Cortical Microtubules Form a Dynamic Mechanism That Helps Regulate the Direction of Plant Growth

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ABSTRACT

Plants form an axis by controlling the direction of cell expansion; this depends on the way in which cellulose microfibrils in the wall resist stretching in particular directions. In turn, the alignment of cellulose microfibrils correlates strongly with the alignment of plasma membrane-associated microtubules, which therefore seem to act as templates for laying down the wall fibrils. Microtubules are now known to be quite dynamic, and to reorient themselves between transverse and longitudinal alignments. Plants "steer" the direction of growth by reorienting the cellulose/microtubule machinery. For example, the model predicts that a transverse reorientation on one flank of an organ and a longitudinal orientation on the other should lead to bending. This response has recently been observed in living, gravistimulated maize coleoptiles microinjected with fluorescent microtubule protein. This paper reviews the idea of the dynamic microtubule template and discusses possible mechanisms of reorientation. Recent biochemical work has shown that microtubules are decorated with different classes of associated proteins, whose potential roles are outlined.

INTRODUCTION

The ability of wooden artefacts to maintain their shape depends on the organization and chemistry of the cellulose-based walls that surround dead cells. In the living state, however, plants change their shape over time, and this plasticity depends on an important contribution from the living cytoplasm in remodelling the structure of the wall. The cell wall can yield and grow in different directions. Although the chemistry of the wall is an important key to understanding how this happens, it is not the only one. The *texture* of the wall is also important. The wall is composed of several, sometimes many, layers of cellulose microfibrils. The fibrils follow the same overall direction within any one layer, but they can change from layer to layer, and it seems that the direction of the layer(s) nearest the plasma membrane determines the direction in which the cell will grow. The direction in which that innermost layer is deposited is believed to depend on cues from a complementary set of proteinaceous fibres just inside the plasma membrane. It is now known that the fibres that constitute this so-called cytoskeleton—particularly the microtubules—are highly dynamic. This dynamicity, which appears to be linked to the ability of the cell to respond to factors such as light and gravity, is the subject of this article.

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MICROTUBULES

Microtubules are usually composed of 13 protofilaments folded into a hollow tube, approximately 25nm in diameter (see reviews in Hyams and Lloyd, 1994). Each protofilament is composed of alternating dimers of á and áâ tubulin, which, since they all enter the polymer with the same sense (áâ, áâ, áâ. . .), imparts a polarity to the microtubule. This is reflected in the fact that subunits add on faster to one end (the plus end) than to the other. Microtubules, therefore, have an intrinsic polarity that imparts directionality to the cytoplasm, which is at the core of the many structural properties of these cytoskeletal elements. Molecules—such as the microtubule motor proteins, kinesin and dynein—"read" the directionality of microtubules and help transport vesicles and organelles to one end or the other. Microtubules are found in virtually all eukaryotic cells, and the relatedness of the tubulin proteins across large phylogenetic distances is demonstrated by the fact that tubulin from vertebrate brains is readily incorporated into the microtubule structures of plants.

Microtubules are employed in constructing the mitotic spindle for the separation of the chromosomal material. However, when the cell is not dividing, microtubules disassemble and reconstruct other assemblies, such as the cilia and flagella of unicellular algae and the bundles that support the long processes of nerve cells. In higher land plants, the microtubules are found immediately beneath the plasma membrane (to which they are attached by poorly characterized cross-bridges). Spread along the entire cell, hundreds of microtubules of variable length circumnavigate the cell in overlapping relays. They are generally pictured as encircling the cell. However, as described below, the microtubule array is highly dynamic and can adopt different orientations.

