Differential Regulation of Cellulose Orientation at the Inner and Outer Face of Epidermal Cells in the Arabidopsis Hypocotyl

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It is generally believed that cell elongation is regulated by cortical microtubules, which guide the movement of cellulose synthase complexes as they secrete cellulose microfibrils into the periplasmic space. Transversely oriented microtubules are predicted to direct the deposition of a parallel array of microfibrils, thus generating a mechanically anisotropic cell wall that will favor elongation and prevent radial swelling. Thus far, support for this model has been most convincingly demonstrated in filamentous algae. We found that in etiolated Arabidopsis thaliana hypocotyls, microtubules and cellulose synthase trajectories are transversely oriented on the outer surface of the epidermis for only a short period during growth and that anisotropic growth continues after this transverse organization is lost. Our data support previous findings that the outer epidermal wall is polylamellate in structure, with little or no anisotropy. By contrast, we observed perfectly transverse microtubules and microfibrils at the inner face of the epidermis during all stages of cell expansion. Experimental perturbation of cortical microtubule organization preferentially at the inner face led to increased radial swelling. Our study highlights the previously underestimated complexity of cortical microtubule organization in the shoot epidermis and underscores a role for the inner tissues in the regulation of growth anisotropy.

INTRODUCTION

Plant growth and development depend on the coordinated expansion of individual cells. In order for cell expansion to occur, the plant cell wall must yield to the force exerted by hydrostatic pressure (reviewed in Cosgrove, 2005). Since hydrostatic pressure is thought to be constant and uniform in normal growth conditions, it follows that the rate and direction of cell expansion is primarily determined by the properties of the cell wall. The plant cell wall is composed of long cellulose microfibrils embedded in a viscoelastic matrix. Cellulose microfibrils are deposited in parallel arrays and confer anisotropic properties to the cell wall (Probine and Preston, 1961, 1962). A widely accepted hypothesis states that cell expansion occurs along the axis perpendicular to the orientation of the cellulose microfibrils (Green, 1962). Thus, cell elongation coincides with the deposition of cellulose microfibrils in transversely oriented arrays, as has been observed, for instance, in the filamentous internodes of the alga Nitella (Roelofsen and Houwink, 1953) and Arabidopsis thaliana root epidermal cells (Sugimoto et al., 2000).

The microtubule-microfibril alignment hypothesis (Ledbetter and Porter, 1963; Green, 1965; Heath, 1974) posits that cortical microtubules guide motile cellulose synthase complexes (CSCs) in the plasma membrane as they secrete cellulose microfibrils into the wall. A large body of evidence shows that the orientation of the cortical microtubule array reflects the orientation of the cellulose microfibrils deposited in the cell wall (reviewed in Baskin, 2001), and CSCs have been shown to move along cortical microtubules in living cells (Paredez et al., 2006). Consequently, the orientation of cortical microtubules determines the orientation of newly deposited cellulose microfibrils in the cell wall, ultimately determining the preferential direction of cell expansion.

The initial studies on the regulation of cell elongation focused on the filamentous internodes of algae like Nitella (Roelofsen and Houwink, 1953; Green, 1960), which lack the complexity and mechanical constraints of organs of higher plants, in which cell layers with extensible walls are connected to layers with less extensible walls. This generates compression and tension forces (referred to as tissue tension), in addition to the local forces of turgor pressure. External epidermal walls are generally the thickest and probably the least extensible and hence are thought to bear the forces generated in internal tissue layers (Kutschera, 1992). Recent genetic studies indeed support this idea (Savaldi-Goldstein et al., 2007), but thus far it remains unclear how the architecture of the external epidermal cell wall relates to the growth anisotropy of the organ. If the epidermis controls growth anisotropy, one would expect to find transversely oriented cellulose microfibrils in epidermal cell walls, as predicted by the experiments in Nitella. This seems to be the case for instance in Arabidopsis roots (Sugimoto et al., 2000). Instead, in hypocotyls...
or coleoptyls, it has been repeatedly shown that cell walls of growing epidermal cells have a crossed polylamellate or helicoidal architecture, containing arrays of cellulose microfibrils deposited in all orientations (Roland et al., 1975; Neville and Levy, 1984; Refrégier et al., 2004; reviewed in Neville et al., 1976). This polylamellate architecture is, at least in the Arabidopsis light-grown hypocotyl, generated by continuously rotating microtubule arrays that correspondingly cause rotation of cellulose synthase trajectories in the plasma membrane (Chan et al., 2010). The resulting wall architecture is predicted to be isotropic, suggesting that the cell walls of inner tissues rather than the outer epidermal walls may play a dominant role in regulating the growth anisotropy of the organ (Baskin, 2005).

