression in animal cells. Tubulin detyrosination, resulting from suppression of the tubulin-tyrosine ligase, and the resulting unbalanced activity of TCP correlate with tumor aggressiveness (Mialhe et al. 2001; Lafanechere et al. 1998). Thus, a clear line is drawn in animal cells between growth regulation, the status of tyrosination, and hence stability of microtubules. This is a correlation that calls for further attention in plant science, highly due after the initial report on GA-induced MT reorientation and modulation of growth rate, two

events that are associated to changes in the tyrosination status of α-tubulin (Duckett and Lloyd 1994). Another element of interest is the finding that tyrosination of the C-terminus of rice α-tubulin greatly reduces the affinity and the binding of ethyl-*N*-phenylcarbamate, a herbicide of the aryl-carbamate class (Wiesler et al. 2002). Finally, but not conclusively, further directions of research for plant biologists should also be pursued after the finding that TTL may actually constitute a domain protein family whose members can catalyze ligation of different amino acids, i.e., tyrosine in case of TTLL1. Quite interestingly, tubulin polyglutamylase enzymes are members of this TLL family (Janke et al. 2005).

3 Tubulin Isotypes and Stochiometry

Multiple-tubulin isotypes and isoforms (post-translationally modified forms of translation products or isotypes) may be the tools by which plants increase their options for regulation of microtubule dynamics (Gull et al. 1996). Microtubule organization, dynamic properties, and interaction with proteins and ligands may depend on the tubulin isotype or isoform composition as dramatically shown for the *Arabidopsis lefty* mutants (Thitamadee et al. 2002). These mutants are characterized by an altered right/left symmetry of their organs that causes left-handed helical growth. The altered phenotype is due to a single dominant negative mutation occurring in either of the α-tubulin genes *TUA4* or *TUA6*. The resultant serine to phenylalanine substitution at position 180 is located at the intradimer interface of tubulin. Thus, a single amino acid change in one specific α-tubulin isotype can heavily affect the morphology of the whole plant. Furthermore, this point is reinforced by the finding that suppressor mutations of the *lefty* phenotype map to the same α-tubulin gene, although to a different location. These suppressor mutations are capable of restoring the original growth properties to the extent that they are indistinguishable from the parent ecotype (Thitamadee et al. 2002). *Lefty* mutations predominantly affect function and organization of cortical microtubules as compared to mitotic or cytokinetic microtubule arrays. In the *lefty* mutants, the cortical MT form right-handed helices that result in left-handed helical growth. This is indirect evidence that cell wall deposition is affected by, and dependent on, microtubule organization, which in turn is influenced by the specific amino acid sequence of the tubulin. Microtubules in the *lefty* mutations also demonstrate an increased sensitivity to treatments with antimicrotubular drugs like taxol, dinitroaniline, and pronamide. Thus, a single amino acid substitution in a tubulin isotype can cause changes in drug sensitivity, cortical MT arrangement, cell-wall deposition and global morphology of a plant. A reasonable prediction derived from these findings is that other, slight differences in the primary sequence of the various tubulin isotypes of

Table 1 Principal outputs of plant transformation experiments done with tubulin genes. The expression of all these constructs is provided by the 35S-CMV promotor. The table summarizes the results substantially obtained in three different laboratories (Key references: Bao et al. 2001; Abe and Hashimoto 2005; Anthony et al. 1999 and related works). EiR = Eleusine indica Resistance (to dinitroanilines). None of the constructs contained either a tubulin promotor or a tubulin intron

Construct	Balanced expression	mRNA	Protein	MT organization	MT dynamics	Plant features
GFP-TUB6	NO	OK	20/30% of endogenous	OK	OK	OK GFP expression restricted to aerial tissues
GFP-TUA6	NO	OK	20/30% of endogenous	Left-handed	Low	Right handed helical growth. GFP expression restricted to aerial tissues
HA-TUB6	NO	OK	NR	OK	OK	OK
HA-TUA6	NO	NR	NR	Left-handed	Low	Right handed helical growth, abnormal roots. Plants semi dwarf, low fertility
cmyc-TUB6	NO	NR	NR	NR	NR	OK
TUB6-GFP	NO	Degraded	NO	OK	OK	OK
TUA6-GFP	NO	NO	NO	OK	OK	OK
TUA6^{mGTPase}cmyc	NO	NR	NR	Left-handed	Moderately low	Right handed helical growth
TUA6-cmyc	NO	NR	NR	NR	NR	OK
EiR TUA1	NO	NO	NO	NO	NO	Lethal
Zm TUB2	NO	NO	NO	NO	NO	Lethal
EiRTUA1-HA+ ZmTUB2-cmyc	YES	66% replacement of endogenous	66% replacement of endogenous	OK	OK	OK
TUA6/antisense	NO	50% less α-tub	Reduced α-tub	Randomly arranged, defects	Altered	Abnormal root growth and morphology

a plant could likewise influence the dynamics of binding of MAPs and ligands to tubulin and microtubules.

These data suggest that is beneficial to understand tubulin isotype-specific differential expression at the protein level as well as to design new mutational approaches that could pinpoint the role that any specific isotype plays in a given tubulin gene family. Many examples that have accumulated over the past several years indicate that transcripts of various members of plant α- and β-tubulin gene families are differentially accumulated in different tissues, organs, and developmental stages, and in response to external signals (reviewed in Breviario and Nick 2000). In the few cases where β-tubulin isotype-specific or α-tubulin subfamily-specific antibodies are available, differential accumulation and usage of specific α- and β-tubulins could be demonstrated (Goddard et al. 1998; Eun and Wick 1998). What is the reason for differential expression of tubulin isotypes? Is it just a matter of maintaining finer regulatory control over gene expression or has it evolved to fulfill specific functional requirements? In other words, is the enrichment of a specific tubulin isotype in a given tissue or cell type caused by a specific signal-triggered expression pattern, possibly based on promotor motives, or is it caused by specific amino-acid motives that confer a specific function or interaction to the resulting microtubule? Why are the Arabidopsis α-tubulin gene TUA1, and the β-tubulin gene TUB9 almost exclusively expressed in the male gametophyte (Carpenter et al. 1992; Chang et al. 2001)? Is it reasonable to think that the combination of α1-tubulin and β9-tubulin confers some specificity to the biochemistry or dynamic properties of pollen tube microtubules or their ability to interact with motor proteins to facilitate sperm migration to the pollen tube tip? Why would one single tubulin isotype not suffice? So far, functional specificity for structurally similar α-tubulins could be demonstrated only in animals such as *Drosophila* (Hutchens et al. 1997), in plants, not yet conclusively.

These are old yet unanswered questions that may have found some new, surprising, answers in recent experiments involving transgenic plants. When maize calli were transformed with exogenous tubulin genes carrying a molecular tag (c-myc or HA) at the C-terminus for recognition, in addition to a specific mutation in the α-tubulin gene conferring resistance to dinitroanilines, the endogenous amounts of tubulin mRNA and protein are progressively and smoothly replaced by those encoded by transfected genes as elegantly demonstrated by Anthony and Hussey (1998). The replacement of the endogenous tubulin by the transfected tubulin was linearly dependent on the strength of the promotor and the copy number with 35S-CMV performing better than MFS-14. The intracellular total content of tubulin could increase up to three fold and endogenous α- and β-tubulin synthesis could be cut down by almost 50% (Anthony and Hussey 1998; Anthony et al. 1999). The kinetics of this molecular replacement were quite impressive in their regularity. A further very important aspect of those studies was the finding that the α- and

β-tubulin monomers had to be expressed in a balanced manner to achieve successful transformation and viable progeny. In fact, transformation of maize calli or tobacco leaf discs was only successful when cells were transfected with a construct that, under the same promotor, was concomitantly expressing both the mutated α-tubulin and a wild-type β-tubulin (Anthony and Hussey 1999). No transformants were recovered when the cells were transfected with the mutated α-tubulin gene alone (Anthony and Hussey 1998) nor if cells were co-transformed with α- and β-tubulin genes under control of the same promotor but located on different, separate plasmids (Hussey, pers. comm.).

In view of data discussed below, it is also of relevance that the one vector-two tubulin genes strategy of transformation was successful with the two polypetides carrying tags at their C-terminal end (HA for α-tubulin and c-myc for β-tubulin). Thus, as long as a balanced expression of α- and β-tubulin is ensured, plants incorporate the transgenic tubulin into all the different MT arrays (cortical microtubules, preprophase band, spindle and phragmoplast) without any apparent damage or phenotype. Under these conditions, the expression of the two α- and β-tubulin transgenes proceeds without any difficulty and proceeds straight up to the incorporation of the two monomers into the microtubules. This type of experiment infers the following reasonings and future directions of research. First, the molecular regulatory mechanism that leads to this amazingly efficient endogenous/exogenous replacement is unknown. There is some speculation on a possible control through the 5′-UTR tubulin sequence, but experimental evidence is lacking. Second, systematic total replacement of the mixed population of endogenous tubulins with different exogenous tubulin of a single type and regeneration of the resulting transgenic plants from calli would allow to conclude, which combinations of an α- and a β-tubulin isotype can give rise to normal plants. This should reveal to what extent a plant needs differential expression of tubulin genes and production of different tubulin isotypes. Finally, if tubulin replacement could be efficiently achieved, one might be able to design simple methods for the selection of transgenic plants containing particular tubulin isotypes or mutations endowed with resistance to drugs, herbicides, or adverse physiological conditions such as cold.

The proposed requirement for balanced expression of tubulin calls for further elucidations, though, because it could not be confirmed by recent experiments in plants (Ueda et al. 1999; Abe and Hashimoto 2005), because it is only partially present in the yeast *Saccharomyces cerevisiae*, where overexpression of β-tubulin but not of α-tubulin is lethal (Weinstein and Solomon 1990; Katz et al. 1990), and because it seems absent in animal cells. At the moment, this is certainly a contradictory issue since, on the other hand, it has been already discussed that in *Arabidopsis*, the *Kis* mutation could be rescued by overexpression of α-tubulin (Kirik et al. 2002). This is consistent with the idea that the overexpression of α-tubulin in *kis* mutants counterbalances the excess of free monomeric β-tubulin resulting from a reduced activity of

the chaperone protein TFC A. A similar situation occurs in yeast where the toxic effect of β-tubulin accumulation could be rescued by overexpression of α-tubulin (Weinstein and Solomon 1990).

The requirement for a control of the stoichiometric balance of α- and β-tubulin for microtubule assembly is further challenged by the experiments where modified α-tubulins were transfected into *A. thaliana*.

A chimeric GFP-TUA6 protein is readily incorporated into all microtubules of transgenic Arabidopsis transformed with a gene construct driven by the 35S-CaMV promotor (Ueda et al. 1999). This transgene could be maintained and expressed up to at least three viable generations of plants and the microtubules that have incorporated the GFP-TUA6 fusion protein did not only assemble correctly but responded normally to treatment with antimicrotubular drugs (Ueda et al. 1999). A subsequent detailed analysis of transcript and protein expression patterns, microtubule dynamics and plant phenotype (Abe and Hashimoto 2005) did not reveal any impact of unbalanced tubulin expression due to overexpression of a monomeric tubulin (included in Table I which summarizes the many aspects concerning tubulin expression). Abe and Hashimoto transformed *A. thaliana* with no less than nine different constructs, each carrying just a single monomeric tubulin (either α- or β-tubulin) fused at the N-terminus or at the C-terminus either to GFP or to tags like c-myc or HA. The resulting transgenic plants were all reported to be viable, even those where no transgenic tubulin or GFP RNA and proteins were detected because they were degraded (constructs carrying the 35S-CMV promotor driving the expression of TUA6-GFP or TUB6-GFP). They even recovered healthy and viable transgenic plants from a transformation with a c-myc-tag attached to the C-terminus of TUA6. This is remarkable, because a very similar construct based on a mutated α-tubulin from *Eleusine indica* (conferring resistance to dinitroanilines) had failed to produce viable transformants in maize or tobacco (Anthony and Hussey 1998). The data set from Abe and Hashimoto (2005) is intriguing from many other aspects of tubulin gene expression and regulation. N-terminal tagging of α- or β-tubulin led to a differential response: TUB6 could be N-terminal tagged by GFP or HA with no effect on plant morphology or MT dynamics, whereas its chimeric counterpart with α-tubulins caused right-handed helical growth and reduced MT dynamics that was further accentuated in HA-TUA6 to such an extent that plants were semi-dwarfs and sterile. Right-handed helical growth that was restricted to aerial tissues in transgenic GFP-TUA6 plants, extended to roots in HA-TUA6 transgenics (Abe and Hashimoto 2005). Right-handed growth is attributed to a microtubule population with reduced dynamics, caused by a decreased GTPase activity and concomitant increase of the GTP capping. This is documented by enhanced decoration of microtubule plus ends by End Binding Factor 1 (EBF1) and by a transgenic tubulin mutant TUA6 D251A/E254A that was able to suppress the phenotype of the HA-TUA6 transgenic (Abe and Hashimoto 2005).