MICROTUBULES AND DIRECTIONAL CELL GROWTH

One of the key features of plant development is the ability both to direct growth up into the atmosphere—to display photosynthetic and reproductive organs—and to send roots down into the ground. This necessarily involves limiting growth to a particular axis, and it is in this capacity that microtubules are believed to have an important role. Naked protoplasts placed in a hypotonic solution will swell isotropically. However, when the cell wall grows back, the turgor pressure is directed along an axis, producing an asymmetric cell (Wymer et al., 1996). Only the inelastic cellulose microfibrils in the wall are believed to have the necessary strength to resist turgor pressure. It was established a long time ago that the cellulose microfibrils in the wall share the same direction as the cortical microtubules, and it is this

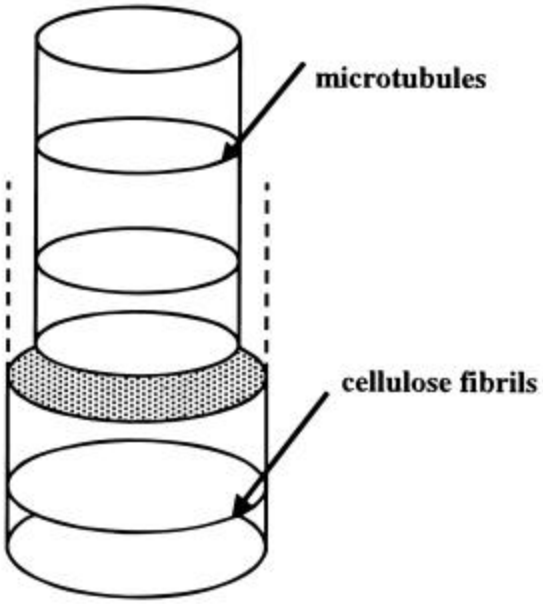

Figure 1. Parallelism Between the Cortical Microtubules, Here Circumnavigating the Cell as Transverse "Hoops," and the Cellulose Microfibrils in the Cell Wall. *Although these two linear elements are separated by the plasma membrane, they are thought to be functionally related by microtubules that form tracks for the movement of the plasma-membranous cellulose that synthesizes enzyme complexes. Transversely arrayed cellulose microfibrils resist increase in cell girth and convert turgor pressure into an elongating force.*

co-linearity that forms the basis of directed growth (Ledbetter and Porter, 1963). Transverse "hoops" of cytoplasmic microtubules follow the same general direction as the cellulose microfibrils in the wall (Figure 1). When cellulose microfibrils are wound around a cell in transverse layers like this, their lack of stretch acts like a girdle that resists increases in diameter. However, because the swelling force of turgor pressure can tease apart adjacent microfibrils in any one layer, the cell stretches perpendicularly to the direction of the microfibrils. (In an oversimplified way, this can be thought of as the stretching of a coiled spring in which adjacent windings are pulled apart). On the other hand, cellulose microfibrils can be arranged parallel to the long axis of the cell. Lack of stretch along this axis should lead to inhibition of cell elongation and encourage lateral (radial) expansion instead. The alignment of the cellulose fibrils therefore influences the direction in which the turgid cell expands. If the microfibrils in epidermal cells are longitudinal on one flank of a shoot and transverse on the other, subsequent unequal growth should lead to bending. As discussed below, this is what seems to occur in gravitropic and phototropic responses.

Returning to the question of microtubule/microfibril parallelism, the microtubules on the inner face of the plasma membrane are believed to form the template for the deposition of cellulose microfibrils on the external face. In brief, large multi-enzyme complexes that sit within the plasma membrane are hypothesized to track along the underlying microtubules, spinning out cellulose microfibrils as they move (for review see Giddings and Staehelin, 1991). Over the years, it has been difficult to test this hypothesis, because its credibility rests entirely on the coincidence of microtubule and microfibril parallelism. There is a large weight of evidence for this parallelism. However, it has not been possible to fully reconstitute cellulose biosynthesis *in vitro* or to completely identify the components of the multi-enzyme complexes in higher plants. This situation has contributed to the lack of probes to test exactly how the cellulose synthases interact (if at all) with the microtubules. One idea is that the microtubules merely define lanes within which the intramembranous synthesizing complexes glide, propelled by the act of extruding the cellulose fibre. However, the possibility cannot be formally excluded that the synthases are somehow linked through the membrane to either the contractile actin ("muscle") filaments or to microtubules, which—if they could use microtubule motor proteins like kinesin and dynein to slide against each other—could generate movement of the cellulose synthases in a more direct way.

