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# Morphology and Microtubule Organization in Arabidopsis Roots Exposed to Oryzalin or Taxol

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In roots of *Arabidopsis thaliana*, we examined the effects of low concentrations of microtubule inhibitors on the polarity of growth and on the organization of microtubule arrays. Intact 6 d old seedlings were transplanted onto plates containing inhibitors, and sampled 12 h, 24 h and 48 h later. Oryzalin, a compound that causes microtubule depolymerization, stimulates the radial expansion of roots. The amount of radial swelling is linearly proportional to the logarithm of the oryzalin concentration, from the response threshold, 170 nM, to 1 μM. Cells in the zone of division were slightly more sensitive to oryzalin than were cells in the zone of pure elongation. Radial swelling is also stimulated by taxol, a compound that causes microtubule polymerization. Taxol at 1 μM causes little swelling, but at 10 μM causes extensive radial swelling of cells in the elongation zone, and does not affect cells in the division zone. To examine the microtubules in these roots, we used methacrylate sections with immunofluorescence microscopy. At all concentrations of oryzalin, cortical arrays are disorganized and depleted of microtubules, and the microtubules themselves often appear fragmented. These effects increase in severity with concentration, but are unmistakable at 170 nM. In taxol, cortical arrays appear to be more intensely stained than those of controls. At 10 μM, many cells in growing regions of the stele have longitudinal microtubules, whereas many cells in the cortex appear to have transversely aligned microtubules. Taxol affects microtubules in cells of division and elongation zones to the same extent, despite the observed difference in growth. We conclude that the precise, spatial pattern of cortical microtubules may not be primarily responsible for controlling growth anisotropy; and that control over growth anisotropy may differ between dividing and non-dividing cells.

**Key words:** *Arabidopsis thaliana* — Cortical microtubules — Growth — Oryzalin — Root morphology — Taxol.

Plant morphogenesis depends on the ability of cells to grow anisotropically. To produce organs with defined shapes, the degree of anisotropic growth must be precisely controlled among the constituent cells. In general, it is known that the occurrence of anisotropic growth requires the presence of the cortical array of microtubules (Gunning and Hardham 1982, Shibaoka 1991). When the cortical array is removed from growing plant cells either artificially by means of inhibitors (Green 1962, Upadhyaya and Noodén 1977), or naturally during certain developmental transitions (Mita and Shibaoka 1983, Wernicke et al. 1993), a common result is that anisotropic growth becomes isotropic. But it is not known how, or even if, the cortical array specifies the different degrees of anisotropic growth that are needed for morphogenesis.

It may be that the spatial pattern of microtubules is important. In this view, highly aligned microtubules would give rise to highly anisotropic growth, and as the alignment among microtubules becomes less then so too the degree of anisotropic growth would become less. However, other hypotheses can also be made. For example, microtubule dynamics are known to be under cellular control (Sheldon and Wadsworth 1993) and the dynamics among cortical mi-

Abbreviation: d, day(s).

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crotubules may influence membrane fluidity and hence cellulose synthesis and growth anisotropy. Finally, it may be that the cortical microtubules specify the axis of major expansion but do not specify the degree of growth anisotropy.

To obtain evidence to decide between these possibilities we have exposed roots to a series of concentrations of oryzalin, a compound that binds to plant tubulin, preventing its polymerization and thus making extant microtubules more likely to depolymerize (Morejohn and Fosket 1991, Hugdahl and Morejohn 1993). Following treatment, we measured root elongation and radial expansion, and examined the cortical arrays. Our rationale was that at certain concentrations of oryzalin intermediate effects on the cortical array should occur, and the relation between these altered arrays and the resulting growth could be explored. To extend further this type of experiment, we have also used taxol, which is known to stimulate polymerization of plant and animal microtubules via a similar mechanism (Morejohn and Fosket 1991, Bokros et al. 1993). Although the effects of taxol on mitosis and growth of cultured plant cells have been reported (Molè-Bajer and Bajer 1983, Weerdenburg et al. 1986, Weerdenburg and Seagull 1988, Kuss-Wymer and Cyr 1992, Yasuhara et al. 1993), this is to our knowledge the first report of the effects of taxol on growth and microtubules in intact plants.

