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Higher Plant Cortical Microtubule Array Analyzed In Vitro in the Presence of the Cell Wall

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Plant morphogenesis depends on an array of microtubules in the cell cortex, the cortical array. Although the cortical array is known to be essential for morphogenesis, it is not known how the array becomes organized or how it functions mechanistically. Here, we report the development of an in vitro model that provides good access to the cortical array while preserving the array's organization and, importantly, its association with the cell wall. Primary roots of maize (*Zea mays*) are sectioned, without fixation, in a drop of buffer and then incubated as desired before eventual fixation. Sectioning removes cytoplasm except for a residuum comprising cortical microtubules, vesicles, and fragments of plasma membrane underlying the microtubules. The majority of the cortical microtubules remain in the cut-open cells for more than 1 h, fully accessible to the incubation solution. The growth zone or more mature tissue can be sectioned, providing access to cortical arrays that are oriented either transversely or obliquely to the long axis of the root. Using this assay, we report, first, that cortical microtubule stability is regulated by protein phosphorylation; second, that cortical microtubule stability is a function of orientation, with divergent microtubules within the array depolymerizing within minutes of sectioning; and third, that the polarity of microtubules in the cortical array is not uniform. These results suggest that the organization of the cortical array involves random nucleation followed by selective stabilization of microtubules formed at the appropriate orientation, and that the signal specifying alignment must treat orientations of ± 180° as equivalent. Cell Motil. Cytoskeleton 57:26–36, 2004. © 2003 Wiley-Liss, Inc.

Key words: cortical microtubules; in vitro assay; plant roots; phosphorylation; polarity; *Zea mays*

INTRODUCTION

Plant morphogenesis depends in part on a special array of microtubules within the cell cortex, the "cortical array." This array lies adjacent to the plasma membrane, and contains many short, partially overlapping microtubules, present in a highly organized pattern [Gunning and Hardham, 1982]. The pattern of the array changes predictably during development and cell differentiation [Shibaoka, 1994]. For example, in the rapidly elongating cells of the maize root, the microtubules are transverse to the long axis of the root, and as the cells stop elongating, the microtubules take on a helical organization [Baskin et al., 1999].

The array functions to control plant cell and organ form, seen by the malformed plants that invariably result when cortical microtubules are disrupted [Mathur and Hülskamp, 2002; Smith, 2003]. The importance of cortical microtubules for morphogenesis was discovered 40 years ago at the same time as microtubules themselves were first seen in the electron microscope [Green, 1962;

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Ledbetter and Porter, 1963]; nevertheless, we remain surprisingly ignorant of how the cortical array influences anisotropic expansion. The leading theory is that the microtubules influence the deposition of cellulose microfibrils but this is not accepted universally and even with acceptance, it is not known mechanistically how microtubules influence cellulose [Baskin, 2001].

Likewise, we know little about the mechanism by which the cortical microtubules become oriented [Williamson, 1991; Hush and Overall, 1996; Baskin, 2001; Hasezawa and Kumagai, 2002; Schmit, 2002; Wasteneys, 2002]. The plant cortical array is unusual because it lacks a conspicuous microtubule organizing center, such as a centrosome or basal body. Other eukaryotes typically use conspicuous organizing centers to control the spatial disposition of microtubule nucleation (because the ambient tubulin concentration is too low for spontaneous polymerization). The plant cortical array may possess analogous organizing centers but, if so, they are present in a cryptic form. Alternatively, models have been suggested in which nucleation and orientation are separated. One model is "selective stabilization," which posits that microtubules are nucleated in the cell cortex without any control over orientation: microtubules happening to run in the correct orientation are stabilized, but others running in unwanted orientations remain labile and are lost [Wasteneys and Williamson, 1989a,b]. Another model invokes reorientation, and states that the randomly nucleated microtubules are translocated into the correct orientation by means of motor proteins [Palevitz, 1991]. Although each model explains some observations, decisive experimental tests are lacking.

A principal problem for elucidating the behavior of the cortical array has been developing an in vitro model. Approaches that rely on permeabilization, common for animal cells, are of little use for the cortical array because the cell wall hinders the exchange of components. There are plant cell types that do not make a cell wall, such as the coenobial endosperm of developing seeds; however, along with their lack of a cell wall, such cell types also lack cortical microtubules.

An in vitro model used previously to characterize cortical microtubules is the protoplast ghost. In this approach, protoplasts are adhered to a glass slide and lysed, so that patches of membrane, i.e., ghosts, remain on the slide, with their cytosolic surface retaining some microtubules and accessible to experimentation. This preparation was developed more than 20 years ago [Marchant, 1978; van der Valk et al., 1980] and has been used informatively to study microtubule behavior [Sonobe, 1997]. However, the preparation is limited for two reasons. First, cortical microtubules in protoplasts are relatively random and so the ghost does not provide access to a cortical array in a native state of organization. Second, it has become increasingly clear that cortical microtubules are bound to the plasma membrane by trans-membrane proteins that bind microtubules at their cytosolic domains and bind the cell wall at their external domains [Shibaoka, 1994; Kohorn, 2000], but in the ghost preparation, the cell wall is absent, and external protein domains are subject to degradation by proteases that contaminate protoplasting enzymes.

Both problems with ghosts are overcome in the in vitro model reported here. The model uses the root tip of *Zea mays* (maize). A root is immersed in a droplet of buffer and sectioned, *without fixation*, on a Vibratome. In the sections, the cytoplasm of the cut-open cells is lost except for a parietal layer that includes cortical microtubules. Cutting open the cell provides access to organized cortical arrays, in which the microtubules retain their native association with not only the plasma membrane but also the cell wall. Furthermore, arrays with distinct orientations are present in the same section: in the meristem and most of the elongation zone, cortical microtubules are transverse to the long axis of the root, whereas at the base of the elongation zone microtubule orientation becomes oblique [Baskin et al., 1999]. Using this model, we show that microtubule stability is highly sensitive to ATP, that microtubule stability is a function of the angle of orientation, and finally that the polarity of microtubules in the cortical array is non-uniform.

