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Cell expansion in the epidermis: microtubules, cellulose orientation and wall loosening enzymes

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Summary

Two models of isolated epidermis were used to demonstrate that the net orientation of cellulose microfibrils in the cell wall is related to mechanical properties of the tissue, and can be used as an indicator for wall anisotropy. In the developing plant epidermis, cells expand in one or two directions in the plane of the plant surface. In epidermis cells actively expanding in one direction (elongation), the orientation of cortical microtubules closely matches the net cellulose orientation. In epidermis cells expanding in two directions, the orientation of the parallel microtubules does not coincide with the net cellulose orientation in the adjacent cell wall. The orientation of cortical microtubules is thus not always a reliable indicator of wall characteristics. In both types of epidermis, a high rate of expansion correlates with a high activity of xyloglucan endotransglycosylase (XET), as determined *in situ*. This high activity alone cannot explain unidirectional wall expansion.

Key words: cellulose – cell wall – congo red – microtubules – polarization confocal microscopy – XET

Abbreviations: XET xyloglucan endotransglycosylase

Introduction

Since the early days of plant anatomy, it has been accepted that the mechanical properties of cell walls are a keystone of plant development. In herbaceous species and in meristematic zones, the epidermis in particular is considered to play a crucial role in determining the development of organ form (Hofmeister 1859, Green 1980). Cellulose microfibrils consist of parallel β-1,4-linked glucan chains, and are known to be stiffer and stronger along their length than in the perpendicular direction (Niklas 1992, Preston 1974, Vincent 1990, Wainwright et al. 1976). Elongating plant cells have in their walls parallel arrays of cellulose microfibrils orthogonal to the growth axis of the cell (e.g. Verbelen and Kerstens 2000, Vissenberg et al. 2000 a), while isotropically growing cells do not. Given the characteristics of cellulose, such a specific cellulose orientation is generally extrapolated into distinct physical properties of the complete cell wall, in elongating cells facilitating extension in the longitudinal axis but prohibiting a gain in cell width. In such statements, the link between cellulose orientation and mechanical strength of the wall is accepted a priori, but has not been proven conclusively. It has been postulated that cortical microtubules control the orientation in which new cellulose microfibrils are laid down (reviewed by Giddings and Staehelin 1991, Gunning and Hardham 1982, Robinson and Quader 1982). This view is based

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on the parallel orientation of the cortical microtubules and the cellulose microfibrils, and on the effect of microtubule destabilizing drugs on wall microfibril orientation.

In studies of the relation between wall mechanics and growth patterns, information on the cell wall architecture is consequently often deduced from the specific pattern of cortical microtubules (Fischer and Schopfer 1998). This generally accepted relation between microtubules and microfibrils has, however, continued to be challenged (e.g. Baskin et al. 1999, Emons and Mulder 2000, Fisher and Cyr 1998, Shibaoka 1994, Williamson 1990, Wilms et al. 1990, Zandomeni and Schopfer 1993). Therefore, the question remains of whether or not a record of microtubule orientation is a reliable indicator for the cellulose orientation in the cell wall.

The model of Carpita and Gibeaut (1993) is still a reference in the matter growing cell walls of dicots. They proposed that not cellulose alone, but the cellulose–xyloglucan network is the load-bearing compartment counteracting the cellular turgor pressure, although the pectin matrix could interact with this framework. In the current view of an expanding cell wall, expansins are believed to loosen the cell wall, in close concert with XETs and β-glucanases, allowing turgor-mediated expansion (McQueen-Mason and Rochange 1999).

Recently, we developed an easy and fast method for the determination of the mean or net cellulose microfibril orientation in cell walls *in planta* (Verbelen and Kerstens 2000), and we are currently defining the relation between mean cellulose orientation and the mechanical properties of the epidermal cell layer. A method to visualize XET action *in situ* was also developed (Vissenberg et al. 2000 b). Here we present evidence that the mean cellulose microfibril orientation defines cell wall mechanics. We compare the orientation of microtubules with the net cellulose microfibril orientation in the outer epidermal wall of dicotyledonous plant organs expanding in one or two directions, and we correlate cell expansion with specific activity of the wall enzyme XET.

Materials and Methods

Plants

Plants of *Arabidopsis thaliana* (L.) Heynh. Wild-type were grown from seed under sterile conditions on a Murashige and Skoog medium without hormones (4.7 g/L; Duchefa, The Netherlands), supplemented with 10 g/L sucrose and solidified with 4 g/L Gelrite (Duchefa, The Netherlands), pH 5.7.