We report here that treatment with a low concentration of oryzalin, at the threshold for affecting growth anisotropy, leads to notable disturbances of the cortical array; and that treatment with taxol decreases growth anisotropy but apparently does not disorganize the cortical microtubules. Our results suggest that the exact spatial pattern of microtubule deployment may not be fundamental for controlling the degree of growth anisotropy.

## Materials and Methods

*Arabidopsis thaliana* L. (Heynh) cv. Columbia was used throughout. Seedlings were grown on nutrient-solidified agar, under constant light and at 18°C as described in Baskin et al. (1992a) (except that paper gave $K_2HPO_4$ as 1 mM instead of the correct 2 mM). Agar plates containing given concentrations of oryzalin (3,5-dinitro-$N^4$,$N^4$-dipropylsulfanilamide; obtained from DowElanco, Indianapolis, IN, U.S.A.) or taxol (obtained from Dr. M. Suffness and the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, U.S. National Institutes of Health, Bethesda, MD, U.S.A.) were prepared by adding a small volume of stock solution to freshly melted agar. Stocks were made in DMSO, and were diluted approximately 1,000-fold into the agar. Controls received the maximum amount of pure DMSO. Separate experiments have shown that DMSO at 1% or below has no effect on root growth (not shown). Seeds were plated on control agar, allowed to grow for 6 d and then transplanted onto plates containing inhibitor. Plants were then placed at 31°C in constant light and sampled at the indicated times subsequently. This high temperature was used to facilitate comparison to a series of temperature-sensitive root morphology mutants that express at 31°C (Baskin et al. 1992a). Preliminary results with drug treatment at 19°C are similar to those reported here. Roots were observed with brightfield optics at low magnification, and root diameters were measured from photographs of the apex, as described in Baskin et al. (1992a). The boundary between the initials of the root cap and the stele is prominent even in brightfield and was defined as "zero" distance from the tip. Root diameter data report means of 8–10 plants from a single representative experiment. Results of other experiments are similar, but done under conditions different enough to prevent pooling.

For examination of microtubules, plants were fixed, following 12 h treatment with inhibitors, by pouring fixative directly on the plates. Fixation was at room temperature for 2 h with 4% paraformaldehyde in 50 mM PIPES. Tissue was prepared for microscopy as given by Baskin et al. (1992b), except as described otherwise here. Briefly, roots were encased in a pellet of agarose, dehydrated in an ethanol series containing 1 mM DTT, infiltrated in a mixture of butyl-methylmethacrylate containing 10 mM DTT, polymerized for 4–8 h under long wavelength UV, and sectioned at 1.75 μm on a microtome (Reichert Ultracut-S). Roots were serially sectioned, with alternate sections being collected for immunofluorescence or cell wall staining. Sections were collected in a drop of water on silanized slides, exposed to heat (60°C) for 1–2 min, and allowed to become fully dry at room temperature.

For immunofluorescence, sections were extracted in acetone for 10 min, hydrated immediately in PBS, blocked in PBS containing BSA and 0.05% (v/v) tween-20, treated with primary antibody against tubulin (see below) for 2 h at 37°C, rinsed well in PBS-tween, incubated in secondary antibody (see below) for 2 h at 37°C, rinsed well in PBS-tween and mounted in a commercial anti-fading agent (Vectasheild: Vector Labs, Burlingame, CA, U.S.A.). At least 100 sections from 5 different roots were examined for each treatment. The anti-tubulin antibody was a mouse monoclonal raised against chicken β-tubulin (Amersham, Arlington Heights, IL, U.S.A.) and was used at 1 : 200. The secondary was a goat anti-mouse Fab fragment conjugated to CY-3 (Jackson ImmunoResearch, West Grove, PA, U.S.A.) and used at 1 : 200. Dilution for both antibodies was with the blocking solution mentioned above. Fluorescence from the CY-3 chromophore was observed with standard filter sets for rhodamine, on a Zeiss Axioplan microscope, and was photographed with Tmax 100 film.

