Disorganization of Cortical Microtubules Stimulates Tangential Expansion and Reduces the Uniformity of Cellulose Microfibril Alignment Among Cells in the Root of Arabidopsis Thaliana

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Disorganization of Cortical Microtubules Stimulates Tangential Expansion and Reduces the Uniformity of Cellulose Microfibril Alignment among Cells in the Root of Arabidopsis

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To test the role of cortical microtubules in aligning cellulose microfibrils and controlling anisotropic expansion, we exposed Arabidopsis thaliana roots to moderate levels of the microtubule inhibitor, oryzalin. After 2 d of treatment, roots grow at approximately steady state. At that time, the spatial profiles of relative expansion rate in length and diameter were quantified, and roots were cryofixed, freeze-substituted, embedded in plastic, and sectioned. The angular distribution of microtubules as a function of distance from the tip was quantified from antitubulin immunofluorescence images. In alternate sections, the overall amount of alignment among microfibrils and their mean orientation as a function of position was quantified with polarized-light microscopy. The spatial profiles of relative expansion show that the drug affects relative elongation and tangential expansion rates independently. The microtubule distributions averaged to transverse in the growth zone for all treatments, but on oryzalin the distributions became broad, indicating poorly organized arrays. At a subcellular scale, cellulose microfibrils in oryzalin-treated roots were as well aligned as in controls; however, the mean alignment direction, while consistently transverse in the controls, was increasingly variable with oryzalin concentration, meaning that microfibril orientation in one location tended to differ from that of a neighboring location. This conclusion was confirmed by direct observations of microfibrils with field-emission scanning electron microscopy. Taken together, these results suggest that cortical microtubules ensure microfibrils are aligned consistently across the organ, thereby endowing the organ with a uniform mechanical structure.

How do plants build organs with specific and heritable shapes? A part of the answer to this question lies in the control of growth. It is not growth rate per se that is crucial for morphogenesis but the directionality of growth. If growth rate were the same in all directions, i.e. isotropic, plant organs would be spherical; organs attain shapes other than spherical because their component cells grow at different rates in different directions, i.e. anisotropically. Understanding how cells control the anisotropy of their expansion is essential for understanding morphogenesis.

Expansion anisotropy is characterized by the direction in which the maximal growth rate occurs and by the degree to which the maximum differs from the minimum. The direction of maximal expansion rate is known to be controlled by the direction of net alignment of cellulose microfibrils. Within a growing cell wall, microfibrils are aligned, on average, perpendicularly to the direction of maximal expansion rate, and the aligned cellulose microfibrils confer a mechanical anisotropy on the cell wall, which translates into expansion anisotropy (Green, 1980; Taiz, 1984). The wall can be considered a composite material with strong, rod-shaped particles (microfibrils) embedded in a compliant matrix. Such materials deform perpendicular to the rod’s long axis more readily than parallel, giving rise to the anisotropic yielding behavior of the cell wall (Mark, 1967).

In contrast to the direction of maximal expansion rate, it is not known whether the degree of anisotropy is also controlled by the alignment of microfibrils. The direction of maximal expansion rate can be safely inferred for a straight-growing cylindrical organ, such as a root; however, there is no way to know the degree of anisotropy without measuring it. Surprisingly, there have been few published measurements of growth anisotropy in plant organs. Those that have been made show that the degree of anisotropy differs with position, species, and treatment (Erickson, 1966; Silk and Abou Haidar, 1986; Liang et al., 1997). One of our aims here was to quantify the spatial profiles of expansion in length and radius and compare them to the underlying profile of microfibril alignment. In that way, microfibril alignment might be discovered to specify the degree of growth anisotropy.

In cylindrical organs, rates of expansion perpendicular to elongation (i.e., radial or tangential expansion) are generally small and difficult to quantify. To increase the magnitude of tangential expansion rate, we inhibited the function of cortical microtubules partially. In contrast to inhibiting microtubules totally, which gives rise to more or less isotropic expansion, we hypothesized that inhibiting them partially would stimulate tangential expansion modestly, making it easier to measure, but not enough to make expansion isotropic. The biochemistry of interaction between the microtubule inhibitor, oryzalin, and plant tubulin has been well characterized (Hugdahl and Morejohn, 1993) and partial inhibition at low concentration minimizes the possibility of nonspecific effects. We used low concentrations of oryzalin, which allowed growth to continue at a reduced but virtually steady rate for several days, and quantified profiles of longitudinal and tangential expansion rate and, concomitantly, the spatial profile of microfibril alignment.

