Onion epidermis as a new model to study the control of growth anisotropy in higher plants

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Abstract
To elucidate the role of cellulose microfibrils in the control of growth anisotropy, a link between their net orientation, in vitro cell wall extensibility, and anisotropic cell expansion was studied during development of the adaxial epidermis of onion (Allium cepa) bulb scales using polarization confocal microscopy, creep tests, and light microscopy. During growth the net cellulose alignment across the whole thickness of the outer epidermal wall changed from transverse through random to longitudinal and back to transverse relative to the bulb axis. Cell wall extension in vitro was always higher transverse than parallel to the net cellulose alignment. The direction of growth anisotropy was perpendicular to the net microfibril orientation and changed during development from longitudinal to transverse to the bulb axis. The correlation between the degree of growth anisotropy and the net cellulose alignment was poor. Thus the net cellulose microfibril orientation across the whole thickness of the outer periclinal epidermis wall defines the direction but not the degree of growth anisotropy. Strips isolated from the epidermis in the directions perpendicular and transverse to a net cellulose orientation can be used as an extensiometric model to prove a protein involvement in the control of growth anisotropy.

Key words: Cellulose orientation, cell wall, extensiometry, growth anisotropy, onion.

Introduction
The huge variety of plant shapes is achieved through a combination of oriented cell divisions and anisotropic cell expansion. Anisotropy in cell growth means that a plant cell expands at different rates in different directions, and it is characterized by the direction and the degree of anisotropy. The former indicates the direction in which the maximal expansion rate occurs while the latter specifies the degree to which this maximal rate exceeds the minimal rate at a given time point (Baskin et al., 1999).

Anisotropic expansion results from anisotropic cell wall structure and/or anisotropic stress distribution in the wall. Due to methodological problems the role of stress distribution in the control of growth anisotropy is still poorly investigated, whereas the role of anisotropic cell wall structure is well documented (Baskin, 2005). Cellulose microfibrils are considered as key elements conferring structural anisotropy and reinforcing the wall parallel to their orientation. As a result, the cell wall will extend more easily transverse to the net cellulose alignment (Taiz, 1984).

Several lines of evidence confirm that the specific alignment of cellulose microfibrils controls the direction of expansion anisotropy. First, it is deduced from the correlative data indicating that plant cells usually grow faster in the direction transverse to the net cellulose orientation in their walls (Sugimoto et al., 2000). Secondly, biomechanical experiments with isolated cell walls show that they are more extensible in the direction transverse to the net microfibril alignment (Richmond et al., 1980; Kerstens et al., 2001; Suslov and Verbelen, 2006). Finally, suppression of cellulose synthesis genetically or by inhibitors usually disorganizes the microfibril orientation in the cell wall and disturbs normal anisotropic expansion (Desprez et al., 2002; Pagant...
et al., 2002; Himmelspach et al., 2003; Roudier et al., 2005; Schopfer, 2006).

The role of cellulose microfibrils in the control of the degree of anisotropy is still debatable. Originally it was postulated that the degree of expansion anisotropy in Nitella cell walls depends on the extent to which microfibrils are aligned (Green, 1964). The higher the microfibril alignment in the cell wall the more anisotropic growth takes place. However, this hypothesis was questioned for higher plants since strong changes in the degree of expansion anisotropy were not associated with changes in the extent of microfibril alignment (Baskin et al., 1999; Wiedemeier et al., 2002; Sugimoto et al., 2003). There were several explanations for this discrepancy. First, it was hypothesized that the degree of expansion anisotropy could be controlled via cellulose-independent mechanisms, such as deposition, formation, and/or rearrangement of other wall polysaccharides or changes in the net rate of secretion and activity of wall proteins (Sugimoto et al., 2003). In line with this assumption genetic evidence indicated that different non-cellulosic factors were responsible for growth perpendicular and parallel to the net cellulose orientation (Wiedemeier et al., 2002). According to an alternative point of view the degree of expansion anisotropy is controlled by a fine arrangement or microfibril misalignment in the cell wall the more anisotropic growth takes place. However, this hypothesis was questioned for higher plants due to considerable differences in the wall composition and molecular mechanisms of cell expansion of higher plants and charophycean green algae (Cosgrove, 2000; Popper and Fry, 2003; Proseus and Boyer, 2006).

This work demonstrates that the adaxial epidermis of onion (Allium cepa) bulb scales can be used as a novel model for unravelling the mechanisms of control of anisotropic expansion in higher plants. By using the correlative approach described above, the model allows checks to be made on whether cellulose microfibrils or other factors define the degree of growth anisotropy. Biomechanical experiments with strips of the adaxial epidermis extended in vitro can be used to establish an involvement of different cell wall loosening/tightening factors in the control of the degree of growth anisotropy.