## Results

*Effects of oryzalin and taxol on radial expansion*—To study the effects of oryzalin on growth, we transplanted 6 d old arabidopsis seedlings onto medium containing oryzalin, and then observed roots at known times after transfer. Examples of roots treated for 2 d are shown in Figure 1. At 1 $\mu$M oryzalin, roots are extremely swollen, and at lesser concentrations there is progressively less radial swelling. To quantify the swelling, we measured root diameter as a function of distance from the root tip. The resulting data are shown in Figure 2. For control roots, radial expansion is confined to the apical 300 $\mu$m of the root, where cell division occurs, and there is no significant radial expansion for cells at the basal part of the zone of cell division or in the zone of rapid elongation (which extends roughly to 1–2 mm from the tip under these conditions; TIB unpublished). However, at 170 nM oryzalin, the apical region expands slightly more than in the controls, but the elongation zone remains able to elongate without radial expansion (Figs. 1, 2). As the concentration of oryzalin increases, both zones (division and elongation) undergo extensive radial expansion. At the higher concentrations by 48 h, the distinction between dividing and elongating zones is essentially lost. To summarize the effect of oryzalin on radial expansion, we plotted the maximum mean root diameter vs. the logarithm of oryzalin concentration (Fig. 3). There is a nearly log/linear relation between oryzalin concentration and radial swelling.

To explore further the relation between cortical microtubules and growth, we used taxol, a compound that drives microtubule polymerization. Taxol, like oryzalin, stimulates radial expansion (Fig. 4). Taxol at 10 $\mu$M also leads to particularly acute distortions of epidermal cell and root hair morphology (Fig. 4). Profiles of root diameter vs. position show that 1 $\mu$M taxol has only a small effect on radial swelling, and that 10 $\mu$M taxol causes extensive swelling in the elongation zone (Fig. 5). Despite the large amount of swelling in the elongation zone, 10 $\mu$M taxol did not cause any detectable swelling in the apical 300 $\mu$m of the root (Fig. 5). The profiles shown for 24 h are essentially the same as those for 48 h, and measurements over this time interval of the elongation of these roots show that at 10 $\mu$M taxol, roots elongate slowly but steadily.

*Effects of oryzalin and taxol on cortical microtubules*—Figure 6 shows cortical microtubules of cells in control roots and those treated with inhibitors for 12 h. In controls, cells of the root cortex and epidermis have dense cortical arrays, which appear to have an overall transverse alignment (Fig. 6a). Roots treated with 1 $\mu$M oryzalin have a few, scattered microtubules remaining, along with diffuse background staining, especially high for cells of the stele (Fig. 6d). At 300 nM oryzalin, cortical microtubules are present, but the overall staining intensity is much less than that of controls, and the organization of the cortical arrays appears disturbed (Fig. 6c). At 170 nM oryzalin, the stain-