Although it has been noted that cortical microtubules are not always organized in the same manner on the outer tangential face and the inner tangential face of epidermal cells (Busby and Gunning, 1983; Flanders et al., 1989; Yuan et al., 1995; Hejnowicz et al., 2000; Barton and Overall, 2010), all studies of cellulose synthase proteins thus far have focused on the outer face due to difficulty in accessing the deeper regions of the tissue (for example, Paredez et al., 2006; DeBolt et al., 2007; Desprez et al., 2007; Harris and DeBolt, 2008; Paredez et al., 2008; Crowell et al., 2009; Gutierrez et al., 2009). To study how cellulose deposition is related to hypocotyl elongation, we found it pertinent to characterize both the inner and outer tangential walls of the epidermal cells (see Supplemental Figure 1 online) and used spinning disk microscopy and field emission scanning electron microscopy (FESEM) to do so. Here, we present a detailed quantitative study of the orientation of microtubules, CSC trajectories, and newly deposited microfibrils throughout the whole volume of the epidermis in etiolated Arabidopsis hypocotyls. Our results show that microtubules and cellulose synthase trajectories at the inner face of the epidermis consistently show a transverse orientation in the growing zone, whereas on the outer face, the transverse orientation occurs only transiently, after which elongation growth continues despite the absence of a preferential microtubule orientation. In addition, a reduced degree of growth anisotropy was observed in plants in which microtubules had been perturbed primarily on the inner epidermal face. The results show that epidermal cells are polarized for the orientation of the cortical microtubules, cellulose synthase trajectories, and newly deposited microfibrils. Furthermore, our data support a model in which internal cell walls and not the outer epidermal cell walls regulate the directionality of the expansion of the organ.

**RESULTS**

**Orientations of Cortical Microtubules and CSC Trajectories on the Outer Face of the Hypocotyl Epidermis**

We focused on the epidermal cells of etiolated hypocotyls of Arabidopsis, which follow an acropetal growth gradient (Gendreau et al., 1997; Refrégier et al., 2004). We chose to characterize seedlings starting at 71 h after induction of germination (hours after induction [HAI]; see Methods), at which time kinematic analysis under infrared light showed that hypocotyls elongated at the maximum rate of 370 μm/h (Figure 1A). We additionally analyzed hypocotyls at 76 HAI, just as the growth rate began to decline (Figure 1A). At both time points, hypocotyls displayed distinct zones with differing growth rates (Figure 1B). The upper quarter of the hypocotyl, termed zone 1, had the highest relative elemental growth rate (REGR) at either time point (Figure 1C). The second quarter, termed zone 2, displayed a 50% decrease in the REGR compared with zone 1 (Figure 1C), and in the lower half of the hypocotyl (zones 3 and 4) the growth rate declined to near zero. Henceforth, we will refer to zone 1 as the rapid elongation zone, since cells in this part of the hypocotyl showed the highest elongation rate.

To explore how cortical microtubule organization might relate to these differing growth rates, we quantified the orientations of cortical microtubules (hereafter simply referred to as microtubules) in living cells expressing green fluorescent protein–tagged α-tubulin 6 (GFP-TUA6) or Arabidopsis end binding protein 1a (EB1-GFP). Global microtubule orientations per cell were calculated from measurements of all visible microtubules (see Methods and Supplemental Figure 2 online). As predicted by Green’s hypothesis (Green, 1962) and based on previous reports (Le et al., 2005; Gu et al., 2008), we expected that the rapidly elongating cells in zone 1 would have primarily transverse microtubules.
However, only a subpopulation of the epidermal cells in zone 1 exhibited transverse alignment of microtubules on their outer surface (Figure 2A). A collar of cells with transversely aligned microtubules was observed to encompass the entire circumference of the hypocotyl; however, this collar was surprisingly short, never exceeding two cells in a file along the length of the hypocotyl (see below; Figure 3). The cells with synchronized transverse microtubules were found ~1.2 mm below the apical hook (for a total hypocotyl length of 9.35 ± 0.3 mm). Immediately above or below this region, microtubule orientations were variable and unsynchronized between cells (Figures 2B and 3). The pooled measurements of microtubule orientations from all cells in zone 1 were plotted to visualize the distribution of angles (Figure 2C). The peak in the distribution corresponded to the transverse angles, indicating that transversely oriented microtubules were slightly more abundant in this population of cells. Cells just a few millimeters farther from the apical hook, in zone 2, were still elongating with an REGR of 0.10 h⁻¹ (Figure 1C). However, the microtubule orientations on the outer surface were unsynchronized among these cells and variable. The plot of the distribution of all measured microtubules illustrated the absence of a dominant peak at the transverse angles (Figure 2D).

We next examined how the situation evolved over the course of the next 5 h. Although the hypocotyl continued to elongate at a high rate of 345 μm/h at 76 HAI (Figure 1A) and zone 1 exhibited a high REGR of 0.16 h⁻¹ (Figure 1C), the cells with synchronized transverse microtubules was no longer present. At this time point, only 23% of the cells in zone 1 had transverse microtubules (Table 1). Relatively equal proportions of cells with transverse, oblique, and longitudinal microtubule arrays were found in the rapid elongation zone (Table 1). Indeed, the pooled microtubule measurements from all cells in this zone yielded an isotropic distribution of orientations, where no statistically significant peaks could be detected (Figures 4A and 4B). Total microtubule orientations on the outer face were isotropic both in the small cells in the apical hook (Figure 4A) and in the cells of the rapid elongation zone (Figure 4B). Considered together, these results indicate that transversely oriented microtubules do not always predominate on the outer face of rapidly elongating cells.