The use of low concentrations of a microtubule inhibitor also allows us to address the question of whether cellulose microfibrils are aligned by cortical microtubules, which was suggested when microtubules were discovered (Ledbetter and Porter, 1963; Hepler and Newcomb, 1964) but has lately become controversial (Wasteneys and Galway, 2003). A role for microtubules in aligning microfibrils is supported by a substantial body of evidence (Baskin, 2001; Burk and Ye, 2002; Gardiner et al., 2003). Nevertheless, some have argued that the parallelism between microtubules and microfibrils represents a correlated response to an unknown polarizing principle rather than a causal relation (Emons et al., 1992). Furthermore, microfibril alignment by microtubules is at odds with some recent results. For example, most cells in the maturation zone of the water-stressed maize (Zea mays) root have microtubule arrays in right-handed helices but microfibrils in left-handed helices (Baskin et al., 1999). Similarly, the Arabidopsis (Arabidopsis thaliana) mutant, microtubule organization 1 (mor1), has aberrant microtubule arrays but apparently unaltered microfibril alignment (Himmelspach et al., 2003; Sugimoto et al., 2003).

We hypothesized that in material treated with low concentrations of a microtubule inhibitor, a substantial population of cortical microtubules would remain, and, if so, then their organization could be correlated with that of the microfibrils. This would make a useful contrast to mor1, assuming that chemical inhibition of microtubule polymerization affects cortical microtubules differently than does genetic ablation of the MOR1 protein. Hence, the contention that microtubules align microfibrils would either be further refuted or instead supported with a counterexample.

For this work, we used Arabidopsis roots. The small size of the root facilitates cytological analysis and, surprisingly, is also convenient for growth analysis (Beemster and Baskin, 1998). Furthermore, this species in general and the root in particular have been used in much recent work bearing on the role of microtubules in aligning microfibrils. To examine microfibrils, we used ambient-pressure cryofixation, maximizing the chances of preserving labile microtubules induced by oryzalin. Cryofixing multicellular samples is customarily done at high pressure to avoid ice crystal damage; however, for light microscopy, it has been shown that ambient-pressure cryofixation of Arabidopsis roots performs better than chemical fixation by several criteria (Baskin et al., 1996).

We report evidence suggesting that the degree of anisotropy may be controlled by differences in the net alignment among microfibrils in neighboring cells. Additionally, we report that microtubules have a role in orienting microfibrils; however, rather than influencing cellulose alignment on the subcellular scale, the cortical array appears to act through imposing a uniform order on microfibril alignment among neighboring cells in the root.

RESULTS

Determination of Suitable Inhibitor Concentrations

To inhibit microtubule function partially, we chose oryzalin, because its interaction with plant tubulin has been well characterized (Hugdahl and Morejohn, 1993). Nevertheless, with any inhibitor, there may be nonspecific effects; therefore, we compared oryzalin to microtubule inhibitors with other chemical structures (Vaughn and Lehnen, 1991). One-week-old seedlings were transplanted onto plates containing an inhibitor, exposed for 2 d, and root elongation rate over the 2nd d of treatment and diameter at the end of treatment were measured. Two compounds, clorpropahm and nocodazole, inhibited elongation at lower concentrations than needed to stimulate radial expansion (Fig. 1), possibly because they affected mitotic microtubules more actively than cortical ones (Hoffman and Vaughn, 1994). The remaining compounds had the same threshold for inhibiting elongation and stimulating radial expansion. The compounds differed in the extent of swelling and in the steepness of the dose-response curve. Oryzalin, like most of the compounds, increased diameter at least 3-fold and had a saturating concentration about 10 times the threshold. Colchicine and RH-4032 (Young and Lewandowski, 2000) increased diameter 5-fold and had a steeper dose-response curve, which suggests that these two compounds may be preferable for work at high doses. We selected 170 nM oryzalin as the low dose (just above threshold) and 300 nM as the moderate dose.