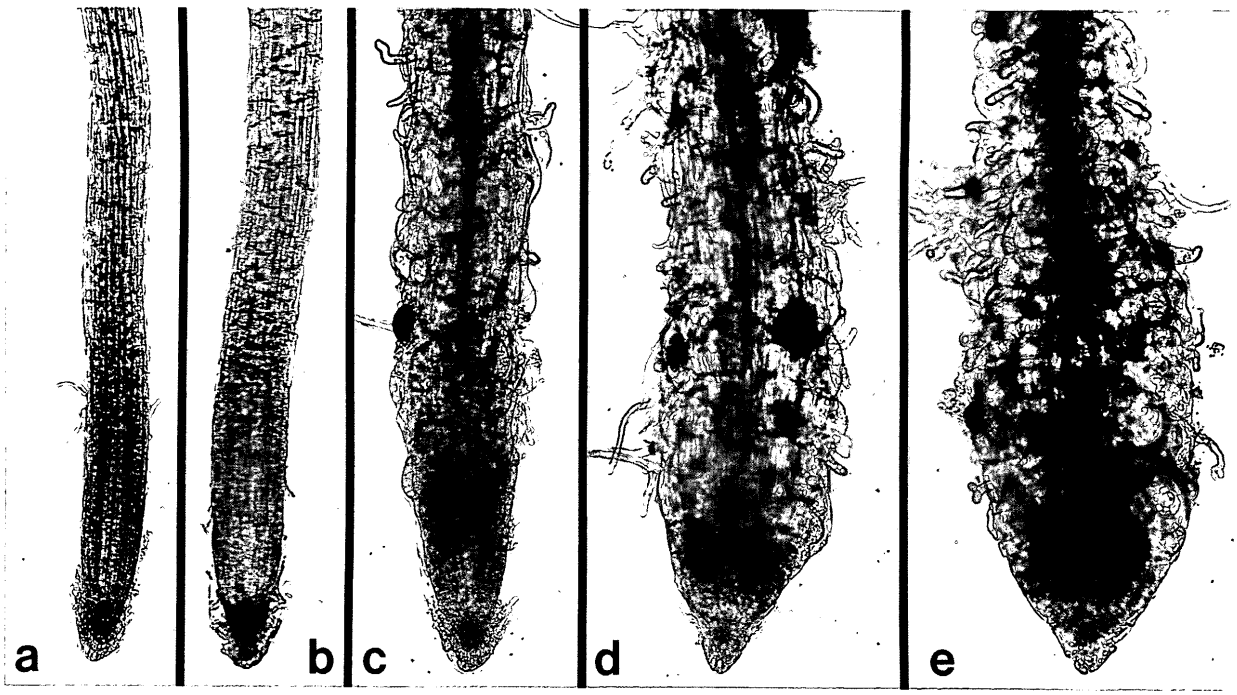


**Fig. 1** Photomicrographs of 8 day old arabidopsis primary roots exposed for 2 days to various concentrations of oryzalin at 31°C. Concentration increases from left to right: (a) control, (b) 170 nM, (c) 300 nM, (d) 560 nM and (e) 1 $\mu$M. All panels are at the same magnification, 64×.

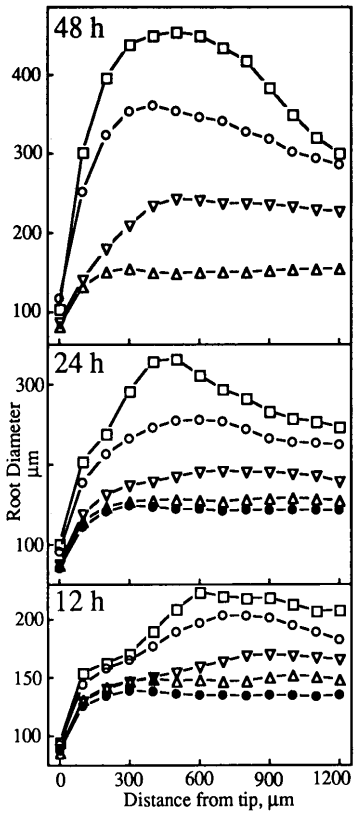


**Fig. 2** Kinetics of root swelling in response to oryzalin. Filled circles (●) plot control, empty symbols plot oryzalin-treated: △=170 nM, ▽=300 nM, ○=560 nM, □=1 µM. The y-axis scale for the 24 and 48 h plots is the same size, and is smaller for the 12 h plot. Data points are means for 8–10 plants, with standard deviations less than 4% of the means. Six day old plants were transferred to oryzalin and 31°C at time zero. Controls at 48 h were lost to contamination, but other experiments show that the difference between 170 nM and control at 48 h is the same as or larger as at 24 h.