GFP-TUA6 transgenics actually mimic microtubule structures and dynamics originally observed in the *lefty* mutants. This similarity is commonly explained by preferential incorporation of the modified α-tubulins into cortical microtubules (although efficiently occurring in other microtubule arrays as well) causing a right-handed helical arrangement that represents a transitional state toward a more fragmented and destabilized organization of microtubules. In fact, the cortical microtubules in the GFP-TUA6-*lefty* double mutant are highly fragmented and randomly oriented similar to those observed in the temperature sensitive *mor1* mutant (Abe et al. 2004; Whittington et al. 2001). This intermediate helical state of the microtubules associates in a still unexplained relationship with cell-wall deposition although the simple and mechanistic explanation that elongation occurs along the main axis that is orthogonal to the distribution of the cortical microtubule array, still holds. In fact, right-handed cortical microtubules promote left-handed helical growth and the other way round, although this does not clarify the functional relationships between microtubules and cellulose synthase complex (Paredez et al. 2006; Chu et al. 2007). *Lefty* mutants are also interesting for an additional aspect of tubulin gene expression, because the *lefty* phenotype can arise from mutations in two α-tubulin isotypes (TUA4 and TUA6) that belong to the same α-tubulin sub-family that carry the N-terminal MRECI pentapeptide and are abundantly expressed in all tissues and organs of *Arabidopsis thaliana* as shown by measuring the mRNA levels of the wild type TUA4 and TUA6 genes and by promotor-GFP-reporter studies (Abe et al. 2004). Authors report that more than 80% of the total α-tubulin transcripts is made up by mRNA of the TUA2/4/6 subfamily. This leads back to the initial question: were the *lefty* mutants discovered simply because they occurred in abundantly expressed isotypes or because these isotypes perform a unique function? The redundancy of α-tubulin genes precludes simple answers to this question (see below).

4 Microtubule Construction: MAPs and Deviant Tubulins

In addition to tubulins themselves, great effort is attributed to the extrinsic proteins that interact with tubulin dimers or with microtubules in a specific, orderly and dynamic way to control microtubules dynamics, influence their organization, or exploit microtubules as tracks for intracellular trafficking. The protein complexes that link microtubules to the spindle, the centrosome, the kinetochore, or the plasma membrane have been reviewed elsewhere (Dhonukshe et al. 2006; Cleveland et al. 2003; Cai et al. 2005), and therefore are not treated here. Included are instead the γ-tubulin complex, structural and regulatory Microtubule Associated Proteins (MAPs), tubulin-end binding proteins, microtubule-severing proteins, viral movement proteins and motor

proteins (Fig. 1). As a matter of fact, the function, turn-over, re-organization and dynamic instability of microtubules can be attributed to the interaction of tubulin with these specific proteins and with exogenous ligands (Downing et al. 2000). Some of these interactions are specific for the tubulin heterodimer, others for tubulins that have been assembled into microtubules. A few are even of clinical and pharmacological relevance.

New MTs are formed through severing of existing microtubules or from nucleation complexes (Ehrhardt and Shaw 2006). Microtubule nucleation triggers further elongation of microtubules via the association of γ-tubulin with α/β tubulin dimers as also shown by co-immunoprecipitation studies (Drykova et al. 2003). This may occur at the level of pre-existing microtubules as in the case of the inter-phase cortical array, or for microtubule-dependent microtubule nucleation (Murata et al. 2005; Murata and Hasebe 2007). Alternatively, microtubules can be nucleated at the nuclear envelope through the recruitment of the nucleation proteins Spc97p and Spc98p (Erhardt et al. 2002). Both types of microtubule nucleation are consistent with the wide distribution of γ-tubulin within the plant cell that localizes at the nuclear envelop, throughout the cytoplasm and along the microtubules of the cortical arrays in inter-phase cells (Ehradt et al. 2002; Ovechkina and Oakley 2001; Hoffman et al. 1994). Depletion of γ-tubulin in *A. thaliana* through disruption of the two γ-tubulin genes, causes profound aberrancies in the formation of the spindle, the phragmoplast, and the cortical array illustrating the central role of γ-tubulin for structure and organization of microtubules (Pastuglia et al. 2006).

γ-Tubulin is a key molecule for microtubule-dependent microtubule nucleation: its binding depends on the presence of pre-existing microtubules and nucleation is probably mediated through accessory proteins forming the γ-tubulin complex. The nucleation of new microtubules from the surface of pre-existing microtubules was a key step in the eukaryotic evolution of microtubular function and organization. This key step may have resulted from differences that occurred in γ-tubulin or γ-tubulin complexes (Murata and Hasebe 2007). If so, microtubule nucleation in the mitotic spindle may depend on pre-existing template microtubules as well. Concordant with this idea γ-tubulin has been found to decorate spindle microtubules (Liu et al. 1993). The ubiquitous decoration of both interphase and mitotic microtubules by γ-tubulin may actually reflect the ancestral situation from which the functional separation of the interphase and mitotic microtubular function evolved. To this regard, the finding of intranuclear α/β tubulin in cold-stressed BY-2 tobacco cells is of relevance. α- and β-tubulin are shown to conserve putative nuclear-export sequences that may still be used to rapidly cycle tubulin in and out of the nucleus in response to low temperatures and re-warming. This was proposed to be reminiscent of an ancient intracellular tubulin function active during the cell cycle progression (Schwarzerova et al. 2006).

MAPs (microtubule-associated proteins) stabilize microtubules by binding to their surface and bridging several tubulin subunits, thus neutralizing

their repulsive negative charges (Wasteneys 2002; Hussey et al. 2002). In particular, basic (positively charged) MAPs interact strongly with the acidic C-terminal region of tubulin, which protrudes from the surface of polymerized MTs (Smertenko et al. 2004). Plant MAPs, with few exceptions such as XMAP215, kinesins and katanin, do not share any relevant homology with animal and fungal MAPs. This sequence divergence, clearly correlated with the specialized functions of plant MAPs, has somewhat hampered the analysis on plant MAPs until recently. Plant MAP65 represents a family of related proteins originally found in microtubule preparations of taxol-treated tobacco BY2 cells (Chang-Jie and Sonobe 1993). The *A. thaliana* genome contains nine genes encoding proteins with homology to the tobacco MAP65. Two of them, AtMAP65-1 and AtMAP65-6, were recently shown to fulfill different functions on microtubules (Mao et al. 2005). While they both bind to microtubules in vitro, it is AtMAP65-1 that activates nucleation, and enhances polymerization of microtubules and renders them cold stable (Mao et al. 2005). On the other hand, At-MAP65-6 is shown to preferentially associate with mitochondria, and induces a mesh-like microtubule network, in contrast to AtMAP65-1 that bundles microtubules by stimulating tubulin polymerization and promoting crossbridging (Chang et al. 2005; Smertenko et al. 2006; Mao et al. 2005). The bundling activity of AtMAP65-1 is inhibited by phosporylation (Smertenko et al. 2006).

The *MOR1* gene is conserved across kingdoms and is essential for the organization of cortical microtubule. It promotes tubulin polymerization and increases number and length of microtubules in vitro (Whittington et al. 2001; Hamada et al. 2004). The binding of MOR1 is probably controlled by phosphorylation, whereas its effect on microtubules is mediated by the suppression of catastrophic decay. This suppression is achieved through a specific binding, mediated at the N-terminal HEAT repeat, to the Kin1-kinesin like protein, an inducer of microtubule catastrophes (Hussey and Hawkins 2001). Point mutations that affect amino acid composition of this N-terminal HEAT repeat lead to the disruption of the cortical array of microtubule. This is reversible in *temperature sensitive* (*ts*) *Arabidopsis* mutants that exhibit left-handed twisting of organs and isotropic cell expansion when cultivated at permissive temperatures (Whittington et al. 2001; Hussey and Hawkins 2001). This phenotype mimics the situation in the Arabidopsis mutants *lefty 1* and *lefty 2* (Thitamadee et al. 2002) that both contain the same amino acid change (Phe replacing Ser at the intradimer contact region of the α-tubulin genes TUA6 and TUA4, respectively).

The two *lefty* mutants were actually identified originally as suppressors of a right-handed spiral mutant, *spr1* that causes right-handed helical growth of roots and etiolated hypocotyls (Furutani et al. 2000; Ishida et al. 2007). SPR1 and five additional, related SPIRAL1-LIKE (*SPLL*) genes encode small-sized proteins (11–14 kDa) that associate to microtubules, probably in a complex with additional microtubule-binding proteins (Sedbrook et al. 2004;

Hamada et al. 2006). Right-handed helical growth progressively aggravates in double or multiple *spr1* and *sp1ls* mutants (Nakajima et al. 2004, 2006). Similarly, mutations in the *TORTIFOLIA1* gene of *Arabidopsis* (synonymously reported as *SPIRAL2*) cause right-handed helical growth in elongating tissues (Buschmann et al. 2004). TORTIFOLIA/SPIRAL2 is an HEAT-repeat containing protein capable of binding taxol-stabilized microtubules in vitro, decorating microtubules in Arabidopsis cells, and recovered from highly purified MAP fractions in extracts of tobacco BY-2 cells (Buschmann et al. 2004; Shoji et al. 2004).

Microtubules can not only twist but they can also be disassembled by severing proteins like katanin that binds to microtubules and provokes their catastrophe by generating new ends that lack GTP (Stoppin-Mellet 2002). The small subunit of the Arabidopsis katanin AtKSS was shown to localize at the perinuclear region of interphase cells, and to the spindle poles during mitosis. This is in accordance with the proposed role of severing microtubules near their minus ends (McClinton et al. 2001).

Motor proteins interact with both α- and β-tubulin monomers when they bind (Downing 2000). The binding of the kinesin KIF1A involves the interaction of its basic, lysine-rich domain with the acidic C-terminus of tubulin (Kikkawa et al. 2000); interaction with the tubulin C-terminus is a general feature of motor proteins. Kinesin and kinesin-like motor proteins have been shown to associate with different microtubule arrays (spindle, phragmoplast) thus affecting the dynamics and the stability of microtubles (Chen et al. 2002; Liu and Lee 2001; Hunter and Wordeman 2000).

A special member in the world of MAPs are the viral movement proteins (MPs) that are considered in more detail in the chapter "Microtubules and Viral Movement" of this book. Lateral contacts between similar amino acid domains of tubulin and viral MPs may influence virus propagation either by favoring vRNA trafficking to plasmodesmata or by facilitating degradation of the MPs (Boyko et al. 2000; Gillespie et al. 2002). The heat-shock protein 70 (Hsp70) homolog encoded by BYV (Beet yellow closterovirus) functions as a viral MP and binds to microtubules (Karasev et al. 1992), and the direct interaction of the TMV (tobacco mosaic virus) movement protein with microtubules may even be required to nucleate microtubules (Seemanpillai et al. 2006; Ferralli et al. 2006).

Finally, a deviant α-tubulin from sunflower (tubπ) was proposed to suppress microtubule assembly in pollen tubes by interfering with microtubule nucleation and elongation through its unusual, basic C-terminal end (Evrard et al. 2002). Similar with γ-tubulin, π-tubulin represents an unusual plant tubulin, and might be the counterpart of the δ-, ε-, ζ- and η-tubulins that have been found in some eukaryotes different than plants, and presumably are associated with the centriole or the basal body (McKean et al. 2001). The fact that these deviant tubulins are not ubiquitously present represents a challenge for evolutionary studies.

5
Concluding Remarks: Novel Approaches and Open Questions

The presence of multiple tubulin genes that are transcribed differentially and encode different isoforms in plants and other multicellular organisms provokes the question how this situation has evolved. Are multiple tubulin genes a means to facilitate quantitative control over the amount of tubulin available at a given developmental stage or in a given tissue or organ? Is it possible that some of the individual tubulin species, either in the form encoded by the gene (isotypes) or in post-translationally modified forms (isoforms), confer unique properties or subtle qualitative differences to the microtubules formed from these tubulins? Evidence from other organisms, notably *Drosophila*, indicate that not all tubulins are functionally equivalent (Hutchens et al. 1997) and that many physical and biochemical properties of tubulin dimers and microtubules generated in vitro or in vivo depend on the composition of isoforms present (Derry et al. 1997; Schwartz and Ludueña 1998). Even single amino acid changes in one of the α- or β-tubulins can profoundly affect the sensitivity of an organism to cold or to microtubule-active herbicides or drugs (Baird et al. 2000). To dissect the role that individual tubulin isotypes may have during plant growth and development, experimental approaches complementary to the production of transgenic plants expressing reporter products under the control of tubulin regulatory sequences should be carried out, but difficulties may be encountered.

In fact, with the notable exception of the Arabidopsis *lefty* mutants described above, forward genetics screening of mutagenized populations for developmental abnormalities generally have failed to identify tubulin mutations that provide insight into tubulin functions or the roles of particular tubulins. This suggests that mutations in tubulin genes tend to be lethal, subtle, masked by gene redundancy, or not apparent under the particular screening conditions employed. However, when the tubulin gene sequences for a given species are known, functional analysis is possible by various reverse genetical means that allow detection of plants containing a knocked-out, silenced, or mutated tubulin gene, even if the mutant phenotype cannot be predicted. So far, reports on this type of approach are scarce, and T-DNA or transposon tagging resulting in phenotype clearly associated with mutations in specific tubulin genes are still missing. The closest example of this is the T-DNA tagged Arabidopsis mutant *diminuto* where the expression of the *TUB1* gene is affected (Takahashi et al. 1995). Other mutations in cytoskeleton components have been reported for such an approach but none has been mapped to any tubulin gene.