We also analyzed microtubule orientations in other regions of the hypocotyl. Cells in zone 2 likewise showed no bias toward transversely oriented microtubules (Figure 4C, Table 1). Finally, microtubules in cells nearer the base of the hypocotyl (zones 3 and 4), which at this stage had ceased elongation (Gendreau et al., 1997), showed a strong, statistically significant bias toward longitudinal orientations (Figure 4D, Table 1). Together, our data show that at 76 HAI, no zone with a preferential transverse microtubule orientation could be observed on the outer face of the epidermis, contrary to expectations based on the relatively high elongation rate of these hypocotyls (Figure 1A).

It has been convincingly demonstrated that microtubules guide the movement of CSCs in etiolated hypocotyl epidermal cells (Paredez et al., 2006). Consequently, we expect that the trajectories of CSCs on the outer epidermal face should also be widely distributed over many angles. To test this hypothesis, we acquired time series of the movement of GFP-CESA3–labeled complexes (Desprez et al., 2007) at 76 HAI (see Supplemental Figure 3 online). In data from 15 plants, only 20 cells out of 113 (18%) showed transverse CSC trajectories (between 22.5° and –22.5°) on the outer surface of zone 1. These results further support the close relationship between CSC movement and microtubule orientation. In conclusion, cellulose microfibrils are not deposited strictly in a transverse orientation on the outer wall of elongating cells.

**Orientations of Cortical Microtubules, CSC Trajectories, and Cellulose Microfibrils on the Inner Face of the Hypocotyl Epidermis**

We next asked if microtubule organization on the inner epidermal face is correlated with the growth anisotropy of the hypocotyl. Remarkably, microtubules on the inner face in zone 1 at 76 HAI were consistently found in a transverse orientation (22.5° to –22.5°) (Table 2, Figure 4F) and were particularly well aligned and parallel to each other (average variance per cell = 427, compared with 2508 for the outer face). Cells in the apical hook likewise had well-aligned, transverse microtubules (Table 2, Figure 4E). Every cell examined throughout zone 1 had a statistically significant bias toward the transverse orientation (Table 2). Even the more slowly elongating cells in zone 2 (Figure 1C) exhibited a strong bias toward transverse microtubules on the inner face (Figure 4G). This result was further confirmed for elongating cells at 71 HAI (Figures 2E, 2F, and 3). The only cells in which transverse microtubules did not predominate on the inner face were the cells in zones 3 and 4 at the base of the hypocotyl (Figure 4H), which grew slowly or had stopped growing (Figure 1C). In sum, only a small fraction of elongating cells had transversely oriented microtubules on the outer face, while all elongating cells had transversely oriented microtubules on the inner face (Figure 4). This indicates that microtubule orientation is consistently biased toward the transverse on the inner epidermal face in elongating cells, while the orientation varies widely on the outer face and is not correlated with growth.

The transgenic lines and imaging methods used did not allow the visualization of the microtubule orientation in cortical tissue layers. We therefore performed antitubulin immunolabeling of longitudinal sections through the hypocotyl. In all sections, a transverse microtubule alignment was observed in the cortical cells (see Supplemental Figure 4 online), thus confirming previous observations for microtubules (Furutani et al., 2000) and cellulose microfibrils (Refregier et al., 2004) in this cell type.

In addition to drawing inferences from populations of cells, we also acquired confocal z-stacks of entire cells to determine if outer and inner surfaces shared the same alignments (175 cells). No correlation was found between the preferred orientation on either face of the epidermis in zone 1 (Figures 5A and 5B), although some microtubules were longitudinal or oblique on both faces of the cell in the mature cells of zones 3 and 4 (Figures 5C and 5D). By generating orthogonal projections of the z-stacks, we could observe continuous, presumably bundled microtubules that extended from the outer face to the inner face (Figure 6A). Microinjection of fluorescent tubulin and immunofluorescence staining of tubulin in other plant species led to similar observations (Flanders et al., 1989; Yuan et al., 1995). While the microtubules facing the inner tangential and anticlinal walls exhibited similar orientations, the curvature of the continuous
Figure 2. Microtubules at the Inner Face Align Independently of Those on the Outer Wall of Etiolated Hypocotyls.

Quantifications were performed in zones 1 and 2 of etiolated hypocotyls at 71 HAI in GFP-TUA6–expressing plants. Bars = 10 μm.

(A) A collar of synchronized cells with transversely oriented microtubules is found in zone 1.

(B) Cells just below those pictured in (A) lack synchronization and have variably oriented microtubules.

(C) and (D) Distributions of microtubule orientations at 71 HAI, in zone 1 on the outer epidermal face ([C], 34 cells from nine plants) and in zone 2 on the outer epidermal face ([D], 53 cells from 14 plants). The total number of microtubules measured (N) is indicated in the top right corner of each graph.

Angles corresponding to the transverse orientation (22.5° < θ > 22.5°) are highlighted in gray at the center of each distribution (see Supplemental Figure 2 online for the definition of orientations). In zone 2, no dominant peak is found at the transverse angles, and the distribution is relatively isotropic.

(E) Transversely oriented microtubules on the inner face in zone 1 at 71 HAI.