Bean (*Phaseolus vulgaris* L. cv. Purple Queen) seeds were grown on vermiculite, whereas onions (*Allium cepa* L.) and *Kalanchoe blossfeldiana* plants were obtained commercially.

Force – extension measurements

Epidermal peels were fixed between clamps and kept in a bath of tap water. The force generated by the samples to counteract an imposed extension (Brüel & Kjær shaker) was measured with a force transducer (Kistler) coupled to a computer. The shaker was steered with a function generator. The force–extension curves shown are the result of a linear extension at a velocity of 0.5 %/s, after two conditioning cycles.

Cytoskeleton and cell wall labelling

For microtubule staining, we used an indirect immunofluorescent method adapted from Vissenberg et al. (2000 a). Material was fixed in PEM-buffer (50 mmol/L Pipes, 5 mmol/L EGTA, and 5 mmol/L $MgSO_4$, pH 6.9) supplemented with 4 % paraformaldehyde and treated with a detergent (Triton-X–100, Sera Lab). FITC-conjugated goat-anti-rat IgGs (Sigma) secondary antibodies were used to detect the primary monoclonal anti-tubulin antibodies (MAS078s, Sera Lab). Primary leaves of bean seedlings were treated similarly. Sections (8 µm) were made after embedding in Steedman's wax (Vitha et al. 1997).

Cellulose microfibrils in the cell wall were stained with 1 % solutions of congo red (Merck CI22120) in culture medium for 30 min, and the net or mean cellulose fibril orientation was determined by measuring the fluorescence dichroism of the wall using confocal polarization microscopy (Verbelen and Kerstens 2000). As the difference in fluorescence intensity cannot be readily visualized in black/white micrographs, the axiality ratio is also mentioned. This is the ratio between the fluorescence intensity of the cell wall with the polarization vector of the laser beam parallel to the long axis of the cell, and the value registered with the vector perpendicular to the cell axis (see number and boxed area in the figures).

XET activity was visualized according to the method described in Vissenberg et al. (2000 b). Briefly, *Arabidopsis* roots and epidermal leaf peels of lamb's lettuce (*Valerianella locusta*) were incubated in 25 mmol/L MES buffer (pH 5.7) containing 6.5 µmol/L sulforhodamine-labelled xyloglucan oligosaccharides (XGO-SRs) for 1 hr. The peels were then washed in ethanol/formic acid/water (15 : 1 : 4 [v/v/v]) for 10 minutes and overnight in 5 % formic acid before being observed.

Fluorescence and confocal microscopy

Fluorescence micrographs were made with a confocal laser scanning microscope (CLSM, Bio-Rad MRC 600 mounted on a Zeiss Axioskop) with a 20× (NA 0.5) dry objective, a 40× (NA 0.9) water-immersion objective or a 63× (NA 1.25) oil-immersion objective, and a co-axial rotating object table.

Results

The net cellulose microfibril orientation and wall mechanics *in vitro*

The relation between net cellulose microfibril orientation and mechanical properties of the epidermis can only be determined on relatively large strips of tissue that are homogenous with respect to the cell type content. Therefore, we used the isolated epidermis of two typical model plants. The adaxial epidermis of onion bulb scales has a very pronounced longitudinal orientation of the cellulose, with an axiality ratio of 1.44 (Fig. 1A, C). The pulling force needed to create a 4 % extension of the tissue is 5 times larger in the longitudinal direction