Tubulin anti-sense constructs are an alternative option, and there is one report in *Arabidopsis* where the expression of the *TUA6* gene was reduced by this approach (Bao et al. 2001). Massive alterations of root morphology and growth were reported although the aerial parts were apparently not affected. This is interesting because once more it is TUA6, one of the abundantly

expressed Arabidopsis α-tubulin isotypes, to be the target for a mutational approach that leads to a clear phenotype as it was the case for *lefty 1* and for the chimeric GFP-TUA6 overexpressor. However, it remains enigmatic, why in case of the antisense strategy it was the roots and not the aerial parts, where the mutant phenotype became manifest, whereas in case of the *lefty 1* mutant or the GFP-TUA6 overexpressor it was the aerial parts that were predominantly affected.

Gene regulation by post-transcriptional gene silencing (PTGS) or virus-induced gene silencing (VIGS, originally known as co-suppression) can also be added to the toolkit as a way to knock out a specific tubulin gene to analyze its potentially specific cellular function. In fact, VIGS has been successfully achieved to inhibit β-tubulin expression in roots of *A. thaliana* using the Tobacco Rattle Virus (Valentine et al. 2004). The expression of *TUB8* was reduced (although not exclusively) with concomitant strong effects on root development and growth. Interestingly, co-expression of an α-tubulin-GFP fusion protein was not affected as verified by confocal microscopy demonstrating that α-tubulin-GFP was not reduced although the roots were stunted (Valentine et al. 2004).

A further approach to isolate mutants of interest is TILLING of plants that contain EMS-induced point mutations that result in mis-sense or, more rarely, nonsense mutations of a gene (Colbert et al. 2001). If the TILLING population is large enough, this approach theoretically would yield allelic series of mutations, and thus also permit the functional analysis of specific regions on the tubulin molecule such as putative MAP binding regions in the carboxyl terminus versus regions involved in intra-dimer or interdimer interactions.

This journey through the microTUBULOMICS world has reached the end. We have learned that this journey is interrupted and sometimes diverted by numerous stations and that each tubulin monomer is never sure where it will stop, how long it will take to reach the final destination and how long it will remain incorporated into a microtubule. Something marvellous, but still unresolved, is to control the whole matter. After 6 years have elapsed since a previous contribution on this subject (Breviario 2000), we have to modify our more simplified conclusions in which we proposed the VMTK (Versatile Molecular Tubulin Kits) as a programmed combination of tubulin promotor and coding sequence to be transferred into a given plant to convey desired traits (Breviario 2000). It is clearly not that easy, but this makes the whole subject even more fascinating. We are now exposed to new glimpses of truth, but will need to continue to delve even deeper to unravel the whole picture.

microTUBULOMICS has also taught me an additional lesson. Many think that the systematic dissection of genomes in its multiple variants called genomics, proteomics, metabolomics, and other "-omics", will eventually lead to the comprehension of the whole. I am not sure of that, but I certainly know that already a small gene family is sufficient to provide you with a tremendous amount of work and thousands of challenging questions.

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Microtubules and the Evolution of Mitosis

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Abstract The microtubular cytoskeleton of higher plants diverges considerably from its animal counterpart. This divergence involves a fundamentally different organization with microtubule arrays, which are specific to higher plants, such as cortical microtubules or the phragmoplast. On the other hand, centrioles, which are central organizers of microtubules in cells of animals and lower plants, have been progressively reduced in the course of plant evolution, and are now absent, as in most seed plants. In addition to these structural differences, the molecular composition of associated proteins also deviates to a degree that sequence homologues of important animal microtubule associated proteins (MAPs) seem to be absent in higher plants. The transition towards multicellular plants was intimately linked to rhythmic changes of division axis, and, thus, to the spatial regulation of mitotic microtubule arrays. To understand the peculiarities of plant microtubules, we have to combine cell biology with a more phylogenetic viewpoint. Therefore, this chapter is dedicated to the role of microtubules in the evolution of mitosis. It is shown that the microtubule divergence between plants and animals is already laid down in the prokaryotic ancestors, when the walled eubacteria are compared to the mycoplasms that lack a cell wall. The complex situation in lower eukaryotes can be understood as variations of this theme. The relation between chromatin/nucleus and the so-called nucleus associated organelle (NAO), which organizes microtubules, is central for the realization of mitosis. The extensive variability observed in algae and fungi is then progressively channeled, when, during the evolution of the higher Chlorophyta, most microtubule structures characteristic for higher plants develop. However, the interpretation of these mitotic structures has remained ambiguous, due to possible convergent developments, and due to our limited understanding of the phylogenetic relationships in many taxa of the algae. At the time when plants shifted to a terrestrial lifestyle, the microtubule arrays from higher plants have already been worked out. This was accompanied by a progressive reduction of centriolar functions and the increasing predominance of acentriolar microtubule organization, which could be followed on the structural level and also by a redistribution of microtubule-nucleating proteins, such as γ-tubulin, during recent years. Indicative of the evolutionary processes towards the highly divergent microtubular cytoskeleton of higher plants, interesting evolutionary footprints still exist, and are difficult to interpret merely in terms of cellular function. These include the cytoplasmic occurrence of the tubulin ancestor, FtsZ, in mosses, or the recent discovery of intranuclear tubulin. If one merely attempts to explain them in terms of current function, these phenomena appear to be unusual and difficult to understand. However, they can be readily interpreted as rudiments from a long evolutionary path, driven by the necessity to divide cells that are surrounded by rigid cell walls.

1 The Cytoskeleton as Target of Evolution

For decades, textbooks have cited the cytoskeleton as a classical example for the achievements reached by the eukaryotes. However, this view has been shattered by exciting discoveries in bacterial cells made at the turn of the century. It began with the striking news that FtsZ, a protein essential for the fission of bacterial cells, is the prokaryotic ancestor of tubulin (Löwe and Amos 1998). It was later shown that MreB, a bacterial protein with homology to actin, is required for the maintenance of cell polarity during asymmetric cell division (Gitai et al. 2004), and is, therefore, also functionally equivalent to its eukaryotic counterpart. Eventually, CreS, a coiled-coiled protein, which, similar to intermediate-filament proteins of eukaryotes, can polymerize into filaments, was shown to be responsible for the shaping of bacterial cells, but not their polarity (Ausmees et al. 2003). Thus, precursors of all three major cytoskeletal elements (microtubules, microfilaments, and intermediate filaments) already do exist in prokaryotes, and they seem to fulfill similar functions.

These functions are linked to cell division – to the fission process itself (FtsZ), to its organization in space (MreB), or to the shaping of the daughter cells (CreS). The evolution of the prokaryotic cytoskeleton was most likely driven by the necessity to increase mitotic efficiency. With the transition to multicellularity, additional requirements were posed upon mitosis – in addition to the correct duplication and separation of chromosomes, it became essential to control the separation of the two daughter cells in space and time. This requirement is especially evident in walled cells, where irregularities of cytokinesis cannot be adjusted by movements of the daughter cells. Here, mitosis is actually used to organize the *Bauplan* of the resulting organism.

By rhythmic changes in the axis or symmetry of individual cell divisions, it became possible to produce defined patterns of cell files. This is impressively illustrated by the modular structure, which is characteristic for the transition from higher algae to the primitive land plants (Fig. 1):

In the primary situation (Fig. 1A, 1), an apical stem cell divides asymmetrically, yielding: a proximal cell, which will elongate without dividing further, and a distal progenitor cell, which will repeat the stem-cell program of its mother (unimodal stem cell). The axis of division is aligned with the axis of the cell file leading to the filaments, which is characteristic for many green algae. However, this situation is also found in the protonemata of mosses and fern plants, which seem to recapitulate the development of their ancestors.

In a more advanced situation, the mitotic activity of the proximal daughter cell will not be irreversibly inactivated. It will recover in defined intervals, whereby the division axis is tilted perpendicular to the file axis (Fig. 1A, 2). This will result in branching. If the mitotic activity rhythmically returns in the offspring of such a branching division, a progressively hierarchical system of axes, branches of first and second order, emerges (Fig. 1A, 2–4). This

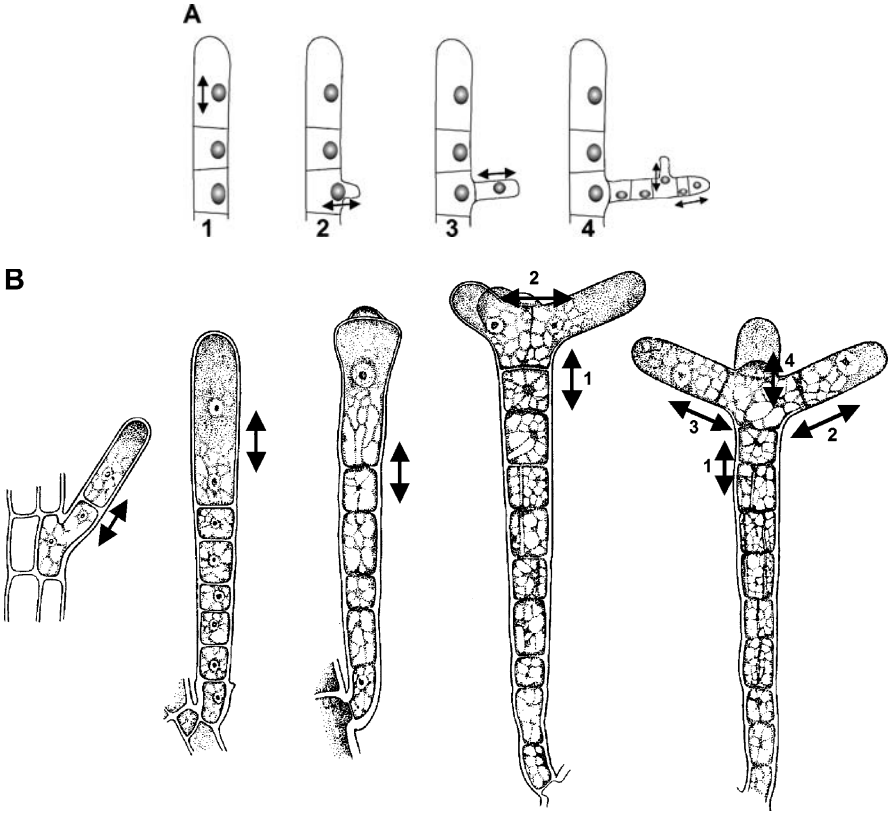

Fig. 1 Impact of spatial regulation of mitosis on the morphogenesis of lower plants. **A** Generation of complex hierarchical morphologies by rhythmic changes in the axis of cell division. *1* filamentous morphology generated by an unimodal stem cell; *2*, *3* branching produced by transient stem cell activity in a proximal cell; *4* secondary branching generated by transient stem cell activity in a second-order cell. **B** Hierarchical whorl formation in *Sphacelaria fusca*, according to Zimmermann and Heller (1956)

is observed in many of the higher green, red, and brown algae. Branching in *Sphacelaria fusca* has been a classical object of studies for this aspect (Fig. 1B). By confining this rhythmic appearance of tilted divisions to the primary stem cell, it is possible to generate dichotomous branching, as observed in the brown alga *Dictyota* and in several pteridophytes. When the intervals between the tilting events are reduced and eventually eliminated, one arrives at the bimodal stem cells, characteristic for the prothallia of ferns.

This example illustrates how relatively simple, rhythmic changes in axis and symmetry of cell division produce a panel of diverse body plans. It further demonstrates that cell division, in a certain sense, has to be placed upstream of cell expansion, because it will define the axis of the daughter cells, and, thus, the framework in which expansion can occur.

In addition, cell division in plants can even interfere with cell differentiation, and, thus, with the developmental fate of a given cell. During cell division, there are two aspects that are under tight spatial control in the walled plant cells: the axis of division and the symmetry of division (Fig. 2). In most divisions, the axis does not change and most divisions are symmetrical. However, occasionally, axis and symmetry do change, and this is usually the earliest manifestation of developmental switches, which often occur in response to environmental stimuli. In protonemata of ferns, which is a classical system for the study of unimodal stem cells, the axis of division is parallel to the axis of the protonema, as long as it grows either in darkness or under red light. However, upon irradiation with blue light, this axis is rhythmically tilted by 90° during each of the subsequent divisions, yielding two-dimensional growth and the formation of a prothallium (Mohr 1956; Wada and Furuya 1970). When the cell is returned to red light, the division will assume the initial state, and a new protonema will emerge from the front edge of the prothallium.