(F) The distributions of orientations of microtubules on the inner epidermal face in zones 1 and 2 (pooled measurements from 94 cells in 11 plants). The total number of microtubules measured (N) is indicated in the top right of each graph. Angles corresponding to the transverse orientation (22.5° < θ > 22.5°) are highlighted in gray at the center of the graph.
bundles changed abruptly at the limit between the outer face and anticlinal walls (Figure 6A). It seems therefore unlikely that microtubule orientation is regulated by a property of the microtubules themselves and suggests that their orientation may be regulated by a property of the local environment.

Differences in microtubule orientation on the outer and inner faces were consistent regardless of whether we visualized the GFP-TUA6 marker or the EB1-GFP marker (Figure 6B). Interestingly, our observations of EB1-GFP served to demonstrate that microtubules are also highly dynamic on the inner face. We quantified the velocity of EB1 plus-end comets on both faces of the epidermis (Figure 6C). The mean velocity on the outer face was $5.4 \pm 1.5 \mu m/min$ ($n = 1119$ comets), similar to previous reports (Chan et al., 2003; Crowell et al., 2009). Similarly, the mean velocity on the inner face was $5.2 \pm 1.5 \mu m/min$ ($n = 1825$ comets), which was not significantly different from the velocity measured on the outer face at a 99% confidence interval. Together, this indicates that a mechanism must continually operate to orient nascent microtubules in the transverse orientation on the inner face, but the dynamics of microtubule polymerization are unlikely to be at the origin of these differences in orientation.

We next sought to determine if CSC trajectories on the inner epidermal face reflect the observed orientations of microtubules. In 10 independent experiments (15 cells from 12 different plants), and regardless of the orientation of the trajectories on the outer face (Figure 7A), we consistently observed CSCs migrating along perfectly transverse trajectories on the inner face of cells in the rapid elongation zone (Figure 7B). This result suggests that cellulose microfibrils are deposited in the transverse orientation on the inner face of these cells.

We further investigated the organization of the cellulose microfibrils themselves using scanning electron microscopy and FESEM, techniques that permit visualization of cellulose microfibril orientation in the most recently deposited cell wall layer. Transverse arrays of cellulose microfibrils were evident on the inner walls of epidermal cells in zones 1 and 2 (see Supplemental Figure 5 online), in accordance with previously published results (Sugimoto et al., 2000; Refrégier et al., 2004). We were able to occasionally obtain epidermal cells in which the wall had torn open and was retained after fixation (Figure 7C). In these cases, it was possible to visualize the cellulose microfibrils on all faces of the same cell, from the outer wall to the inner wall (Figure 7D). Interestingly, cellulose microfibrils could be found in oblique or longitudinal orientations on the outer wall, quickly transitioned through mixed orientations, and finally were found in perfectly transverse orientations on the inner wall and portions of the radial side wall (Figure 7D). These findings confirm the predictions based on our measurements of microtubule orientation and illustrate that cellulose microfibrils can be deposited in strikingly different orientations on the outer and inner walls of the same cell.

**Relationship between Cortical Microtubules in the Inner Tissues and Cell Expansion**

Our data indicate that deposition of transversely oriented cellulose microfibrils in the outer epidermal wall is spatially and

| Table 1. Results of the Classification of Individual Cells According to the Major Orientation of Their Microtubules at 76 HAI in the Outer Face |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Outer Face      | Apical hook     | Zone            | Longitudinal Oblique | Transverse Oblique + Mixed |
| Apical hook     | 12 (17%)        | 1               | 14 (20%)           | 17 (24%)         |
| Zone            | 1               | 14 (20%)        | 14 (20%)           | 16 (23%)         |
|                 | 2               | 11 (16%)        | 16 (23%)           | 14 (20%)         |
|                 | 3 and 4        | 12 (26%)        | 12 (26%)           | 10 (23%)         |
|                 | 49 (83%)        | 3               | 5 (8%)             | 1 (2%)           |
|                 | 5 (8%)          | 1 (2%)          | 3 (5%)             | 1 (2%)           |

See Supplemental Figure 2 online for a definition of the orientations denoted in the tables. Each cell was classified by examination of its microtubule distribution and by testing if any major peaks were significant (see Methods). The number of cells in each class is noted, with the percentage of the total number of cells shown in parentheses. Cells with more than one peak in their distribution were counted according to which peak was significant or counted as “mixed” if two peaks were significant.
temporally restricted. By contrast, deposition of transverse cellulose microfibrils is maintained in the inner epidermal wall over a much larger region of the hypocotyl for longer periods of growth. The microtubule orientation on the inner face is perpendicular to the cell elongation axis, as would be predicted for elongating cells. Furthermore, the degree of anisotropy on the inner face is closely correlated with the growth status of the cells. Deposition of transversely oriented cortical microtubules on the inner epidermal wall precedes rapid cell elongation (in the apical hook, Figure 4E) and is maintained during cell elongation (Figures 4F and 4G), but gradually dissipates as the cells cease to grow (Figure 4H).

