Microtubules and CESA tracks at the inner epidermal wall align independently of those on the outer wall of light-grown Arabidopsis hypocotyls

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Summary
Microtubules are classically described as being transverse, which is perpendicular to the direction of cell elongation. However, fixation studies have indicated that microtubules can be variably aligned across the epidermis of elongating shoots. In addition, microtubules are reported to have different orientations on inner and outer epidermal surfaces, undermining the idea of hoop-reinforcement. Here, long-term movies of Arabidopsis seedlings expressing GFP–TUA6 allowed microtubule alignment to be directly correlated with the rate of elongation within individual growing cells. We also investigated whether microtubule alignment at the inner or the outer epidermal wall better reflected the growth rate. Movies confirmed that transverse microtubules form on the inner wall throughout elongation, but orientation of microtubules is variable at the outer wall, where they tend to become transverse only during episodes of accelerated growth. Because this appears to contradict the concept that circumferential arrays of transverse microtubules or microfibrils are essential for cell elongation, we checked the organisation of cellulose synthase tracks using GFP–CESA3 and found a similar mismatch between trajectories on inner and outer epidermal surfaces. We conclude that microtubule alignment on the inner wall appears to be a more stable predictor of growth anisotropy, whereas outer-wall alignment is more sensitive to the elongation rate.

Key words: Microtubules, Cell wall, Cellulose microfibrils, Cellulose synthases, Cell elongation

Introduction
When they were first seen in the electron microscope, cortical microtubules were described as transverse ‘hoops’, matching the alignment of overlying cellulose microfibrils in the cell wall (Ledbetter and Porter, 1963). This agreed with the biophysical concept of hoop-reinforcement (Green, 1962), in which transversely aligned, load-bearing cellulose microfibrils maintained the girth of the expanding cell, directing growth into elongation orthogonal to the net alignment. This picture seems to apply to the root where microtubules are transverse in rapidly elongating cells, reorienting to oblique or longitudinal as growth declines (Baskin, 2001; Granger and Cyr, 2001; Sugimoto et al., 2000). However, reports suggest that the situation is not so straightforward in shoots, where cortical microtubules beneath the outermost epidermal wall can be oblique or longitudinal or mixed, as well as transverse, apparently during cell elongation (Duckett and Lloyd, 1994; Le et al., 2005; Sawano et al., 2000; Takeda and Shibaoka, 1981; Yuan et al., 1995; Zandomeni and Schopfer, 1993).

Various studies have indicated that rather than representing stable configurations, microtubule arrays in shoots might undergo a regular programme of reorientation (e.g. Mayumi and Shibaoka, 1996). Microtubules in pea shoot epidermal cells, which had been microinjected with fluorescent tubulin, have been seen reorienting from transverse to longitudinal (Yuan et al., 1995) and in the reverse direction upon addition of gibberellic acid (Lloyd et al., 1996). In their immunofluorescence studies on mung bean shoots, the Shibaoka laboratory observed longitudinal or oblique, as well as transverse microtubules in elongating tissue and suggested this variability to be an expression of a cyclic reorientation physiologically regulated by a range of hormonal factors (Mayumi and Shibaoka, 1996; Takesue and Shibaoka, 1998). In his immunofluorescence study of rapidly elongating epidermal cells in the sunflower hypocotyl, Hejnowicz (Hejnowicz, 2005) also saw microtubule arrays in a variety of alignments. He hypothesised that the variability of alignment reflected stages sampled from a rotational clock-like cycle, rather than the oscillatory cycle proposed by the Shibaoka laboratory. Chan and colleagues (Chan et al., 2007) then made long-term movies of Arabidopsis hypocotyl epidermal cells, confirming that microtubules beneath the outer epidermal wall do indeed undergo both discontinuous shifts (‘jumps’) in alignment and continuous rotary movements in a clockwise or anticlockwise direction (which are collectively termed ‘persistent reorientation’). However, neither rotations like this, nor Shibaoka-like oscillatory cycles have been seen in living Arabidopsis root epidermal cells imaged with GFP–MBD (Granger and Cyr, 2001), underlining the differences between roots and shoots.