Similar changes in division symmetry accompany differentiation events in all land plants. For instance, the formation of guard cells (Bünning 1965), the generation of water-storing cells in peat mosses (Zepf 1952), or the first cell division that separates prospective thallus from prospective rhizoid fate in many spores and zygotes of lower land plants, provide ample evidence for

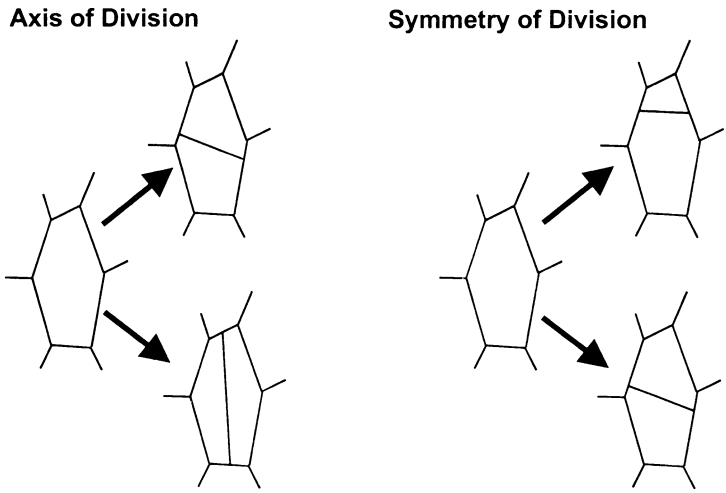

Fig. 2 Impact of division axis and symmetry on the shape of plant cells. In most cases, the division axis is parallel to the main axis of cell expansion (*left*); however, in a few defined cell divisions, it can be tilted by 90°. These tilted divisions in most cases give rise to cell lineages of differing developmental fate. Most cell divisions are symmetric (*right*); however, asymmetries can occur, but are relatively rare and are characteristic for differentiation events, similar to the tilted divisions

such asymmetric divisions. In some cases, it is possible to modify these asymmetric divisions by either pharmacological or genetic manipulation, such that the cells separate symmetrically. This determines whether these asymmetric divisions are formative in character, i.e., whether they assign differential developmental fates to the daughter cells.

As an example, it is possible to shift the first division of the spore in the fern *Onoclea sensibilis* from asymmetry to symmetry by treatment with antimicrotubule compounds (Vogelmann et al. 1981). Instead of the normal separation of a protonema from a rhizoid precursor, this will result in the formation of two protonemata-like organs.

Similar to the situation in lower land plants, the first zygotic division of higher plants is asymmetric as well, and it separates suspensor from embryo fate. By mutation in the *GNOM* gene, it is possible to obtain a symmetric division. The subsequent development is affected by dramatic defects in apicobasal polarity and the differentiation of daughter cells (Mayer et al. 1993). The GNOM protein was later shown to encode an accessory protein that regulates the activity of the small GTPase ARF, which is involved in the budding of vesicles at the endoplasmatic reticulum (Geldner et al. 2003). This finding indicates a link between the spatial regulation of vesicle fluxes, the spatial regulation of cell division, and developmental fate.

Thus, plant mitosis is peculiar, not only with respect to its cellular details, but also because it plays a tremendous role in establishing a developmental pattern. This peculiarity is connected with the presence of a cell wall, excluding cell migration as the morphogenetic mechanism (central to development in animal development). Thus, in order to understand plant mitosis, it is necessary to consider how it evolved parallel and in concert with a plant-specific developmental strategy.

2
The Starting Point – Prokaryotic Mitosis

The evolution of mitosis reaches back beyond the discovery and research on bacteria. Remainders of this time persist today. They suggest that the most primitive cells are those of the mycoplasmas (Wallace and Morowitz 1972), because their genomes are much smaller than those found in other bacteria. They lack many of the genes typically present in bacteria, including those used in wall synthesis, signaling, and even housekeeping activities, such as *de-novo* synthesis of nucleotides and amino-acids (Himmelreich et al. 1996). The typical, large, bacterial chromosomes are thought to derive from mycoplasma-like ancestors by end-to-end annealing, whereas duplications of the genome unaccompanied by such end-to-end annealing gave rise to small and separate chromosomes, which is characteristic for lower eucaryotes. The formation of numerous separate genetic entities was the driving force for the

evolution of more efficient mechanisms of segregation and, thus, for the evolution of mitosis. Moreover, generating a specialized chromosome-containing compartment and creating a specific environment favorable for replication and transcription, would be advantageous in such a multi-chromosome constellation. This might have been the driving force for the development of a nuclear envelope.

The mechanisms of chromosomal segregation in mycoplasm should, therefore, recapitulate some aspects of this very early stage of mitotic evolution. The genome of mycoplasmas is tethered to the plasma membrane by its replication point. In some mycoplasmas, specialized discoid structures adjacent to the membrane have been detected by electron microscopy (Maniloff and Quinlan 1974), and later designated as terminal organelles (for a recent review, see Balish and Krause 2006). Mutant analysis, in combination with in vivo microscopy of fluorescently labeled components of these terminal organelles (Hasselbring et al. 2005, 2006), has shown that they are not only responsible for the adhesion of the parasitic mycoplasms to the host cell, but also for gliding and cell division. The terminal organelles are singular in non-dividing cells, but duplicate prior to division. They constitute a type of polarity marker, because they are situated at opposite poles. Interestingly, the division process is blocked by cytochalasin B, a well-know inhibitor of actin filaments (Ghosh et al. 1978). This indicates that actin filaments are used to push the replicated daughter genomes apart and constrict the two daughter cells. It should be mentioned, however, that FtsZ, a central component of bacterial cell division and ancestor of eukaryotic tubulin, exists in mycoplasmas and is able to functionally complement *E. coli* strains deficient in FtsZ (Osawa and Erickson 2006). Nevertheless, in this very primitive situation, the central role is played by actin, and not by microtubules. This might reflect the hypothetical initial stage in the evolution of mitosis. When the plasma membrane bearing the disc-like structures invaginated this hypothetical ancestor, such that the terminal organelle was internalized, this would generate a typical eukaryotic nucleus with intranuclear genome organizers.

It seems that the mycoplasmic type of mitosis mirrors the primordial situation for those organisms that lack a cell wall. A detailed model for the evolution of mitosis has been elaborated for such cells (Heath 1974) and it is also valid for the evolution of animal mitosis. For walled cells, the controlled segregation of genetic material is only one of the tasks to be solved – in addition, a walled cell has to be divided. A septation of the daughter cells by constriction through the actomyosin system (or its functional homologues in prokaryotes) is not sufficient; walled cells have to establish a new cross wall, and this poses specific challenges to the mitotic machinery. Already walled prokaryotes – such as eubacteria and cyanobacteria – were confronted with this task. They used the FtsZ system to cope with it.

FtsZ is present in almost all recent prokaryotes (including the mycoplasmas). It has been identified as the essential component of the division

machinery (for review, see Errington et al. 2003). Prior to cytokinesis, FtsZ assembles into a ring, accompanying the inner membrane, and it recruits additional proteins to this ring. This ring then progressively constricts and organizes the synthesis of a new cross wall (which is, thus, formed in a centripetal fashion).

FtsZ harbors a GTP-binding activity. It can assemble into polymers, and it shares striking similarities to tubulin in three-dimensional structure (Löwe and Amos 1998). Therefore, it is thought to represent the ancestor of eukaryotic tubulin. The formation of a contractile FtsZ ring underneath the plasma membrane heralds the ensuing bacterial cell division (Sun and Margolin 1998). This FtsZ ring recruits accessory proteins to the division site (for review, see Addinall and Holland 2002), which partially act as stabilizers of FtsZ in the division site, or suppress the assembly of FtsZ at ectopic locations. For instance, the division factors ZipA, FtsA (Pichoff and Lutkenhaus 2002), or the recently discovered protein, ZapA (Gueiros-Filho and Losick 2002), seem to stabilize the FtsZ ring, whereas the MinC and MinD proteins suppress the assembly of FtsZ (Hu et al. 1999), and are, in turn, inhibited by MinE (Fu et al. 2001). MinE forms a ring independently of FtsZ, and, thus, it seems to be the upstream factor that defines the prospective site of cell division (Raskin and De Boer 1997). Thus, the division plane appears to be determined by an interplay between non-linear self-amplifying circuits (FtsZ, ZipA, ZapA) and lateral inhibition (MinC, MinD), which is, in turn, suppressed by the inhibitory activity of MinE heralding the division site (Fig. 3). Consistent with this model, overexpression of MinC and MinD blocks cell division and phenocopies a FtsZ-null mutation, whereas mutation or deletion of MinC or MinD results in ectopic division sites.

In contrast to cell division in eubacteria, relatively little is known about cyanobacterial mitosis (for review, see Hashimoto 2003). The central player

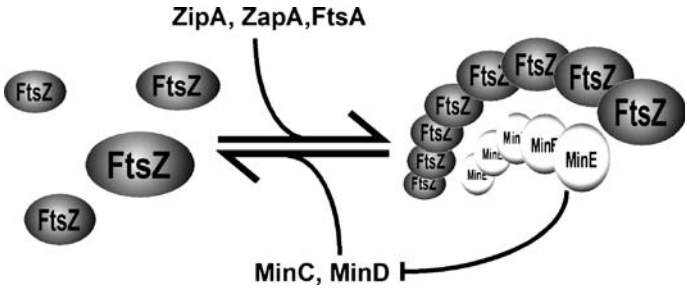

Fig. 3 Control of the bacterial FtsZ division ring by accessory proteins. The assembly of FtsZ is promoted by ZipA, ZapA, and FtsA, but suppressed by MinC and MinD. MinC and MinD, in turn, are suppressed by MinE, which forms a ring at the division site independently of FtsZ. This ensures that FtsZ cannot assemble outside the prospective division site, such that FtsZ monomers are preferentially recruited to the FtsZ ring

seems to be FtsZ (Osteryoung and Vierling 1995). The FtsZ transcription is regulated by DNA-replication. When replication is inhibited by hydroxyurea (a blocker of ribonucleotide reductase) or nalidixic acid (a blocker of DNA gyrase) in the cyanobacterium *Microcystis aeruginosa*, the transcription of FtsZ and cytokinesis are arrested (Yoshida et al. 2005). However, the associated proteins seem to differ. Proteins that are specific for cyanobacteria and plastids, such as ARTEMIS (Fulgosi et al. 2002) or the Arc proteins (Vitha et al. 2003) not found in *E. coli*, exist, whereas MinC seems to be absent from cyanobacteria and plastids. Some of those seem to be functionally equivalent to the eubacterial Min proteins. For instance, the arc6 protein appears to stabilize the FtsZ ring in a manner similar to the eubacterial ZipA protein (Vitha et al. 2003). Since chloroplasts arose in a long-lasting process of endosymbiontic domestication from free-living cyanobacteria (Hashimoto 2003), they are more relevant to our understanding of plastid division than the much more intensively studied division systems in *Escherichia coli* or *Bacillus subtilis*.

Thus, already at the prokaryotic stage, three at least partially different division systems were available. They were adapted to the specific needs of the corresponding cells: (i) The terminal organelles that seem to interact mainly with an actin-related force-generating system in the unwalled mycoplasmas and may have been the ancestral situation for mitosis in primitve eukaroytes; (ii) the FtsZ-Min-ring system that constricts the daughter cells by a converging ring in the walled eubacteria; and (iii) a FtsZ-dependent system utilizing a different set of accessory proteins that evolved in the walled cyanobacteria and was adopted during endosymbiosis for the division of chloroplasts in the plant kingdom. It should be noted that FtsZ, the tubulin ancestor, is present in all three division systems, but differs with respect to its impact on mitosis. The primordial eukaryotic mitosis was probably much more actin-dominated than in modern eukaryotes. This situation changed dramatically when the eukaryotic cells, probably through an endosymbiontic interaction, acquired a new organelle – the flagellum. This acquisition subsequently shaped the evolution of mitosis in many of the lower eukaryotes.

3
Mitosis in Lower Eukaryotes

3.1
Structural Aspects of Mitosis and Cytokinesis

In a recent review, Cavalier-Smith (2006) established an Eukaryote phylogeny partially based on the diversification of the microtubular cytoskeleton. The tree was rooted in the unikonts. Eukaryotic motility was postulated to originate from an ectosymbiosis of a spirochete (Margulis 1993). However, this

hypothesis was recently revised (Li and Wu 2005). Polymers composed of α/β-tubulin dimers observed in epixenosomes, ectosymbionts of *Euplotidium* ciliates (Petroni et al. 2000), place the possible origin of tubulin into the newly discovered bacterial division of Verrucomicrobia (Li and Wu 2005).