These findings support the hypothesis that the walls inside the organ primarily control the growth direction, whereas the extensibility of the thick external epidermal walls limits growth independently of the growth direction. To test this hypothesis, one has to envisage a treatment that reduces the anisotropy of inner walls, including the internal tangential epidermal wall, without changing the outer epidermal wall. Such a treatment should lead to increased radial organ expansion or not depending on whether the growth anisotropy is regulated by the inner or outer walls of the organ, respectively. Previous observations of changes in microtubule organization in plants expressing a GFP-fused microtubule binding domain of the mammalian microtubule-associated protein 4 (GFP-MBD) prompted us to examine in detail transgenic lines stably overexpressing this protein (Marc et al., 1998). On the outer epidermal face, microtubules in GFP-MBD–expressing plants showed, like the GFP-TUA6 or EB1-GFP control lines at the same growth stage, parallel arrays that were widely distributed over all angles, but with a slight bias toward negative oblique angles (Figure 8A). By contrast, on the inner epidermal face of the GFP-MBD line, microtubule orientations showed a highly reduced anisotropy: they were less transverse and less parallel than in the GFP-TUA6 line (average variance per cell = 911 for GFP-MBD and 427 for GFP-TUA6), with a bias toward oblique angles (Figure 8B, compare with Figure 5B). In conclusion, overexpression of GFP-MBD reduced the anisotropy

![Figure 4. Microtubules Are Transverse on the Inner Face of Elongating Cells.](image)

The distribution of orientations of microtubules at 76 HAI on the outer epidermal face ([A] to [D]) and inner epidermal face ([E] to [H]; see Supplemental Figure 1 online). The distributions present pooled measurements of microtubules from multiple cells in multiple plants (outer face: n = 243 cells, n = 33 plants; inner face: n = 205 cells, n = 28 plants). The total number of microtubules measured (N) is indicated in the top right corner of each graph. The growth zones correspond to those outlined in Figure 1B. Microtubule orientations are shown for the apical hook region of zone 1 ([A] and [E]), the rapid elongation zone ([B] and [F]), zone 2 ([C] and [G]), and zones 3 and 4 ([D] and [H]). Angles corresponding to the transverse orientation (22.5° ± θ > ±22.5°) are highlighted in gray at the center of each distribution (see Supplemental Figure 2 online for the definition of orientations).

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<th>Table 2. Results of the Classification of Individual Cells According to the Major Orientation of Their Microtubules at 76 HAI in the Inner Face</th>
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expansion in GFP-MBD hypocotyls compared with wild-type and GFP-TUA6 lines (Figures 8D and 8E; see Supplemental Figure 6 online), suggesting that the anisotropy of internal cell walls is indeed required to maintain the growth direction of the organ. Cortical cells showed the largest surface increase (75%) on sections, compared with epidermal cells, which showed a 50 or 20% surface increase, respectively (Figure 8E), depending on whether or not they faced anticlinal cortical walls (Figure 8F). These increases in radial expansion were accompanied by a reduced cell elongation (in zone 1, mean cell length GFP-MBD = 233 μm; GFP-TUA6 = 312 μm; P value = 10^{-12}) and a reduced hypocotyl length (mean length GFP-MBD = 8.62 mm; GFP-TUA6 = 9.35 mm; P value = 0.01).

DISCUSSION

In this report, we provide a full characterization of microtubule orientations, CSC trajectories, and newly deposited cellulose microfibril orientations in the epidermis of Arabidopsis etiolated hypocotyls. This led to a number of interesting observations: First, we observed a perfect parallel between the orientation of microtubule orientations, CSC trajectories, and newly deposited cellulose microfibrils on the external and internal faces of the epidermal cells. Although technical limitations prevented us from being able to visualize all three components simultaneously, our quantitative statistical approach allowed us to draw firm conclusions. These new data confirm and extend previous findings that microtubules guide the trajectories of GFP-labeled CSCs, which in turn orient the deposition of the microfibrils. By imaging CSCs and microtubules on the inner epidermal surface, we were able to visualize cellulose deposition on the same wall surface that has been characterized by FESEM (Sugimoto et al., 2000; Refrégier et al., 2004). It should be kept in mind that after deposition, the orientation of microfibrils could change during growth according to the multinet growth theory (Roelofsen and Houwink, 1953). This is also true for the Arabidopsis dark-grown hypocotyl, in which, during a rapid elongation phase, the outer cell wall thins and cellulose microfibrils in older wall layers reorient toward a longitudinal orientation (Refrégier et al., 2004; Derbyshire et al., 2007; Anderson et al., 2010).

Second, microtubules, CSC trajectories, and newly deposited cellulose microfibrils on the outer wall of epidermis cells did not show a preferential orientation during most of the elongation period. This fits with observations on pea (Pisum sativum) internodes, dandelion (Taraxacum officinale) peduncles, oat (Avena sativa) coleoptiles, and soybean (Glycine max) hypocotyls, which showed that these organs were able to elongate despite a net longitudinal orientation of cellulose microfibrils in the outer epidermal walls (Iwata and Hogetsu, 1989; Paolillo, 2000). This is also in agreement with the recent study on the mor1 mutant showing that there are different orientations of cellulose microfibrils in outer walls (Fujita et al., 2011). In addition, the outer walls of the hypocotyl epidermis in multiple species have been shown to consist of layers of cellulose in a complete range of orientations (Roland et al., 1975; Neville and Levy, 1984; Refrégier et al., 2004; reviewed in Neville et al., 1976). A recent study on light-grown Arabidopsis seedlings shows that microtubules on the...
outer face of the epidermis and cellulose synthase trajectories in the plasma membrane cycle through a number of different orientations, thus producing a polylamellate cell wall architecture (Chan et al., 2010). The rotary movements of the microtubule array have also been proposed for the hypocotyl epidermis of etiolated sunflower (Helianthus annuus; Hejnowicz, 2005). Although observation of long-term microtubule behavior is not feasible on dark-grown seedlings, our observations of the wide distribution in angles on the outer face of elongating epidermal cells suggest that microtubules may also undergo rotary movements in etiolated conditions and thus generate the polylamellate external cell wall found in immature hypocotyl epidermal cells.