MICROTUBULES REORIENT THEMSELVES

Microtubules can be transverse, oblique, or parallel to the organ axis, and it is known that they must reorient themselves between these different configurations. Reorientation can be affected by a range of environmental agents (like gravity) and by plant growth regulators. This is illustrated in Figure 2, which shows that factors work antagonistically to stimulate transverse or longitudinal microtubules and to shift the direction of cell expansion. For instance, the plant growth regulator ethylene is known to stunt growth and, when added to pea stem cells, to cause the microtubules to reorient through 90^0 so they become parallel to the cell's long axis (Roberts, Lloyd and Roberts, 1985). Dwarf peas with the *le* mutation have short internodes and an insufficiency of the hormone gibberellic acid. Experimental addition of exogenous gibberellic acid to segments of pea stems causes the cells to elongate measurably within two hours, during which the originally longitudinal microtubules reorient to the transverse (Duckett and Lloyd, 1994).

As templates for the co-alignment of cellulose microfibrils, longitudinal microtubules should lead to the deposition of new layers of wall in which the longitudinal cellulose microfibrils resist cell elongation. From examining the pattern of cellulose in successive layers of the cell wall, Shibaoka's group has suggested that cells with a cross-ply structure (alternating layers of longitudinal and transverse microfibrils) should display a corresponding rhythmic alternation of the microtubular template (Shibaoka, 1994; Mayumi and Shibaoka, 1996). This implicit reorientation was also long-suspected from immunofluorescence and electron-microscopic images of fixed cells, but the mechanism was unclear. In the 1990s, Peter Hepler's colleagues (Wasteneys, Gunning and Hepler, 1993; Hush et al., 1994) microinjected fluorescently tagged vertebrate brain tubulin into plants to study microtubule dynamics in living plant cells. In this way, Hepler and his colleagues showed that the fluorescent protein cycled through some of the other assemblies (mitotic spindle and cytokinetic

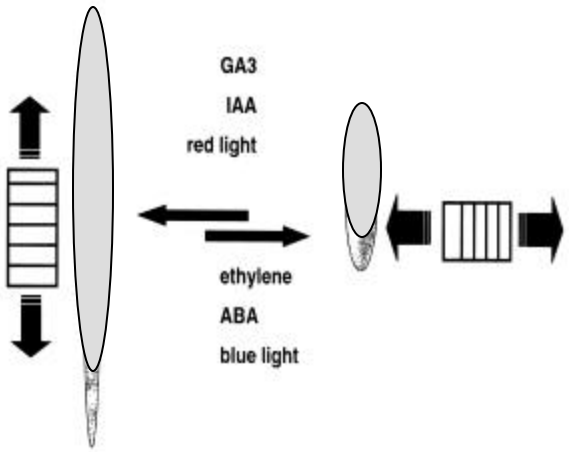

Figure 2. Antagonistic Factors Affect the Alignment of Microtubules and the Direction of Cell Expansion. *Red light and the plant hormones, gibberellic acid and auxin, encourage transverse microtubules, which are generally found in elongating tissue. Blue light, ethylene, and abscissic acid encourage longitudinal microtubules, which are often found in cells that are swelling laterally instead of elongating. There may be a rhythmic switching between these two configurations in some cells, building up a cross-ply pattern of wall lamellation.*

phragmoplast) that microtubules form in dividing plant cells.

Our group microinjected rhodamine-conjugated pig brain tubulin into pea epidermal cells to see whether this would label the cortical microtubules of nondividing cells (Yuan et al., 1994). Some of these cells are very large—several hundred micrometers long—but the cortical microtubule array was labelled as soon as the stem tissue could be transferred from the microscope used for injection to the confocal laser-scanning microscope. This suggests that the soluble tubulin is incorporated into the polymer and that the microtubules must be exchanging subunits with the cytoplasmic pool. One possibility, however, is that this may label only a dynamic subset, so that any stable microtubules would go unlabelled. To test this, we correlated the image obtained from microinjected cells with the image of the same area fixed and subsequently labelled with anti-tubulin antibodies that should recognise dynamic and stable microtubules alike (Wymer et al., 1997). The limited resolution of light microscopy is unable to distinguish between single microtubules and small groups of parallel microtubules. Nevertheless, the coincidence of the microinjected and the fixed patterns indicates that there is no hidden subset of stable microtubules in a different orientation, demonstrating that microinjection does produce a representative picture of the array.