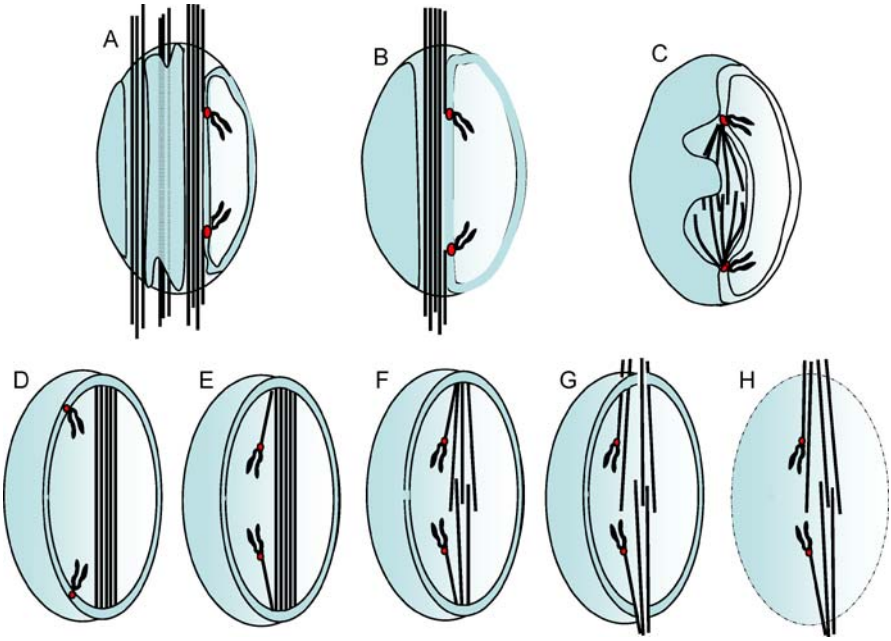

Fig. 4 Organization of the mitotic spindle in lower Eukaryotes. Extranuclear (**A**–**C**) and intranuclear spindles (**D**–**H**). **A**–**B** Cytoplasmic microtubule arrays are formed in one or more grooves of the nucleus. Chromosomes are directly attached to the nuclear envelope and they separate along this axis. Kinetochores and NAOs form the same entity (i.e., *Cryptothecodinium* and *Syndinium*, Dinoflagellata, Alveolata). **C** A cytoplasmic spindle links kinetochores/NAOs to chromosomes and drives karyokinesis (i.e., *Trichomonas*, Parabasilida, Excavata). **D** An intranuclear bundle of continuous contiguous pole-to-pole microtubules forms a central spindle. Its elongation during anaphase splits the chromosomes in two batches (i.e., *Trypanosoma*, Kinetoplastida, Excavata). **E** Kinetochores have dissociated from NAOs and kinetochore microtubules are formed. A central spindle of continuous microtubules elongates during anaphase (i.e., *Polysphondilium*, Myxomycetes, Amobozoa). **F** Elongation of interdigitating polar microtubules allows segregation with or without poleward migration of chromosome. NAOs are part of the nuclear envelope or intranuclear structures (i.e., *Paramecium*, Ciliates, Alveolates). **G** NAOs are cytoplasmic, and the spindle forms through fenestrae within the nuclear envelope which partially opens (i.e., *Euglena*, Euglenida, Discicristates; *Toxoplasma*, Apicomplexa, Alveolates; *Plasmodium*, Phytomyxa, Rhizaria and *Phytophtora*, Oomycota, Stramenopiles). **H** Open mitosis with complete breakdown of the nuclear envelope. When at all present, NAOs take highly variable forms (i.e., *Navicula*, Diatoms, Stramenopiles and *Ochromonas*, Chrysophyta, Stramenopiles; *Pelomyxa*, Rhizopoda, Amoebozoa and *Cyanophora*, Glaucophyta, Rhodophyta)

The putative ancestral, heterotrophic, aerobic, uniciliate eukaryote would first have developed a microtubular cytoskeleton associated with a basal body. This organizer of the flagellar apparatus would have been designed to ensure multiple functions: locomotion, sensory perception, as well as cell division. Given optimal survival value of the primitive eukaryote cells, this integration of three basic processes would have optimally increased the fitness, because increase in size was linked to the acquisition of subcellular compartment (Azimzadeh and Bornens 2004). During evolution, lower eukaryotes largely diversified their spindle apparatus, in order to ensure equational symmetric partitioning of chromosomes (Fig. 4). A detailed review on the structural aspects of mitosis was given by Heath (1980). These morphologic variations bear on polar structures, nuclear envelope, distribution and activity of spindle microtubules, and chromosome behavior.

The evolution of microtubule mitotic structures in relation to actin needs to be considered. For a long time, it has been known that actin microfilaments are present in an acto-myosin ring, which constricts at the cell equator in telophase and is centrally involved in cytokinesis (Guertin et al. 2002). Therefore, the loss of myosin functions affects cytokinesis in budding and fission yeast. In *Dictyostelium*, this may be accompanied by furrowing, which is independent from myosin II, but dependent on adhesion.

Protists, algae, and fungi are polyphyletic. They are dispersed among the five to 11 eukaryotic groups (superkingdoms or kingdoms). We will examine structural mitotic aspects of these groups. The polar structures closely resembling the nine triplet-microtubule arrangement will be named centrioles, whereas for other structures related to the organization of microtubules we will use the term nucleus associated organelle (NAO).

3.1.1
Excavata and Discicristata (Ciliate and Flagellate Protists)

The nuclear membrane remains intact (closed) during mitosis; in the Excavata, the anaphase spindle is cytoplasmic, and it is located in a groove of the nucleus (i.e., *Barbulanympha*). The central spindle extends across one side of the nucleus, with chromosomes connected to microtubules at kinetochores situated within the nuclear envelope; the spindle poles are often associated with "atractophores" (fibrous extensions from specific basal bodies) (i.e., *Gigantomonas herculea*).

Discicristata (i.e., Euglenida, Kinetoplastida) also maintain an intact nuclear envelope, but form an internal spindle, connecting separate nucleoplasmic chromosomes to poles via kinetochore microtubules. The rhizoplast, a striated band, elongates from duplicated flagellar basal bodies (blepharoplasts) to the nuclear envelope at undifferentiated polar regions or at NAOs.

3.1.2
Stramenopila (Heterokonts)

Among other taxa, this phylum includes the Oomycota, Xanthophyta, Chrysophyta, Phaeophyta, and the diatoms. In the majority of studied species, one microtubule per kinetochore is observed, but the chromatin does not condense. Mitotic microtubules form only an intranuclear spindle, without a central non-kinetochore array. This spindle elongates between anaphase and telophase. Chromosomes do not separate; they form a joint mass (i.e., in Cryptophyta and diatoms). In the Xantophyta, chromosomes are well-separated, with exception of one taxon where the chromatin was observed to be uncondensed.

The nuclear envelope disperses and a bundle of closely spaced interdigitating microtubules connect both poles in diatoms. A central spindle is only present in the Bacillariophyta (diatoms) and one taxon of the Xantophyta. In diatoms and chrysophytes, the NAO forms a striated bar or plaque resembling a rhizoplast. In oomycetes, centrioles are aligned in parallel (Fig. 5C), and the nuclear envelope remains intact during mitosis.

3.1.3
Alveolata

Dinoflagellates maintain an intact nuclear envelope. Chromosomes are mostly well-separated, but they sometimes remain aggregated in a joint mass. Non-kinetochore microtubule bundles are located in extranuclear tunnels or channels (i.e., *Cryptothecodinium*). In this species, the chromosomes become temporarily attached to the nuclear membrane surrounding these channels without distinguishable kinetochores. In the Ciliophora (ciliates), polar structures are absent, but spindle microtubules still elongate in anaphase. The micronucleus performs a closed mitosis with an intranuclear spindle and chromosomes with kinetochores. In Apicomplexes, a closed mitosis with an intranuclear spindle is mostly observed. Nuclear pores associated with cytoplasmic NAOs have also been described (i.e., *Plasmodium*). Alternatively, in the absence of any structured NAO, schizogony occurs through nuclear budding (i.e., *Toxoplasma*). In *Eimeria*, a cytoplasmic ring nucleates cortical microtubules, whereas spindle microtubules are nucleated from centrosomes.

3.1.4
Rhizaria and Cercozoa

In a majority of these protists, the nuclear envelope remains intact during mitosis and the spindle is intranuclear.

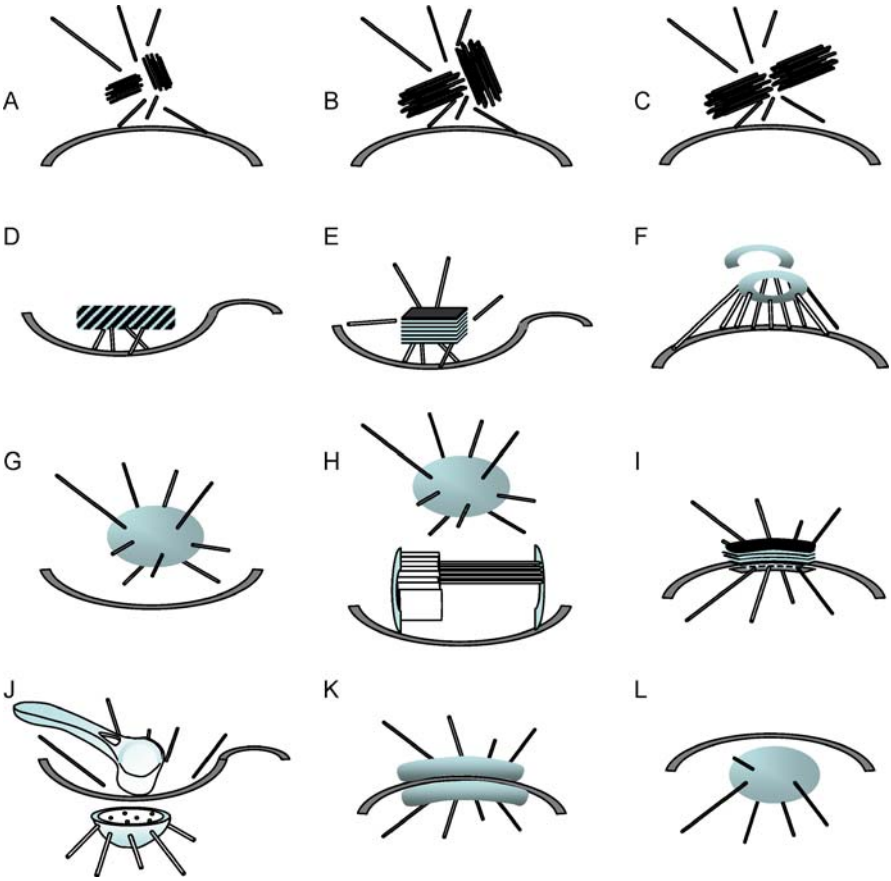

Fig. 5 Organization of centrosome and NAOs in lower Eukaryotes. Extranuclear (**A**–**H**), mixed (**I**–**K**), and intranuclear (**L**) microtubule organizing centres are observed. **A** Procentrioles (i.e., *Cladophora* and *Labyrinthula*). **B** Canonical centrosome as found in the chytrids. **C** Aligned, antiparallel centrosomes of the oomycetes. **D** Rhizoplast (i.e., *Ochromonas*) or plaques of Cnidosporidia. **E** Eight-tier core NAO in *Dictyostelium*. **F** Ring–shaped NAO (i.e., *Polysyphonia*, *Polysphondilium*). **G** Attractophore or sphere (i.e., *Filobasidiella*, Basidiomycota, and Zoomastigina). **H** Striated bar appearing beneath a cytoplasmic sphere in the prophase of diatoms (i.e., *Surirella*). It splits into two sets and forms a central spindle as soon as the nuclear envelopes breaks down. **I** Typical spindle pole body of yeast, forming dense plaques within the nuclear envelope. **J** Specialized structured NAO of *Erynia neoaphidis* (Zygomycota, opistokonts). **K** Slightly electron dense material distributed on both sides of the nuclear envelope (i.e., *Mucor*, Zygomycota). **L** Intranuclear NAO organized in a sphere (i.e., *Minchinia*, *Rhizaria*, plasmodial form of *Physarum*, *Tocoplasma tachyzoites* and *Oedogonium*)

In the Phytomyxa (i.e., *Plasmodiophora brassicae*), an unusual type of chromosome segregation, called cruciform division (chromatin divides in a clump and moves to the poles, while the nucleolus elongates), is observed.

In interphase, the centrioles are aligned antiparallel in one axis. The nuclear envelope partially breaks down in prometaphase, and large amounts of membrane cisternae become clearly visible in the interior of the spindle during metaphase (Garber and Aist 1979). In the Radiolaria, adjacent fan-shaped microtubule arrays form simultaneously at the surface of duplicated polar structures (plaque and orthogonal centrioles). Bipolarization occurs in a subsequent step. Acantharia (i.e., *Acantholithium*) develop a central spindle, whereas polycistines (i.e., *Theopilium*) do not.

3.1.5 Amoebozoa

There are three types of mitotic divisions: **promitotic**, with an intranuclear spindle and a persistent nucleolus (i.e., *Naegleria gruberi*, *Entamoeba histolitica*, *Acanthamoeba*); **mesomitotic**, with the absence of centrioles and delayed breakdown of the nuclear envelope and nucleolus (i.e., *Pelomyxa carolinensis*); and **metamitotic**, with absent centrioles and early breakdown of both nuclear envelope and nucleoli. In the Myxomycota (i.e., *Physarum*) and Dictyostela (i.e., *Dictyostelium*), mitosis is closed with an intranuclear spindle that is connected with a ring or a cubical extranuclear NAO, respectively.

3.1.6 Opisthokonta (Including Fungi and Animals)

Orthogonal centrioles are observed in the hypochytrids and chytrids, whereas Ascomycota and Basidiomycota integrated the microtubule-nucleating components closer to the nuclear envelope in various forms of NAOs, such as plaques, spheres, bars, or rings. Fungi and animal cells present mostly well-defined, separate, distinguishable chromosomes. They exhibit a clear central intranuclear pole-to-pole microtubule bundle, which is absent in the hypochytrids and chytrids, but is present in some zygomycetes. The fungi display a great variability with regard to the persistence of the nuclear envelope. Their variability range includes: a nuclear rim that remains intact throughout mitosis; nuclei that are polarly fenestrated (i.e., in the chytrids); as well as a situation where the nuclear envelope becomes entirely dispersed.