Similarly to microtubules, cellulose microfibrils are classically considered to be transverse in rapidly elongating tissue, providing hoop-reinforcement during expansion. Baskin (Baskin, 2005), however, concludes that the behaviour of the stem epidermis is ‘paradoxical’ because the epidermis of aerial tissue has been
reported to contain longitudinal microfibrils, even in rapidly elongating areas. Non-hoop-like behaviour is also suggested by reports that microtubules on the outer epidermal surface are not necessarily co-aligned with microtubules on the inner epidermal surface (Busby and Gunning, 1983; Flanders et al., 1989).

This major difference in apparent behaviour of cortical microtubules in shoot and root tissues prompted us to look at the relationship between the alignment of microtubules and cell elongation in light-grown hypocotyls, paying attention to both outer and inner tangential walls of epidermal cells. Arabidopsis seedlings expressing GFP–TUA6 were grown in special growth chambers (Chan et al., 2007) and live changes in microtubule alignment were monitored over several hours. In this way, instead of basing conclusions on populations of cells with potentially unsynchronised microtubule orientations, it was possible to dynamically correlate the behaviour of microtubules with the variable growth rate for up to 24 hours at a time, within individual cells. This showed that microtubules on the outer epidermal wall behaved differently to microtubules on the inner wall of the same cell; a finding that we confirmed with a cellulose synthase marker. This allows us to discuss the role of microtubules in growth anisotropy.

Results
Relationship between growth and microtubule alignment at the outer epidermal face
In a previous study (Chan et al., 2007), persistent microtubule reorientation was observed in Arabidopsis seedlings expressing GFP–EB1a. Here, we used the same experimental procedure except that microtubules in 2- to 5-day-old seedlings were labelled with GFP–TUA6 (Ueda et al., 1999). This probe maintains bright fluorescence, enabling confocal z-stacks of epidermal cells in the

![Fig. 1. Changes in microtubule alignment during elongation of epidermal cells of Arabidopsis hypocotyl expressing TUA6–GFP.](image)
middle portions of hypocotyls to be captured every 15–30 minutes for up to 24 hours.

Three different general patterns of microtubules were seen at the outer epidermal surface of the hypocotyl, which appeared to change in a growth-related fashion. Most commonly, microtubule alignment differed between neighbouring cells (Fig. 1A). This particular pattern was associated with relatively slow growth rates (average cellular elongation rate of 1.27µm/hour; s.d.=1.4, s.e.=0.14 or an average relative elongation rate of 0.02/hour; s.d.=0.02, s.e.=0.002, n=99 cells; 12 hypocotyls). Movies confirmed that microtubules undergo persistent reorientation in such tissues (Chan et al., 2007). Importantly, although microtubules could be transiently transverse in these persistently reorienting arrays, in slower-growing cells the phase of reorientation was not synchronised across the epidermis (Hejnowicz, 2005; Chan et al., 2007).

A different pattern of microtubule alignment was observed in relatively fast-growing hypocotyls (with an average cellular elongation rate of 3.19µm/hour; s.d.=3.78; s.e.=0.54 or an average relative elongation rate of 0.06/hour; s.d.=0.07, s.e.=0.01, n=49 cells). In these tissues, transverse alignments of microtubules were observed that were coordinated across fields of neighbouring cells (Fig. 1B). Transverse alignment could be maintained over several hours, illustrating that it is not a fixed stage extracted from the rotary process (Fig. 1C).

A third pattern was observed between days four and five, when growth ceased. In such tissues, coordinated alignments were also sustained between neighbouring cells, except in these cases, microtubules were arranged in oblique or longitudinal arrays (Fig. 1D). Such oblique alignments – similarly to the transverse alignment of faster-growing cells (Fig. 1B) – could be maintained over several hours.

