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Tensile Tissue Stress Affects the Orientation of Cortical Microtubules in the Epidermis of Sunflower Hypocotyl

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Abstract

In turgid multicellular organs, it is convenient to differentiate between the two kinds of tensile forces acting in cell walls as a result of turgor pressure. The primary forces occur both *in situ* and in cells isolated from the organ, whereas the secondary forces occur only *in situ*. The latter are an unavoidable physical consequence of the variation in mechanical parameters of tissues forming layers or strands. The most rigid tissue is under maximal tensile force, whereas the least rigid is under maximal compressive force. These forces cause tissue stresses (that is, certain tissues are under tensile stress, whereas others are under compressive stress in the organ). The primary and secondary forces result in primary and secondary stress in cell walls, respectively. The anisotropy of the primary stress is a function of cell shape. For instance, in cylindric cells the anisotropy expressed as the ratio of longitudinal to transverse stresses is 0.5. The anisotropy of the secondary stress is a function of the compound structure of the organ. For example, in the epidermis of sunflower hypocotyl, the longitudinal secondary stress is much higher than the transverse stress. The primary and secondary stresses are superimposed, and, as a consequence, the stress anisotropy in the outer thick walls of epidermal cells is greater than 1. These outer epidermal walls transmit most of the tissue stress. When the epidermis is peeled but remains turgid, only primary stress remains, but loading of the peel can reestablish the original stress anisotropy. We studied the effect of stress anisotropy changes on the orientation of cortical microtubules (CMTs) in the sunflower hypocotyl epidermis. We showed that changes in stress anisotropy cause the CMT orientation to change in the direction of maximal wall stress. *In situ*, the relatively high tensile tissue stress in the epidermis causes maximal stress in the longitudinal direction and relatively steep CMT orientation. When the tissue stress is removed from the epidermis by peeling, the CMTs tend to reorient toward the transverse direction, which is the direction of maximal stress in the primary component. On application of external longitudinal stress, to substitute for tissue stress, CMTs tend to reorient in the longitudinal direction. However, a relatively high rate of plastic strain is caused by the stress applied to the peel in an acid medium. This produces a less steep orientation of CMTs. It appears that the change in stress anisotropy orients the CMT in the direction in which the stress is maximal after the change, but there is also some effect of the growth rate on the orientation.

Key words: Cortical microtubules; Epidermis; Helicoidal wall; Mechanical stress; Tissue stress; Stress anisotropy

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INTRODUCTION

It was Paul Green who, in an article written in 1962 and published in 1963, proposed that long cytoplasmic elements, sensitive to colchicine, which appear to be identical to mitotic spindle fibers (that is, microtubules [name introduced by Ledbetter and Porter in 1963], determine the axis of cell growth by providing a template for cellulose microfibril deposition. In the last two decades, evidence has accumulated in support of the idea that cortical microtubule (CMT) arrays are involved in the orientation of nascent cellulose microfibrils in cell walls (Lloyd 1984; Prodham and others 1995; Seagull 1991). Evidence also exists that the microfibrils may be the source of directional information for CMT orientation. They affect the directional tensile forces in the wall, which provide a spatial cue to orient the CMTs (reviewed by Williamson 1991; Fisher and Cyr 1998). On the basis of these observations, Fisher and Cyr (1998) restated Paul Green's microtubule/microfibril paradigm as follows, "Cortical microtubules can direct the orientation of cellulose microfibrils, which, because of their great tensile strength, typically limit growth to one major axis. As a result of this unidirectional wall compliance, biophysical forces are generated parallel to the major strain axis and are relayed back to the plasma membrane and to the attached cortical microtubules . . . . Thus, the cell wall, plasma membrane, and underlying cortical microtubules provide a self-reinforcing system to ensure that plant cell expansion occurs continuously in the proper direction." Such a self-reinforcing system can respond to either a change of the tensile forces acting in the cell wall or to a change in CMT orientation (for example, when hormone levels are varied or light is applied). Here we shall consider the type of response initiated by changing the forces in cell walls.

The tensile forces, which act in the walls of turgid cells, are due to turgor pressure. In turgid, multicellular organs, it is convenient to distinguish between two types of tensile forces: one that occurs both *in situ* and in cells isolated from the organ and another that occurs only *in situ*. The latter is an unavoidable physical consequence of the variation in mechanical parameters of tissues (cells arranged in layers or strands) within turgid organs, both elongating and nonelongating (Hejnowicz 1997, Hejnowicz and Sievers 1996a, Vincent and Jeronimidis 1991). The most rigid (most densely packed with cell walls) layer or strand of cells is under maximal tensile force, whereas the least dense is under maximal compressive force. These forces cause tissue stresses. Certain tissues are under tensile stress, whereas others are under compressive stress. Traditionally, it is assumed that tissue stresses are generated by differential growth of tissues (see for example, Peters and Tomos 1996). Also, tissue stresses may occur in an organ as a result of externally imposed forces (for example, like those causing organ bending). It is thus reasonable to distinguish between several types of tissue stresses: structure-based, differential growth-based, and tissue stresses with external origin. Measurements of tissue stresses in stems and comparison of the results with values calculated from the structural differences in the tissues allow one to conclude that the structure-based tissue stresses readily explain the stresses occurring in stems when no external forces are acting on them (Hejnowicz and Sievers 1996a).

As mentioned previously, the forces acting in cell walls and related to structure-based tissue stresses are a consequence of turgor pressure. This, however, is only an indirect effect of the pressure and takes place exclusively *in situ*, in contrast to the forces that would also act in the wall of isolated cells. For convenience, we shall call the cell wall stress representing the direct effect of turgor pressure (that is, that occurring both *in situ* and in isolated cells) the "primary stress component" or "primary stress (σ_p)" and the stress representing the indirect turgor effect, which occurs only *in situ*, the "secondary stress (σ_s)." These two components are superimposed in cell walls. The primary component can be calculated from turgor pressure and cell geometry. The secondary component can be calculated from the tissue stress, T, which is expressed as force per cross-sectional area of the tissue and therefore is comparable with turgor pressure. The primary and secondary stress components (expressed as force per cross-sectional area of cell wall) are products of the turgor pressure and tissue stress, respectively, with the same coefficient.

We note that cell wall stress in a given direction is equal to the ratio of the forces acting in this direction and the cell wall cross-sectional area in the plane perpendicular to this direction. We denote force as *F*, the cross-sectional area on which the force acts as *A*, tissue stress as *T*, turgor pressure as *P*, cell wall stress as σ, and introduce subscripts *p* for primary, *s* for secondary, *lum* as referring to cell lumen, *tis* to tissue, *cell* to a single cell, *cw* to cell wall. Then, because, in general, $\sigma = F/A$, we have: $\sigma_p = F_p/(A_{cw})_{cell}$; $\sigma_s = F_s/(A_{cw})_{tis}$. Substituting $F_p = P \cdot A_{lum}$; $F_s = T \cdot A_{tis}$, we obtain $\sigma_p = P(A_{lum}/A_{cw})_{cell}$; $\sigma_s = T(A_{tis}/A_{cw})_{tis}$. Because $(A_{lum}/A_{cw})_{cell} \approx (A_{tis}/A_{cw})_{tis}$, the primary and secondary stress components are products of P and T, respectively, with nearly the same coefficients.