Canonical centrosomes are present in all animal classes, with the exception of Euarthropodae (i.e., *Drosophila*), in which they are replaced by cytoplasmic ring-like structures, and nematodes (i.e., *Caenorhabditis elegans*), in which only orthogonal procentriole-like structures are visible. In the zygomycete *Erynia*, the NAO consists of both a saucepan-shaped extranuclear and a saucer-shaped intranuclear plaque.

3.1.7
Lower Plants

Uncondensed chromatin and a central spindle were only described for one taxon of the Rhodophyta. Otherwise, well-defined chromosomes are condensing in G2 phase, and the nuclear envelope breaks down in prophase (i.e., *Cyanophora*, Glaucophyta, Rhodophyta). In the green algae *Cladophora*, the NAO is composed of one centriole and a procentriole, which is only formed by microtubule singlets in place of triplets and persists during mitosis. Otherwise, centrosomes are maintained from green algae to *Gingko* in their motile male gametophytes.

3.2
Molecular Aspects of Mitotic Evolution

Most of the genes involved in mitosis and their control were discovered using **yeast** models. This was possible by the strong conservation, preserved up to mammalian systems, of the major players and regulators of mitosis, due to the fundamental importance of cell division.

3.2.1
Microtubule Motors

Among other functions, the kinesin superfamily participates in chromosome movement and spindle functions during mitosis. The kinesins, which have the catalytic domain located at the N- or C-terminus are responsible for: generating forces for spindle assembly, bipolarity, stabilization, and dynamics; maintaining antagonistic forces within the spindle; and allowing for cross-linking and bundling of microtubules. On the other hand, kinesins, which have the catalytic domain located in the center, link the microtubule end and stimulate its depolymerization.

Kinesins are found in protists, yeasts, fungi, plants, invertebrates, and vertebrates. They are distributed over at least 14 subfamilies. Among the seven kinesins found in *Saccharomyces cerevisiae*, six play a role in mitosis in association with one dynein. In *Caenorhabditis elegans*, 19 kinesins and two dynein havy chains have been characterized. The number of kinesin species raises up to 45 for humans, and even 60 for *Arabidopsis* kinesins. Protozoan kinesins diverge quite considerably. Additional kinesin-like proteins, which could not be attributed to any of the above mentioned 14 classes of canonical kinesins, have been identified in *Cenorhabditis*, *Plasmodium*, *Tetrahymena*, *Giardia*, and *Leishmania*.

Interestingly, kinesins have acquired multiple additonal functions during their evolution from their first ancestors. There are classical microtubule plus-end directed motors, such as kinesin-5 (formerly BimC); microtubule

minus-end directed motors, like kinesin-14 (formerly C-term motors); and I-type kinesins, like kinesin-6 (formerly MKLP1) or kinesin-3 (formerly Unc104/KIF1). All of them are involved in spindle maintenance or cytokinesis. However, there are chromosome-associated kinesins, connecting kinetochores and kinetochore microtubules or associated along chromosome arms, and contributing to chromosome condensation or motility, such as kinesin-7 (formerly CENP-E), kinesin-13 (formerly MCAK/KIF2), and kinesin-4 (formerly Chromokinesin or KIF4), respectively (Miki et al. 2005; Mazumdar and Misteli 2005; Wordeman 2005).

Dyneins are strictly microtubule minus-end motors. They have been first characterized in *Tetrahymena*. Members of this family were found in most phyla, including slime molds, fungi, *Chlamydomonas*, sea urchin, *Drosophila*, or mammals. Among higher plants which have lost cilia and flagella, four dynein heavy chains have been reported only for *Oryza sativa* (King 2002). However, a recent comparative genomic analysis came to the conclusion that dyneins have been lost during the evolution of higher plants (Wickstead and Gull 2007).

3.2.2 Centrosomal Proteins

As mentioned above, the (9 + 2) centriolar structure was acquired early. It was primarily associated with cell motility, sensing, and division (Azimzadeh and Bornens 2005). Although the architecture of microtubule organizing centers has undergone divergent evolution, many of their molecular components have remain conserved.

This is the case for γ-tubulin and the γ-tubulin complex proteins (GCPs), which are the essential components of microtubule nucleation (Erhardt et al. 2002). However, for a long time, the γ-tubulin orthologues of budding yeast and *C. elegans* had not been identified, due to a high divergence in their sequence. This could either be the consequence of rapid evolution rate, or of an adaptation to specific partners associating the γ-tubulin small complex into a larger nucleation complex (like the γ-TuRC found in *Drosophila*, *Xenopus*, humans, and higher plants).

Centrins, a second important group of centrosomal proteins, are closely related to calmodulin and have been proposed to act as regulators. They form two largely conserved subfamilies that are defined by the founding members, Cdc31p of *S. cerevisiae* (Cdc31p) and the centrin of *Chlamydomonas reinhardtii*. The sequence specificities of these subfamilies do not necessarily mirror functional differences, since both centrin types play a role in centrosome duplication, segregation, and cytokinesis. These canonical centrins are complemented by centrin subfamilies in the Alveolata and the angiosperms, which are, apparently, no longer linked with motility (Azimzadeh and Bornens 2005).

3.2.3
Kinetochore Proteins

Kinetochore proteins are multiproteic complexes binding to centromeric DNA and connecting chromatids to the spindle poles via kinetochore microtubules. Phylogenetic and structural analysis of kinetochore proteins (Meraldi et al. 2006) revealed that the kinetochore core proteins have conserved short motives, which have been conserved throughout the eukaryotes, despite a huge divergence within centromeric DNA sequences. The simplest kinetochore has been characterized in *Encephalitozoon cuniculi*, a microsporidial parasite, which reduced its genome under selection pressure. Three of the four multiproteic complexes characterized in higher eukaryotes link DNA-binding components to microtubule binding components (including motors) and regulatory elements (including the kinases, Mps1 and Bub3, both playing a role in the mitotic checkpoint). The proteins constituting these complexes have been identified all over the eukaryotes, from yeast to humans (Meraldi et al. 2006).

3.2.4
Microtubule Associated Proteins

Structural Microtubule Associated Proteins (MAPs) are proteins whose binding to microtubule modulates their dynamic properties (Cassimeris and Spittle 2001). Several classes of MAPs have been implied in microtubule nucleation and spindle organization (for instance, the γ-TuRC components and TPX2); monomer binding (e.g., OP18/stathmin); polymer stabilization (e.g., MAP U); and destabilization or severing (e.g., I-type kinesins and katanin complexes, respectively). Among these, the MAP215/Dis1 family of MAPs are highly conserved microtubule regulators in the mitotic spindle (Gard et al. 2004), and have been described under various names in a large number of organims (Dis1 and Alp14 in *S. pombe*, Stu2 in *S. cerevisiae*, DdCP224 in *D. discoidum*, Zyg9 in *C. elegans*, Msps in *Drosophila*, XMAP215 in *Xenopus*, ch-TOG in humans, MOR1 in *Arabidopsis*, and TMBP200 in tobacco). In the meantime, more than 30 additional related genes have been discovered through database searching. All these homologues are targeted to kinetochores in metaphase and to their respective microtubule organizing center (MTOC) in anaphase.

3.2.5
Mitotic Kinases and Phosphatases

Protein kinases are one of the largest gene families in all eukaryotes, accounting for 2–4% of all genes (Manning et al. 2002). This is to be expected, considering the large number of biological processes that are regulated through

phosphorylation. Mitotic kinases, such as the Cdk/cyclins, Aurora-kinases, polokinases, and the "Never In Mitosis A"-kinases play a role in the condensation of chromatin, the disassembly of nuclear lamina, nuclear envelope, nuclear bodies and nucleoli, as well as in spindle assembly, microtubule-kinetochore attachment, and spindle checkpoint (for review, see Li and Li 2006).

The serine/threonine phosphatases, PP1 and PP2A/CDC55, and the dual-specificity protein tyrosine phosphatases (DUSPs), CDC25 and CDC14, are key regulators of mitosis (for review, see Trinkle-Mulcahy and Lamond 2006). PP1 is involved in the maintenance of chromatin achitecture during metaphase-anaphase (Trinkle-Mulcahy et al. 2006; Vagnarelli et al. 2006). It regulates both chromatid decondensation and nuclear envelope reassembly in telophase (Steen et al. 2003; Landsverk et al. 2005). PP2A participates in the maintenance of chromosome cohesion (Kitajima et al. 2006). It regulates both chromosome segregation (Tang et al. 2006) and mitotic exit (Queralt et al. 2006). Animal and plant PP2A have evolved independently (Terol et al. 2002), but members of this family still share the same functions.

CDC25 is a family of three phosphatases, which are generally involved in all cell-cycle checkpoints (Donzelli et al. 2003). CDC14 has also been conserved from yeast to mammals. Some of their mitotic functions are species specific, but their role in spindle stabilization and cytokinesis seems to be conserved among eukaryotes (Trinkle-Mulcahy and Lamond 2006).

4 Milestones Towards Acentriolar Plant Mitosis

Land plants are generally thought to originate from green algae, where structured NAOs dominate. Centrosomes or organelles deriving from basal bodies are found in the unicellular flagellates of monadoid algae or the spermatozoids of multicellular green algae. The transition from a mobile aquatic life style to a sessile terrestrial life style was accompanied by profound changes of mitosis, which directly influenced morphogenesis. These evolutionary modifications went through a progressive loss of the typical (9 + 2) centrioles, and they were associated with a diversification of body plans, varying across unicellular forms, organisms composed of multinucleated modules, and mononucleated cells, which are organized into functional contexts capable of functional differentiation (first primitive parenchymes, which subsequently developed into differentiated tissues). In all cases, nucleocytoplasmic domains containing microtubule organizing complexes represent the basic cellular unit (Pickett-Heaps et al. 1999).

From green algae up to the Gingkophyta, the canonical centrioles generated de novo in gametes are replaced by a variety of more or less structured NAOs in somatic or meiotic cells. Prior to division of these cells, the micro-

tubule interphase network is usually disassembled and replaced by a polarized mitotic spindle. In terms of evolutionary relationships, emphasizing the structural differences of spindle poles can be more misleading than revealing. Instead, it is more fruitful to consider the proteins or protein complexes involved in spindle assembly and organization.

4.1 Localization of γ-tubulin

Gamma-tubulin has been often used as a marker for microtubule organization centers. It is mostly found to segregate at each mitotic pole. It has been shown to concentrate around the centrosomes at the spindle poles in all cells endowed with centriole-driven mitosis, to mark the periphery of polar plastids, and to label acentrosomal spindle poles. All these different conformations are present in Bryophytes (Shimamura et al. 2004). For example, during the final spermatogenous mitotic division of liverwort *Makinoa crispata*,

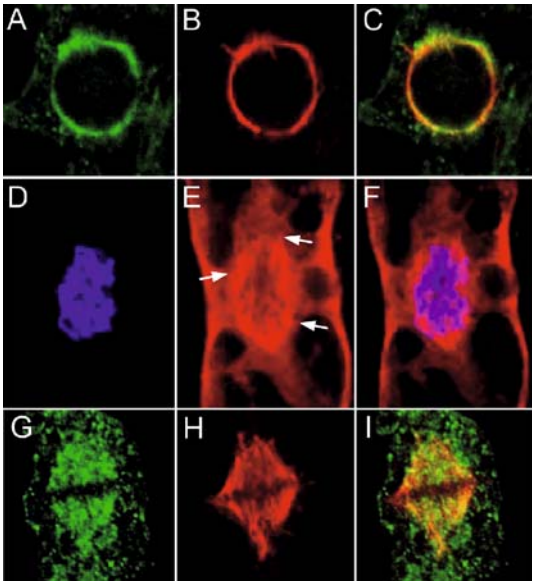

Fig. 6 Dynamic localization of γ-tubulin (*green*), α-tubulin (*red*), and chromosomes (*blue*) in the tobacco cell line, BY-2, during the transition between prophase and metaphase. **A–C** late G2 phase: A majority of perinuclear microtubules is polarized into the future spindle axis and γ-tubulin concentrates into two polar caps. Microtubular plus-ends penetrating into the nucleus are void of γ-tubulin at their tips. **D–F** Breakdown of the nuclear envelope: chromosomes disperse into the cytoplasm and microtubule fibers are not clearly aligned with the division axis (*arrows*). **G–I** During metaphase, the spindle is characterized by broad poles and γ-tubulin is not localized to the microtubular plus-ends at the kinetochores

γ-tubulin surrounds polar Centrosomes. Conversely, during archesporial mitosis of liverwort *Marchantia polymorpha*, γ-tubulin reorganizes from initial foci around discrete microtubule organizing centers in prophase to a broader polar region in metaphase. Monoplastidic meiotic cells of *Dumortiera hirsuta* present periplastidial γ-tubulin in prophase I and II; a focused polar distribution in late prophase I; a broader localization on spindle fibers in metaphase I; and localization to the nuclear rim as well as the minus-ends of phragmoplast microtubules in telophase I and II.