Spatial Distribution of Longitudinal and Tangential Relative Expansion Rate

We examined root elongation over time for seedlings transplanted onto control medium or medium containing 170 nM or 300 nM oryzalin (Fig. 2). In controls, root elongation rate accelerated, as previously reported (Beemster and Baskin, 1998). Roots on
170 nM oryzalin reached a steady-state growth rate after 1 d, which continued for at least several days (longer term experiments not shown). Roots on 300 nM oryzalin were considerably inhibited but nevertheless continued to elongate at a finite rate for more than 5 d. For all following experiments, we fixed drug-treated seedlings 2 d after transfer, when elongation rate was steady or nearly so, and control seedlings 1 d after transfer, to have roots that were shorter and hence easier to handle (Fig. 2, arrows).

We quantified the spatial distribution of relative expansion kinematically (Liang et al., 1997; Beemster and Baskin, 1998). Sample images from the analysis are shown in Figure 3. The black spots are the graphite particles used for measurement. Roots on oryzalin were swollen and root hairs differentiated close to the tip. On 300 nM oryzalin, roots were less swollen than roots grown on a saturating concentration, 1.7 μM, which have almost no microtubules and which cease growth by 2 d of exposure (Baskin et al., 1994).

The spatial profile of relative elongation rate is shown in Figure 4 (top). Oryzalin truncated the growth zone progressively with concentration. Surprisingly, elongation rates in the apical portion of the root were indistinguishable among the three treatments. In the initial 200 μm or so of the profile (which is mainly root cap) rates differed, probably because the ends of the data set are prone to curve-fitting errors (Beemster and Baskin, 1998).

Relative tangential expansion rate quantifies the rate of expansion of the root’s circumference. In controls, tangential expansion rate was low, 1% h⁻¹ or less, in the first 400 μm of the root, and then became negative for the next 500 μm and possibly farther (Fig. 4). Negative rates occur because root diameter decreases,
starting at about the location where cell length begins to increase markedly. Even where tangential expansion rates were positive, they were much smaller than elongation rates, showing that growth was highly anisotropic throughout the growth zone, including the meristem. Oryzalin promoted tangential expansion, with the maximal rate occurring a little basal of the relative elongation rate maximum. Interestingly, maximal tangential rate was the same on 300 and 170 nM oryzalin, but moved apically under the higher dose. The apical shift led to thicker roots because cells at the more apical locations moved more slowly than those more basal; therefore on 300 nM oryzalin, cells experienced the maximal tangential expansion rate for a longer time and thus swelled to a greater extent. Even on 300 nM oryzalin, growth remained anisotropic, but the degree of anisotropy was less than at 170 nM and much less than that of controls.

Patterns of Microtubule Organization

To examine microtubules, roots were cryofixed at the times indicated by Figure 2, freeze-substituted, embedded, sectioned, and stained with antitubulin (Baskin et al., 1996). Fluorescence was dim in the maturation zone and microtubules there were not examined. In the growth zone, oryzalin appeared to affect microtubules similarly in all tissues. In dividing cells, preprophase bands and phragmoplasts were often misaligned. Additionally, cell shapes were distorted, as if from the deposition of cell walls at oblique angles (see below). Cortical arrays in control were dense and transverse to the root’s long axis; whereas on oryzalin, the cortical array appeared to contain fewer microtubules and to be partially disorganized (Fig. 5). Particularly on 300 nM oryzalin, microtubules appeared thicker and shorter, but light microscopy has insufficient resolution to determine length or bundling unambiguously (Williamson, 1991).

We quantified the angular distribution of microtubules for cortex and epidermis (Fig. 6). The frequency plots divided angles into 10-degree classes, with 90° representing transverse. The angular distribution of cortical microtubules in control was sharp and centered on 90° for the first 900 μm from the quiescent center, coinciding with increasing relative elongation rate (Fig. 4). With greater distance, where elongation rate decreases, 90° orientation was replaced gradually by a bimodal distribution, with peaks at 45° and 135°, indicating oblique microtubules (Fig. 6). Oblique angles occur where microtubule arrays are helical, and even though the helices are nearly all right-handed (Liang et al., 1996; Thitamadee et al., 2002), orientations of 45° and 135° are seen equally because sectioning presents views of the helices from inside and outside. On oryzalin, the means of the
distributions were consistently 90° but the distributions were flattened, particularly at 300 nM, and the reorientation to oblique angles was minimal or absent.