MATERIALS AND METHODS

Preparation and Handling of Un-Fixed Sections

Seeds of maize (*Zea mays* L., cv. FR27 X FRMo17) were washed with a 5% (v/v) solution of household bleach for 15 min and then germinated in a growth chamber at 30°C for 2 days in wet germination paper, held vertical. Apical or sub-apical segments (10 to 15 mm) from straight primary roots, approximately 2 to 3 cm long, were cut and affixed to the stub of a Vibratome-1000 (Technical Products International, St. Louis, MO) by means of gel-type Superglue. Longitudinal sections were made at a nominal 100μm thickness in a drop of a buffer containing 50 mM Pipes pH 7.0, 4 mM $MgSO_4$, and 20 mM EGTA (PME) containing 0.02% Brij 58. The concentrations of PME were optimized in preliminary experiments, and the Brij 58 was found to reduce non-specific background for fluorescence observation. Sections were then incubated in a 1-mL volume of test solutions. In some cases, smaller volumes (0.3 to 0.5 mL) were used to conserve reagents.

Field-Emission Scanning Electron Microscopy (FESEM)

Sections were fixed in a solution containing 2% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in PME

buffer (pH 7.0) for 1.5 h at room temperature. After rinsing in PME, sections were placed on Formvar-coated copper wire loops and encased in a second Formvar film, as described for arabidopsis roots by Baskin and Wilson [1997]. Encasing the samples between Formvar films rigidifies the samples and prevents cytoplasm from being lost from the cut-open cells during processing. Sections (on loops) were postfixed in 0.5% (w/v) aqueous OsO_4 for 30 min, rinsed thrice in distilled water for 10 min each, dehydrated in ethanol, and critically point dried. While viewing through a stereomicroscope, sections were carefully removed from between the Formvar films and mounted on stubs with double-sided sticky carbon tape. The samples were sputter coated with platinum (to a nominal thickness of 2 nm) and were observed on a FESEM (Hitachi 4700S) at 5 kV with a working distance of 6 to 8 mm.

Hook Decoration Assay and Transmission Electron Microscopy

To determine the polarity of cortical microtubules, we used hook decoration [McIntosh and Euteneuer, 1984; Heidemann, 1991]. Vibratome sections were incubated in PME buffer containing 0.02% Brij 58 for 10 min. Sections were then incubated in hook buffer (500 mM Pipes, 1 mM EGTA, 1 mM GTP, 1 mM $MgCl_2$, 2.5% DMSO, 0.05% DTT, pH 6.90, and 0 to 2 mg ml^{-1} pig brain tubulin at 37°C for 45 min. The tubulin was purified by two cycles of assembly-disassembly followed by phosphocellulose chromatography by standard methods [Williams and Lee, 1982]. Prior to use in the assay, pooled column fractions were spun at 4°C at high speed (100,000*g*) to pellet oligomers. After incubation in hook buffer, the sections were immediately fixed at room temperature for 60 min in 50 mM Pipes (pH 6.90) containing 6% glutaraldehyde and 0.1% (v/w) tannic acid (Mallinckrodt, Paris, KY). Sections were encased within Formvar on wire loops (as described above) and were rinsed twice in 50 mM Pipes for 10 min each, and once in 100 mM cacodylate, pH 7.2, for 15 min, postfixed in 1% (w/v) aqueous OsO_4 for 2 h at room temperature, dehydrated in a graded ethanol series, and embedded in Spurr's resin. Blocks were sectioned (approximately 70 nm) parallel to the long axis of the root to transect cortical microtubules. To control for section orientation, blocks were marked by cutting one end obliquely. To increase the sampled area, approximately 100 μm was trimmed from the block face after each collecting each grid. Sections were stained with uranyl acetate for 30 min, lead citrate for 10 min, and examined using a JEM-1200EX transmission electron microscope at 60 to 80 kV. Hooks were scored on 10 grids, several utra-thin sections per grid, from three embedded Vibratome sections.

Immunofluorescence Staining

Sections were fixed in 4% (v/v) formaldehyde in PME buffer for 90 min. Following this, the sections were handled by a protocol modified from Liang et al. [1996]. Sections were transferred to silanized slides, dried at 40°C for 15 min, and then peeled away by means of a fine forceps, leaving the cortical cytoplasm of the cut-open cells, including microtubules, affixed to the slide. We will refer to this as a "microtubule print" because, in a sense, the cortical microtubules are printed onto the slide.

The material on the slide was incubated in primary antibody (mouse monoclonal antibody against sea urchin-sperm axoneme alpha-tubulin, diluted 1:1,000; Sigma, St Louis, MO) at 37°C for 1 to 2 h. Subsequently, the slides were rinsed in phosphate-buffered saline (PBS) three times for 10 min each and then incubated in secondary antibody (Cy3-conjugated goat anti-mouse IgG, diluted 1:400, Jackson Immunoresearch Laboratories, West Grove, PA) at 37°C for 1 h. The slides were then rinsed three times for 10 min each in PBS, and mounted in antifade reagent (Vectashield; Vector Laboratories, Burlingame, CA) and sealed with nail polish.