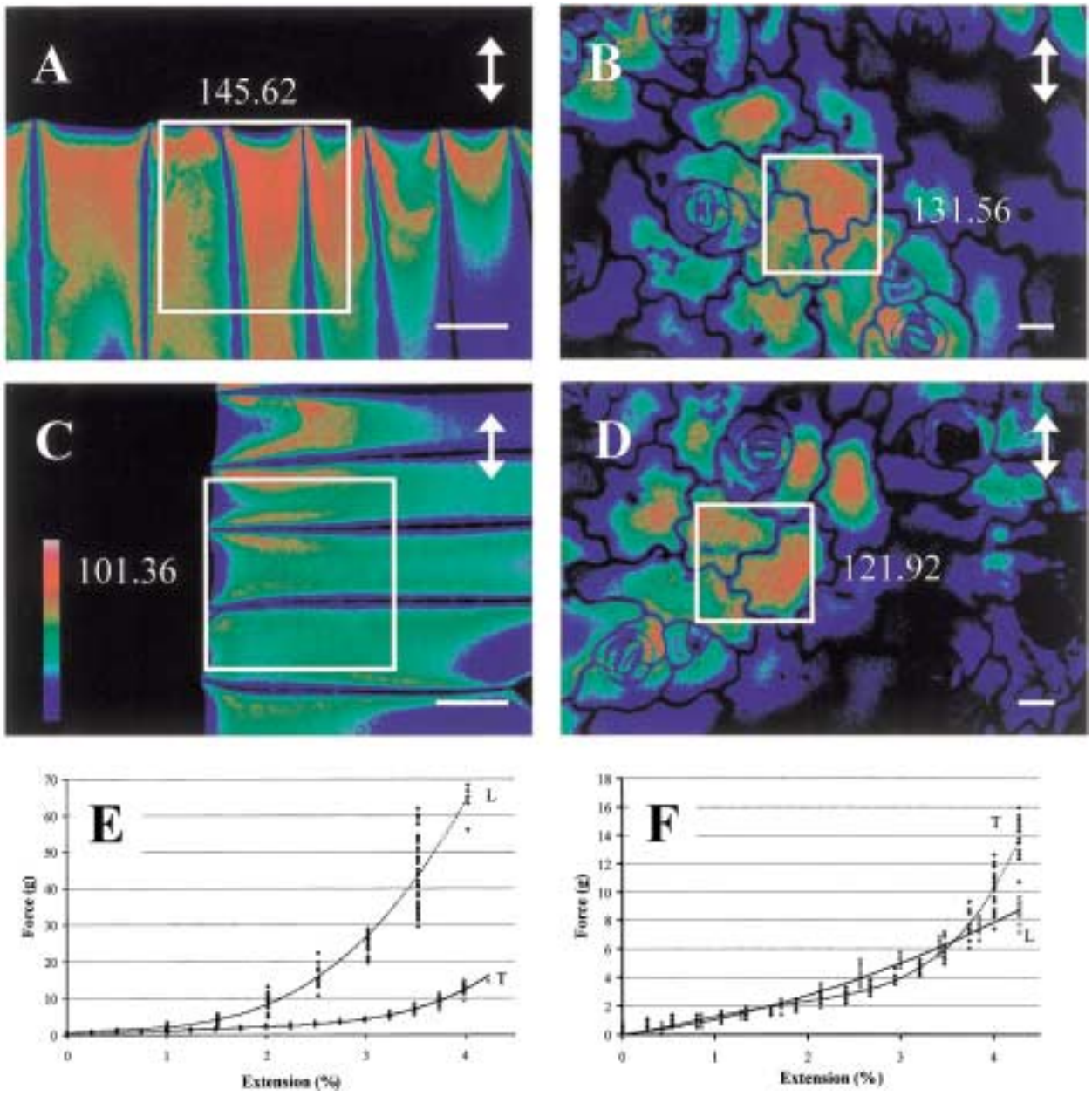

Figure 1. Mean cellulose microfibril orientation and mechanical cell wall anisotropy. The net cellulose orientation is visualized by congo red and polarization CLSM. In the adaxial onion bulb scale epidermis, the fluorescence intensity is maximal when the polarization vector of the excitation light is parallel to the cells long axis (**A**), whereas a rotation of 90 degrees reduces the fluorescence to a minumum (**C**). In *Kalanchoe* there is no significant difference in intensity (**B** and **D**). Graphs in **E** and **F** represent force–extension curves. In the onion peel (Fig. 1E), the force needed to extend the peel in the longitudinal direction (L on figure) is much larger than the force needed for transverse extension (T on figure), whereas in *Kalanchoe* (Fig. 1F) the required forces are more or less equal. For *Kalanchoe*, longitudinal and transverse extension (L and T) are relative to the main vein of the leaf. Bar is 50 µm.

than in the transverse direction (Fig. 1E). The adaxial epidermis of *Kalanchoe* leaves has a net random orientation of cellulose fibrils with an axiality ratio of 1.08 (Fig. 1B, D). In this tissue, the required forces to reach a 4% extension in the directions parallel or transverse to the main vein are nearly equal (Fig. 1F).