In higher plants, as exemplarily shown for the acentrosomal tobacco BY-2 cells, γ-tubulin is dispersed at the nuclear surface of interphase cells. In late G2, (Fig. 6), the division axis becomes progressively manifested by a corresponding reorientation of perinuclear microtubules. Simultaneously, γ-tubulin is redistributed along perinuclear microtubules, and it concentrates into two polar caps (Fig. 6A). Then, the nuclear envelope breaks down at polar sites and microtubule plus-ends rapidly elongate toward chromatin (Fig. 6B,E). γ-tubulin does not colocalize with these fast growing sites, but it shifts towards the proximal minus-ends of the polar microtubules (Fig. 6A,G).

4.2 Evolution of Cytokinesis

Cytokinesis is the mechanisms leading to cellularization. Usually, it directly follows karyokinesis. Structurally, it is achieved through different mechanisms (Fig. 7): a centripetal cleavage furrow between daughter cells (e.g., *Nephroselmis*, Prasinophycaea, Fig. 7A); a cleavage furrow forming a phycoplast, consisting of a set of microtubules oriented parallel to the plane of the new cell wall (i.e., *Chlamydomonas*, Clamydomonadales, Chlorophycaea, Fig. 7B); a central fusion of vesicles with the phycoplast (i.e., *Oedogonium*, Oedogoniales, *Cylindrocapsa*, Chlorococcales and *Uronema*, Chaetophorales, and Chlorophycaea, Fig. 7C); a cleavage furrow with simultaneous persistence of central spindle microtubules (i.e., *Pyra-mimonas*, Prasinophyceae; *Ulothrix*, Ulvophycaea; *Klebsormidium*, Klebsormidiophycaea, Fig. 7D); or a centrifugally established cell plate organized by a phragmoplast (i.e., *Trentepohlia*, Ulvophyceae; *Spirogyra*, Zygnematophyceae; *Chara*, Charophyceae, *Chlorokybus*, Choleochaetophycaea, and also all Embryophytes, Fig. 7E). In multinucleated cytoplasts, cytokinesis may be delayed after karyokinesis and phragmoplasts form wherever overlapping microtubules are present, i.e., between sister and non-sister nuclei (i.e., sporogenesis of *Conocephalum*, Bryophyta; endosperm of higher plants, Fig. 7F).

It is puzzling that a phragmoplast is found not only in the Streptophyta (e.g., some Zygnematophycaea, Charophycaea, Choleochaetophycaea) and the Embryophyta (e.g., *Bazzania*, Marchantiophytes), but also in Chlorophyta, such as the Trentepohliales (Mattox and Stewart 1984). It either corresponds to a structural convergence evolved in parallel or, in case it is of mono-

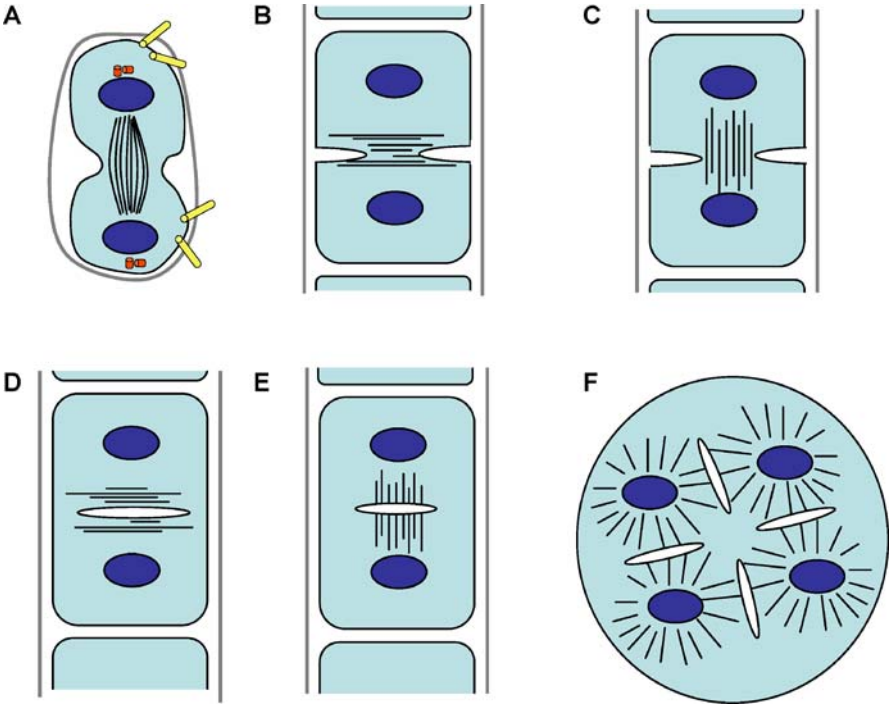

Fig. 7 Variability of cytokinetic mechanisms in algae and embryophytes. **A** Microtubule-independent cleavage furrow in an unicellular organism (e.g. Nephroselmis). **B**, **D** Phycoplast microtubules that are oriented perpendicular to the spindle axis, are involved in cell wall formation either through cell wall ingrowth (**B**) or outgrowth (**D**). **C**, **E** Phragmoplast microtubules that are oriented parallel to the spindle axis associated with a cleavage furrow (**C**) or with centrifugal vesicle fusion leading to the formatkion of a cell plate development (**E**). **F** Cellularization of coenocytes with phragmoplasts developing between sister and non-sister nuclei

phyletic origin, it necessitates a revision of the current views on taxonomy and evolution of the Chlorophyta. To address this question, Lopez-Bautista et al. (2003) analyzed the phylogeny of phragmoplastin, a protein found to play a role in squeezing Golgi-derived vesicles, which fuse at the cell plate and form a tubulovesicular pattern during early cell plate development. This protein could be identified in *Trentepohlia* and *Cephaleuros*, consistent with the model of a putative common ancestor expressing phragmoplastin and using a primitive form of phragmoplast-dependent cytokinesis. However, this data set is still too limited for a conclusive answer. With respect to phragmoplast structure, the Trentepohliales are close to the Charophycea. However, in many other aspects, they behave more like Chlorophyta, such that they seem to represent a chimera comprising traits from both Chlorophyta and Streptophyta.

4.3
Cytokinesis – The Role of Actin and Spindle Polarity

In the absence of microtubules, a ring of actin is involved in the formation of the cleavage furrow (e.g., *Spirogyra*, Goto and Ueda 1988). Moreover, actin filaments are also found in the phycoplasts of the Chlorophyta (e.g., in *Chlamydomonas*, Harper et al. 1992; *Nannochlorys*, Yamamoto et al. 2001). In phragmoplasts, actin filaments are mostly aligned parallel to microtubules, forming a wreath or a belt encircling the extending microtubule ring. Inhibitors of F-actin, such as cytochalasins or latrunculin, impede the centrifugal extension of the microtubule phragmoplast, suggesting that the actin cytoskeleton cooperates with microtubules in the development of the cell plate (Schmit and Lambert 1988).

In walled, sessile cells, the polarity of the mitotic spindles and the subsequent deposition of the new cell walls are of crucial importance for the control of morphogenesis. In higher plants, the phragmoplast is laid down in close proximity to the site where, prior to the mitotic prophase, a cortical preprophase band consisting of microtubules is formed. It is interesting to determine whether the cleavage plane is similarly predictable in lower eukaryotes.

Cortical microtubules encircling the nucleus in higher plants are absent in algae, including *Coleochaete* and *Chara*. In these cells, the polarity of division is mostly determined by polar location of NAOs or plastids.

In primitive Embryophyta, the future plane of cell division is predicted by a microtubule preprophase band (PPB). Actin filaments are associated with this PPB. When the PPB disappears at the onset of the prophase, it leaves behind a cytoplasmic region, which is also depleted of actin. This reorganization of both cytoskeletal components predicts the boundaries of the prospective daughter cytoplasts (Pickett-Heaps et al. 1999). However, the formation of a PPB seems to be confined to somatic cells (Brown and Lemmon 2001), and it seems to be frequently incomplete in the fern *Azolla* (Gunning et al. 1978), and even absent in the filamentous moss protonema (Doonan 1991).

The reproductive lineage of most land plants (Bryophytes, Lycophytes, and certain ferns) utilizes different modes of cellular polarization. In the sporogenesis of monoplastidic cells, a bipolar NAO first appears during early meiotic prophase. Subsequently, a quadripolar microtubule system (QMS) originates from the divided plastids, delineating the position where the four haploid spores will segregate in telophase II (Pickett-Heaps et al. 1999; Shimamura et al. 2004). Recently, Brown and Lemmon (2006) described sporogenesis in liverwort *Aneura pinguis*, in which perpendicular girdling bands of microtubules encircle the prophase nucleus and delimit the prospective cytokinetic partition planes of each individual spore. This organization may correspond to ancestral PPBs. A difference noticed in this case is the as-

Table 1 Phylogeny used in this chapter

Kingdom	Glaucophyta			
	Rhodophyta			
	Chlorobionta			
	Division	Chlorophyta		
		Class	Prasinophycaea	
			Order	Prasinococcales
				Pyramimonadales
				Mamiellales
				Nephroselmidiales
				Pseudoscourfieldiales
				Picocystidales
				Chlorodendrales
			Trebouxiophycaea	
				Trebouxiales
				Microthamniales
				Prasiolales
				Chlorellales
			Chlorophycaea	
				Oedogoniales
				Chaetophorales
				Dunaliellales
				Tetrasporales
				Chlamydomonadales
				Chaetopeltidales
				Sphaeropleales
				Volvocales
				Chlorococcales
				Chlorasarcinales
				Microsporales
				Cylindrocapsales
			Ulvophycaea	
				Ulotricales
				Ulvales
				Trentepohliales
				Cladophorales/Siphonocladales
				Oltmannsiellopsidales
				Bryopsidales
				Caulerpales/Halimedales
				Dasycladales
		Streptophyta		
			Mesostigmatophycaea	
			Chlorokybophycaea	
			Klebsormidiophycaea	
			Phragmoplastophytes	
			Zygnemophycaea	
				Zygnematales
				Desmidiales

Table 1 (continued)

Plasmodesmophytes
Charophycaea
Charales
Chaetospheridiophycaea
Parenchymophytes
Coleochaetophycaea
Coleochaetales
Embryophyta (land plants)
Bryopsideae
Marchantiophytes (Hepatics = liverworts)
Bryophytes (Mosses)
Anthocerophytes (Hornworts)
Tracheophytes (Vascular plants)
Polysporangiophytes
Lycophytes
Euphyllophytes
Monilophytes
Sphenophytes
Filicophytes
Spermatophytes
Coniferophytes
Ginkgophytes
Pinophytes
Cycadophytes
Anthophytes
Gnetophytes
Angiosperms

sociated protrusion of four distinct lobes, restricting the cytoplasm earlier than what is found in other Bryophytes and higher plants. The mechanism of lobing remains unknown. However, it seems not to involve F-actin constrictions, as observed in animal cell cleavage (Brown and Lemmon 2006). In the liverwort *Reboulia*, microtubules originating from polar NAOs are organized in a criss-cross pattern, constituting a PPB-like structure coexisting with the prophase spindle (Brown and Lemmon 1990). In flowering plants, a triploid coenocytic endosperm is only later cellularized (e.g. in *Haemanthus*), such that karyokinesis is uncoupled from the largely delayed cytokinesis. These cells do not form PPBs either (Mole-Bajer and Bajer 1963). This is not surprising if one considers that these cells lack cortical microtubules. Instead, the G2 nuclei are efficiently separated by perinuclear microtubules that maintain a cytoplasmic area around the individual nuclei, which enter mitosis in a synchronized manner, such that a wave of division

can be observed to travel through the syncytium (e.g., in *Ginkgo*, Brown et al. 2002).

5
Evolutionary Footprints 1: Division of Plastids and Mitochondria

The qualitative difference between eukaryotic and prokaryotic cells is generally accepted to have derived from multiple endosymbiotic events, whereby a larger "host" cell integrated a prokaryotic prey and failed to digest it. During the subsequent phase of "domestication", numerous genes were transferred from the endosymbiont into the host genome, such that the endosymbiont progressively became dependent on the eukaryotic host. This process, in general, seems to be more advanced for the mitochondria, as compared to the plastids. However, both organelles have preserved their generic identity, because they cannot be assembled by the host de novo. Instead, they arise exclusively ex suis generis. This evokes the question whether or not organelle divisions are coordinated with the division of the host cell. It seems that plastids and mitochondria represent different steps on this evolutionary path. Plastids have not yielded to domestication to the same degree as mitochondria (for review, see Osteryoung and Nunnari 2003).

Mitochondria have lost FtsZ, whereas plastids still utilize the original prokaryotic FtsZ-machinery for division. However, mitochondrial FtsZ can still be detected in primitive Eukaryotes, such as the chromophyte alga *Mallomonas* (Beech et al. 2000). In animals and fungi, the division of mitochondria involves the activity of dynamin-related proteins, i.e., GTPases that can generate force and associate with the cytoplasmic surface of the outer membrane in form of a ring. This indicates that the originally autonomous (FtsZ-based) division function of mitochondria has been completely replaced by a host function. However, dynamin-related proteins have been identified in plastids as well. They participate there in the late phases of division. For instance, the dynamin-related proteins, Arc5 (*Arabidopsis*, Miyagishima et al. 2006) or Dnm2 (Rhodophyta, Miyagishima et al. 2003), interact with the plastid division machinery during later phases of septation. Thus, the dynamin-related proteins represent a general eukaryotic tool for organelle division. In the case of plastids, they interact with FtsZ, whereas, in mitochondria, the function carried by FtsZ has been taken over by other unknown host proteins.