Photography and Image Analysis

Samples were examined on a fluorescence microscope (Axioplan; Zeiss, Thornwood, NY). Fluorescence from Cy3 was observed with standard filter sets for rhodamine. Images were captured using a charge-coupled device video camera (VI-470; Optronics Engineering, Goleta, CA) and transferred to a Macintosh computer. To quantify the abundance of microtubules, we used NIH Image, version 1.62C (U.S. National Institutes of Health; available on the internet at http://rsb.info.nih.gov/nih-image). Image contrast was inverted and a rectangular region of interest was selected spanning an appreciable portion of an array. The selected region was thresholded to separate fluorescent from background pixels, and the resulting binary image was then skeletonized. The percentage of black (i.e., fluorescent) pixels within the region was taken as the measure of microtubule abundance. Compared to the original binary image, measuring the skeleton reduced the dependence of the result on the choice of threshold. For the reported treatments, microtubule abundance data represent the average of 4 roots ± SE, with each root measured at 4 different locations. Microtubule orientation was measured by hand with NIH image. The frequency plots shown represent a total 500 microtubules selected arbitrarily from 8 to 10 cells.

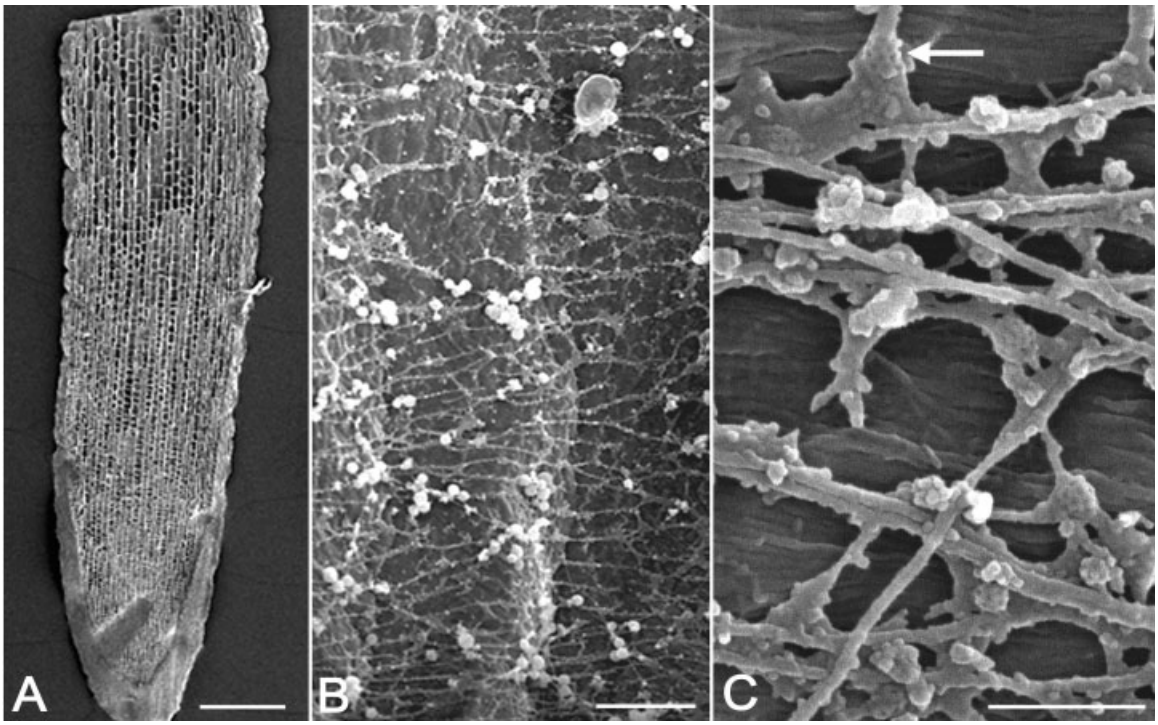

Fig. 1. FESEM micrographs of a Vibratome section of a maize root. The root was sectioned without fixation and was immediately fixed and processed. **A:** Survey view showing many cut-open cells, apparently empty. **B:** View of the lumen of a cut-open cell, showing a filamentous network and vesicular material adhering to the cell wall. **C:** Enlarged view of the cell cortex, showing microtubules, single or bundled, surmounting residual patches of membrane. *Arrow* points to a polygonal structure indicative of a clathrin coated pit. In gaps between microtubules, the microfibrillar texture of the cell wall is visible. Bars = 500 μm in A, 5 μm in B, and 0.3 μm in C.

RESULTS

A Novel In Vitro System for Studying Cortical Microtubules

Our in vitro model uses Vibratome sections of living maize roots. To examine the sections, we used FESEM (Fig. 1). At low magnification, many cells had been cut open and appeared empty. At higher magnification, some material remained within the cell cortex, including vesicles and a skein of filaments with a net transverse orientation, as expected for microtubules. At still higher magnification, the filaments had a diameter of approximately 25 nm and are presumably microtubules. We have confirmed their identity by staining with an anti-α-tubulin antibody followed by a gold-conjugated secondary (data not shown). The microtubules surmounted detergent-resistant remnants of plasma membrane, identified by the polygonal formations indicative of clathrin-coated pits (Fig. 1C, arrow). In between the microtubules, plasma membrane was lost, revealing the microfibrillar texture of the cell wall. Because cortical microtubules were not ejected at sectioning, it appears that they are bound to the cell wall tightly, presumably by trans-membrane proteins.

While the FESEM preparations provide high-resolution images, they are time consuming and difficult to use for examining many cells. As an alternative, we used immunofluorescence, taking advantage of a "printing" method, which transfers the cortical microtubules and remaining cytoplasm from the cut open sections onto a slide, thereby removing interference from the overhanging cell walls and placing all of the microtubules in a single focal plane [Liang et al., 1996]. Un-fixed sections were incubated in PME for various times and then fixed, printed, and the microtubules stained (Fig. 2A–D). Even with 30-min incubation, many cortical microtubules remained. The presence of a significant fraction of stabilized microtubules in the cut-open cells establishes the sectioned maize root as suitable for probing the behavior of cortical microtubules.