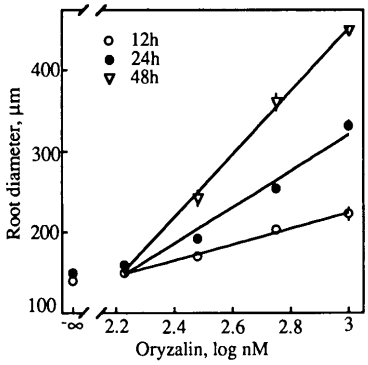


**Fig. 3** Relationship between root diameter and oryzalin at three different exposure times. Data points are maximum mean root diameters found in the experiment shown in Figure 2, (with standard deviations shown when larger than the symbols).

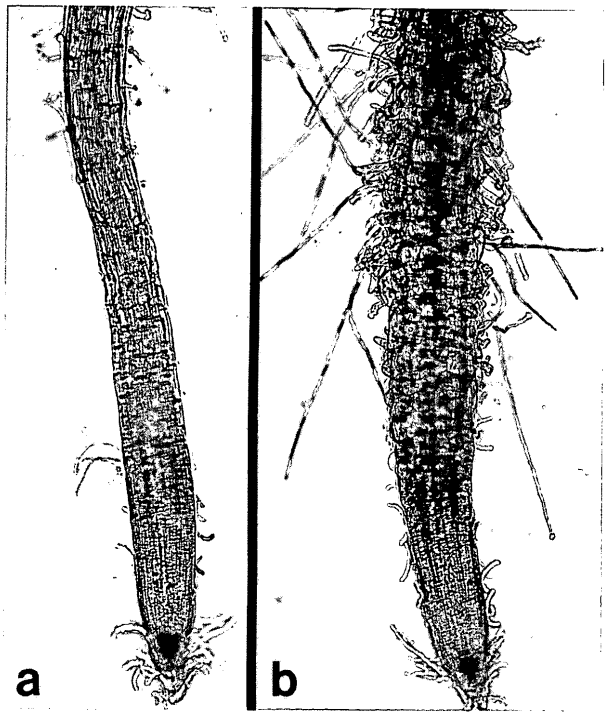


**Fig. 4** Photomicrographs of 8 day old arabidopsis primary roots exposed for 2 days to taxol at 31°C. (a) 1 µM, (b) 10 µM; both panels are at the same magnification, 64×.

ing intensity is less than that of controls, with photographic exposure times being typically twice as long as for controls.

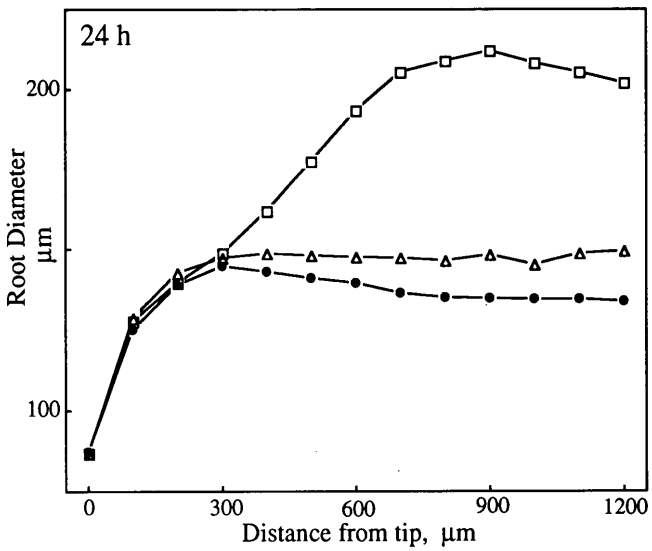


**Fig. 5** Pattern of root swelling in response to taxol, 24 h after exposure to taxol at 31°C. Filled circles (●) plot control, empty symbols plot taxol treated: △=1 µM, □=10 µM. Data points are the mean of 8–10 plants, with the standard deviations the same magnitude as shown in Figure 2.