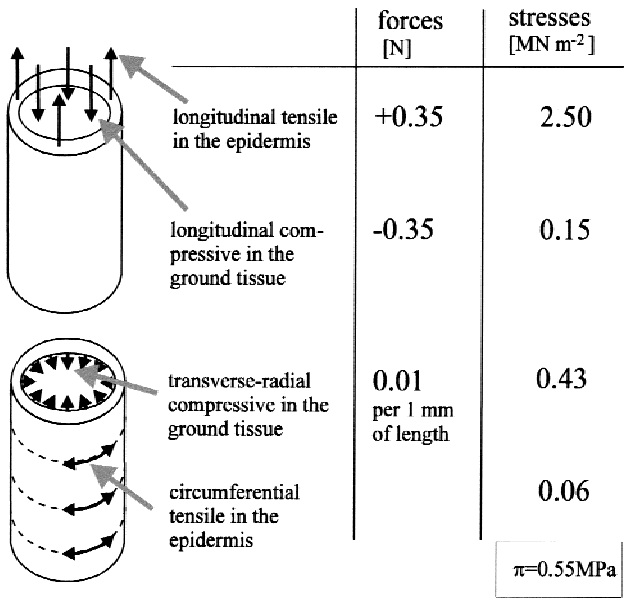

Figure 1. Structure-based tissue stresses and corresponding forces acting on the epidermis and the ground tissue in different directions in sunflower hypocotyl. For comparison the osmotic pressure of cell sap (π) is indicated. (Data from Hejnowicz and Sievers 1995).

How are the stress components related quantitatively in sunflower hypocotyl, the object of this study? Figure 1 shows that the longitudinal tensile tissue stress in its epidermis is nearly five times higher than the osmotic pressure (π), whereas the transverse tissue stress in this tissue is only a small fraction of the pressure. Formally, the osmotic pressure is equal to turgor pressure (P) when the osmolarity of the apoplasmic solution is zero. Therefore, in a real tissue $P \leq \pi$. This means that the secondary stress component (longitudinal) attains an impressive value (in comparison to P) in the hypocotyl epidermis.

Primary stress depends on turgor pressure, cell wall thickness, cell shape, and the direction in which the stress is measured. For example, in a cylindric cell with radius R and cell wall thickness D, the longitudinal stress is given by $\sigma_{p(long)} = P \cdot R/2 \cdot D$, but the transverse stress is $\sigma_{p(trans)} = P \cdot R/D$. This implies that the primary stress in cell walls is usually anisotropic. Characterizing the anisotropy as the ratio of longitudinal to transverse stress we obtain 0.5 as the anisotropy value of the primary stress in cylindric cells. In relevant literature on stress anisotropy in cell walls of an organ, only primary stress in the walls is taken under consideration.

In most cases, the secondary stress is superimposed in a wall, and this stress may also be anisotropic, depending on the structure of the organ. In the peripheral tissues (epidermis, collenchyma) of turgid organs of a shoot type, the relatively high longitudinal tensile tissue stress is accompanied by weak transverse tensile tissue stress. The longitudinal tissue stress may be high enough to invert the anisotropy of primary stress in cylindric cells.

The overall stress anisotropy in cell walls has been suggested to offer the cell a piece of information important for the arrangement of CMTs or cellulose microfibrils (Richmond 1983; Williamson 1990, 1991).

From these considerations the following working hypothesis emerges: the structure-based tissue stresses have an influence on the stress anisotropy in cell walls that in turn affects the orientation of CMTs. However, until now, the tissue stresses were not considered in this respect. The only stresses considered were the stresses imposed by lateral compression (applied to the root tip (Hush and Overall 1991), by bending (Zandomeni and Schopfer 1994; Fischer and Schopfer 1997), and by centrifugal forces applied to protoplasts (Wymer and others 1996). These stresses were shown to affect CMT orientation. The studies performed by Schopfer's group showed that imposed mechanical stresses lead to changes in IAA-dependent strain rate (Fischer and Schopfer 1997).

The epidermal cells of sunflower hypocotyl are cylindric. Thus, in cells of the isolated (but still viable and turgid) epidermis, there is an anisotropy of 0.5 (1:2), but in the same cells *in situ* the anisotropy is greater than 1 because of the effect of the tissue stress. Applying external longitudinal force on an isolated epidermis may also change the stress anisotropy. Our studies indicate that stress anisotropy is important for CMT orientation in cells of sunflower hypocotyl. Here we report that CMTs respond to anisotropy changes when the secondary stress component in the epidermis of sunflower hypocotyl is varied.

Material and Methods

Plant Material

Sunflower achenes were germinated in moist vermiculite in containers kept in darkness at a temperature 22 ± 2°C. Seedlings 5–6 days old (after sowing), with hypocotyls approximately 6-cm long, were used for the experiments. All manipulations were done under dim green safety light.

Incubation Media

One percent concentration of agar was used for solidification of all experimental media. Three differ-

ent media were used in experiments: (i) an unbuffered medium containing microelements, macroelements, and organic compounds according to Murashige and Skoog (1962), supplemented with 2% sucrose, pH adjusted to 5.8 before autoclaving; (ii) MES buffer (4 mM, titrated with KOH with pH 6.5; 3) acetate buffer (4 mM) with pH 4.5.

Experimental Treatments

Experiments were performed on the outer tissue of the hypocotyl peeled from the slowly elongating zone located 2–4 cm below the cotyledons. The zone was marked with India ink on hypocotyl before peeling. The peels containing the outer tissue were obtained in the following way. One end of a fine forceps was inserted beneath the epidermis perpendicular to the hypocotyl axis 5 cm below the cotyledons and then moved acropetally to 1 cm below the cotyledons. Peels approximately 2-mm wide were obtained connected to the hypocotyl on both ends. This procedure avoids stretching the epidermis, which would occur if an isolated end of the nascent peel was grasped by the forceps and pulled. The peels were subjected to different experimental manipulations. To determine CMT orientation *in situ*, the peel ends were cut off transversely from the hypocotyl (with a razor) at the zone limits, and a piece of adhesive tape was attached to the outer surface of the peel, so the cuticle was glued to the tape. The tape facilitated further handling of the peel (fixation, and so on). In other experimental treatments, the peel was cut 5 mm outside the zone limits, and the isolated peel was placed on the surface of an agar medium, with the inner peel surface touching the medium. To examine the viability of peels, or the CMT orientation in unstretched peels, the peels were incubated on agar medium in sets and examined simultaneously. In other experiments the effect of applied tensile force was estimated. Then, small containers prepared from plastic sheets were filled with agar medium. Thin aluminum strips (cut from a can), 2-mm wide and 10-mm long, were glued with cyanoacrylate adhesive to peel ends to ensure proper holding by the grips applying the force. When force was exerted by means of a weight (load), the upper end of the peel and the agar container were attached to the upper grip in a vertical position so that the peel could slide on the agar surface while being stretched by the load attached to its lower end. When force was exerted by means of a tensiometer (material testing machine MTS SYNERGIE 100, MN, equipped with 10 N load cell and software TestWorks 4.0), the agar container was fixed to the lower grip and the peel was held by the lower and upper grip.