Photobleaching small patches of fluorescence in microinjected cells has made it possible to show that the fluorescence recovers rather rapidly (Hush et al., 1994; Yuan et al., 1994). That the fluorescence is "killed" when the spot is bleached implies that new fluorescence must invade from beyond the bleach zone. There are two ways to account for this phenomenon. (1) Plant microtubules undergo dynamic instability (Mitchison and Kirschner, 1984). This concept, first introduced in animal cell biology, speaks in terms of the biased behavior of microtubule growth, mentioned earlier. Tubulin subunits increase at the plus end faster than at the minus end. (2) Individual microtubules show stochastic transitions between growing and shrinking phases—i.e., some microtubules grow while others shrink. However, depending on the conditions, microtubules tend not to shrink completely, but to undergo "rescue," whereby they stop and regrow. The rapid recovery of fluorescence within bleach zones of the plant cortical array can therefore be ascribed to dynamic instability—the addition of new fluorescent tubulin to the ends of microtubules within the area. Because we cannot be sure that a fluorescent element under the microscope is actually a single microtubule, there is the formal possibility, which we cannot yet rule out, that some contribution to the fluorescence recovery is made by groups of microtubules sliding against each other ("telescoping") in the zone. Nevertheless, the microtubules must be turning over rapidly, given the speed of the initial labelling. Certainly, the half-times for recovery from photobleaching put plant microtubules ahead of most figures for dynamic animal microtubules. This apparently paradoxical behavior of highly dynamic microtubules in stationary cells could account for the ability of sessile organisms to respond rapidly to changing environmental conditions.

MICROTUBULE REORIENTATION IN LIVING CELLS

By microinjecting rhodamine-tubulin into pea epidermal cells we have seen how such reorientations might occur. Yuan et al. (1994) reported that new microtubules could first be seen along the edge of a cell—at the junction between the outer circumferential face of the epidermal cell and the radial side wall. These microtubules could be newly nucleated at that locus, or they could have grown along the radial wall and then spilled over onto the outer surface. Either way, they do not share the alignment of the existing array. These "discordant" microtubules increase in number in the new direction, while the existing microtubules either depolymerize or re-grow in the new direction. Normally, microtubules are sufficiently well organized that populations can be classified as "transverse," "oblique," or "longitudinal." That is, there is an approximate, general uniformity. We now believe that the transitional stages of the reorganization represent the random-looking mixtures of new and previously unclassified, old microtubules (Lloyd, 1994). After the microtubules in the new direction have replaced those in the old, the array must undergo some kind of smoothing process to restore overall order. We hypothesize that proteins involved in the parallel packing of microtubules are likely to play an important role in this process. By taking serial optical sections through microinjected cells, then rotating the 3-D computer reconstructions, it is possible to see that microtubules on the outer face of pea epidermal cells can sometimes be in a different orientation to the microtubules along the radial side walls (Yuan et al., 1995). This tends to suggest that reorientation occurs cell face by cell face (although there is no rule

that microtubules need always share the same orientation on different facets of the same cell). However, oblique arrays have been seen in microinjected cells where microtubules wind in 45º helices around all surfaces of the cell. Since these arrays tend to occur in the older parts of the internode, it is possible that they represent the final stage of the reorganization process, whereby the parallelization process imposes an order that spreads to all cell surfaces. They could represent the "best-fit" configuration for cells no longer subjected to the strains of cell elongation.