Patterns of Microfibril Orientation

To examine cellulose microfibrils, we used polarized-light microscopy. To ensure congruence of the data sets, alternate sections were collected for analysis of microtubules and microfibrils. As described in “Materials and Methods,” the quantitative polarized-light attachment produces a pair of images, one showing retardance and the other optical azimuth of the retarding elements. We quantified both parameters for epidermal and cortical cell walls lying parallel to the section plane (i.e. longitudinal-radial cell walls).

Retardance images reveal cell shape clearly, highlighting the misplacement of cell walls on oryzalin (Fig. 7). The misaligned cell walls meant that in oryzalin-treated material there was less cell wall lying parallel to the section plane available for measurement; nevertheless, some was present and virtually all suitable areas were sampled per image (see “Materials and Methods”). Retardance of controls reached a peak in the meristem and then gradually declined (Fig. 8A). On oryzalin, retardance became significantly greater than control; this happened for 170 nM beyond about 750 μm from the quiescent center and for 300 nM beyond about 250 μm. The locations where retardance surpassed the control level also had elevated rates of tangential expansion; therefore, increased tangential expansion, and hence root swelling on oryzalin, was accompanied by there being denser or better-aligned microfibrils. In contrast to retardance, the average azimuth was approximately 90° for all positions and did not differ significantly among treatments (Fig. 8B). This appears to indicate that microfibril directionality was unaltered despite the pronounced deterioration in microtubule organization.

However, Figure 8, A and B, plot means and s.e.s. These errors reflect the variability among roots. We then assessed the variability among measurements by looking at the s.d.s, which were obtained for each root, and averaged (Fig. 8, C and D). The deviations for the retardance data tended to be proportional to the retardance values themselves, a scaling behavior that typifies many parameters. In contrast, the deviations for the azimuth data differed; compared to controls, deviations for the oryzalin-treated roots were larger and, what’s more, appeared to increase at around the location where tangential expansion rate increased. These data show that although the net orientation among all microfibrils remained transverse (Fig. 8B), local regions of cell wall had divergent microfibril orientation. Similarly, saturating oryzalin for 24 h altered microfibril orientation globally but not locally (Sugimoto et al., 2003).

To confirm the polarized-light data, we examined the innermost layer of the cell wall with field-emission scanning electron microscopy (FESEM). Care was taken to collect images of cortical or epidermal cells from within the growth zone (within 1,500 μm from the quiescent center in controls, 1,000 μm for 170 nM oryzalin, 600 μm for 300 nM). Cell walls in control roots had microfibrils with uniformly transverse orientation (Fig. 9, A and B); however, cells on 170 nM (Fig. 9, C and D) and 300 nM oryzalin (Fig. 9, E and F) often had microfibril alignments that deviated from the transverse. Although microfibril alignment was usually coherent at the level of a single image, occasionally...
in the 300 nM samples, cell walls had bands of divergent microfibrils (Fig. 9F). As described in “Materials and Methods,” the average degree of alignment among microfibrils, as well as their net angular orientation, was quantified by means of their fast Fourier transforms (FFTs; Table I). The eccentricity of the transforms at three spatial frequencies (at about the size of the prominent fibrillar structures in the micrographs) was essentially the same for the treatments, indicating that there was little difference in the angular divergence among the microfibrils (divergence would tend to make the transform less eccentric). On the other hand, the angle defined by the major axis of the ellipse and the long axis of the root was significantly more variable on oryzalin than in controls. These results support the polarized light data; microfibril orientation in roughly 1 \( \mu m^2 \) patches was not degraded on oryzalin, but orientations in different cells were less consistent.

DISCUSSION

Growth Patterns

To our knowledge, this is the first report quantifying tangential expansion rates for the Arabidopsis root, and for the root of any species exposed to a microtubule inhibitor. For calculation, we assumed steady-state growth, even though diameter increases with time (van der Weele et al., 2000). This gives rise to a time-dependent expansion rate, without which tangential rates are underestimated. By measuring the spatial profile of diameter on days 1, 2, and 3, we found that the term is about 0.3% h\(^{-1}\) for controls, slightly larger for 170 nM, and smaller for 300 nM oryzalin. Importantly, the term is essentially the same at all positions in the growth zone. Spatial constancy was also reported for the time-dependent change in the diameter of the well-watered maize root (Liang et al., 1997). Therefore, neglecting the time-dependent term underestimates tangential rate inconsequentially.