Immunocytochemistry

Peels attached to adhesive tape were fixed for 1 h in 3.7% paraformaldehyde in microtubule stabilizing buffer (MSB; 50 mM PIPES, 5 mM Mg SO_4, 5 mM EGTA) supplemented with 1% DMSO and washed with three changes of MSB (for 10 min each). Cortical parenchyma cells were carefully removed under a dissecting microscope with the aid of fine forceps to expose the inner surface of epidermal cells. These epidermal strips (peels with partially removed cortical cells), still attached to the adhesive tape, were incubated for 45 min at 37°C in digestive enzyme solution in a humid chamber. The enzyme solution was prepared on the basis of 67 mM phosphate buffer with pH 5.6 and contained the following: 1% Hemicellulase; 1% Cellulase (Onozuka); 5 mM EGTA; 2 mM mannitol; 1%Triton X-100; and 0.3 mM PMSF (as protease inhibitor). After the enzyme treatment, strips were washed with MSB for 20 min, then with MSB containing 1% TritonX-100 for 10 min, and again with MSB for 20 min. Then they were incubated in monoclonal anti α-tubulin (clone DM1A, raised in mouse, Sigma, St. Louis, MO) diluted to 1:200 with phosphate-buffered saline (PBS including: 0.14 M NaCl, 3×10^{-3}M KCl, 8×10^{-3}M Na_2HPO_4, 15×10^{-3} KH_2PO_4) with addition of bovine serum albumin (BSA; 1 mg/mL) in a humid chamber at 37°C for 1.5 h. After washing with three changes of MSB (10 min each), strips were incubated with a secondary antibody (FITC-conjugated anti-mouse Fab specific fragment of goat antibody, Sigma), diluted to 1:100 with PBS with addition of BSA (1 mg/mL). Strips were mounted with 90% glycerol containing *p*-phenylenediamine as an antifading agent.

Fluorescence Microscopy and Quantification of CMT Orientation

Strips with stained CMT were viewed under an epifluorescence microscope (Olympus AX70 PROVIS, Japan) equipped with a CCD camera (C5810 Hamamatsu Photonics K.K., Japan) and the AnalySIS software (Soft Imaging System GmbH, Münster, Germany) designed for image acquisition and analysis. The inner surface of a strip was placed toward the microscope objective. In some experiments, we acquired the images of CMTs underlying both outer and inner tangential walls of the same cell. In other experiments, only the CMTs underlying the outer wall were considered. The orientation of CMTs was

often nonuniform along the cell axis. In these cases the angle between the array of parallel CMTs and the cell axis within a cell fragment whose length was two to three times bigger than cell width (cell length was more than 10 times cell width) was measured. These angles were measured on the digital images. The measurements were done for five cell fragments in each of 30 randomly selected cells on a strip. These five measurements gave a mean angle for every cell. Six strips were examined in each experimental treatment, giving a total of 180 mean angles (each for an individual cell) for a treatment. Distribution of the angles and the statistical analysis were performed with Statistica software (STATSOFT, Tulsa, OK). Images selected for illustration of the CMT orientation were printed on a video-printer (CP700D Mitsubishi, Tokyo, Japan).

Cell Viability Test in Peels

Peels were incubated on agar medium in a humid chamber in darkness. After 6 h or more, they were immersed for a moment in a solution of fluorescein diacetate (Franklin and Dixon 1994) and the percentage of dead (nonstained) cells in the total number of cells was assessed under the epifluorescence microscope.

Measurements of Elongation of Intact Hypocotyls

Hypocotyls of intact seedlings grown singly in small containers were held by special grips of the tensiometer. The upper grip held the hypocotyl 2 cm below the cotyledons, and the lower grip 4 cm below cotyledons (corresponding to the position studied for CMT orientation). Measurements were performed in a vertical position. To facilitate tensiometric (isotonic) measurement, the hypocotyls had to be slightly stretched, and for this reason a 0.01 N force was applied. In other hypocotyls, growth rate was measured visually by use of a color camera (Hitachi VK-C77E, Tokyo, Japan). Images were acquired every 30 min for 24 h. At the beginning of each experiment, hypocotyls were marked with India ink (with 1 cm scale) and the marks were then used to calculate the growth rate for the zone 2–4 cm below cotyledons using AnalySIS 3.0 software (Soft Imaging System GmbH, Münster, Germany). Hypocotyls were illuminated with dim green safety light.

Results

Viability of Cells in Peels

The peels contained the epidermis and one layer of cortical cells (Figure 2). In some peels or peel parts there were three layers of cortical cells. A high percentage (97 ± 3%) of epidermal cells survived peeling and their viability did not change during the 24 h they were kept on agar medium. This estimation does not include the dead cells at the transverse margins of the strips that were killed by transverse cuts through the epidermis, but it does include the cells at the longitudinal margins. In fact, most of the cells that did not survive peeling occurred at these margins. They were probably killed during peeling. Examination of the inner peel surface (on transverse sections like that in Figure 2 and on the surface of fresh peels when viewed under the epifluorescence microscopy after staining with fluorescein diacetate) showed that the peeling occurred mostly by disruption of the tangential middle lamellae. Most of the cortical cells were viable, and there was no accumulation of dead cells at the inner surface of a peel. Because of the occurrence of damaged cells at peel margins, these portions of peels were excluded from the surface from which the cells for determining CMT orientation were randomly chosen.

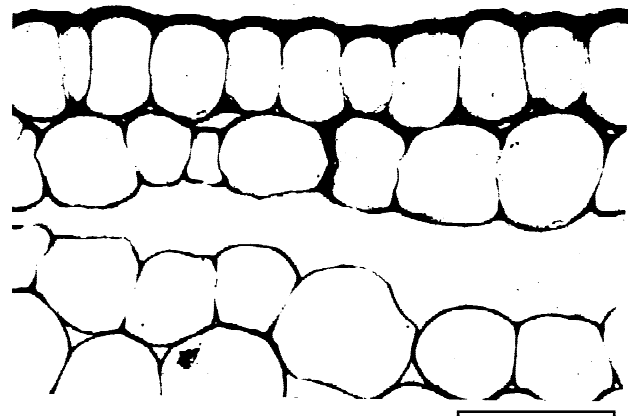

Figure 2. Representative transverse section through the peripheral part of sunflower hypocotyl after peeling, showing the peel and the corresponding surface of the cortex. The 2-µm-thick section was cut from material embedded in Epon 812 resin and was stained with toluidine blue "O" after PAS reaction. Bar = 50 µm.