This proposed method of reorientation—in which one set appears to be replaced by another in a different orientation—may not be the only method. It is important to keep open the possibility that other mechanisms may occur in cells not traumatized by excision from the stem, artificial increase in the size of the tubulin pool through microinjection, physical damage from the microneedle, horizontal placement, etc. Yuan et al. (1994) and Wymer and Lloyd (1996) have seen another kind of reorientation. This occurs in long epidermal cells where microtubules at one end of the outer circumferential cell face are oriented differently from those at the other end. In such cases, we have sometimes seen one set gradually adopt the alignment of the other. At the moment, we do not know whether this is an alternative mechanism of reorientation or part of the "smoothing" process that restores order after reorientation.

MICROTUBULES AND GRAVITY

Because the reorientation of microtubules in microinjected cells occurs spontaneously and sporadically, it has been important to find factors that trigger this process. Plant growth regulators are known to do this (the effect of one of them is discussed below), but more recently we have obtained data on the effect of gravity, based on photo- and gravitropic studies pursued in Peter Nick's laboratory. Himmelspach et al. (1999) germinated maize seed under continuous red light, and the coleoptiles were excised at three days. Fixation and immunofluorescence studies have shown that cortical microtubules are generally transverse to the organ axis in vertically grown coleoptiles; but that, when they are gravistimulated by horizontal placement of the coleoptile, they reorient themselves from transverse to longitudinal on the upper side and adopt a transverse alignment on the lower side (Nick et al., 1990). This is consistent with the increased elongation of the cells on the lower side, such that the growing coleoptile then bends upwards.

Figure 3 shows how this bending is thought to occur. When maize coleoptiles grown in red light are placed horizontally, the coleoptile bends upward in response to gravity. The microtubules in the epidermis on the upper side reorient themselves to the long cell axis (resulting in inhibited cell elongation), and the transverse microtubules on the lower side encourage the elongation that results in the bending. For the purposes of microinjection, so that this reorientation could be observed in living cells, Himmelspach et al. (1999) glued coleoptiles horizontally to microscope slides using medical adhesive. Conventional fixation studies showed that more than 75% of the microtubules in epidermal cells on the upper flank reoriented to steeply oblique/longitudinal within one hour of gravistimulation—hence the graviresponse was not inhibited by the attachment process. However, it was formally possible that attachment could set up stresses that could themselves trigger the microtubule reorientation. To control for this, coleoptiles were attached to slides, exactly as they would have been for microinjection, then placed vertically, i.e., without gravitropic stimulation. No effect could be detected on microtubule reorientation, indicating that gravity was the trigger. Using this set-up, it was possible to microinject gravistimulated epidermal cells on the upper surface of the coleoptile and observe them undergo transverse-to-longitudinal reorientation in response to 1-*g*. The microtubule response could be subdivided into three categories:

- About 30 minutes after the onset of stimulation, longitudinal microtubules appeared among the previously transverse microtubules, giving the array a patchwork appearance.

- The microtubules sorted out a new oblique orientation in which the mixed order became replaced by a new parallel organization in the new direction.

- The angle of microtubule alignment often increased smoothly over time to give a steeper, near-longitudinal pattern. This sequence was similar to that seen in the earlier microinjection studies on pea epidermis (Yuan et al., 1994) and in the gibberellic acid-induced reorientation described below (except that this was in the opposite longitudinal-transverse direction) (Lloyd, et al., 1996).

As discussed, the plant growth regulator gibberellic acid can stimulate stem elongation and convert longitudinal microtubules to the transverse axis. Using pea epidermal cells microinjected with fluorescent tubulin, Lloyd et al. (1996) first identified living cells with longitudinal microtubules and then added exogenous gibberellic acid to the cells under the microscope. A reverse transition to the transverse orientation resulted. As before, transverse "discordant" microtubules appeared, and the existing longitudinal microtubules seemed to depolymerize as these microtubules grew. That is, microtubules appear in the new direction, and there is a mixed alignment for a while before the new, parallel array sorts out the new direction. In all cases, it seems paradoxical that one set of microtubules grows as the old set shrinks. Therefore, to understand the mechanism involved, it will probably be necessary to understand the molecular basis of microtubule stabilization. The literature indicates that gibberellic acid physically stabilizes microtubules. For instance, Mita and Shibaoka (1984) showed that pretreatment of onion leaf sheath cells with gibberellic acid stabilized the cortical microtubules against the depolymerizing effects of cold or of an anti-microtubule herbicide. In dwarf pea, the gibberellic acid-induced, longitudinal-to-transverse reorientation is accompanied by modification of the tubulin polypeptides (Duckett and Lloyd, 1994). Immunoblotting 2-D gels with