Materials and methods

Sampling of plant material

The onions (A. cepa L. cv. Bonkajuin) used for this study were planted in a field at the end of March as sets (small bulblets). The developing bulbs were collected at week 6, 8, 10, and 12 after planting and are referred to in the text as stages I, II, III, and IV, respectively. Five bulbs were chosen for analysis from the material collected at each stage in such a way that their diameters were: (i) approximately equal and (ii) close to the maximal for that stage. Lower parts (5–40 mm from the scale base) of all green leaf-carrying scales of each bulb were excised and cut into two (three) vertical segments. The first segment of each scale was used for cell size measurements. The four 4×4 mm areas spaced evenly along its vertical axis were excised, and their adaxial epidermis was peeled and photographed immediately. The second segment of each scale was used for analysis of the net cellulose orientation. Again the four 4×4 mm areas were excised and peeled as above, and their adaxial epidermis was immediately frozen in liquid nitrogen and stored at −20 °C before analysis. The third segment was excised only from the sufficiently large scales (the majority of scales at stages III and IV) and used for extensometry. The 25–30 mm long and 4 mm wide epidermal strips were peeled from it in directions parallel and perpendicular to the
axis of a bulb. These ‘longitudinal’ and ‘transverse’ epidermal strips were immediately frozen in liquid nitrogen, stored at −20 °C, and used for analysis within 1 week after isolation. As the adaxial epidermis adheres very weakly to the underlying parenchyma in young onion bulbs, the epidermal strips used here always consisted of one cell layer.

Numbering of the scales reflects the actual order of their formation during bulb development. Number 1 is the most external scale at stage I, being the first green leaf-carrying scale formed; the higher numbers were assigned to subsequent more internal (and younger) scales. Despite the fact that scale 1 was dead after stage I, this leaf-carrying scale was clearly visible up to stage IV. Using scale 1 as a reference allows establishment of the unified scale numbering at stages I–IV.

**Cell size measurements and determination of degree of growth anisotropy**

Micrographs of the 4×4 mm areas of the adaxial epidermis were made with a digital camera (Nikon DFM1200) mounted on a Zeiss Axioskop microscope using a ×5 or ×10 objective. All cells have a more or less rectangular shape in the plane of the epidermis. The length and diameter of 12 cells in each 4×4 mm area were measured using ScionImage software (www.scioncorp.com). Given that the four areas were studied in each scale of a bulb, and a total of five bulbs were analysed, the reported cell size data are based on 240 repeats (12×4×5=240).

The rate of cell growth in length (or diameter) in the epidermis was calculated according to the formula (\(\ln D_n - \ln D_{n-1} / T\times100\%\)) where \(n-1\) and \(n\) indicate, respectively, times corresponding to the beginning and the end of the specified growth interval; \(D\) represents the average cell length (or diameter) for \(n=240\); and \(T\) is the time between \(n-1\) and \(n\) (Green, 1976). The degree of growth anisotropy was calculated as a ratio of cell growth rate in the two dimensions.

**Cell wall staining and analysis of the net cellulose microfibril orientation**

The net or mean orientation of cellulose microfibrils in the outer periclinal wall of the adaxial epidermis was determined using polarization confocal microscopy and fluorescent Congo Red staining as described previously (Verbelen and Stickens, 1995; Suslov and Verbelen, 2006). The thawed 4×4 mm areas of the epidermis were stained in a 1% (w/v) solution of Congo Red (Sigma) in water for 2 h. After rinsing in water, the Congo Red-mediated fluorescence of the outer cell walls was studied in 2–3 cells of each 4×4 mm area using a confocal laser scanning microscope (Nikon D-Eclipse C1) with a ×20 dry objective and a co-axial rotating object table. Given that the four areas were studied in each scale of a bulb, and a total of five bulbs were analysed, the reported data on the net cellulose orientation are based on 50 repeats (10 repeats in each scale of a bulb).

The dichroic fluorescent dye Congo Red preferentially absorbs light directed parallel to the dipole moment of its chromophoric group. It has an ability to bind specifically to β-1,4-linked glucan polymers, such as cellulose, which results in aligning the Congo Red chromophoric group dipole moments in the direction of the cellulose polymer orientation. In the case where the cellulose fibrils have a preferential orientation, Congo Red fluorescence will be maximal when the vector of the polarized exciting laser beam is parallel to the net cellulose orientation (Fig. 1A, D, E). When the vector is perpendicular to the net microfibril orientation, fluorescence intensity will be minimal (Fig. 1B, C, F). In the case of a net random cellulose orientation, wall fluorescence intensity will be equal for all orientations of the polarized light vector (Fig. 1G, H).