Until relatively recently, it was unknown whether the division of plant mitochondria was driven by dynamin-related proteins, as is the case in animals and fungi. However, a screen for *Arabidopsis* mutants with altered morphology of mitochondria and peroxisomes led to the identification of the dynamin-related protein, DRP3A, which participates in the division of mitochondria and peroxisomes. Mutants of the corresponding gene produce misshaped and partially giant mitochondria and peroxisomes, but contain

perfectly normal chloroplasts (Mano et al. 2004). This suggests an evolutionary scenario, where a GTPase responsible for a host-cell activity (peroxisomal division) has acquired a dual function accepting mitochondrial function as "moonlighting" job.

Similar to mitochondria, chloroplasts have lost most of their genetic autonomy by transfer of genes into the nuclear genome. The cyanobacterium *Synechocystis* encodes more than 3000 proteins, whereas most chloroplast genomes harbor less than 200 coding sequences (Martin and Herrmann 1998), which means that most proteins have to be imported from the cytoplasm into the chloroplast, including FtsZ and other components of plastid division. The first indication for the activity of a prokaryotic type of division machinery came from the identification of a plant FtsZ (Osteryoung and Vierling 1995). When this FtsZ was specifically knocked down in moss *Physcomitrella patens* (Strepp et al. 1998), vermiform and giant chloroplasts were produced, demonstrating that this FtsZ was essential for plastid division. This conclusion was supported by similar findings in *Arabidopsis* (Osteryoung et al. 1998). In contrast to the situation in prokaryotes, where Ftsz is almost exclusively encoded by a single-copy gene, plant FtsZ contain multiple homologues of FtsZ, which cluster into two subfamilies (Rensing et al. 2004). The respective gene products participate in chloroplast division. However, most of the bacterial division proteins could not be identified in plant chloroplasts. Moreover, the cause for the duplication and diversification of the plant FtsZ family has remained unknown.

To gain insight into these questions, it is necessary to enlarge the spectrum of the usual model plants (mostly *Arabidopsis*, rice, and tobacco), by including model plants that are more distant in terms of phylogeny. Mosses, such as *Physcomitrella patens*, are very well suited in this context, because they appeared as early as 450 million years ago (Theißen et al. 2001), and, judging from fossil records, they have remained unchanged, at least in terms of morphology (Miller 1984). Thus, the bryophytes represent the most conservative group of land plants. They are ideal candidates for evolutionary studies.

During a study on the localization FtsZ isoforms in *Physcomitrella patens*, one isoform, FtsZ1-2, was found in the cytoplasm (Kiessling et al. 2004). So far, plant FtsZ isoforms have been exclusively reported in filamentous structures, whereas rings and networks inside the chloroplasts, GFP fusions of FtsZ1-2, could be found in form of rings in both compartments. This peculiar FtsZ isotype is endowed with two alternative start codons, whereby the chloroplast signature present in the longer transcript (which has been demonstrated to be imported into the chloroplast) is absent in the shorter transcript (which forms peculiar filamentous structures in the cytoplasm). The nature of these cytoplasmic filaments has not yet been uncovered. However, they seem to be specific for this FtsZ-isotype, because ectopic expression of a different FtsZ-isotype by removal of the plastidic import signature yielded punctate structures instead of filaments. It is tempting to interpret

FtsZ1-2 as a component with dual function. In addition to its participation in the division of chloroplasts, it might have acquired a second "moonlighting" function that involves an interaction with unknown partners of the host cytoskeleton.

So far, cytoplasmic FtsZ isoforms have not been reported outside the Bryophyta. Therefore, they might represent an exotic footprint of evolution. However, from a functional point of view, proteins of analogous function must exist in the other land plants as well, because the division of chloroplasts has to be tuned with the division of the host cells, in order to ensure that each cell remains with at least a minimal set of plastids. If one takes into account that most components of the prokaryotic division machinery have been replaced by functionally equivalent, but phylogenetically unrelated proteins, carrying the respective functions in organelle division, it is to be expected that the cytoplasmic function carried by FtsZ1-2 in mosses has been taken over by other (still unknown) proteins during the evolution of the cormophytes.

6 Evolutionary Footprints 2: The Plant Nucleus Revisited

As described in detail above, the open mitosis found in animals and higher plants has evolved from mitotic forms where the nuclear envelope persists, at least partially. In organisms that follow this so-called closed mitosis, the entry of tubulin and microtubule-nucleating components into the nucleus at the onset of mitosis, as well as their exclusion at the end, must be under strict cell-cycle control. For example, the spindle pole body of Saccharomyces cerevisiae is assembled in the cytoplasm and subsequently transported in a cell-cycle-dependent manner into the nucleus via the nuclear-localization sequence (NLS) of the Spc98p protein (Pereira et al. 1998). Interestingly, α- and β-tubulins are not found in interphasic nuclei of cells with closed mitosis. Therefore, in these organisms, tubulin has to be transported in and out of the nucleus. Indeed, it has been shown in *Aspergillus nidulans* that tubulin enters the nucleus immediately before the onset of closed mitosis, and it is rapidly excluded at the end (Ovechkina et al. 2003). Although the molecular mechanisms of nuclear tubulin transport remain to be elucidated, these results suggest that the movement of tubulin through the nuclear envelope during the cell-cycle is an active and highly regulated process.

In contrast to closed mitosis, in the open mitosis of animals, higher plants, and Charophyceae, the interaction of (cytoplasmic) tubulin with (intranuclear) chromatin occurs spontaneously at the breakdown of the nuclear envelope. Consequently, directed movement of tubulin through the nuclear envelope is not required for formation of the mitotic spindle. After disintegration of the nuclear envelope, the microtubule spindle can exploit the pool

of cytoplasmic tubulin that is no longer withdrawn by compartmentalization. As discussed above, the spindle microtubules are nucleated from centrosomes in animal and algal cells, but from rather diffuse microtubule-organizing centers (MTOCs) in the acentriolar cells of higher plants. In addition, the kinetochores of both animal and plant cells are endowed with a microtubule-nucleating activity (Cande 1990). γ-Tubulin, an indispensable component of both centrosomes and plant MTOCs (Pereira and Schiebel 1997, Stoppin-Mellet et al. 2000), is already interacting with the prospective kinetochore sites during the G2 phase and, thus, prior to the disintegration of nuclear envelope (Binarová et al. 2000). This indicates that, like the SPB of *S. cerevisiae*, the microtubule-nucleating component γ-tubulin must be actively transported through the intact nuclear envelope before mitosis. Again, neither has the molecular mechanism of this nuclear import been identified, nor is it known whether other components of MTOCs persist in the nucleus during the whole cell-cycle or have to be imported as well. A nuclear-localization sequence (NLS) is present in GCP3, one of the three components of the small gamma-tubulin nucleation complex (γ-TuSC), and this protein was detected by immunolabeling in G2 nuclei of BY-2 cells (Schmit, unpublished results). Moreover, sequences conferring targeting to the nuclear envelope have been found in GCP2 and GCP3 of *Arabidopsis thaliana* (Seltzer et al. 2007). This suggests that perinuclear microtubule nucleation initiates spindle formation in early G2, and that, upon breakdown of the nuclear envelope, microtubules are newly assembled around chromosomes from nucleation complexes already present in the vicinity of chromosomes.

Not only nucleation components, but also the bulk tubulins themselves, can be imported into the nucleus. Circumstantial evidence of intranuclear tubulin comes from mammalian tumour lines (Menko and Tan 1980; Walss et al. 1999; Walss-Bass et al. 2003) and *Aesculus hippocastanum* by electron microscopy (Barnett 1991). However, the functional context of nuclear tubulin import remained enigmatic. One possibility might be the sequestration of soluble tubulin heterodimers to circumvent the induction of regulatory circuits that otherwise would downregulate tubulin abundance (see chapter "Plant tubulin genes: regulatory and evolutionary aspects" of this book). If this working hypothesis was correct, the formation of intranuclear tubulin should be initiated by factors that trigger the disassembly of interphase microtubules. To test this idea, tobacco BY-2 cells were subjected to a chilling treatment that efficiently eliminated cortical and radial microtubule arrays (Schwarzerová et al. 2006). In fact, tubulin progressively accumulated in the nuclei with the disassembly of cytoplasmic microtubules. Upon re-warming, this intranuclear tubulin was rapidly, within minutes, excluded from the nuclei, and it immediately polymerized into cytoplasmic microtubules. This cold-induced tubulin accumulation was not only shown by immunofluorescence, but could also be followed in vivo in cells expressing a GFP-fusion of one tobacco tubulin isotype. By confocal microscopy it was verified that

this tubulin is localized inside the nucleus. When nuclei were purified from cold-treated cells and stringently stripped of cytosolic proteins, the tubulin remained in the nuclear fraction, nevertheless. Nuclei from unchilled control cells were void of intranuclear tubulin demonstrating the stringency of this assay. Interestingly, *bona-fide* nuclear-export sequences (NES) could be identified in plant tubulins. Some of them were specific for plant tubulins, which would explain the rapid exclusion of intranuclear tubulin upon return to the permissive temperature.

The exclusion mechanism that sequesters tubulin from the chromatin during interphase in those cells seems to be highly sensitive to temperature. Nuclear import is mediated by nuclear pore complexes residing in the nuclear envelope. Passive diffusion through these pores is almost impossible for proteins exceeding 50 kDa (for review, see Talcott and Moore 1999). Therefore, the import of $\alpha\beta$-tubulin heterodimers (110 kDa) must involve specific interaction with the nuclear transport machinery. The massive increase in the pool of free tubulin dimers in the cytoplasm, as a consequence of cold-induced elimination of assembled microtubules, might simply overrun the highly selective transport through the nuclear envelope, such that tubulin "leaks" into the nucleus. However, when such an excess of tubulin dimers was generated by oryzalin, tubulin did not enter the nucleus (Schwarzerová et al. 2006). Moreover, intranuclear tubulin accumulated against a concentration gradient, suggesting active transport and binding to an unknown intranuclear lattice. The affinity to this lattice seems to be rather strong, since tubulin remained attached even during the isolation of nuclei.

Tubulin is exclusively cytoplasmic during interphase, probably because its presence in the interphasic nucleus would induce serious disorders of cell-cycle regulation. During interphase, when nuclear pores control the transport of molecules, the integrity of the nuclear membrane seems to be the main mechanism that ensures the sequestration of tubulin to the cytoplasm.

However, the interaction of tubulin with chromatin is crucial during mitosis. For closed mitosis, tubulin is imported into the nucleus by an unknown mechanism, whereas for open mitosis, no tubulin transport through the nuclear membrane is required, due to the breakdown of the nuclear envelope. However, when daughter nuclei are formed at the end of mitosis, new nuclear envelopes have to be reestablished and the pool of tubulin molecules from the disintegrated spindle has to be removed from the karyoplasm. At this stage, tubulin is excluded from nuclei, probably utilizing the NES export signals conserved in all tubulins. This exclusion mechanism seems to depend on active metabolism. Therefore, it is impaired during cold treatment, resulting in intranuclear accumulation of tubulin.

This study on intranuclear tubulin suggests that, in spite of substantial differences between open and closed mitosis, some mechanisms must be preserved in all organisms in order to ensure proper progression of the cell-cycle. Some of these mechanisms include the import of cytoskeletal components

regulating polymerization of microtubules at the beginning of mitosis and export of tubulin at the end.

Whereas the biological function of tubulin shuttling in interphase nuclei is still enigmatic, intranuclear actin and even type-I myosin have been reported for mammalian cells to participate in chromatin modeling and the regulation of gene expression (for review, see Pederson and Aebi 2005). This seems to be true for plant cells as well, because silencing of an intranuclear actin-related protein identified in *Arabidopsis* produced marked changes in plant development (Kandasamy et al. 2005). Thus, we have to get used to the idea of an intranuclear cytoskeleton sharing at least some of the components that build cytoplasmic actin filaments and microtubules. As noted in this article, open mitosis represents a highly advanced situation, and it derives from ancestral precursors, where the nuclear envelope persists during mitosis. The evolutionary origin of the eukaryotic nucleus is still under debate (Pennisi 2004). However, recent findings (for review, see López-García and Moreira 2006) seem to rehabilitate the original idea that the nucleus derives from an endosymbiontic event (Margulis 1993), suggesting a scenario where a methanogenic archaeon is ingested by a fermentative myxobacterium. This is, for instance, supported by similarities between nuclear-core complexes and components of coated vesicles (Devos et al. 2004).

If the ancestor of the eukaryotic nucleus was an endosymbiont, it must have been endowed with an autonomous division machinery. The variant forms of mitosis in lower eukaryotes can be understood as variations on the theme of closed mitosis, where the (presumably endosymbiotic) nucleus maintains a high degree of integrity. The original function of the cytoskeleton – mitosis was, therefore, located inside of the nucleus. Intranuclear tubulin and actin might be nothing else than evolutionary footprints, rudiments dating back to the times when eukaryotes began their victorious existence. A few years from now, once molecular cell biology extends from the relatively small group of currently studied model organisms to the more distanly related group of species, we will be able to determine whether this is more than a speculation.

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