Unidirectional expansion *in planta* – cells in the root epidermis

Epidermis cells of *Arabidopsis* roots exhibit cortical microtubules in parallel arrays. These appear as parallel hoops orthogonal to the long axis of the cells from the end of the di-

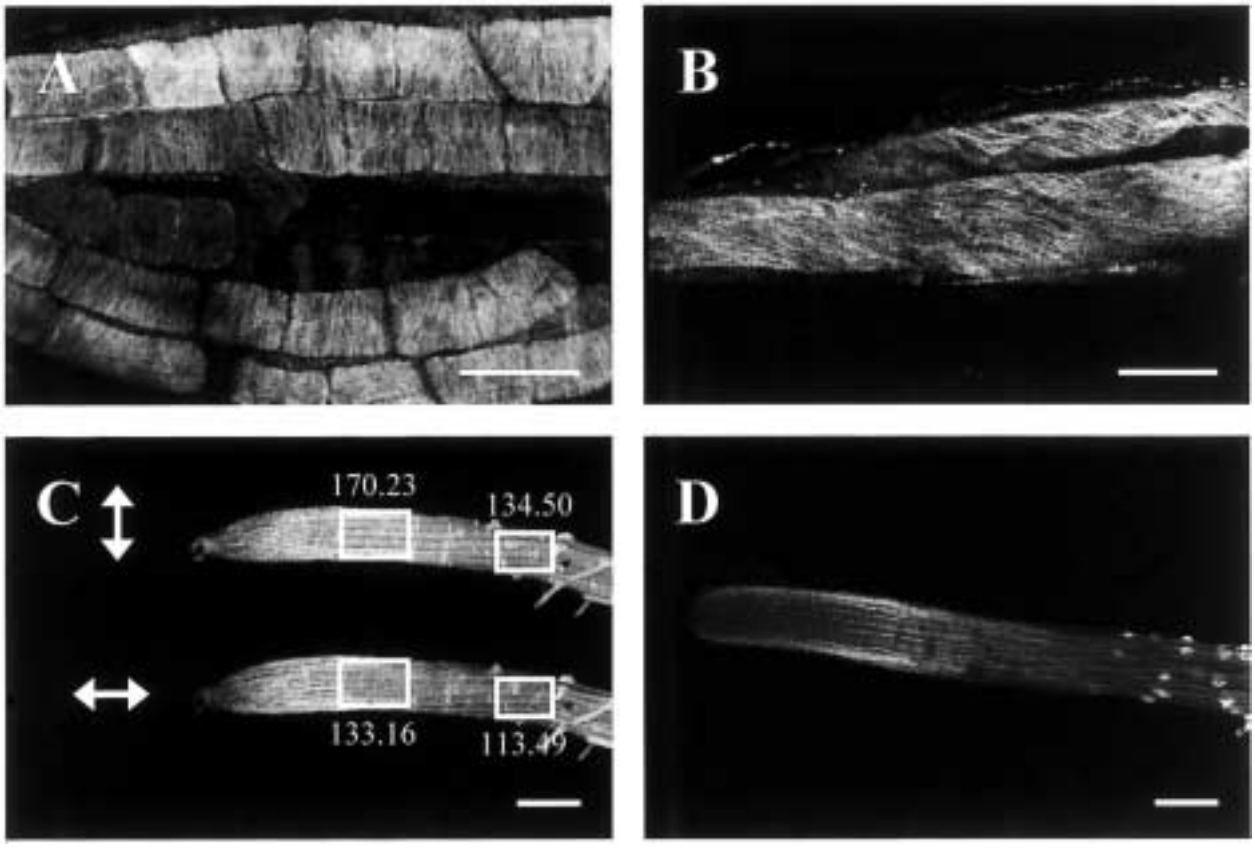

Figure 2. Microtubules, mean cellulose microfibril orientation, and xyloglucan endotransglycosylase activity in a tissue expanding in one direction. Microtubules lie parallel and in transverse hoops throughout the elongation zone of the *Arabidopsis* root (**A**), and become obliquely oriented when the cells enter the differentiation zone (**B**). Similarly, the net cellulose microfibril orientation in the elongation zone is clearly transverse, while the transverse, orientation is less pronounced in the differentiation zone (**C**). On the other hand, XET activity is preferentially confined to the elongation zone proper (**D**). Bar is 20 µm in A and B, 100 µm in C and D.

vision zone throughout the elongation zone (Fig. 2 A). In the differentiation zone, microtubules are still parallel but oblique in different angles to the long axis of the cell (Fig. 2 B).