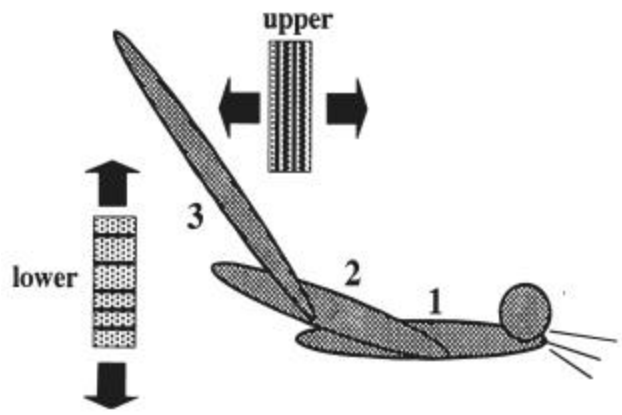

Figure 3. Different Microtubule Orientations on Opposite Flanks Are Found in Maize Coleoptiles Bending in Response to Gravity. *Maize coleoptiles placed horizontally bend upwards. The epidermal cells on the lower side of the coleoptile elongate more than those on the upper side. When the coleoptile is placed horizontally, transverse microtubules in the vertically grown tissue reorient themselves through 90^0 on the upper flank, consistent with the decreased rate of cell elongation.*

antibodies specific for particular tubulin epitopes has shown that gibberellic acid causes the probable detyrosination of an alpha tubulin. Throughout eukaryotic cell biology, this posttranslational modification usually accompanies conversion of microtubules to the stable state. The idea is that stable tubules are a good substrate for a carboxypeptidase that removes the terminal tyrosine residue more efficiently than it does from dynamic microtubules. More recently, it has been shown that pretreatment of a maize cell suspension with gibberellic acid stabilizes the protoplast microtubules against being chilled (Huang and Lloyd, 1999). This effect was accompanied by acetylation of a tubulin isotype—another posttranslational modification associated with microtubule stabilization. Neither modification is thought to directly affect the microtubule; instead such modifications are thought to indirectly indicate changes in dynamicity imposed by other factors, such as microtubule-associated proteins.

MICROTUBULE-ASSOCIATED PROTEINS (MAPs)

MAPs are generally defined as the proteins that co-polymerize with microtubules as they go through rounds of temperature-dependent cycles of assembly/dis-assembly. This definition originally came from the animal field, where microtubules were first biochemically isolated. Brains contain a high proportion of tubulin, and it was found that—when calcium was chelated and temperature increased—the tubulin in brain homogenates self-assembled into microtubules that could be pelleted at low speed. After several rounds of assembly/dissembly, MAPs (these include the low- and high-molecular-weight MAPs, tau and MAP2, respectively) could also be seen to be purified. Since then, other MAPs have been identified. For instance, the microtubule motor protein kinesin is now a key player in microtubule biology. However, it is noteworthy that this protein was not isolated by the classical methods. Accordingly, definitions should be sufficiently flexible to include, for example, enzymes that could attach to microtubules and modify their functionality.