Arrangement of CMTs *In Situ*

In epidermal cells, the CMTs underlying the tangential walls tended to be mutually parallel and inclined at a particular angle to the cell axis (Figure 3). The inclination is or can be variable in neighboring cells or even in different parts of a single cell. It was also different in the same cells at the outer and inner walls (Figure 3); the CMTs underlying the outer wall were steeply inclined and showed maximal angular distribution at 35 degrees, whereas the CMTs under-

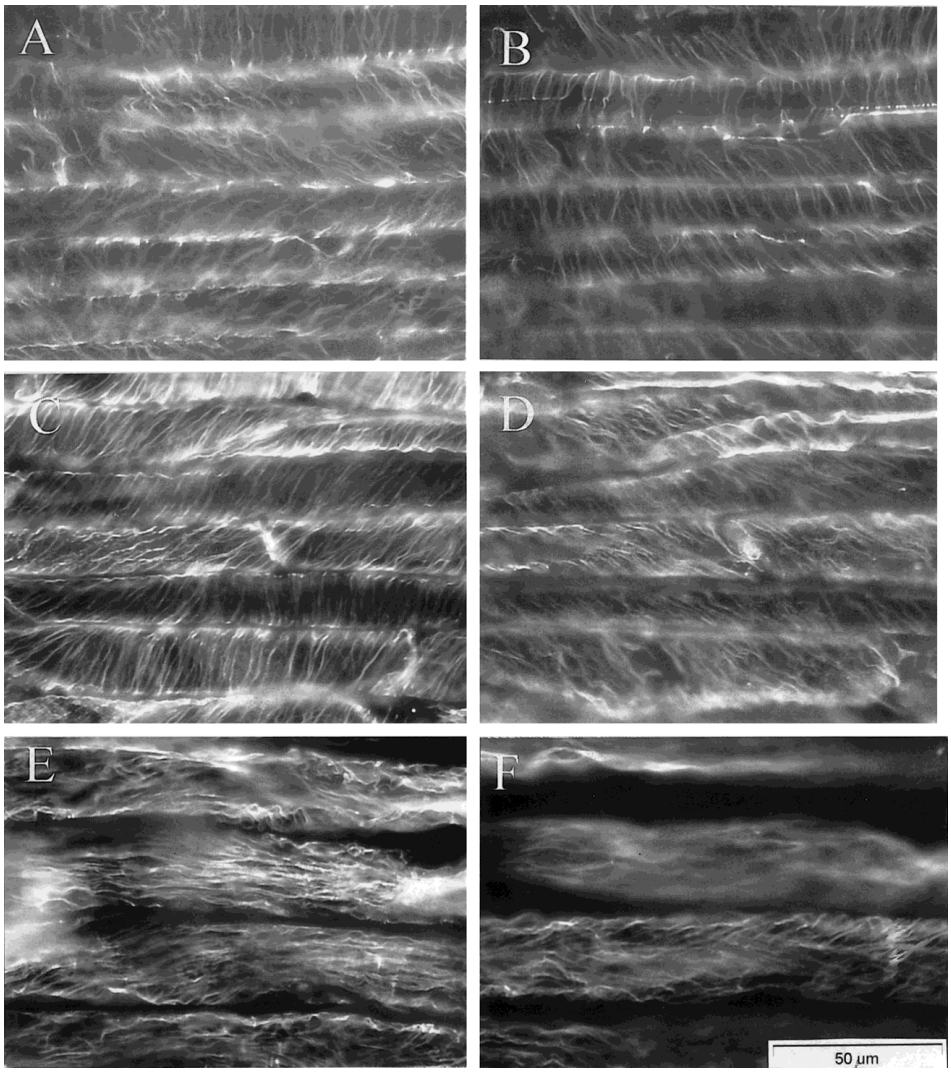

Figure 3. Arrays of CMTs underlying the outer **(A,C,E)** and the inner **(B,D,F)** epidermal walls in sunflower hypocotyl (each horizontal pair of images is from the same cell. (**A** and **B**) *In situ*; (**C** and **D**) in a peel on unbuffered medium, 90 min after peeling; (**E** and **F**) in a loaded peel on unbuffered medium, 90 min after peeling.

lying the inner wall showed maximal distribution at 75 degrees (Figure 4A).

The orientation of the CMTs underlying radial walls could not be studied in detail. However, CMTs from tangential walls apparently continued onto the radial walls. CMTs underlying the two opposite tangential walls (two facets of one cell) but seen from the same side (that is, as projections from the facets) always crisscrossed. This indicates the existence of whole-cell helical CMT arrays, although with different inclination on the two tangential facets. There was no statistically important difference between the number of cells with clockwise and counterclockwise inclination of CMTs.

Arrangement of CMTs in Peels on Unbuffered Medium

The orientation of CMTs underlying the outer wall examined 90 min after peeling was less steep than the orientation *in situ* (Figure 4B). However, the reverse was true for the CMTs underlying the inner wall.

Arrangement of CMTs in Loaded Peels in Unbuffered Medium

The applied load was such that the resulting longitudinal stress excessively compensated for the average structure-based longitudinal tensile tissue stress that acted in the epidermis *in situ*.

The orientation of CMTs in the loaded peels is shown in Figure 4C. The orientation of CMTs underlying the outer wall was much steeper than in unloaded peels and even steeper than *in situ*. The applied stress tended to invert, with an excess, the change caused by the isolation. In CMTs underlying the inner wall, there was only a slight increase in steepness as a result of the load application.