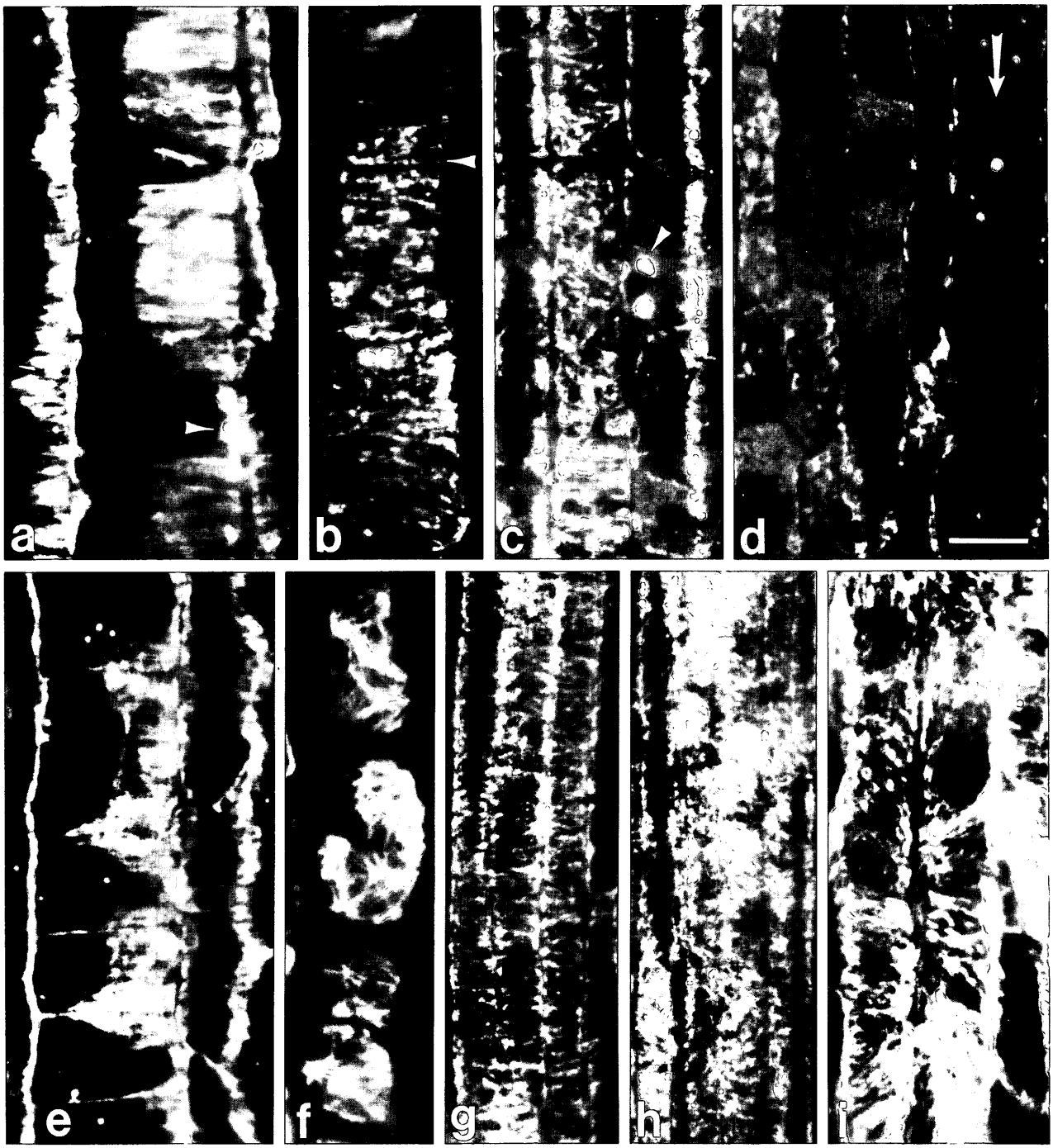


**Fig. 6** Cortical microtubule arrays. (a) Control cells from the root cortex at the start of the zone of elongation. Arrowhead marks one of several starch grains, which bind the anti-tubulin antibody used here. (b) 170 nM oryzalin; cell shown is from the epidermis in the zone of elongation. Arrowhead exemplifies a microtubule bundle that appears fragmented. (c) 300 nM oryzalin; cells shown are from the cortex. Arrowhead marks a remnant of the mitotic spindle, as judged from condensed chromosomes (not shown). (d) 1 $\mu$M oryzalin; cells shown are from the stele (left 4 files) and cortex (file marked with an arrow). (e, f) 10 $\mu$M taxol; cells in (e) are from the root cortex at the start of the zone of elongation, and those in (f) are from the zone of cell division. (g–i) cells of the root stele in the zone of elongation; (g) control; (h) 170 nM oryzalin; (i) 10 $\mu$M taxol. Magnifications: (a–e, g–i) Bar = 10 $\mu$M, (1,320×); (f) Bar = 6.9 $\mu$M (1,900×).

In cells of the root cortex and epidermis, an overall transverse alignment is usually present, but compared to controls, the cortical array appears fragmented and sparse (Fig. 6b).

By contrast, in roots exposed to 10 $\mu$M taxol, the overall staining level is high, with photographic exposure times typically half those of controls. Indeed, the density of the cortical array makes it difficult to determine the alignment of component microtubules, but it appears for cells of the root cortex and epidermis as though an overall transverse alignment remains present (Fig. 6e). Taxol treatment occasionally leads to cells with prominent, random microtubule bundles (Fig. 6f). Such cells are observed only in the epidermis within the zone of cell division; and they occur in groups of 2–4 cells, with only one or two such groups present per root. No difference in the effect of taxol on microtubules was observed between zones of division and rapid elongation, despite their different growth responses.