Plant MAPs may be sufficiently different from the well-known animal MAPs to make searching for sequence homologies in the database unproductive. Our own approach has been to biochemically identify proteins that associate with the cytoskeleton. By extracting protoplasts with detergent, we have produced resistant fibrous cytoskeletons that contain microtubules, actin bundles, and the nucleus. We can then depolymerize the cytoskeletal proteins in cold, calcium-containing buffer and isolate any potential plant MAPs by pelleting them on taxol-stabilized brain microtubules (Chan et al., 1996). In this way, we have identified two groups of carrot proteins homologous to those isolated by Jiang and Sonobe (1993) from tobacco cells. The so-called 65kDa proteins are a family of three or more polypeptides from 62-68kDa that appear to be antigenically related. In immunofluorescence studies, anti-MAP65 antibodies stain the four microtubule arrays around the cell cycle. Recently, we have separated the 65kDa MAPs from the 120kDa MAP in our carrot MAP fraction and, in add-back experiments, shown that MAP65 causes brain microtubules to form into tight, parallel bundles (Chan et al., 1999). In the electron microscope, one can see the microtubules cross-bridged by 25-30nm filaments, which are regularly spaced along the length of the microtubule. In EM studies of plant cells, this is the spacing between microtubules, and it would appear that MAP65 is responsible for the parallel arrangement *in vivo*. A combination of 1) attachment to the plasma membrane and 2) maintenance of a regular intermicrotubule distance by MAP65 goes a long way to explaining the typical organization of the plant cell's cortical array. Microtubules from the microinjection studies appear in a mixed organization before they realign and become smoothed in the new direction, and it is probable that MAP65 plays a part in this parallelization process.

We have seen three, sometimes four polypeptides in immunoblots of the 65kDa family, and further studies are required to determine the individual role of the members. We were previously unable to demonstrate a bundling effect with the 60kDa MAP, and it is therefore unclear at present whether

- only one or some of the proteins can cross-link microtubules,
- their activity is modified by posttranslational modification,
- they form complexes,
- their patterns of expression and activity change according to the stage of the cell cycle.

For instance, with the onset of cell division, cortical microtubules disappear from the ends of the cell and bunch up into the preprophase band (which foretells the plane of cell division), and it seems likely that some modification of the cross-linking activity of

MAP65 must occur in the process. Concerning the interphase array itself, the ability of MAP65 to form stable microtubule bundles *in vitro* is ostensibly inconsistent with the observed dynamicity of the cortical microtubules *in vivo*. For this reason, it is possible that some process, such as posttranslational modification, regulates the activity of the 65kDa MAPs, switching them on and off. Such signalling processes may be part not only of cell cycle progression but also of the response to plant growth regulators, such as gibberellic acid, which are known to modulate the behavior of plant microtubules.

Models of the influence of microtubules on cellulose biosynthesis must be updated to account for the presence of filamentous MAPs on the cortical array (see Giddings and Staehelin,1991). We know too little to decide definitively between direct models (in which microtubules and cellulose synthases are directly linked) and indirect models (in which microtubules form the channels above which the synthases freely move). However, in both models the cross-bridging of adjacent microtubules would have an effect on the hypothetical mechanisms. According to the indirect models, synthases move in the membranous lanes between microtubules. In principle, there could be two sorts of "lanes," depending on the precise organization of the microtubules. According to the first model, the synthases could move in lanes between adjacent bundles of microtubules. In this case, the interbundle channels would almost certainly be variable if the bundles are not bridged to each other. In such a loose model, there would be an approximate relationship, instead of a 1:1 relationship, between tubule and microfibril. However, it is possible to envisage a tight version of the indirect model, in which synthases move in the narrower lanes directly above pairs of microtubules bridged by MAP65. The ca. 25nm cellulose-synthesizing rosettes would be accommodated by the 25-30nm intermicrotubule space maintained by MAP65 cross-bridges. In this tight, indirect model, paired microtubules form a defined "railroad track," for which MAP65 molecules form the ties (or sleepers). This version predicts that exact 1:1 matches will be found between pairs of microtubules and individual microfibril *as they are being deposited*. However, this will be sensitive to the visualization technique. It is unlikely that such a relationship would be revealed by simply comparing an already-deposited lamella's pattern of fibrils with a fixed image of the microtubule array. The latter, we now know, is so dynamic that, by the time an entire lamella is deposited, fine details of patterning will have been lost by turnover.

Although it is still too early to decide between direct and indirect models for the influence of microtubules on cellulose alignment, it seems increasingly likely that MAPs play an important part in the determination of wall texture.

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