In the control, tangential rates peak at about 1% h\(^{-1}\) in the meristem and fall to about −4% h\(^{-1}\) in the elongation zone (Fig. 4). Negative expansion rates of any kind are unusual, reported previously for regions of the shoot apical meristem in folds between emerging primordia (Kwiatkowska and Dumais, 2003) and for the concave side of bending organs, where the strong growth of the convex side may compress the weakly growing concave side. In a straight-growing organ like the root, differential stresses between inner and outer tissues could conceivably cause tangential shrinkage. Another potential cause is the loss of the lateral root cap, which sheathes the root around the apex but disappears at about the location where shrinkage occurs.

Expansion throughout the Arabidopsis root’s growth zone, including the meristem, is anisotropic, although the degree of anisotropy varies with position. Some authors have observed the roughly isodiametric cell shapes in the Arabidopsis root meristem and have mistakenly concluded that expansion there is isotropic (e.g. Bichet et al., 2001). Cell shape is formed by division as well as expansion; therefore, static images of meristem cell shape reveal nothing about expansion anisotropy (Green, 1976; Baskin and Beemster, 1998).

Microtubule inhibitors are often thought to reduce elongation because they stimulate lateral expansion, as if the rate of cell wall area expansion were constant.
This explanation is untenable here because, for example, at 300 μm from the quiescent center, elongation rates for the three treatments are identical but tangential expansion rates differ by a factor of 5 (Fig. 4). Instead, microtubule inhibition appears to affect elongation and tangential expansion by different mechanisms. Elongation rate is truncated apically, as also reported over time for wild-type roots responding to saturating oryzalin and for mor1 roots responding to a shift in temperature (Sugimoto et al., 2003). Because elongation zones are truncated apically when a root responds to deficits of water (Sharp et al., 1988) and phosphorus (Ma et al., 2003), we postulate that the truncation of the elongation rate profile reflects a response to stress, whereas the stimulation of tangential expansion is specific to the disruption of microtubule function.

Microtubule Organization

Hoffman and Vaughn (1994) reported that low doses of various microtubule inhibitors, including oryzalin, disorganize all classes of microtubule array in oat (Avena sativa) roots, although their paper focused on mitotic arrays. Here, in addition to disorganizing the cortical array, low oryzalin prevented cortical microtubule reorientation from transverse to oblique and led to misplaced preprophase bands, phragmoplasts, and cell walls. In fact, aberrant phragmoplasts and

Figure 9. FESEM micrographs of the innermost cell wall layer taken within the growth zone. A and B, Control; C and D, 170 nM oryzalin; E and F, 300 nM oryzalin. The long axis of the root is parallel to the long side of the page. For the oryzalin treatments, images are representative of least affected (upper) and most affected (lower) cell walls. All images are from cortex or epidermis. Material was sampled according to Figure 2. Bar represents 250 nm.
misplaced cell walls are seen frequently in roots exposed
to microtubule inhibitors (Ennis, 1948; Hardham
and Gunning, 1980; Holmsen and Hess, 1985; Cleary
and Hardham, 1988; Galatis and Apostolakos, 1991;
Hoffman and Vaughan, 1994). Given that the pre-
prophase band helps to determine the placement of the
cell plate (Mineyuki, 1999), it is reasonable that
misaligned preprophase bands be followed by mis-
aligned cell walls. We hypothesize that disorder in
both preprophase and cortical arrays arises from de-
creased microtubule stability. Regardless of mecha-
nism, these results highlight the importance of
microtubules for the alignment of the cell plate.

Conceivably, the misdirected cross walls impair the
plant’s ability to align cellulose microfibrils, but evi-
dence suggests otherwise. The disposition of cross
walls appears to have little influence on anisotropic
expansion and microfibril alignment. For example, the
tangled mutant of maize has aberrant cross walls but
expansion and microfibril alignment. For example, the
walls appears to have little influence on anisotropic
diffraction suggests otherwise. The disposition of cross
walls in FESEM images of cell walls

These parameters were obtained from the Fourier transform, as
described in “Materials and Methods.” Number of cells examined: 20
(control), 14 (170 nM oryzalin), and 12 (300 nM oryzalin).