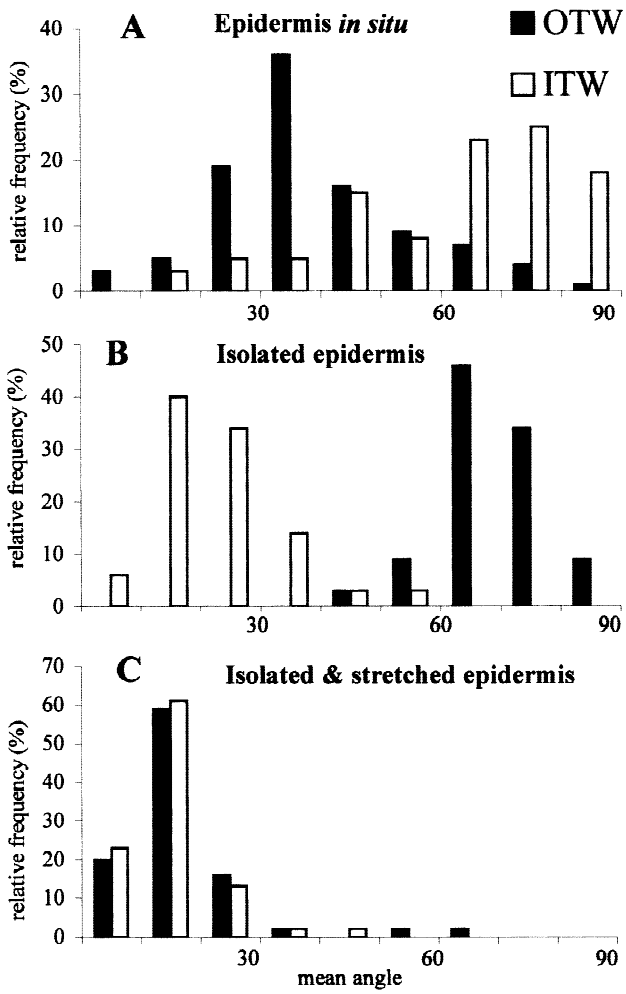

Figure 4. Orientation of CMTs underlying the outer *(OTW)* and the inner *(ITW)* tangential walls of the epidermis of sunflower hypocotyl *in situ* and in peels (2-mm wide) unloaded and loaded (stretched) by 10 g. The peels were incubated either unloaded or loaded on unbuffered medium for 90 min after peeling. The 90° angle corresponds to transverse orientation.

Arrangement of CMTs Underlying the Outer Epidermal Cell Wall in Peels Stressed by a Tensiometer on Buffered Media

The two tensile forces, for the sake of convenience referred to as weak and strong, applied to peels 2-mm wide, were 0.01 N (corresponding to approximately 1 g weight) and 0.1 N. The strong force compensated (with excess) for the tissue stress removed during peeling. The weak force (resulting in stress much lower than the tissue stress) assured proper functioning of the tensiometer in the case of control-type experiments.

When a weak force was applied, the CMT orientation was less steep than *in situ*, and the decrease in steepness was more pronounced at pH 4.5 (Figure 5A, C). However, there was a difference in expansion rate depending on pH (see next section), so we cannot infer that pH alone had some effect on the CMT orientation.

When the peels were stressed with 0.1 N, they experienced tissue stress much greater than that in the intact epidermis (see Discussion), and orientation of the CMTs on the buffered medium was more steep than under the weak force and *in situ*. However, on pH 4.5 medium steep angles (less than 20 degrees) were avoided (Figure 5B, D). Again, the effect of the mechanical stress on CMT orientation was evident: the orientation tended to change in the direction of the applied tensile stress.

Extension Rates

Representative time courses of length change of the hypocotyl and the peels obtained from tensiometric measurements are presented in Figure 6. Extension rates of peels depended on the applied force: peels under the weak force extended more slowly than the intact hypocotyl in the region from which the peels were taken, whereas those under the strong force extended much faster. Apparently, the weak force caused less stress in the epidermis than the longitudinal tissue stress occurring *in situ*. However, the strong force greatly exceeded the missing tissue stress. The average rate of elongation of hypocotyl in the region from which the peels were taken was 1.01% h^{-1} ($n = 3$, tensiometric data), and 1.14% h^{-1} ($n = 3$ photographic data for 24-h period). The average extension rate of peels under strong force on the medium with pH 4.5 and 6.5 was 23.3% h^{-1} ($n = 3$) and 1.42% h^{-1} ($n = 3$), respectively.

Discussion

Mechanical Stress and CMT Orientation

Considering the changes in CMT orientation caused by isolation, one must keep in mind that the isolation caused severe wounding, removal of the tensile tissue stress occurring *in situ*, and cessation of the longitudinal extension of the tissue. Because the stress applied to the peels tended to reverse, with an excess, the stress change caused by the isolation, we infer that change of CMT orientation at the outer wall occurring as a result of peeling was mainly due to the removal of the tissue stress and not to the wounding.

This study clearly shows that the changes in longitudinal tensile stress in the outer epidermal wall affect the orientation of CMTs underlying the wall. The prevailing orientation of CMTs approaches the

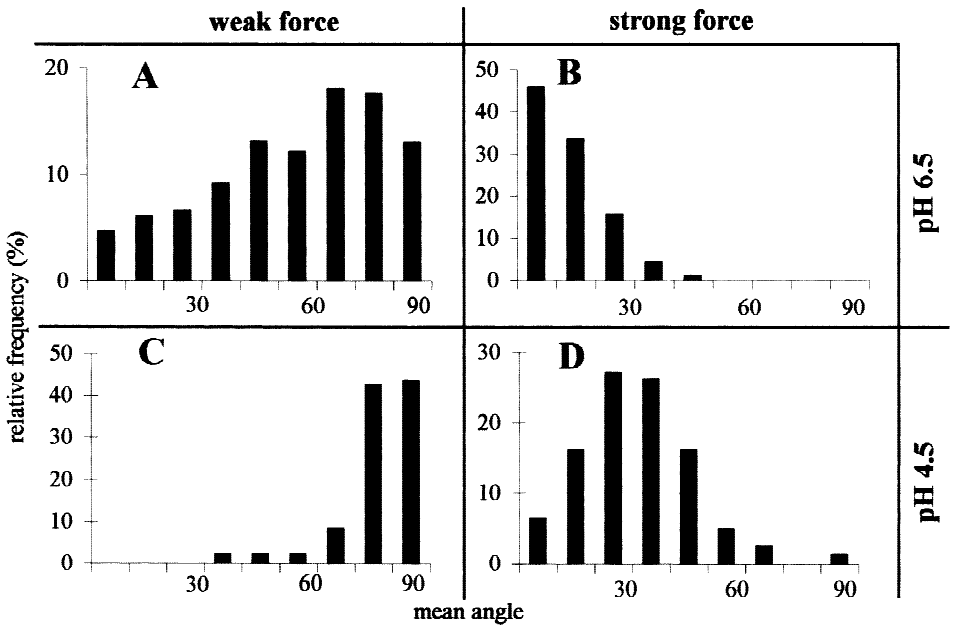

Figure 5. Orientation of CMTs underlying the outer epidermal wall in peels (2-mm wide) incubated on buffered media (pH 6.5 and 4.5) under weak (0.01 N) or strong (0.1 N) tensile longitudinal force for 90 min after peeling.

direction of the maximal stress in the wall. *In situ*, the relatively high tensile tissue stress in the epidermis causes the maximal stress to be in the longitudinal direction, and therefore CMT orientation is relatively steep. When tissue stress is removed in the course of peeling, CMTs tend to reorient toward the transverse direction, which is the direction of the maximal primary stress component. On application of longitudinal stress to peels, CMTs tend to reorient toward the longitudinal direction. It should be emphasized that it is mainly the outer wall that transmits either the tissue stress in the intact organ epidermis or the stress applied to the peel by loading (see the fourth section of the discussion).