Analysis of movies showed that hypocotyls did not maintain constant growth rates and transitions between relatively fast and slow elongation rates (and vice-versa) were captured. Such movies were used to analyse the dynamics of the development of transverse alignments with respect to growth (supplementary material Movie 1). Fig. 1F provides an example of microtubule alignment as the growth rate changes. To obtain these data, the length of the cell within a movie frame was measured by projecting a one-pixel-wide line from one end wall to the other. After extracting a time series of such lines from the movie, the bottom ends were aligned horizontally, so that the upper ends traced a curve that shows the rate of cell elongation (Fig. 1E). A corresponding montage of the different microtubule alignments that occurred over the same period of elongation is shown in Fig. 1F (which is best seen by magnifying the image online). During more rapid growth (Fig. 1E), microtubules tended towards transverse alignment (supplementary material Movie 2), which was coordinated across fields of cells (Fig. 1G; t=210, compare with the more variable alignments at t=0). But as the growth rate reached a plateau (Fig. 1E), the corresponding panels of microtubules (which are aligned in Fig. 1F), switch to a more oblique alignment. This alignment was also coordinated across the tissue (Fig. 1G; t=645).

Observation of those transitions where the rate of elongation accelerated revealed two features of the evolution of transverse microtubule alignment. First, transverse arrays are a transient phenomenon and tend to develop before phases of more rapid elongation (Fig. 2A,B). Out of 41 cells monitored long-term in five hypocotyls filmed as they changed from slow to faster elongation, 35 evolved transverse alignment an average of 184 minutes (3.07 hours) (s.d.=170 minutes; s.e.=27) before a measurable burst of elongation, whereas the arrays of six cells became transverse at the transition to more rapid growth (Fig. 2C,D). During more rapid elongation, microtubules spent on average 370 minutes (6.2 hours) in transverse alignment (s.d.=212 minutes; s.e.=42, 25 cells, 4 hypocotyls). Second, transverse alignment is not necessary to maintain rapid elongation. For instance, supplementary material Movie 1 shows transverse arrays reorienting towards the longitudinal axis despite the fact that analysis showed no decrease from the maximum elongation rate. This demonstrates that although arrays might have a tendency to become transverse before rapid elongation, it is not essential for this alignment to be maintained throughout the entire process of more rapid elongation.

In summary, persistent reorientation is mainly seen at the outer epidermal surface during hypocotyl growth (between days two and three and days three and four) but can be interrupted by transient periods of coordinated transverse alignment before rapid elongation. When growth diminishes between days four and five, there is a switch towards an oblique or longitudinal alignment that is also coordinated between groups of cells. Alignment of microtubules
beneath the outer epidermal wall, coupled with the extent to which this is coordinated between neighbouring cells, therefore serves as a general indicator of the elongation status of the tissue.

Relationship between growth and microtubule alignment at the inner epidermal face

Next, we investigated whether microtubules on the inner tangential wall (in contact with the cortical cells) of the epidermis display similar behaviour to those on the outer tangential surface (in contact with the external environment). First we collected z-stacks of 2-, 3- and 4-day-old hypocotyls. By separating the outer and inner epidermal surfaces within z-stacks, it was possible to compare microtubules on inner as well as outer tangential surfaces of the same cell. Arrays were categorised on a ‘one-cell–one-microtubule alignment’ basis (transverse = 0±22.5°, oblique = 45±22.5°, longitudinal = 90±22.5°), but where an overall alignment of domains could not be clearly determined they were classified as ‘mixed’.

Quantification showed that microtubule alignments on the inner epidermal wall were distributed differently to those on the outer surface (Fig. 3A). In 2-day-old hypocotyls, which had just emerged from the seed, microtubules of various orientations (oblique, transverse, mixed and longitudinal) were present on both the inner and outer epidermal walls, consistent with uncoordinated reorientations described above. However, in elongating 3-day-old seedlings, mixed arrays formed the predominant category on the outer wall. By contrast, on the inner epidermal surface, the predominant alignment was transverse, with 62% (s.d.=23%, 78 cells in 7 hypocotyls) of cells organised in this way. This was double the number of transverse arrays sampled at the outer surface (33%, s.d.=18%, 78 cells in 7 hypocotyls). This was consistent with findings from the dynamic studies, which showed that microtubules on the inner epidermal wall of 3-day-old hypocotyl cells had stopped reorienting and became aligned in coordinated transverse alignments. At this stage, none of the cells displayed transverse alignments. At day four, approximately equal proportions of oblique and transverse microtubules were seen on both inner and outer surfaces.