Table I. Analysis of orientation parameters from FESEM images of
cell walls

<table>
<thead>
<tr>
<th>Spacing, nm</th>
<th>Eccentricity, Mean ± so</th>
<th>Control</th>
<th>170 nM Oryzalin</th>
<th>300 nM Oryzalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.6</td>
<td>0.92 ± 0.12</td>
<td>0.93 ± 0.09</td>
<td>0.87 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>23.75</td>
<td>0.91 ± 0.13</td>
<td>0.96 ± 0.06</td>
<td>0.94 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>32.7</td>
<td>0.96 ± 0.05</td>
<td>0.94 ± 0.03</td>
<td>0.94 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Angle, Degrees, Mean ± so</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.6</td>
<td>0 ± 13</td>
<td>2 ± 19</td>
<td>12 ± 28</td>
<td></td>
</tr>
<tr>
<td>23.75</td>
<td>-1 ± 16</td>
<td>2 ± 30</td>
<td>5 ± 25</td>
<td></td>
</tr>
<tr>
<td>32.7</td>
<td>0 ± 17</td>
<td>7 ± 36</td>
<td>-2 ± 30</td>
<td></td>
</tr>
</tbody>
</table>

Microfibril Organization

On oryzalin, the increase in retardance compared to
control, particularly striking for 300 nm, indicates that
microfibrils become better organized or there are more
microfibrils per unit area of cell wall (or both). The
reason for increased retardance is unknown. In prin-
ciple, an increased retardance within the basal part of
the elongation zone in controls could move apically
under oryzalin; however, such an increase was absent
from controls processed on other occasions where the
sections included the basal part of the elongation zone.
Alternatively, oryzalin could enhance microfibril self-
assembly, but, if so, then one would expect to see
evidence of this in FESEM images, which, as analyzed
through the Fourier transforms, are indistinguishable
among the treatments, except in the orientation of the
transform. Finally, retardance may have increased
because the so-called multi-net reorientation de-
creased. Efficient multi-net rotation of transverse mi-
crofibrils depends on highly anisotropic expansion
(Erickson, 1980; Preston, 1982); for 300 nm oryzalin,
where the increase of retardance is largest, expansion
is nearly isotropic. Therefore, the increased retardance
could reflect decreased multi-net reorientation in older
layers of the cell wall, but modeling will be required to
assess this explanation.

Microtubule-Microfibril Syndrome

We show here that cortical microtubules are dis-
ensurable for microfibril alignment locally but not
globally, a conclusion that has been reached previously
(Baskin, 2001; Sugimoto et al., 2003). By local we refer
to subcellular regions, 10 μm² or less, an area typical of
the polarized-light samples and larger than the area
sampled in FESEM (<1 μm²). By global, we refer to
whole cells or groups of cells. Sampling within cells in
polarized light was precluded because a cell rarely had
more than one area suitable for measuring. In FESEM,
in principle, different parts of the same cell could have
been compared; however, for the oryzalin-treated
roots, cell walls tended to wrinkle and the entire cell
wall area was rarely useful. Furthermore, the FESEM
technique is difficult and few samples were processed
successfully, particularly for 300 nm oryzalin. Based on
images we did obtain, on 300 nm oryzalin, the break-
down of coherent microfibril alignment can occur
within the same cell (Fig. 9F); but whether the break-
down is consistently within or between cells will
require further investigation.

On the local scale, for diffuse-growing higher plant
cells, microtubule disruption rarely leads to mis-
aligned microfibrils (Baskin, 2001); however, nearly
all previous experiments assessed microfibril align-
ment visually. In contrast, Sugimoto et al. (2003) mea-
sured the angles of individual microfibrils in FESEM
and here we quantified microfibril alignment in both
FESEM and polarized-light microscopy. That local
microfibril alignment does not require microtubules
is thus demonstrated quantitatively.