Third, and this is the most surprising finding, growing epidermal cells are polarized in the orientation of microtubules, with no preferential orientation on the outer face and a strictly transverse orientation on the inner face in elongating cells. Differences in microtubule orientation on opposing faces of the epidermis have been noted previously (Busby and Gunning, 1983; Flanders et al., 1989; Yuan et al., 1995; Hejnowicz et al., 2000; Barton and Overall, 2010), but their relationship to organ expansion was not established. As discussed below, the transverse alignment of microtubules on the inner epidermal face has implications for the regulation of the growth anisotropy of the organ and raises new questions on the establishment and maintenance of such a polarity in the epidermis.

**Regulation of Growth Anisotropy by Inner Tissues and Not by Outer Epidermal Walls in the Hypocotyl**

The finding that microtubules and microfibrils are transversely oriented on the inner epidermal wall (Figures 4 and 7) extends previous observations that microtubules (Furutani et al., 2000) and newly deposited microfibrils (Refrégier et al., 2004) are transverse in growing cortical cells of the Arabidopsis hypocotyl. Transversely oriented microfibrils have also been observed in the parenchyma of other species (Hogetsu, 1986; Iwata and Hogetsu, 1989; Abe et al., 1995; Baskin et al., 1999; Crow and Murphy, 2000). Likewise, in Arabidopsis roots, transversely
aligned cellulose microfibrils are found on the inner epidermal walls in elongating cells (Sugimoto et al., 2000). However, cortical microtubule organization seems to be distinct in roots and shoots, since microtubules were shown to have a transverse orientation on both inner and outer faces of the root epidermis throughout the cell elongation zone (Sugimoto et al., 2000). When the inner tissue layers are separated from the epidermal layer, the inner tissues have a tendency to elongate, while the epidermis bends toward its outer surface (reviewed in Kutschera and Niklas, 2007). This can be illustrated both in isolated tissues and in epidermal cells of Arabidopsis mutants with cell adhesion defects (Bouton et al., 2002). These observations form the basis of the tissue-tension model of growth (Peters and Tomos, 1996). This model posits that the highly anisotropic cell walls inside the organ transmit a large axial force to the epidermis, which drives elongation of the organ despite an outer cell wall that lacks anisotropy (Baskin, 2005). Interestingly, consistent with a critical role for internal tissues in the regulation of growth directionality, hypocotyl cortex and endodermis show increased radial expansion in mutants for the microtubule-associated protein SPIRAL1, in which the orientation of microtubules is selectively perturbed in ground tissues (Furutani et al., 2000). Furthermore, we showed...
Figure 8. Microtubule Orientation Is Perturbed on the Inner Epidermal Face of GFP-MBD-Expressing Plants.

(A) and (B) Extended depth of field images of GFP-MBD–labeled microtubules on the outer (A) and inner (B) epidermal face of the same cells in zone 1. The microtubules are abnormally oriented in oblique angles on the inner face (B; compare with Figure 5B, right panel). Bar = 10 μm.

(C) The distribution of orientations of microtubules on the outer and inner epidermal face in zone 1 of GFP-MBD–expressing plants at 76 HAI (pooled measurements of 78 cells from three plants). The total number of microtubules measured (N) is indicated in the top right of each graph. Angles corresponding to the transverse orientation (22.5° ± 6 > 22.5°) are highlighted in gray at the center of each distribution. The microtubules on the inner face of the GFP-MBD line are less parallel and more obliquely oriented than in the GFP-TUA6 line (cf. with Figure 4F).

(D) Photographs of hypocotyl cross sections from a Wassilewskija wild-type (top) and GFP-MBD line (bottom). The asterisk shows an epidermal cell facing an anticlinal cortical wall. Bar = 100 μm.

(E) Distributions of epidermal and cortical cell surface areas measured in the Wassilewskija wild-type (wt) and GFP-MBD line (n = 75 to 160 cells, n = 4 plants). Note the significant overall increase in cortical cell surface area in the GFP-MBD line.

(F) Distributions of epidermal cell surface areas for the subpopulation facing anticlinal inner walls (n = 67 cells, n = 4 plants). This subpopulation of epidermal cells undergoes radial swelling in the GFP-MBD line.
in this study that expression of GFP-MBD preferentially perturbed microtubule anisotropy at the inner epidermal face and caused increased radial expansion of the organ. It is interesting to note that epidermal cells in this line also showed increased radial expansion. The extent of the radial expansion (50 or 20% depending on whether or not they faced anticlinal cortical walls) was less pronounced than that of the cortical cells (75%) and was proportional to the fraction of the cell surface that is exposed to the highly anisotropic inner periclinal walls (41 ± 0.03% [SE] and 26 ± 0.03% of their perimeter for the two classes of epidermal cells). These observations are consistent with the hypothesis that the reduced anisotropy of the inner periclinal walls of the epidermis and cortex in the GFP-MBD line is responsible for the increased radial expansion of the organ.