The extent to which cellulose microfibrils were aligned with respect to the cell’s long axis, which is parallel to the axis of the bulb, is expressed as the axiality ratio (AR). This is the ratio between the fluorescence intensities (quantified using Adobe Photoshop 4.0 software) with the vector of the excitation light directed parallel and perpendicular, respectively, to the cell’s longitudinal axis. The AR equals unity if the net cellulose microfibril orientation is random (Fig. 1G, H). It will have values above (Fig. 1C, D) or below (Fig. 1A, B) unity in walls with a net cellulose orientation parallel or perpendicular, respectively, to the cell axis.

**Extensiometry**

The in vitro extensibility of onion epidermal cell walls was studied with a custom-built constant-load extensiometer as described previously (Suslov and Verbelen, 2006). A 10 mm segment of a 4 mm wide epidermal strip was secured between the extensiometer clamps and extended by a 10 g load while being completely submerged in a solution of 10 mM sodium citrate buffer (pH 6.0). Its extension was measured for 15 min and characterized by the average creep rate at the interval 10–15 min after the load application, which represents a good diagnostic step for the extensibility measurement (Suslov and Verbelen, 2006).

**Analysis of the effect of stretching on the net cellulose microfibril orientation**

The AR in the outer wall of a randomly chosen cell in the central part of a transverse epidermal strip (prepared as for extensiometry) was determined as described above. The epidermal tissue next to the chosen cell was marked with black ink to allow easy tracing of the cell taken. A 1 cm segment of the strip containing the marked region was secured between the extensiometer’s clamps and extended under the same conditions as described for extensiometry. Subsequently the strip was removed from the extensiometer, and the AR of the chosen cell (in relaxed state) was determined again and compared with its initial value.
Results

General characterization of growing onion bulbs

Several external scales of the bulblets planted in the field do not produce green leaves. After being used as a source of nutrients they die ~6 weeks after planting. An easily separable envelope of these dead and partially disintegrated scales marks the border of a new developing bulb. It is composed of leaf-carrying scales whose adaxial epidermis was studied in the present work.

The total number of leaf-carrying scales (Fig. II–K) present during the early stages of onion bulb growth is defined by the balance of their formation inside the bulb and their death at the bulb’s periphery (Table 1). Formation of new scales stops between stages III and IV as the total number of live scales at stage IV (from seven to nine) is equal to that found in mature onion bulbs of the cultivar studied (D Suslov et al., unpublished data). The oldest external leaf-carrying scales dying at stages II–IV become dry and form a multilayer envelope protecting the underlying live scales.

Fig. 1. Onion bulb scales and their adaxial epidermis. (A–D) Polarization confocal micrographs of Congo Red-stained adaxial epidermis cell walls demonstrating the mean cellulose microfibril orientation. The intensity of fluorescence is colour-coded (low, blue; high, red). The polarization vector of the laser beam is indicated by arrows. The intensity of fluorescence is maximal when the vector of polarization is parallel to the net cellulose orientation, and it is minimal when the vector of polarization is perpendicular to the net microfibril orientation. Accordingly, cell walls from scale 6 at stage I (A, B) have the net transverse orientation of microfibrils to the bulb’s axis (which is parallel to the long axes of cells) while cell walls from scale 2 at stage I (C, D) have the net longitudinal cellulose orientation. (E–H) Polarization confocal micrographs of Congo Red-stained adaxial epidermis cell walls demonstrating cellulose microfibril reorientation in the direction of strain in vitro. Epidermal cell walls with the net longitudinal cellulose orientation (E, F) from scale 7 at stage III were extended in vitro perpendicular to this orientation by a 10 g load for 15 min. As a result, microfibrils realigned in the direction of strain, and their orientation became random (G, H). (I–K) General view of onion scales at different stages of development: (I) the youngest scale soon after its formation, (J) the same scale at the next early stage of development, and (K) the oldest live scale in a young onion bulb. Upper and lower margins of the scales are marked with arrows. In order to show the scales in (I) and (J) all other scales were removed, the remaining scales were cut longitudinally across their green leaves into two symmetrical halves, and their concave sides with the adaxial epidermis facing the viewer were photographed. (L–N) Cross-sections of the adaxial epidermis of scale 5 at stage III. Optical cross-sections stained with (L) Congo Red [1% (w/v) in water] and (M) propidium iodide [0.001% (w/v) in water] obtained using a confocal microscope. (N) Hand-made sections embedded into a LX112 resin and stained with toluidine blue [0.5% (w/v) in 0.5% (w/v) sodium borate]. Insets in the lower right corners of L–N are ×2 magnifications of the boxed areas. Scale bars are 60 μm (A–D); 100 μm (E–H); 5 mm (I); 10 mm (J, K); 20 μm (L–N).
At stage I each of the leaf-carrying scales represents a tube with the same diameter along its length (Fig. 1J). A localized growth in girth close to the base of these tube-shaped juvenile scales starts at stage II and highlights the beginning of formation of a bulb. The subsequent progressive outgrowth results first in the formation of an axially extended bulb (stage III) and, finally, leads to a spherical bulb (stage IV) (Table 1). The early onion bulb development studied here involves all stages from the first signs of bulb formation to a small spherical bulb containing the final number of live scales typical for this onion cultivar.