On the global scale, the situation is reversed; in
diffuse growing cells, disrupting microtubules rarely
fails to misalign microfibrils globally. In the shoot
epidermis of many species, microfibril alignment
cycles between transverse, oblique, and longitudinal,
creating a layered, so-called polylamellate cell wall;
when microtubules are removed, the layering stops
and microfibrils appear to be deposited in a single
orientation (Srivastava et al., 1977; Takeda and
Shibaoka, 1981; Vian et al., 1982; Satiat-Jeunemaitre,
1984). Additionally, in the growth zone, cells with
transverse alignment outnumber cells with oblique
and longitudinal alignments; but when microtubules
are removed, the three alignment classes become
represented equally (Takeda and Shibaoka 1981; Iwata
and Hogetsu, 1989). Studies like these support the idea
Microtubules, Microfibrils, and Growth Anisotropy

Anisotropy of growth is characterized by the direction of maximal growth rate and by the difference between maximal and minimal growth rates, that is, the degree of anisotropy. The degree of anisotropy was hypothesized by Green (1964) to be proportional to the degree of alignment among microfibrils; that is, the more parallel cellulose microfibrils are laid down, the greater the difference between maximal and minimal expansion rates. This hypothesis was tested for the maize root, which grows more anisotropically under water deficit (Liang et al., 1997) and falsified; despite there being a more than 10-fold difference in the degree of anisotropy, there was no difference in microfibril alignment (Baskin et al., 1999). Likewise, decreased anisotropy occurs in several Arabidopsis mutants without detectable disorganization among microfibrils (Wiedemeier et al., 2002; Sugimoto et al., 2003).

We suggest that Green’s hypothesis is valid, provided that it invokes the degree of alignment among microfibrils considered globally rather than locally (Fig. 10). An organ with a uniform tubular texture among microfibrils plausibly expands more anisotropically than one with patchy reinforcement. This resembles the situation in polylamellate stems where removal of microtubules causes swelling and an increase in the patchiness of microfibril orientation (Takeda and Shibaoaka, 1981; Iwata and Hogetsu, 1989). In the work on roots cited above, apparently falsifying Green’s hypothesis, none of the authors explicitly examined the variability of average microfibril orientation among cells, and a patchy distribution of locally well aligned cellulose may have been present. If so, this would be strong evidence that global microfibril orientation specifies the degree of anisotropy, the degree of alignment among microfibrils, and the role of microtubules in morphogenesis is to align microfibrils globally. Alternatively, the patchy microfibril orientation seen here may be
inconsequential, and, if so, then the reason for the reduced expansion anisotropy would have to be sought in an activity of microtubules other than aligning cellulose. Deciding between these alternatives is a challenge for future research.

MATERIALS AND METHODS

Material, Growth Conditions, and Treatments

Seeds of *Arabidopsis thaliana* L. (Heynh) ecotype Columbia were sterilized in dilute bleach and germinated on nutrient-solidified agar supplemented with 3% Suc in petri plates, sealed with air-permeable bandage tape, and seedlings were grown vertically under continuous yellow light (80 μmol m$^{-2}$ s$^{-1}$) and constant temperature (19°C) for up to 10 d in a growth chamber, as described by Baskin and Wilson (1997). For experiments, 6- to 7-d-old seedlings were transplanted onto plates with the given concentration of inhibitor and returned to the growth chamber. There were three plates for each concentration, and each experiment was usually repeated three times.

Inhibitors were obtained from Sigma Chemical (St. Louis), except for the following: Propyzamide and oryzalin were from Chem Services (West Chester, PA), dithiopyr was a gift from Doug Sammonds (Monsanto, St. Louis), terbutol was a gift from Kevin Vaughn (Agricultural Research Service, Stoneville, MS), RH-402 was a gift from David Young (Rohm and Haas, Philadelphia), and amiprophos-methyl was a gift from Dr. Carl Gregg (Bayer CropScience, Kansas City, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) except colchicines, which was dissolved in growth medium. Stacks were frozen between uses and diluted into measured quantities of melted agar at least 300-fold, and usually 1,000-fold. Control medium was given the maximal amount of DMSO used for a given dose-response curve.

Measurement of Growth

For measuring overall root elongation rate, plates containing seedlings were scored with a razor at the back of the plate at the position of the root tip, once per 24 h, and the plate was photocopied to end the experiment. The arc length of the root between marks, measured on a digitizing tablet interfaced to a computer running SigmaScan (Jandel Scientific, Corte Madera, CA), was divided by the time interval between marks. Root diameter was measured by imaging the roots at low magnification through a compound microscope directly on the agar plate, as described by Baskin and Wilson (1997).