The stress applied to peels should compensate for the average structure-based longitudinal tensile tissue stress acting on the epidermis *in situ*. The studies described here were based on work from Hejnowicz and Sievers (1995) in which the longitudinal force involved in tissue stress was found to be approximately 0.05 N/1 mm of circular length (width) of the epidermis. Thus, we applied a 10 g load or 0.1 N to peels 2-mm wide. However, after obtaining the results shown here, we discovered that the tissue stress in the hypocotyls of this study was, on average, nearly three times lower than that of the previous study (data not shown). The stress applied to peels exceeded the tissue stress *in situ* so that the stress anisotropy in the outer cell wall was similar to that *in situ* only in having the same direction of maximal stress. It appears that it is the change in stress anisotropy that *causes* reorientation of the CMTs to the direction in which the stress becomes maximal after the change.

In tensiometer experiments, it was necessary to not only apply a stress to substitute for the tissue stress (strong force) but to also use a lower stress that would serve as the tensiometric "control" experiment (weak force, 0.01 N/2-mm of peel width). Undoubtedly, the weak force caused lower stress in the epidermis than the longitudinal tissue stress occurring *in situ*, although, as mentioned, the tensile tissue stress in the hypocotyls studied in this article was three times less than that previously determined (Hejnowicz and Sievers 1995). In this study, the force that generated the tensile tissue stress in the epidermis of hypocotyls could be estimated as 0.12 N ≈ 12 g (weight), which gives 2 g/2 mm of epidermis circumference (peel width). The weak force was thus equivalent to 50% of the force, which generated the tissue stress. Assuming that the turgor pressure in hypocotyls in this study was similar to that in the previous one (Hejnowicz and Sievers 1995), the tensile tissue stress in the epidermis of the former was approximately 5/3 P ≈ 1.6 P (that is, it was still able to revert the primary stress anisotropy in the walls of cylindric epidermal cells *in situ)*, but the applied stress caused by the low force (the applied stress formally equal to 0.8 P) was not able to do this in peels.

Regulation of CMT orientation by stress anisotropy explains the experiment of Richmond (1983), in which *Nitella* cells with depolymerized microtubules deposited cellulose microfibrils at random orientations so that transverse cell strain exceeded longitudinal strain. This would be expected for an isotropic wall under the stress anisotropy characteristic for cylindric cells. If CMTs reassembling in the per-

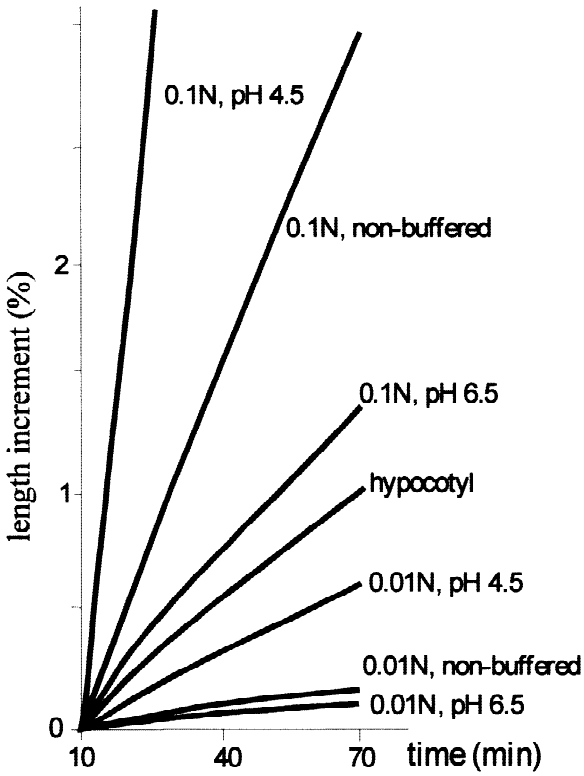

Figure 6. Representative tensiometric graphs of extension of intact hypocotyl in the studied zone (2–4 cm below cotyledons) and peels (2-mm wide) under strong (0.1 N) and weak (0.01 N) tensile forces on buffered (pH 6.5 and 4.5) and unbuffered media.

turbed cells were oriented at right angles to the direction of maximum strain, which is assumed to explain the orientation of CMTs in unperturbed *Nitella* cells (maximum strain longitudinal, CMT transverse), the newly formed microfibrils should be longitudinal. However, these microfibrils were transverse. This means that it was not the direction of maximum strain that oriented the CMTs. Rather the recovered CMTs were oriented in the direction of maximal stress.

The influence of stress anisotropy also explains why CMTs in young internodal characeaen cells remain transverse to the long axis of the cell long after growth cessation, although they become randomly oriented many days after growth cessation (Kropf and others 1997). It also explains the reorientation of microtubules in protoplasts subjected to centrifugal force (Wymer and others 1996).

Mechanical stress in a solid has two effects: (i) according to Hooke's law, it always causes reversible (elastic) strain; and (ii) surpassing of the threshold for plastic deformation results in irreversible strain. In (ii) a relationship exists between the stress and the rate of the strain and not between the stress and the strain itself because the latter depends on the time during which it is measured. In the case of growing plant organs, the rate of irreversible strain is the same as the growth rate. Tensile tissue stress in the epidermis increases elastic strain, but it also increases the "driving force" for the irreversible elongation of this tissue. Tissue stress may be necessary to make the "force" strong enough to surpass the yield threshold of epidermis for elongation (Hejnowicz and Sievers 1996b). In our experiments with application of external stress to peels, there was also a strain rate effect presumably similar to the growth of the epidermis in intact hypocotyls.

There is, or may be, a relationship between stress anisotropy and growth anisotropy. This relationship is mediated by at least two additional factors that may be modulated by cells—wall extensibility and yield threshold. Assuming that CMT alignment depends on stress anisotropy, dependence of alignment on growth anisotropy may be expected; however, it may be much more complicated (Baskin and others 1999).

In peels loaded or under strong force, the applied stress was much larger than the longitudinal secondary stress because of the tensile tissue stress *in situ*. Thus, it is not surprising that the peels on unbuffered medium and even those on buffered medium with pH 6.5 extended faster under such a force than in the case of natural elongation (under the structure-based tensile tissue stress in the epidermis *in situ*). Despite this relatively high extension rate, CMTs underlying the outer cell wall were oriented more steeply than those in unloaded peels or those under the weaker force, which extended slower than the intact hypocotyl. However, there was an interesting difference between the peels under strong force on media with different pH; on the medium with pH 4.5, the CMT arrays tended to avoid steep orientation (less than 20 degrees) in contrast to the peels on pH 6.5 medium. What may be the reason(s) behind this tendency? It may be an effect of pH alone; perhaps higher pH shifts to a steeper orientation. However, a more probable reason may be the large difference in the longitudinal extension rates of peels under the strong force between media with different pHs. Peels stressed by 0.1 N showed a low extension rate (1.4% h^{-1}) at pH 6.5, and a high rate (23% h^{-1}) at pH 4.5 (Figure 6). The normal elongation rate of intact hypocotyls in the region from which the peels were taken was 1% h^{-1}.

How Can a Cell Measure the Mechanical Stress in its Walls?