We quantified the amount of microtubules present in the prints by means of an image processing routine, modified from that of Fisher et al. [1996] and described in Materials and Methods. Strictly speaking, this method measures only a relative amount of microtubule polymer; nevertheless, it permits different treatments to be compared objectively, and increases the usefulness of the in vitro model. About 20% of the microtubules depolymerized in the first 5 to 10 min of incubation and after that microtubules were highly stable (Fig. 2E). As shown, transverse microtubules (which are present up to approx-

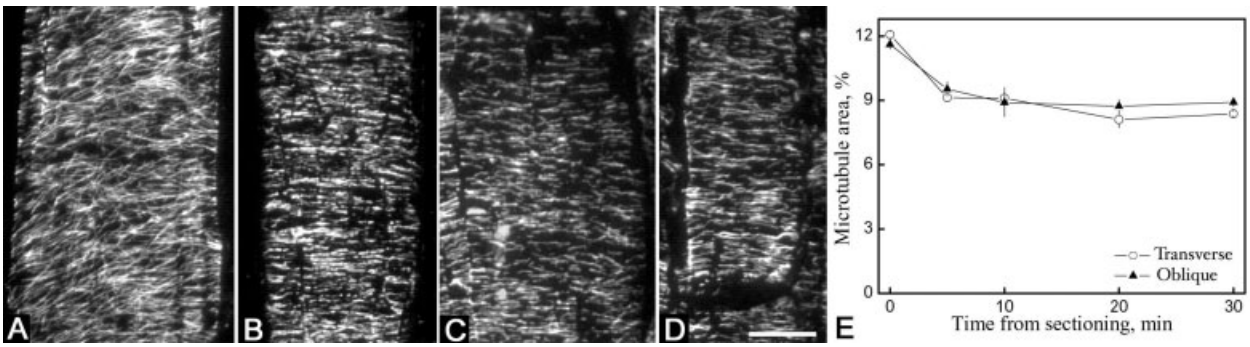

Fig. 2. Time course of microtubule abundance in un-fixed sections. A–D: Fluorescence micrographs of microtubule prints obtained from sections fixed (**A**) immediately, (**B**) 10 min, (**C**) 20 min, or (**D**) 30 min after sectioning. Procedure for obtaining "microtubule prints" is described in Materials and Methods. Bar = 10 μm. **E:** Quantification of microtubule abundance. Transverse microtubules (as shown in A–D) were imaged in the first approximately 7 mm of the root tip and oblique microtubules (not shown) were imaged between 15 and 20 mm from the tip. Symbols plot mean ± SEM (when larger than the symbol) of measurements made on four replicate sections from different roots.

imately 8 mm from the tip) [Baskin et al., 1999] as well as oblique microtubules (present beyond 8 mm from the tip) behaved similarly. There was little additional depolymerization for up to 2 h (data not shown). If the buffers for sectioning and incubation contained 10 μM taxol, then the amount of microtubules present after 30 min was indistinguishable from the amount present at time zero, suggesting that the loss of microtubules in the first 10 min reflects disassembly rather than proteolysis or mechanical dislocation (Table IA).

Microtubule Stability Is a Function of Orientation

To test whether the reduction in microtubule abundance represents a stochastic loss or instead the loss of a specific subset, we looked at the relationship between stability and orientation. We measured the orientation of 500 microtubules in material fixed immediately after sectioning and 10 min later. The frequency distribution after 10 min was significantly narrower than initially, for cortical arrays of both transverse and oblique orientation, and nearly all microtubules in divergent classes were gone (Fig. 3). Oblique arrays were sampled at approximately 15 mm from the tip, because the net angle of the oblique array becomes constant by that position [Baskin et al., 1999]. Because the abundance of microtubules in the cortical array is lower at 10 min compared to time zero (Fig. 2), the frequency distributions were normalized by expressing microtubule number as a percentage of the number in the maximal class. For both transverse and oblique arrays, the area under the curve for 10 min in PME was about 20% less than for time zero, suggesting that few microtubules of the maximal class were lost during the incubation. The sharpened frequency distributions show that microtubules depolymerize more readily when they diverge from the mean orientation. As there was no energy source provided in the buffer, microtubule rotation can be excluded. These data directly support the "selective stabilization" model, in which microtubule stability within an array is a function of angle.

TABLE I. Effects of Various Additions to the Incubation Buffer on the Stability of the Cortical Array

		Microtubule area†	
	Treatment*	Mean ± SEM	% of initial
A	Initial (0′)	11.6 ± 0.14	100
	PME, 20′	8.4 ± 0.12	73
	10 μM taxol, 20′	11.8 ± 0.14	102
	taxol, 10′ → 10 mM ATP, 10′	11.7 ± 0.25	101
B	Initial (0′)	11.3 ± 0.21	100
	PME, 30′	8.4 ± 0.08	74
	1 μM oryzalin, 30′	8.7 ± 0.19	77
	0.1 mM $AlCl_3$, 30′	8.1 ± 0.06	72
	6°C, 30′	7.6 ± 0.21	67
	0°C, 30′	4.5 ± 0.37	40
C	PME, 40′	7.7 ± 0.38	
	1 mM DTT, 40′	7.8 ± 0.9	
	PME, 30′ → ATP, 10′	6.6 ± 0.22	
	DTT, 30′ → DTT + ATP, 10′	0.7 ± 0.07	
D	Initial (0′)	12.1 ± 0.33	100
	PME, 10′	8.9 ± 0.18	73
	1 mM GTP, 10′	6.8 ± 0.2	56
	3 mM GTP, 10′	5.6 ± 0.21	46
	10 mM GTP, 10′	0.8 ± 0.12	7
	1 mM AMPPNP, 10′	8.7 ± 0.07	72

*All treatments contained PME plus the indicated compound.
†Microtubule area was quantified as described in Materials and Methods.