The outer periclinal wall of epidermis cells in the elongation zone clearly has a net transverse cellulose orientation with an axiality ratio of 0.78 (Fig. 2 C, boxed areas). In the differentiation zone, marked by the development of root hairs, the cells have a less pronounced transverse orientation, with an axiality ratio of 0.84 (Fig. 2 C, boxed areas).

XET activity is especially confined to the walls of epidermal cells in the elongation zone of the root. In the differentiation zone there is clearly much less XET activity (Fig. 2 D, see also Vissenberg et al. 2000 b).

Bidirectional expansion *in planta* – leaf epidermis cells covering the palisade mesophyll

In paradermal sections of the adaxial epidermis of expanding primary bean leaves, cortical microtubules are found organized in parallel arrays but with a varying orientation in neighbouring cells. This is illustrated for a young leaf in Figure 3 A and for an older leaf in Figure 3 B.

The outer periclinal wall of epidermal cells of similar leaves exhibits a net random cellulose orientation, with an axiality ratio of 1.06. The fluorescence intensity of the walls of the cells represented in Figure 3 C and D (boxed areas) is indeed very similar.

XET visualization in isolated peels of lamb's lettuce leaves reveals that XET activity is high in young actively growing leaves (Fig. 3 E), whereas in adult non growing leaves, the activity is very low (Fig. 3 F).

Discussion

A long-standing dogma in plant science states that the parallel orientation of the cellulose microfibrils in the plant cell wall reflects the direction of cell expansion and plant growth (e.g. Roelofsen 1951) due to the inherent mechanical anisotropy of cellulose. So it is also thought that in young developing plant parts, the epidermis exerts a key-morphogenic control, pre-