Williamson (1990) proposed that a cell measures directional forces (stresses) in the wall by means of the

anisotropy of elastic strain of molecules associated with cellulose microfibrils. The strain of the molecules, which are present in all orientations within the recently deposited layer of wall, is proportional to the stress they carry. In such a case, the strain anisotropy reflects the stress anisotropy. This information about the anisotropy would be transmitted across the plasma membrane to orient the CMTs. According to Green (personal communication, and Gertel and Green 1977 as an early publication), stress can be measured only by the strain it produces. However, it is known that mechanical stress can be estimated not only directly from the strain but also from other accompanying phenomena (for example, from the frequency of vibration [by analogy to what is recognized by a musician who tunes a stringed instrument]). On the molecular level, stress results in conformational changes that could affect ligand binding properties (Marszalek and others 1998).

Fisher and Schopfer (1997) have shown that CMT reorientations are temporarily correlated with the simultaneous changes in growth rates elicited by auxin, red light, or blue light. They also postulated that "imposed mechanical stresses are not effective as such but only inasmuch as they lead to changes in (IAA-dependent) strain rate." This means that what is sensed is connected with the irreversible, plastic deformation of cell walls rather than the elastic deformation.

Therefore, we propose two concepts on how stresses in cell walls can be sensed: (i) through reversible (elastic) directional strains (let us call this concept the elastic sensing), and (ii) through the rate of irreversible, directional strain (i.e., growth rate [further called the concept of growth sensing]). Taking all the data accumulated in the literature (Fischer and Schopfer 1997; Williamson 1991; Wymer and others 1996) and this study into account, we can infer that both concepts are realistic. Probably both routes of sensing of vectorial forces in cell walls to orient the CMTs were used in the course of evolution. Obviously, growth sensing is not applicable to the cases where temporal or spatial variation in CMT orientation is not accompanied by variation in growth, and it is especially not applicable when CMT orientation changes in nongrowing cells (Prodham and others 1995).

The sunflower hypocotyl region considered in this study was still elongating, although at a low rate. The results indicate the existence of elastic sensing in this region. They also indicate that growth sensing may take place to some extent: a relatively high rate of extension after application of a strong force on acid medium resulted in CMTs avoiding the very steep orientation, which occurred while the same load was applied at neutral medium, which caused much lower extension rate.

Importance of Tissue Stresses for CMT Orientation

This study of CMT orientation; the first one to take tissue stresses into account explicitly, shows that structure-based tissue stresses should be taken into consideration when interpreting the changes of CMT orientation. Other studies did note, however, that the authors were aware of tissue stresses in the organ and of their importance with respect to CMTs (Baluska and others 1996; Ivata and Hogetsu 1988; Lang and others 1982).

We know that usually tissue stresses exist in axial, shoot-type organs, and we can admit occurrence of tissue stresses in the objects studied with respect to the orientation of CMTs. However, it is difficult to interpret the possible importance of the tissue stresses in studies because of the lack of the following information: the relative values of the tissue stresses in the studied organs, the way in which the factors considered with respect to the CMT orientation affected the tissue stresses (for instance, application of auxin partially relaxes tissue stresses), or the way in which the experimental method affected tissue stresses (for instance, cutting a short segment of a coleoptile might cause partial release of stresses in the segment if it was kept in air but might even increase tissue stresses if the segment was immersed in water). Nevertheless, we would like to point to the possible importance of structure-based tissue stresses in CMT orientation in a number of studies. Most of the studies involve the orientation of CMTs in the peripheral cell layers (listed in Fischer and Schopfer 1997) under tensile longitudinal tissue stress. In such layers, CMTs are inclined in elongating cells and tend to become steeper as elongation ceases (Fischer and Schopfer 1997; Ivata and Hogetsu 1988; Lang and others 1982; Laskowski 1990). If the tensile longitudinal tissue stress is strong compared with the turgor pressure and also compared with the transverse tissue stress in the same cells, an anisotropy of stresses is expected in which the longitudinal stress predominates. However, it should be noted that elongation is connected with a partial relaxation of stress in the direction of elongation (Cosgrove 1987). Surely, a cell does not distinguish between the primary and secondary components of overall tensile stress, which is the driving "force" of the elongation; and it is the resultant stress that is lowered by the relaxation. Formally, however, we may interpret stress relaxation as a decrease in its secondary component because turgor pressure is

kept rather constant during growth. Relaxation means a change in the stress anisotropy: the ratio of transverse to longitudinal stress increases as elongation rate increases. When elongation ceases, anisotropy changes in the opposite direction because relaxation of the longitudinal tissue stress does not occur. Assuming that CMTs tend to orient according to anisotropy, changes in anisotropy relative to elongation rate would fit the known correlation between elongation status and CMT orientation. When tissue stresses are taken into account, it is possible to relate this correlation to the casual relationship between the anisotropy and the orientation postulated in this article. A common signal perception chain in the interaction of auxin, light, and mechanical stress, postulated by Fischer and Schopfer (1997), may involve the perception of stress anisotropy change (that is, elastic sensing) and not only growth sensing.

Tissue Stresses and the Orientation of CMTs Underlying the Outer and Inner Tangential Walls of Epidermal Cells

We believe that tissue stresses help to explain the mysterious differences in orientation of CMTs underlying different walls in the same epidermal cell.

In situ, the epidermis of turgid axial shoot organs is a stiffer tissue than the parenchyma, which occupies most of the organ volume. If the two tissues were separate, the existing turgor pressure would cause different elastic axial extension of their walls. The extension in the thin-walled parenchyma would be higher at the same turgor pressure. *In situ*, however, the elastic longitudinal extension must be the same throughout the organ. Because of the symplastic behavior of the tissues within an organ and differences in rigidity of cell walls, the tissue stresses appear: the thin-walled parenchyma is under the compressive tissue stress, whereas the more rigid epidermis is under tensile stress. Uniform extension *in situ* is achieved by tissue stresses (Hejnowicz 1997, Hejnowicz and Sievers 1996a). The same arguments (as for epidermis vs parenchyma) can hold for the different longitudinal walls of epidermal cells. Although epidermis as a whole may be considered as being under a tensile tissue stress, the tensile force involved in the generation of this tissue stress may act mainly in the thick outer wall. This is because the two longitudinal walls of epidermal cells are of different thickness and are probably characterized by different Young's moduli for the longitudinal elastic strain. If walls other than the outer wall are not rigid enough to transmit the tensile force involved in the generation of tissue stress in the epidermis, the anisotropy of stresses in different walls is affected by the tissue stress in different ways. If this is true, tissue stress changes resulting in realignment of CMTs must be a local rather than a general process affecting the whole-cell helical array. Many studies involving the epidermis and observations of CMTs in living plant cells by Yuan and others (1995) support this conclusion.