In conclusion, the results support the hypothesis that the directionality of organ expansion is regulated by the highly anisotropic walls inside the organ rather than the outer epidermal walls. In this view, the controlled yielding of the thick external wall, in part through the secretion of wall remodeling agents (Pelletier et al., 2010), will regulate the growth rate of the organ independently of the growth direction. While this manuscript was under review, Chan et al. (2011) published a similar study on light-grown hypocotyls with essentially the same conclusions as our study on dark-grown hypocotyls. They invariably observed a transient transverse orientation of the cortical microtubules facing the outer tangential wall, concomitant with growth acceleration. In our study, however, we showed that rapid elongation growth could occur even in the absence of such a transient fixation of the transverse orientation on the outer face. In addition, we further corroborate the proposed model for the regulation of growth anisotropy of the hypocotyl by the experimental perturbation of the cortical microtubules facing the inner tangential epidermal wall.

**The Epidermis Is Polarized for Cortical Microtubule Orientation**

One intriguing question raised by this study is how the coordinated transverse alignment of microtubules on the inner face is maintained, while microtubules cycle between different orientations on the outer face. This is not related to differences in the dynamics of microtubule polymerization, since no difference in the motility of the plus end marker EB1-GFP could be observed on the two faces of the epidermal cells. It is also unlikely to be related to intrinsic properties of the microtubules themselves, since the same microtubules can be visualized extending from the outer to inner face. It is possible that mechanical force fields may underlie the synchronous alignment of microtubules transverse to the elongation axis in cortical cells and on the inner epidermal face. For example, in the epidermis of the shoot apical meristem, microtubules align parallel to the axis of maximal stress, resulting in the coordinated alignment of microtubules (Hamant et al., 2008). Microtubules in peeled epidermis were also found to reorient depending on the stress anisotropy applied to the cells (Hejnowicz et al., 2000). Such a mechanical feedback mechanism would explain the maintenance of the cell wall anisotropy of the inner cell walls, but it does not explain the establishment of the transverse orientation of microtubules in inner layers while excluding the outer face. In etiolated hypocotyls, the establishment of the transverse orientation appears to take place well before elongation, since we consistently found that cells in the apical hook also showed transverse microtubules on the inner face before elongating (Figure 4E). Interestingly, the situation in light-grown hypocotyls appears to be different, since microtubules initially rotate on the inner and outer faces preceding elongation (Chan et al., 2011).

In conclusion, our quantitative analysis of microtubules, CSC trajectories, and cellulose microfibril orientations on both the outer and inner faces of the shoot epidermis presents a coherent model for the regulation of growth anisotropy in this tissue. It will now be interesting to see how and when exactly during hypocotyl development the epidermis becomes polarized in its cortical microtubule orientation.

**METHODS**

**Plant Material and in Vitro Growth Conditions**

Transgenic Arabidopsis thaliana plants expressing GFP-TUA6 (Ueda et al., 1999), end binding protein 1a fused to GFP (EB1-GFP; Chan et al., 2003), GFP fused to the cellulose synthase catalytic subunit 3 (GFP-CESA3; Desprez et al., 2007), or GFP fused to the microtubule binding domain of microtubule-associated protein 4 (GFP-MBD; Marc et al., 1998) were sown in chambers, as previously described (Chan et al., 2007), or on plates containing Estelle and Somerville medium (Estelle and Somerville, 1987) without Suc. Seeds were subjected to cold treatment at 4°C for 48 h, exposed to fluorescent white light for 5 h, and cultured for an additional 66 or 73 h in darkness at 20°C. The number of hours after induction was counted from the start of the light exposure. The GFP-TUA6, EB1-GFP, and GFP-CESA3 transgenic lines have been extensively studied previously and exhibit growth patterns indistinguishable from those of the wild type (Ueda et al., 1999; Chan et al., 2003; Desprez et al., 2007).

**Cell and Hypocotyl Length Measurements**

Hypocotyl length measurements were performed on seedlings grown for 71 or 76 h in darkness on Estelle and Somerville medium (Estelle and Somerville, 1987) without Suc. Hypocotyl length was measured on photos in ImageJ. To determine the REGR, the hypocotyls were photographed under infrared light using a Nikon D70 camera with a 50-mm macro-objective, from 71 to 73 HAI or from 76 to 78 HAI. Each hypocotyl was divided into four equivalent zones: the uppermost quarter including the apical hook (zone 1), the second quarter (zone 2), and the lower half (zones 3 and 4). In each zone, the strain was determined by measuring the change in distance between two arbitrarily chosen natural marks at the hypocotyl surface (i.e., anticlinal cell walls) at the two time points, divided by the distance at the first time point. GFP-MBD and GFP-TUA6 seedlings were visualized using a Leica DMRB microscope. Cell lengths in zone 1 were measured using ImageJ.