Microtubule Stability Is Regulated by Reversible Protein Phosphorylation

The marked stability of a majority of the exposed microtubules is surprising, insofar as the cortical array in living cells is thought to be dynamic, turning over within

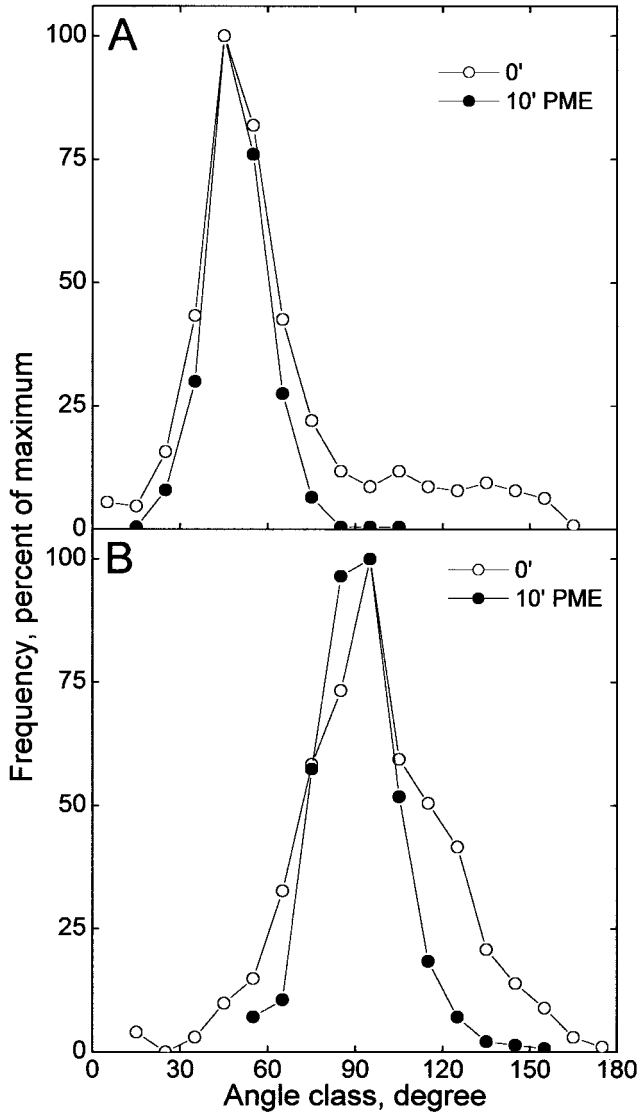

Fig. 3. Frequency plots of microtubule orientation. **A:** Oblique microtubules from mature, non-growing cells, 15 to 20 mm from the tip. **B:** Transverse microtubules from rapidly elongating cells, 1 to 7 mm from the tip. Angles was measured for 500 microtubules from material that had been fixed either immediately after sectioning (0′) or 10 min afterward (10′ PME). Distributions were normalized by expressing the amount in each angle class (10 degree bins) as a percentage of the maximal class. For both A and B, equivalence of the two distributions is rejected at $P < 0.001$ by the Kolmogorov-Smirnov two sample test.

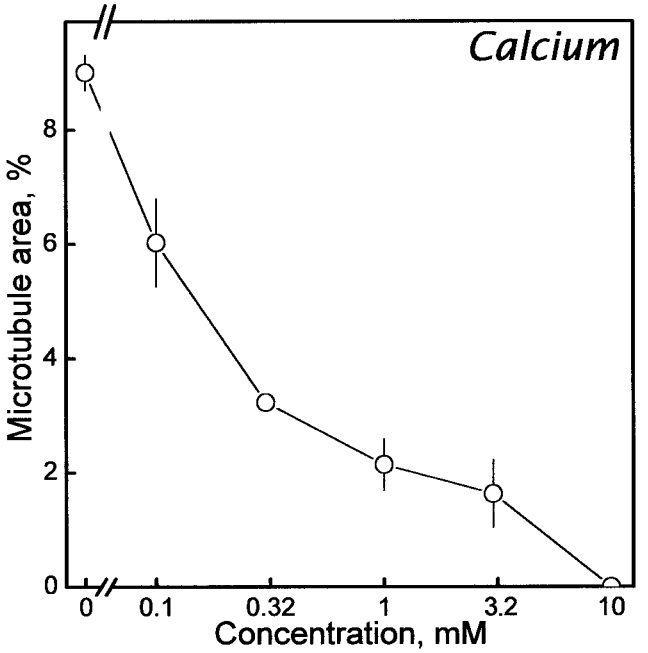

Fig. 4. Microtubule area remaining in the sections as a function of the concentration of calcium added to the incubation buffer (replacing EGTA). Sections were incubated for 30 min, and then microtubule prints were made and quantified. Symbols plot mean ± SEM (when larger than the symbol) of measurements made on four replicate sections from different roots.

minutes [Hush et al., 1994; Yuan et al., 1994; Shaw et al., 2003]. As the sections are incubated without exogenous tubulin, it is doubtful that assembly could balance disassembly. When sections were exposed to oryzalin, a compound that blocks microtubule assembly [Hugdahl and Morejohn, 1993], microtubule abundance was not affected (Table IB), confirming the absence of microtubule assembly in the system. When sections were exposed to even high concentrations of aluminum, microtubule stability was not affected (Table IB), suggesting that plant microtubules depolymerize in the presence of this metal by means of indirect interactions [Sivaguru et al., 2003]. When microtubules were exposed to cold, some additional depolymerization was seen, particularly at 0°C (Table IB), suggesting that the stabilization process is efficient but not invincible. In contrast, cortical microtubules in the maize root depolymerize totally when the intact, living root is exposed to 0°C [Baskin et al., 1999].