This analysis indicated that microtubules could be differently aligned on different surfaces of the same cell, with microtubules having an increased tendency to maintain transverse alignment at the inner wall of three-day-old hypocotyls. Movies made of the inner and outer walls of the same cell supported this conclusion. The key frames from supplementary material Movie 3 (see Fig. 3) show that microtubules upon the inner epidermal wall remain transverse (Fig. 3B, 0–270 minutes) throughout a period when microtubules rotate over time at the outer surface (Fig. 3C). This was consistent with findings from the dynamic studies, which showed that microtubules on the inner epidermal wall of 3-day-old hypocotyl cells had stopped reorienting and became aligned in coordinated transverse alignments. At this stage, none of the cells displayed transverse alignments. At day four, approximately equal proportions of oblique and transverse microtubules were seen on both inner and outer surfaces.

The sensitivity of inner wall alignment to growth was examined between days three and four by making kymographs of hypocotyls cells found to be undergoing fluctuations in the elongation rate. Only cells whose inner tangential wall was not too facetted by contact with subjacent cells had a sufficiently flat surface for this study. In contrast to the upper surface, detailed kymographic analysis of six cells revealed that microtubule alignment on the inner surface was less sensitive to decelerations and accelerations in the rate of growth. The kymograph in Fig. 4A shows the end-wall trace of an elongating cell in which growth plateaus for a while before undergoing a sudden acceleration.

Fig. 4A shows an end-wall trace where growth plateaus then accelerates. In Fig. 4B, each strip shows microtubule alignment at the outer epidermal wall, corresponding to the end-wall trace directly above it (in Fig. 4A). This shows that the alignment of microtubules at the outer epidermal surface varies over time, but that after a period in which the growth rate plateaus, microtubules tend towards transverse alignment as the growth rate accelerates once more. By contrast, the microtubules upon the inner surface of the same cell (Fig. 4C) were notably less variable, remaining mainly transverse throughout. Microtubules upon the inner surface could remain net transverse for prolonged periods of time. Such an array was tracked for 20 hours (Fig. 4C; supplementary material Movie 4), in contrast to the shorter episodes of transverseness on the upper surface of the same cell. This suggests that transverse alignment of microtubules on the inner epidermal wall is relatively
Insensitive to fluctuations in elongation rate. By contrast, microtubules on the outer face rotate in slow-growing cells and only display an increased tendency towards transverseness during phases of accelerating growth.

In addition to examining outer and inner tangential or periclinal epidermal surfaces, we looked at the connecting radial or anticlinal walls. Similarly to the inner surface, these maintained transverse microtubule alignment, whereas a more pronounced realignment was confined to the outer surface (see supplementary material Movie 4).

Orientation of cellulose microfibrils at the inner epidermal surface
The preceding analysis indicated that microtubules can adopt various alignments on the inner epidermal surface at day two, but at day three, they have a stronger tendency than those in the outer wall for transverse alignment. To view the most recently deposited layer of cellulose microfibrils upon this inner epidermal surface, the outer epidermal wall was removed by sectioning, the cell contents and plasma membrane were removed with detergent, and the exposed surface of the inner epidermal wall examined by field emission scanning electron microscopy (FESEM). In 2-day-old hypocotyls (77 cells, 5 hypocotyls), the inner wall showed various alignments of microfibrils that differed from cell to cell, with ‘oblique’ forming the largest category (Fig. 5A–E). In 3-day-old hypocotyls (51 cells, 6 hypocotyls) there was a doubling in the percentage of transversely aligned microfibrils, which now formed the predominant category, followed by oblique, whereas virtually no longitudinal alignments were seen at this stage (Fig. 5A). This pattern was reversed at day four (34 cells, 4 hypocotyls) when transverse alignments were almost absent with oblique or longitudinal forming the predominant category Fig. 5A.