Because of tensile tissue stress in epidermis *in situ*, stress anisotropy in the outer wall is such that the maximal stress is in the longitudinal direction. It is likely, however, that the anisotropy in the inner wall is mainly determined by the primary stress component that in cylindric cells is characterized by the predominance of transverse stress. If stress anisotropy in a cell wall affects the orientation of underlying CMTs, it is not surprising that inclinations of the CMTs underlying different walls are not the same, although the CMTs form an overall helical array. If, as indicated by this study, orientation of CMTs tends to be in the direction of maximal stress, the orientation at the outer wall should be steeper than that of the inner wall in an organ with structure-based tissue stresses *in situ*. On removal of the tensile tissue stress in the epidermis by peeling, orientation at the outer wall should change in the transverse direction. These predictions fit reality in the case of the sunflower hypocotyl. But why does the orientation of CMTs at the inner wall increase in steepness in peels (compare ITW on Figure 4A and B)? In sunflower hypocotyls, the inner wall of an epidermal cell and the outer wall of the subepidermal cell is thicker than the double tangential walls of cortical cells located farther from the epidermis. The peels contain at least one layer of cortical cells. Thus, in a turgid peel, an effect similar to that leading to tissue stresses in an intact organ should occur: the thin-walled cortical cells cause additional stress in the inner thicker wall, although they are not able to affect the stress in the thick outer wall because the anticlinal walls (radial and transverse) are too thin to transmit the additional stress to this wall. *In situ*, the secondary stress in the inner wall may be lower than after isolation (in peels) because it is the outer wall that mainly transmits the tensile tissue stress.

Tissue Stresses and the Multinet and Helicoidal Concepts: the Spatially and Temporally Variable Orientation of CMTs at the Polylamellate Wall

What is the reason for the variability in CMT inclination on a given wall in neighboring cells? To consider this we must start with the two concepts concerning the orientation of the recently deposited cel-

lulose microfibrils. According to the first one, known as the multinet hypothesis, the nascent microfibrils are transverse or make a shallow helix with respect to the major axis of growth. According to the helicoidal wall concept, there is a progressive change in the inclination of the newly deposited microfibrils (continuous or in steps) resulting in a polylamellate helicoidal wall structure. In other words, microfibrils are aligned with changing angles in successively deposited lamellae (Roland and others 1987). Both concepts agree that the deposited microfibrils are passively reoriented during wall expansion so that their initial arrangement is dissipated during elongation, and eventually most microfibrils become nearly parallel to the major axis of the long cell.

The involvement of CMTs in orienting the cellulose microfibrils is well documented in the case of cell walls, to which the multinet concept is applicable. However, even in such cases there may be exceptions; in cortical cells localized in the basal elongation zone of maize root where CMT alignment became helical, microfibrils often formed helices of opposite handedness (Baskin and others 1999). The case of cell walls with polylamellate helicoidal structure is more complicated. Self-assembly of helicoidal systems is well documented in systems other than plant cell walls and may also occur in cell walls with helicoidal texture (Jarvis 1992; Neville 1988). A geometric model for microfibril deposition in cell walls with helicoidal texture has been proposed by Emons (1994). However, in the semihelicoidal walls of the tension-wood fibers of *Fraxinus mandshurica* var. *japonica* the orientations of CMTs and that of nascent microfibrils are related (Prodham and others 1995). Also, in epidermal and peripheral cortical cells of pea (*Pisum sativum*) epicotyls that are depositing polylamellate walls, CMTs are oriented parallel to the most recently deposited microfibrils. The orientation at a given moment may be different for neighboring cells, for different walls within a single cell, and even along a single wall (Lang and others 1982). The micrographs showing divergent microtubule orientation may capture the instantaneous orientation of an array of microtubules that cycles through different orientations, all of which are preserved in the helicoidal structure of the wall (Lang and others 1982). Similarly, the outer epidermal walls of *Vigna angularis* epicotyls have a polylamellate texture, and orientation of microfibrils in the innermost wall layer is the same as the orientation of underlying CMTs. This implies cyclic changes of CMT orientation. The variation in CMT orientation from one cell to another indicates an asynchrony in these changes (Mayumi and Shibaoka 1996).

The outer epidermal wall of maize coleoptiles is helicoidal (Satiat-Jeunemaitre 1984). A detailed kinetic analysis of the CMT orientations beneath the outer epidermal wall of coleoptile segments in red or blue light showed that CMTs did not experience changes in orientation that could be related to helicoidal microfibril rotation with an ultradian oscillatory period (Zandomeni and Schopfer 1994). However, it is not known whether there was a helicoidal change in the arrangement of nascent microfibrils in the examined segments during the irradiation period.

In the epidermis of the sunflower hypocotyl, the outer cell wall is polylamellate, but the cortical cell walls are thin and do not show any lamellation (Hodick and Kutschera 1992). This study clearly indicates that orientation of CMTs in the epidermis is oblique with variable inclination in neighboring cells or even in different parts of a single cell. Therefore, it appears as if these arrays represented different phases of a cycle of inclination changes. It is thus possible that in the sunflower hypocotyl epidermis, the microtubules are involved in orienting the cellulose microfibrils similar to polylamellate walls in *Pisum* or *Vigna* epicotyls.

It is noteworthy that in elongating organs, the helicoidal texture occurs in tissues such as epidermis and collenchyma (Chafe and Wardrop 1972; Neville and Levy 1985) (that is, those that are typically under structure-based tensile tissue stress), whereas the multinet texture usually occurs in the parenchyma, which is typically under compressive tissue stresses. In elongating tissues under tensile tissue stresses, the orientation of CMTs oscillates (that is, the tendency to orient CMTs toward the axial direction is unstable). This instability may be due to the counteracting effects of the two means of sensing forces in the wall: elastic sensing that tends to orient the CMTs in the direction of the maximal stress, in this case longitudinal, and growth sensing that tends to orient the CMTs transversely to the maximal strain rate. In contrast to the tissues under structure-based tensile tissue stress, in elongating tissues under the compressive tissue stress, orientation of CMTs is transverse. In such a tissue, the maximal stress in cell walls is oriented transversely even in isodiametric cells (that is, in the direction perpendicular to the elongation axis) because the compressive tissue stress decreases the longitudinal tensile stress in cell walls. Both ways of sensing forces in the wall lead to transverse orientation of CMTs, and therefore this orientation can be stable, leading to transverse microfibrils typical for the multinet hypothesis.

For elongating tissues under tensile tissue stress

that have polylamellate walls, we hypothesize the existence of two opposite tendencies in orienting CMTs. The "strength" of these tendencies may determine the duration of "longitudinal" and "transverse" phases of consecutive lamellae formation that in turn determine the relative thickness of the longitudinal and transverse lamellae. Duration of the phases probably depends on the stability of the association between CMTs and plasma membranes as shown by Shibaoka (1993) or by mechanisms responsible for directional stability of microtubules (see Nick 1999). The relative thickness of the lamellae determines the ability of a polylamellate wall to grow longitudinally and transversely; increasing thickness of the recently deposited lamella with longitudinal microfibrils brings elongation to a stop (Lang and others 1982). In general, then, the tissue stresses appear to be an element that allows us to unify the contrasting multinet and helicoidal concepts for formation of different textures in cell walls.

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