As a further way to probe microtubule stability, we tested their sensitivity to calcium by replacing the EGTA in the PME buffer with a defined calcium concentration. The amount of polymer remaining after a 30-min incubation was roughly proportional to the logarithm of the calcium concentration (Fig. 4). Active concentrations ranged from 0.1 to 10 mM, which indicates that the observed depolymerization is unlikely to be relevant physiologically, insofar as cytosolic calcium rarely if ever reaches 0.1 mM. Similarly, microtubules in protoplast ghosts are quite insensitive to calcium [Kakimoto and Shibaoka, 1986; Fisher et al., 1996]. Additionally, we tested the sensitivity of the microtubules to ATP, previously reported to depolymerize microtubules in ghosts [Sonobe, 1990; Katsuta and Shibaoka, 1992]. For these experiments, the sections were incubated first in PME for 10 min to allow the divergent microtubules to

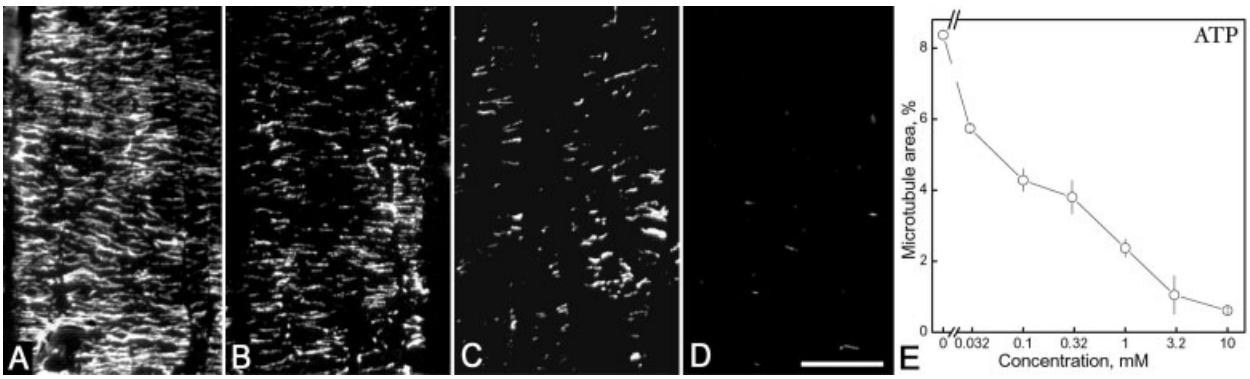

Fig. 5. Microtubule abundance in un-fixed sections as a function of ATP concentration. A–D: Fluorescence micrographs of microtubule prints obtained from sections fixed after a 10-min incubation in PME followed by a second 10-min incubation in PME (**A**) alone, (**B**) + 1 mM ATP (**C**) + 3 mM ATP, or (**D**) + 10 mM ATP. Procedure for obtaining "microtubule prints" is described in Materials and Methods. Bar = 10 μm. **E:** Quantification of microtubule abundance. Symbols plot mean ± SEM (when larger than the symbol) of measurements made on four replicate sections from different roots.

depolymerize, and then exposed to ATP for a further 10 min. ATP depolymerized microtubules readily (Fig. 5A–D), with the amount of remaining polymer being approximately proportional to the logarithm of the ATP concentration (Fig. 5E). Depolymerization activity was lost after a 30-min pre-incubation in PME but was retained when 1 mM DTT was included with the PME (Table IC). Like ATP, GTP also caused depolymerization, although higher concentrations were required (Table ID). Conversely, the non-hydrolysable analog AMP-PMP was without effect (Table ID). That 30 μM ATP depolymerized microtubules appreciably and 1 mM substantially suggests that in living cells, ATP plays an important role regulating microtubule dynamics.

ATP-induced depolymerization was blocked completely when sections were incubated in ATP and taxol (Table IA). This indicates that the disappearance of microtubules is unlikely to be caused by the activity of the microtubule severing protein, katanin, which is known to participate in the organization of the cortical array [Bichet et al., 2001; Burk et al., 2001], but whose severing activity is unaffected by taxol [Burk and Ye, 2002].

As microtubule stability in animal cells can be regulated by the phosphorylation of microtubule-associated proteins, we determined whether the same was true for the plant cortical array. When sections were incubated in a broad-spectrum inhibitor of protein kinases, staurosporine, and then exposed to ATP, still in the presence of the inhibitor, ATP could no longer depolymerize microtubules (Fig. 6). Conversely, an inhibitor of protein phosphatases, cantharidin, promoted microtubule depolymerization (in the absence of ATP). Both staurosporine and cantharidin, at the concentrations used, have been shown to disorganize the cortical array when applied to living arabidopsis roots [Baskin and Wilson, 1997]. In contrast, a different kinase inhibitor, chelerythrine, did not greatly affect microtubule organization in the living root (although it did inhibit root elongation) and, consistently, was far less effective than staurosporine in preventing ATP from depolymerizing microtubules (data not shown). These data suggest that stability