For all growing regions, the cortical arrays in cells of the root cortex and epidermis are better organized than the arrays in cells of the root stele. In control steles, the cortical array usually has a net transverse alignment, but microtubule bundles running in various directions are common (Fig. 6g). In 170 nM oryzalin, cortical microtubules in stelar cells are less abundant and more fragmented and disorganized than those of controls (Fig. 6h). In 10 $\mu$M taxol, staining is commonly so intense as to prevent unambiguous assessment of cortical array alignment, but a longitudinal alignment seems to predominate (not shown). In some cells, microtubule bundles could be resolved and their alignment appears to change frequently (Fig. 6i).

## Discussion

We have explored the role of the cortical microtubule array for controlling root shape in intact plants by using two contrasting microtubule inhibitors, oryzalin and taxol. Recent biochemical studies on plant material have confirmed that taxol promotes microtubule polymerization by lowering the critical tubulin concentration (Bokros et al. 1993), and oryzalin depolymerizes microtubules by binding to tubulin dimers and preventing their incorporation into polymer (Hugdahl and Morejohn 1993). Although we cannot rule out undiscovered, additional activities, we assume that the effects of these two drugs, over the concentration range used here, are mediated via their activity toward tubulin polymerization (Morejohn and Fosket 1991).

*Cortical microtubules and growth anisotropy*—It has been known for many years that the array of cortical microtubules is somehow involved in controlling growth anisotropy, but the mechanism of this control is not known (Giddings and Staehelin 1991, Shibaoka 1991). Our results indicate that the cortical microtubule array is able to exert some control over growth anisotropy even when disorganized and depleted of microtubules. This is particularly clear for 300 nM oryzalin where the cylindrical growth form of the root remains despite widespread disruption of the cortical arrays.