Comparison of cellulose synthase tracks on inner and outer cell surfaces
After observing that microtubules can display different alignments on opposing tangential surfaces of the same cell, we investigated whether this also applies to cellulose synthase tracks on the outer and inner tangential epidermal walls. This was performed with seedlings expressing GFP–CESA3 under control of its own promoter in the cesa3je5 mutant background (Desprez et al., 2007).

The GFP–CESA3 labelling pattern in light-grown hypocotyl cells was consistent with that seen in dark-grown cells, i.e. cellulose-synthesising particles were observed upon the cortex, whereas faster-moving Golgi bodies were seen deeper in the cytoplasm (Desprez et al., 2007).

In particular, the CESA particles moved along the plasma membrane in linear tracks, as previously described (Crowell et al., 2009; Paredez et al., 2006). Although this was difficult to discern upon the inner epidermal wall, as a result of the dimmer fluorescence and basal accumulation of Golgi bodies (particularly in the small cells of 2-day-old seedlings), it was possible to identify cells in which CESA tracks on the inner wall did not mirror the alignment of tracks on the outer wall of the same cell. This was observed more clearly in the longer cells of 3-day-old hypocotyls. In this case, GFP–CESA3 tracks were aligned transversely upon the inner epidermal surface, whereas a variety of alignments were observed on the outer surface (Fig. 6A–D).

In summary, just as microtubules do not necessarily form transverse ‘hoops’ around illuminated epidermal cells, the cellulose synthases also form tracks that can be aligned in different orientations on different surfaces of the same cell.

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**Fig. 4.** Mismatch between inner and outer wall microtubule alignment during changes in growth rate. (A) Kymograph along the long axis of an elongating cell showing a change in the rate of elongation, from slow (0.2 μm/hour) to more rapid elongation (2.0 μm/hour). (B) Corresponding montage of microtubule alignments that occurred along the outer surface of the cell during elongation. (C) Montage of microtubule alignments that occurred along the epidermal surface of the same cell. Note that microtubules remained in a net transverse orientation on the inner wall regardless of changes in growth rate. Scale bar: 298 minutes (A, x axis), 10.5 μm (A, y axis).

**Fig. 5.** Analysis of cellulose microfibrils along the inner walls of hypocotyl epidermal cells. (A) Quantification of cellulose microfibril alignment along the inner walls of hypocotyl epidermal cells at different days of development. Note that cellulose microfibrils along inner walls have a greater tendency towards transverseness on day 3. Results are mean ± s.e. (B–E) FESEM images of cellulose microfibril orientations at the inner wall of Arabidopsis epidermal cells: (B) longitudinal, (C) oblique, (D) transverse and (E) mixed orientation. Scale bars: 200 nm.
Discussion

This study of individual hypocotyl epidermal cells shows that microtubules display complex three-dimensional reorganisations in which alignment of microtubules on different faces of the cell can vary in a growth-sensitive manner. In the early stages of growth, 2-day-old hypocotyls had various alignments (transverse, oblique, longitudinal or mixed) on both inner and outer surfaces. But as growth proceeded, microtubules on the inner walls of 3-day-old hypocotyls tended to display coordinated transverse alignments. These illuminated Arabidopsis seedlings do not grow as fast as etiolated plants (Gendreau et al., 1997; Le et al., 2005) and microtubules at their outer epidermal surface can undergo rotary movements during slow growth, but during phases of accelerating growth, hypocotyls show a tendency to switch to transverse alignment when imaged for long periods. Although this switch in alignment tended to occur before accelerations in growth rate, it was not tightly coupled with the actual change in elongation rate, nor was it essential for this alignment to be maintained throughout the process of rapid elongation. Then, as growth diminished, the pattern changed once more, consisting of arrays in which oblique and steeply oblique or longitudinal microtubules were present on both outer and inner surfaces. The same trend was noted for microtubule alignment on the outer surface of light-grown Arabidopsis seedlings expressing GFP–TUA6 (Le et al., 2005). This pattern is also very similar to that reported in a live cell study of Arabidopsis root epidermal cells expressing GFP–MBD (Granger and Cyr, 2001). There, transverse microtubules were mainly associated with accelerating growth, whereas a variety of orientations was found as the growth rate declined. From their investigation on fixed Arabidopsis root cells, Sugimoto and co-workers (Sugimoto et al., 2000) came to similar conclusions: cortical microtubule alignment can vary between neighbouring cells and microtubules tend to become transverse during phases of accelerating growth and reorient to oblique as the growth rate declines. One difference between Granger and Cyr’s (Granger and Cyr, 2001) investigation of the living Arabidopsis root and ours on the living Arabidopsis hypocotyl is that although they found no evidence for a cyclic, oscillatory reorientation of microtubules of the kind deduced to be occurring in azuki bean epicotyls (Mayumi and Shibaoka, 1996), our observations show that microtubules can rotate or jump. This might be due to inherent differences between roots and light-grown shoots, such as their different rates of growth.