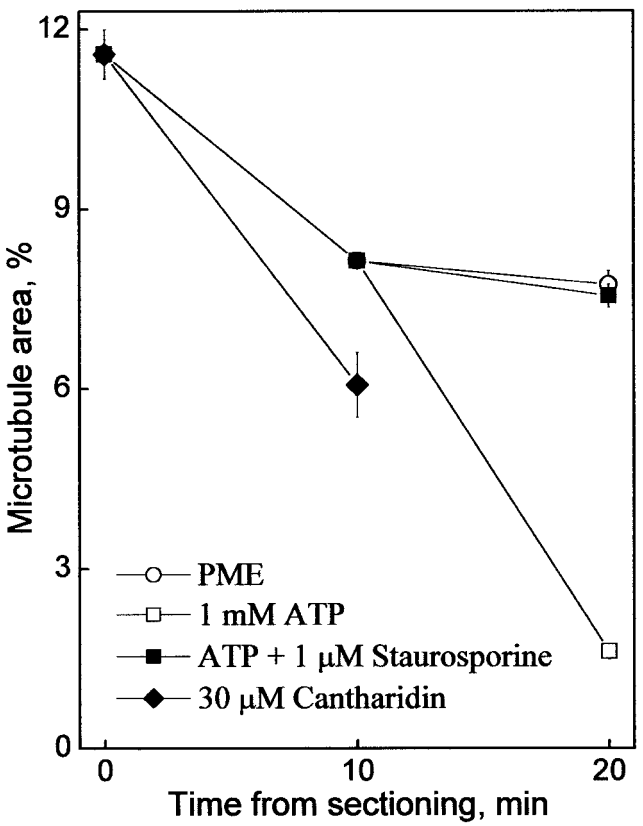

Fig. 6. Microtubule abundance in the presence of inhibitors of protein phosphorylation. Staurosporine alone had no effect (not shown). Symbols plot mean ± SEM (when larger than the symbol) of measurements made on four replicate sections from different roots.

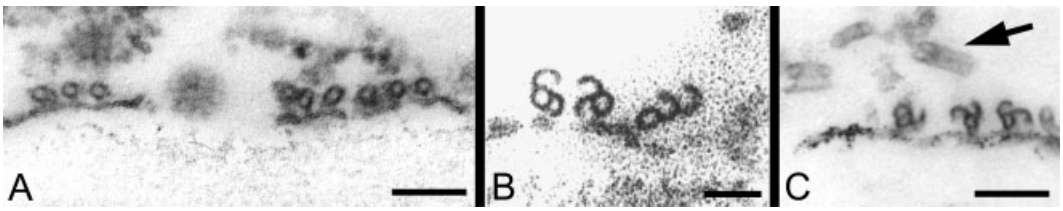

Fig. 7. Transmission electron micrographs of cortical microtubules in the cut open sections. Un-fixed sections were incubated for 10 min in PME to remove divergent microtubules and then for a further 45 min in hook buffer (**A**) alone, or (**B, C**) + 1 mg ml^{-1} porcine brain tubulin at 37°C. Afterwards, sections were fixed and processed for TEM. Section plane was chosen to transect cortical microtubules that were transverse. In A, cortical microtubules can be seen overlying patches of membrane, analogous to the FESEM images (Fig. 1C). B and C show examples of hooked microtubules and the opposite polarity is clear. *Arrow* in C points to ectopic microtubules that formed in the lumens of the cut-open cells. Scale bars = 100 nm in A and C, and 50 nm in B.

of cortical microtubules is regulated in part by reversible phosphorylation of a microtubule-binding protein.

Cortical Array Contains Microtubules of Non-Uniform Polarity

To determine the polarity of the cortical microtubules, we used hook decoration [McIntosh and Euteneuer, 1984]. Sections were incubated first in PME to remove divergent microtubules and then in hook buffer containing porcine brain tubulin. In TEM images, as in FESEM images, the plasma membrane tended to remain in the cut-open cells only in the vicinity of cortical microtubules (Fig. 7A). In sections incubated in 0.5 mg ml^{-1} tubulin, few microtubules were hooked, and in those incubated in 2 mg ml^{-1} tubulin, ectopic microtubules formed abundantly, preventing analysis of the native cortical array; however, in sections incubated in 1 mg ml^{-1} tubulin, although some ectopic microtubules formed (Fig. 7C, arrow), these could be distinguished from the native ones by proximity to the plasma membrane. By this criterion, for a subset of 1 mg ml^{-1} sections, average density of microtubules scored as native (hooked and unhooked) was 1.43 ± 0.46 microtubules μm^{-1} (mean $\pm$ SD), indistinguishable from that of controls, 1.67 ± 0.75. Approximately 30% of cortical microtubules were hooked in a random sample of approximately 500 microtubules. Over all of the scored sections, clockwise and counterclockwise hooks appeared at equal frequency (154 clockwise vs. 162 counterclockwise). Although hooks of each handedness were seen side-by-side in the same cell (Fig. 7B,C), side-by-side parallel hooks were also observed, and the incomplete hooking prevented our determining whether there was a pattern in the polarity of neighboring microtubules. Taken together, these results make it unlikely that the cortical array has a uniform polarity.

DISCUSSION

We have developed an in vitro model that gives access to cortical microtubules in native states of orientation and retaining the native cell-wall association. The model uses maize roots, which are suitable because they grow rapidly and are easy to section. Other plant material can be used, provided that it is thick enough to be sectioned on a Vibratome; for example, we have found cortical arrays remaining in un-fixed sections of cucumber and tobacco hypocotyls.

Previously, work with the ghost assay provided evidence that cortical microtubules are linked to the cell wall via trans-membrane proteins [Sonobe, 1997]. In the ghost preparation, the external domains presumably bind glass substrate in lieu of the cell wall; whereas, in the un-fixed section model, the trans-membrane proteins bind the cell wall itself. The fact that Vibratome sectioning ejects all of the cytoplasm except for cortical microtubules and some associated material supports the idea that cortical microtubules connect tightly through the plasma membrane to the cell wall; otherwise, cortical microtubules would be lost with the rest of the cell's contents. Tight binding between cortical microtubules and cell wall is predicted by some models for how the cortical array orients cellulose microfibrils [Baskin, 2001] and by other models for how the cell wall itself influences microtubule organization [Williamson, 1990; Fisher and Cyr, 1998], but hitherto has been inaccessible experimentally. Experiments where cell wall synthesis is manipulated prior to sectioning will be interesting in this regard.