**Cell Wall Preparation and FESEM**

FESEM was performed as described for Figure 6B (Refrégier et al. 2004). For Figure 7D, seedlings were treated as described by Sugimoto et al. (2000), except for the fixation (seedlings were incubated in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at room temperature for at least 3 h and then seedlings were incubated in 20% Suc for at least 1 h). For scanning electron microscopy, 3-d-old etiolated seedlings were imaged with a Hron SH-1500 (Horx Europe-JYe1) microscope at -30°C.
Image contrast was improved using Photoshop CS 8.0.1 (Adobe Systems).

Immunolocalization Using Indirect Immunofluorescence Analysis

Seedlings were fixed in 1.5% formaldehyde, 0.5% glutaraldehyde, and 0.05% triton in microtubule-stabilizing buffer (MTSB) and put under vacuum for 2 h. Samples were dehydrated, waxed, and dewaxed as described (Masclaux-Daubresse et al., 2006). Longitudinal sections (12 μm) were prepared using a microtome (Jung RM2055; Leica) and air-dried on polylysine-coated glass slides. Samples were blocked with 5% (w/v) BSA in PBS and incubated with mouse antibody against tubulin (Monoclonal B5 1-2; Sigma-Aldrich) diluted at 1:1000. After three washes of 5 min each in PBS, slides were incubated with goat anti-mouse IgG conjugated with Alexa 488 (Molecular Probes) diluted at 1:200.

Cross Sections and Surface Area Measurements

Seedlings were fixed in 2% (w/v) formaldehyde, 1% (w/v) glutaraldehyde, and 1% caffeine and put under vacuum for 15 min. Seedlings were embedded in historesin (Technovit 7100; Kulzer) following the manufacturer’s instructions. Sections 5 μm thick were cut using a microtome (Jung RM2055; Leica). The cross sections were visualized using a Leica DMRB microscope. Surface areas were measured using ImageJ.

Spinning Disk Microscopy

Imaging of 3-d-old, live seedlings was performed on an Axiovert 200M microscope (Zeiss) equipped with a Yokogawa CSU22 spinning disk, Zeiss x100/1.4 numerical aperture oil objective, and Andor EMCCD iXon DU 895 camera (Plateforme d’Imagerie Dynamique, Institut Pasteur) or on an Axio Observer Z1 Zeiss microscope (Zeiss) equipped with a Yokogawa CSU-X1 spinning disk, Zeiss ×100/1.4 numerical aperture oil objective, and Roper EMCCD Quantum 512C (Plateforme d’Imagerie Institut Jean-Pierre Bourgin-Institut National de la Recherche Agronomique, Versailles). A 488-nm diode-pumped solid-state laser was used for excitation of GFP, and emission was collected using a band-pass 488/25 filter (Semrock). Z-stacks were acquired with an interval of 0.3 to 0.5 ms of GFP, and emission was collected using a band-pass 488/25 filter (Semrock). A 488-nm diode-pumped solid-state laser was used for excitation. P. Bourgin-Institut National de la Recherche Agronomique, Ver-

extended depth of field images were prepared using an ImageJ plugin (Biomedical Imaging Group; Forster et al., 2004). Figures and drawings were prepared with Photoshop CS 8.0.1 (Adobe Systems).

Statistical analysis of microtubule orientations was performed using the Physics Analysis Workstation (PAW) software (CERN). Distributions of all microtubule orientations for each cell were generated using PAW. Each cell was classified according to the major orientation of its microtubules using a statistical test based on the position and height of the peaks in the distribution (see Supplemental Figure 3 online for the definition of orientations). Statistically significant peaks or troughs in the distributions were detected using a binning of 18. A peak or trough was considered significant if the difference of the absolute value of the count in that bin and the mean count was >2 times the SD (σ). The statistical test was calibrated visually by examining the distributions of microtubules for each individual cell and the raw images. Only cells for which 30 or more measurements could be obtained were analyzed.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Cross Section through an Arabidopsis Hypocotyl, Showing the Two Distinct Regions of the Epidermis Studied in This Report.

Supplemental Figure 2. Definition of Angles of Orientation Relative to the Cell Long Axis.

Supplemental Figure 3. Representative Average Projected 10-min Time Series Acquired at a 20-s Interval, Showing the Trajectories of GFP-CESA3–Labeled Cellulose Synthase Complexes on the Outer Face of Zone 1 Epidermal Cells at 76 HAI.

Supplemental Figure 4. Representative Maximum Projected z-Stack Showing Antitubulin Immunostaining of Cortical Cells in Zone 1 of the Hypocotyl at 76 HAI.

Supplemental Figure 5. Representative FESEM Images of Transverse Cellulose Microfibrils on the Inner Wall of an Epidermal Cell in Zone 1 (Left) and Zone 2 (Right) at 76 HAI (Courtesy of G. Refrégier).

Supplemental Figure 6. Scanning Electron Microscopy Images of 3-d-Old Etiolated Hypocotyls of Arabidopsis, Expressing Either GFP-TUA6 or GFP-MBD.

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Differential Regulation of Cellulose Orientation at the Inner and Outer Face of Epidermal Cells in the Arabidopsis Hypocotyl

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