Our observations indicate that both microtubule tracks and CESA trajectories can have different alignments in different cells of growing shoot tissue and, furthermore, that alignment can also differ between outer and inner tangential surfaces of the same cell. The classical view of the cortical array as a continuous hoop-like structure is evidently overly simple, because the present observations demonstrate that the cortical array behaves as if it is composed of domains or groups of mobile microtubules that can move independently of one another (Chan et al., 2007). The idea that microtubules and cellulose microfibrils form transverse hoops originated with freely-growing filamentous cells, such as Nitella internodal cells (Green, 1960; Green, 1962; Green, 1963). However, as the above observations indicate, shoot epidermal cells do not necessarily show this strict hoop reinforcement. This mismatch between inner and outer surfaces is seen for CESA tracks, as well as for microtubules. Despite their relatively slow rate of growth, illuminated hypocotyls nonetheless produce a well-defined axis. This study shows that such anisotropic growth can occur without the constantly coordinated alignments of microtubule tracks or CESA trajectories on the outer epidermal surfaces that comprise the ‘organ wall’.

An interesting aspect of this study is that microtubules and CESA tracks on the inner epidermal wall tend to show more highly coordinated transverse alignments than seen with those on the outer wall. This is relevant to discussions about the role of inner wall versus the function of the outer epidermal ‘organ’ wall. Because the epidermis shrinks when the stem is cut whereas inner tissues swell, it has been concluded that the epidermis constrains the driving force provided by the expansion of inner tissues (Baskin, 2005; Hejnowicz et al., 2000; Kutschera and Niklas, 2007; Schopfer, 2006). According to these ideas, the thicker outer epidermal cell wall resists stress isotropically, whereas the inner tissues channel expansion anisotropically and show better ‘hoop reinforcement’ (Schopfer, 2006). This could explain why the microtubules on the inner epidermal wall display transverse microtubules during phases of cell elongation, consistent with organ-level hoop reinforcement, whereas transverse alignment is not necessarily strictly observed on the more isotropic outer face, which forms a thicker, strong mechanical barrier without dictating the direction of organ growth.

Materials and Methods

Plant material

Arabidopsis thaliana plants expressing 35S::GFP:TUA6 and CESA3::CESA3:GFP have been described previously (Chan et al., 2010). Seeds were sterilised in 5% (v/v) sodium hypochlorite before transfer onto Petri dishes containing 0.43% (w/v) Murashige and Skoog medium (Formedium, Hunstanton, UK) containing 1% (w/v) sucrose (Sigma) and 0.5% (w/v) Phytagel (Sigma). Seeds were placed at 4°C for 2 days and then incubated at 25°C under continuous light. 2- to 4-day-old seedlings were transferred to microscopy chambers (Chan et al., 2007) and then imaged under the confocal microscope.
Confocal imaging and image analysis
The middle region of hypocotyls were imaged using either a VisiTech (UK) spinning disc confocal microscope or a Bio-Rad 1024 confocal laser-scanning microscope using a 40×/1.3 NA oil objective lens. GFP was excited using the 488 nm line of an argon ion laser and emitted light filtered through a 500–550 nm band-pass filter. For the spinning disk microscope, fluorescence was detected using a Hamamatsu ORCA-ER cooled CCD camera with hybrid cooled CCD (H11003) or a Bio-Rad 1024 confocal laser-scanning microscope. Confocal imaging and image analysis