In our assay, soluble tubulin concentration falls essentially to zero at the moment of lysis. Such a dilution would depolymerize completely microtubules assembled from pure tubulin; therefore, the prolonged stability of cortical microtubules after lysis means that they are stabilized. We report that microtubule stability is affected by protein phosphorylation, confirming previous work with ghosts: cortical microtubules in ghosts depolymerize in the presence of 1 mM ATP and staurosporine blocks this activity [Sonobe, 1990; Katsuta and Shibaoka, 1992]. Likewise, when cultured animal cells are lysed, microtubules are stabilized, but can be depo-

lymerized by ATP [Lieuvin et al., 1994]. In the lysed animal cells, ATP needs to be added along with a kinase whereas in the maize root sections, the kinase activity was endogenous. That kinase activity remains suggests that the relevant proteins are not exclusively cytosolic. The kinase and its substrate (presumably a microtubule-associated protein) may bind microtubules in both stabilizing and destabilizing states, a behavior reported for MAP-4 in animal cells [Chang et al., 2001]. Alternatively, kinase and substrate could be membrane proteins, anchored to the cell wall through external domains, and thus retaining access to microtubules despite cell lysis.

In addition to the stability of the microtubules depending on protein phosphorylation, we find that microtubule stability within an array is a function of orientation. Differences in stability as a function of orientation have been reported before [Foissner and Wasteneys, 1994; Shibaoka, 1994; Wiesler et al., 2002]; however, these reports compare stability between entire arrays, for example reporting that transverse arrays in growing cells are less stable than oblique arrays in mature cells. In contrast, we have addressed stability among microtubules within a single array. Approximately 20% of the cortical array depolymerized within minutes of sectioning, and the labile microtubules were predominantly ones that were oriented away from the mean orientation, whether transverse for cells of the growth zone or oblique for cells basal of the growth zone.

These observations support the "selective stabilization" model of microtubule organization, in which microtubules are nucleated spatially at random and then those that are oriented appropriately are stabilized. In the living cell, the extent of stabilization may need to be only modest, insofar as the entire cortical array typically turns over within minutes [Hush et al., 1994; Yuan et al., 1994; Shaw et al. 2003].

Previously, evidence for this model came from experiments where cortical microtubules were transiently depolymerized and allowed to reform: arrays with various orientations form with the appropriate order emerging only after several hours [Cyr and Palevitz, 1995; Wasteneys, 2002]. However, those experiments are limited because transient depolymerization presumably increases the level of soluble tubulin and may thus lead to anomalous patterns at re-polymerization. Selective stabilization is also consistent with the observation that polymerization inhibitors, such as colchicine or oryzalin, at sub-saturating levels, disorganize cortical microtubules [Hoffman and Vaughn, 1994], presumably because the microtubules are not sufficiently long-lived to become stabilized.

It has been a longstanding question whether the cortical array has a uniform polarity [Gunning and Hardham, 1982]. Unfettered access to the cortical array offered by the in vitro model allowed us to determine the polarity of cortical microtubules with the classic hook-decoration method. Hook decoration of plant microtubules with brain tubulin, as done here, has been used to determine the polarity of phragmoplast and spindle microtubules, taking advantage of endosperm cells, which lack a cell wall [Euteneuer and McIntosh, 1980; Euteneuer et al., 1982]. We found that cortical arrays contain microtubules of both polarities at equal frequency. However, only 30% of the microtubules were hooked, so the hooked microtubules could in theory reflect a distinct population having random polarity. We discount this possibility for several reasons. The hook assay was initiated after a 10-min incubation in PME to remove divergent microtubules. Furthermore, average density of cortical microtubules in control and hooked samples was indistinguishable, implying that hooked microtubules were endogenous. Most compellingly, results from living cells are consistent: Plus ends of cortical microtubules tagged with fluorescent CLIP-170 in tobacco tissue-culture cells grow in both directions within a single array [Dhonukshe and Gadella, 2003]; similarly in arabidopsis hypocotyls expressing fluorescent tubulin, cortical arrays have dynamic, i.e., "plus" ends running in both directions [Shaw et al., 2003]. Therefore, we conclude that the cortical array lacks a uniform polarity.

This is a surprising finding. Polarity is a fundamental property of microtubules and most if not all arrays contain microtubules of well-defined polarity. Moreover, previous work on roots has shown that, near the base of the growth zone, when the net orientation of cortical microtubules goes from transverse to helical, right-hand helices are formed nearly exclusively [Liang et al., 1996]. Given a random polarity among microtubules, transverse arrays are symmetrical, and therefore the formation of a helical array of a defined handedness represents a symmetry breaking event.

The significance of the symmetry breaking is that it constrains theories about how a plant specifies an axis in space in relation to which microtubules are aligned [Williamson, 1991; Cyr and Palevitz, 1995; Hush and Overall, 1996]. At present, so little is known about this that the theories are abstract. Any such theory must posit an interaction between a microtubule and some structures in the plant containing directional information. The interaction is presumably mediated by a protein (complex) with one domain to bind the microtubule and another domain to bind the informational structure. Because such a protein (complex) must itself be chiral, the lack of uniform polarity among cortical microtubules means a corresponding lack of uniform polarity among the structures conveying the directional information.

Interestingly, this protein (complex) offers a straightforward way to reorient the array from transverse

to helical. If the angle between the two domains of the interacting protein changes from, say, 0° to 45°, with no change in the structures containing directional information, then the inherent chirality of the interacting protein means microtubules would run at 45° and 225°, forming a helical array of a single handedness but containing microtubules of both polarities.

Protein constituents of the cortical array are finally being identified biochemically and genetically [Mayer and Jürgens, 2002]. With the in vitro model reported here, it should now be possible to assay for function in the context of the native cell cortex. For example, it can be determined if antibodies against a given protein block ATP-induced depolymerization. It may also be possible to purify proteins directly from the cut-open cells. This should help inaugurate a new regime for deep understanding of the structure and function of the cortical array of microtubules in higher plants.

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