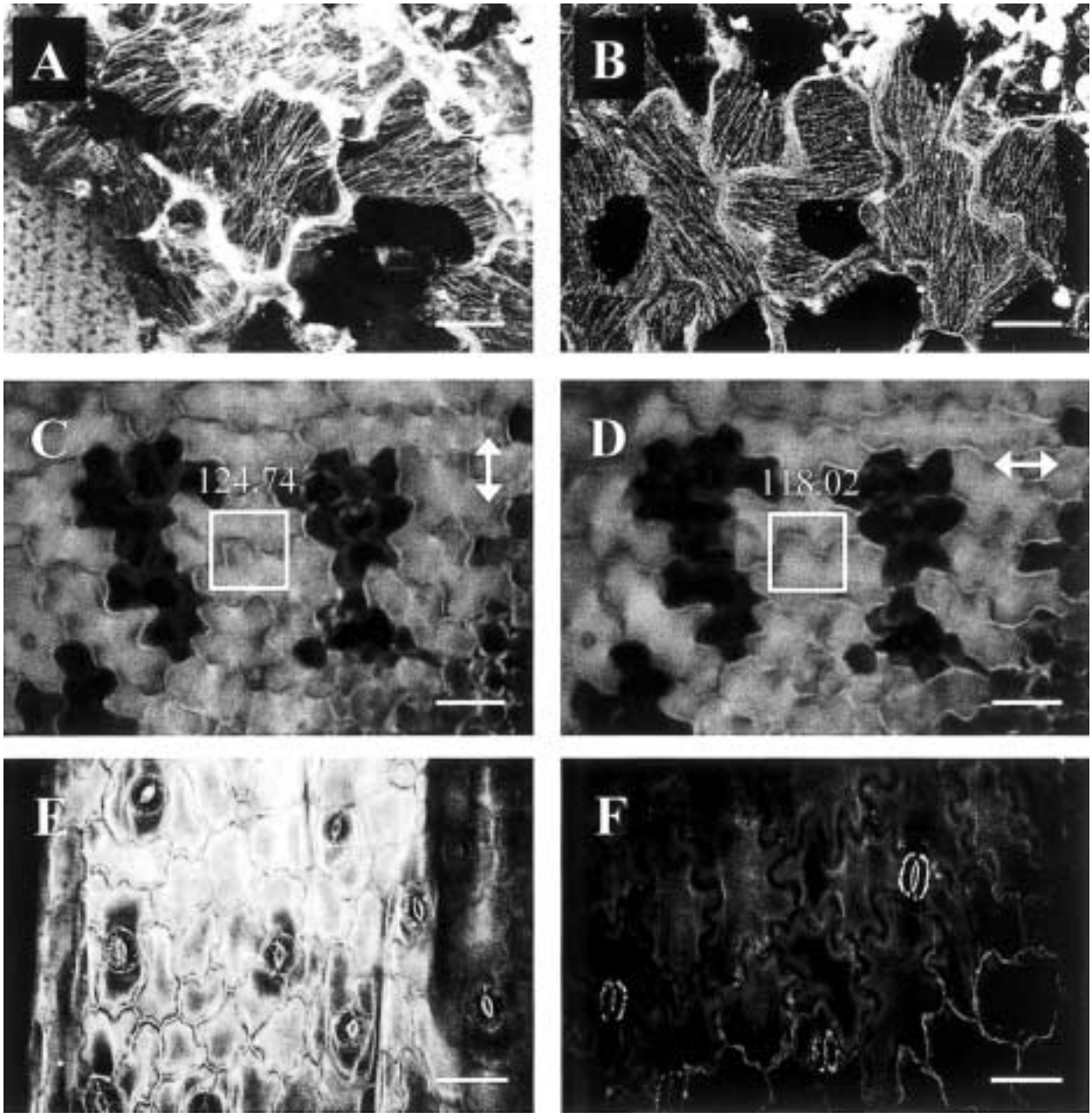

Figure 3. Microtubules, mean cellulose microfibril orientation, and xyloglucan endotransglycosylase activity in tissues expanding in two directions. In adaxial bean leaf epidermis cells, microtubules lie parallel to each other, but with a varying orientation in neighbouring cells. This is shown in a young (**A**) and in an older leaf (**B**). The mean cellulose orientation, however, is random, as no big differences in fluorescence intensity can be found when excited at two different orientations (**C** and **D**). In lamb's lettuce, high XET activity is found especially in the epidermis of young expanding leaves (**E**), whereas the activity is low in old, non-expanding leaves (**F**). Bar is 50 μm.

cisely through the mechanical properties of its wall positioned at the interface plant–environment (Hernández and Green 1993, Peters and Tomos 1996). The force–extension experiments on single cell layers combined with the congo red visualization method provide experimental evidence for both paradigms. A cell wall with net parallel and longitudinal cellulose microfibril orientation resists uniaxial extension in the longitudinal direction better than in the transverse direction. A cell wall with a net random cellulose microfibril orientation is mechanically isotropic.

Following the hypothesis of Heath (1974), cortical microtubules can guide the cellulose synthesising complexes in the plane of the plasma membrane. Microtubules were in this way, considered to control the orientation of the cellulose

microfibrils, the direction of cell expansion (Giddings and Staehelin 1991, Gunning and Hardham 1982, Robinson and Quader 1982). However, this relation between cortical microtubules and wall properties has often been questioned (see Introduction).

In dicotyledonous roots, epidermis cells exhibiting fast anisotropic growth or elongation in general have their cortical microtubules parallel to each other and parallel to the microfibril orientation. In leaf epidermis cells that expand in two directions, microtubules are parallel to each other but with varying orientations, while the overlaying walls exhibit a net random orientation. There is, however, no fundamental contradiction between the orientation patterns of microtubules in the cell cortex and microfibrils in these cell walls, as microtubules regularly change their orientation (Yuan et al. 1994). But a caption of microtubule orientation at a given moment is not necessarily a good reflection of the orientation of the cellulose microfibrils in the cell wall, nor a good method for developing hypotheses concerning cell wall mechanics. We therefore state that net cellulose orientation of the cell walls is always the preferred indicator of mechanical anisotropy of the wall, relevant for cell expansion and thus also for plant growth.

In the current view of cell wall architecture, it is not the cellulose framework but the cellulose–xyloglucan network that is the load-bearing component of cell walls (Carpita and Gibeaut 1993). In such a structure, xyloglucan endotransglycosylase (XET) is believed to cut and rejoin the load-bearing xyloglucan chains during wall extension (e.g. Catalá et al. 1997, Palmer and Davies 1996). We found that high XET activity is indeed correlated with very active cell expansion in the epidermis, in elongating organs as well as in organs with planar expansion. A very recent model specifically states that the xyloglucan domains connecting the cellulose fibrils are enzymatically affected, thus allowing the fibrils to move apart (Pauly et al. 1999). This model suits the isotropic expansion of walls but it does not fully explain elongation in anisotropic walls. In these walls, xyloglucans are the bonding materials that well line up individual fibrils into the parallel bundles or hoops, as forms the kind of spacer arms keeping the hoops parallel to each other. For elongation to occur, the latter xyloglucans have to be affected by wall-loosening enzymes like XET, and the former have to be protected from enzyme activity. Clearly, in this aspect, the actual models have to be refined.

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