However, at 170 nM oryzalin, virtually normal root elongation and radial expansion occur despite the depletion and fragmentation of cortical microtubules. A similar lack of correspondence between a specific distribution of cortical microtubules and growth anisotropy is found in other studies. Throughout the elongating phase of cotton fiber development (Seagull 1992) and during the latter growth phases in pea epicotyls (Laskowski 1990) or Characeaen internodes (Wasteneys and Williamson 1987, 1993) there is a progressive increase in the percentage of cells with longitudinal or oblique microtubule arrays (pea), or in the angular dispersion of microtubules (cotton, Characeae), yet as far as is known, growth anisotropy remains constant, or may even increase. From this we argue that the control of growth anisotropy does not depend entirely on a specific, spatial pattern among cortical microtubules.

Further evidence against thinking that alignment among cortical microtubules alone specifies the degree of growth anisotropy comes from our results with taxol. In cells of the root cortex, taxol treatment induces radial swelling without greatly changing the overall alignment of microtubule arrays, although it may be that the intense staining with taxol masked microtubule misalignment. Nevertheless, Weerdenburg and Seagull (1988) reached a similar conclusion for suspension culture cells. In their study, taxol treatment scarcely affected organization of the cortical array, especially in certain sub-populations of cells, yet caused these cells to swell similarly to colchicine treatment. Therefore, it is possible, as these authors suggested, that microtubule dynamics play an important role in the function of the cortical array.

*Cortical arrays in different root tissues*—We find differences in the behavior of microtubules between cells of the stele and those of the cortex and epidermis. Cortical microtubules in stelar cells are in general less well organized than those of epidermal and cortical cells. Tissue-specific differences in microtubule array structure, broadly similar to those reported here, have also been found in maize roots (Baluška et al. 1992). Also, cortical arrays of stelar cells, as compared to those of cortex and epidermis, are more susceptible to disorganization by taxol or oryzalin, and have a higher background of diffuse staining when treated with oryzalin. Part of this difference may be due to the narrow cylindrical shape of stelar cells. Green (1992) has emphasized the importance of cell shape for controlling the organization of cortical arrays, and Weerdenburg and Seagull (1988) noted differences in microtubule organization between wide and narrow cells in culture. However, there is evidence to indicate that cortical arrays are organized locally, not globally over the whole cell (Hogetsu 1989, Ishida

and Katsumi 1991, Nick et al. 1991). Therefore, the distinct behavior of cortical microtubules in stele and surrounding tissue probably reflects tissue differentiation.

*Control of expansion in different regions of the root*—Studies on the mechanism of growth have traditionally focused on rapidly growing cells; however, dividing cells also expand. To what extent do rapidly elongating and dividing cell types share cellular mechanisms of growth control? For roots, there is evidence that anisotropic expansion in dividing and elongating cells can be independently controlled. Longitudinal sections of roots treated with auxin show dividing regions with normal morphology and elongating regions with considerable swelling (Carlton 1943). Similarly, ethylene treatment is known to cause radial swelling in rapidly elongating cells but not in dividing cells (Whalen and Feldman 1988, Baskin and Williamson 1992, Baluška et al. 1993).

Confirming the difference between dividing and non-dividing cell types, we found that growth in the zone of cell division and the zone of rapid elongation responded differently to microtubule inhibitors. A small effect was observed at 170 nM oryzalin, which stimulated the radial expansion of dividing cells but not of elongating cells. Moreover, a large effect was observed for taxol, which stimulated radial swelling in rapidly elongating cells but not in dividing cells. Differences in the uptake of the drugs could in principle explain these results, but differences in drug-mediated effects on microtubules were not observed between zones of division and rapid elongation. Traas et al. (1984) found that microtubules in the cortical cells of radish roots are on average shorter but denser in dividing cells compared with fully elongated ones. These structural differences in the composition of the microtubule array suggest that microtubule dynamics may vary in different cell types of the root. If so, the differences in dynamics may underlie the complementary differences in growth response to oryzalin and taxol observed for elongating and dividing regions of the root. In any case, the relationship between cortical microtubules and growth differs between dividing and rapidly elongating cells, and the basis for this difference requires elucidation.

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