The spatial profile of relative elongation rate was obtained as described by Beemster and Baskin (1998). Briefly, the root was dusted with fine graphite particles and allowed 1 h to recover. The petri dish containing seedlings was then clamped to the stage of a horizontal microscope, and images of the root captured via a CCD camera and a computer running the public domain NIH Image program (version 1.62; developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Images were captured hourly for 4 h with the dish returned to the growth chamber between times. Five roots were imaged per treatment, each on a different petri dish. The positions of the marks at each time point were measured with NIH-Image. A separate image was also obtained by focusing at the median plane, from which was obtained the spatial profile of diameter and the distance between the root tip and the quiescent center. For each pair of images (five pairs), the measured displacements gave rise to a set of velocity-versus-position data and the five sets were pooled. A final velocity profile was obtained by a piece-wise, iterative polynomial fitting procedure that interpolated points at 25-μm intervals. The spatial profile of diameter was also smoothed and interpolated by the same algorithm. Relative tangential expansion rates were calculated from the spatial profiles of velocity and diameter (Silk and Abou Haidar, 1986; Liang et al., 1997). Longitudinal and tangential profiles were obtained for each root and then averaged over the roots in the treatment.

Examination of Microtubules and Microfibrils

Roots, treated as needed, were cryofixed as described by Baskin et al. (1996). Briefly, a root was mounted on a Formvar film supported on a wire loop and plunged into liquid propane at −180°C. Samples were then substituted in anhydrous ethanol for 2 d at −80°C, raised to room temperature, and embedded in butyl-methyl-methacrylate. Roots were sectioned longitudinally, at 2-μm thickness, with alternate sections collected for immunocytochemical detection of microtubules and polarized-light analysis of the birefringent element. Inset in the azimuth image shows the gray level coding. Boxes shown are typical of those used for measurement. The black (low retardance) throughout most cells in (A) reflects the absence of cell wall. Bar represents 10 μm.

![Figure 11. Polarized-light micrographs of a region of the root cortex illustrating the output of the LC-Pol Scope and the method of sampling.](image-url)
those frequencies. A 256 altitudes; the more circular the shape, the less well oriented the structures at image. The algorithm analyzed the overall shape of the transform at a series of a mountain, with contour levels linking frequencies represented equally in the thickness of 2 nm, and viewed in a FESEM (4700 S; Hitachi, Tokyo).

Samples were treated with a 1:10 dilution of household bleach (0.525% [w/w] hypochlorite) for 30 min to remove cytoplasm, dehydrated in an ethanol water for 30 min followed by 50% DMSO until cryo-sectioning at 20°C. After sectioning, cut-open roots were thawed in phosphate-buffered saline and viewed in a FESEM (Hitachi, Tokyo).

Orientation parameters were quantified from the FESEM images by means of a novel algorithm applied to the FFT, implemented as a plug-in for Image-J (v. 1.31e; developed by the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij) by Chris Coulon (GAIA Group, Novato, CA). In three dimensions (x, y, and z), the FFT can be likened to a mountain, with contour levels linking frequencies represented equally in the image. The algorithm analyzed the overall shape of the transform at a series of altitudes; the more circular the shape, the less well oriented the structures at those frequencies. A 256 × 256 pixel² (840 × 840 nm²) region was chosen from each image and the FFT obtained. The transform was thresholded to generate a binary image, separating pixels containing power (black) from the background (white). An ellipse, a rough approximation to the shape of the transform, was fitted to the black pixels, and the major and minor axes recorded as well as the angle between the major axis and the vertical. Eccentricity (E) was calculated from the major (a) and minor (b) axes as:

\[ E = \sqrt{a^2 - b^2} \]

This was done iteratively, starting at the lowest threshold that gave a distinct shape, incrementing the threshold by five gray levels, and stopping when the area of the black pixels contained less than 200 pixels. The parameters for each threshold were assigned to the frequency of an average ellipse radius, (a + b)/2, and this frequency was converted to a distance by dividing it into the total width of the transform (256 pixels).

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**LITERATURE CITED**


Microtubules, Microfibrils, and Growth Anisotropy