**Cell dimensions and growth**

Cell dimensions in any plant organ are defined by the balance of their expansion and division. The former increases cell size while the latter decreases it (Green, 1976). In the absence of cell divisions the rate and the direction of growth in a plant organ are proportional to the increase in cell dimensions. Staining the adaxial epidermis of onion bulb scales with acridine orange did not reveal mitotic nuclei in any of the scales studied (results not shown). Therefore, the data on cell dimensions in the epidermis at the successive stages of development can be used to calculate the average rate of expansion in length and in diameter, and to determine the direction and the degree of growth anisotropy.

On cross-sections the adaxial epidermis cells look more or less like circles (Fig. 1L–N). In longitudinal sections they have a rectangular to slightly irregular elliptical shape (not shown). Although adjacent cells in the same epidermis can differ considerably in size (Fig. 1A, B), there are no notable cell size gradients in the lower part of the adaxial epidermis that participates in onion bulb formation.

A plot of mean cell dimensions (Fig. 2) shows the main trends of development. At each stage (I–IV) there is a gradual increase in cell size from the youngest to the oldest scales. Between stage I and II cell growth is small or nil. There is only a significant increase \((P<0.001)\) in cell length in the youngest scale 6 (Fig. 2A) while a significant increase in cell diameter \((P<0.001)\) takes place in all scales with the exception of scales 4 and 5 (Fig. 2B). From stage II on the increase in both cell dimensions was much higher.

A detailed analysis of these data demonstrates that cell expansion in the adaxial epidermis is mostly anisotropic (Fig. 2). Moreover not only the degree of growth anisotropy but also its direction changes during onion scale development (Table 2). The youngest scales grow predominantly in length (Fig. 1I, J) while older scales grow predominantly in diameter (Fig. 2, Table 2). The fact that the intermediate scales 4 and 5 do not grow at the interval I–II (Fig. 2) indicates that the phases of preferential growth in length and in diameter are well separated in time and space, at least at the beginning of onion bulb formation. Analysis of many onion bulbs at stages I–III reveals very few young scales having the intermediate length (Fig. 1J) while the majority are either very short (Fig. 1I) or their length is close to that of the oldest scales (Fig. 1K). Apparently the initial elongation of scales is relatively rapid and transient, which makes it more difficult to be measured reliably within the time scale used in the present study. This rapid axial extension of scales is followed by a slow expansion predominantly in width, leading to onion bulb formation. The development of scale 6 at the intervals I–II, II–III, and III–V clearly illustrates these changes in the direction and the degree of growth anisotropy (Table 2).

**Cellulose microfibril orientation**

Cellulose microfibril orientation was studied in the outer periclinal walls of the epidermis cells using polarization confocal microscopy and fluorescent Congo Red staining. In Fig. 1L–N the efficiency of cell wall staining by Congo Red is estimated by comparing its result with propidium iodide and with toluidine blue staining of epidermal cross-sections. The outer periclinal wall is considerably thicker than the other cell walls in the tissue. When stained with the cellulose-specific Congo Red, it looks like a bright band bordered by two fainter bands (Fig. 1L, insert). The outer cell walls stained with propidium iodide or with toluidine blue binding to various wall polymers look like homogenous bands of the same thickness (Fig. 1M, N, inserts) as the bright Congo Red band. This shows that Congo Red penetrates well through the whole thickness of the outer epidermal wall and therefore gives information on the mean cellulose orientation across all wall layers. The faint bordering bands are probably caused by a limited Congo Red diffusion from the wall into the surrounding solution.

The profiles of mean cellulose orientation in the scales at different stages during bulb development are shown in
Fig. 3. This orientation is expressed as the AR and demonstrates striking changes during growth. In scale 6, present at all stages studied, the mean cellulose orientation changed from transverse (stage I, AR = 0.78) through random (stage II, AR = 0.98) to longitudinal (stage III, AR = 1.41) and back to transverse (stage IV, AR = 0.80). Other scales repeated at least part of this scenario during their development (Fig. 3). Apparently there are two phases of cellulose reorientation in the epidermis of developing scales. During the first phase microfibrils attain a more longitudinal orientation, whereas during the second phase they readopt a more transverse orientation. The first phase is more difficult to detect than the second one due to its transient nature. It is not seen between stages III and IV when the formation of new scales proceeds very rapidly (Fig. 3, Table 1).

Cellulose microfibril orientation and direction of growth anisotropy

Does the mean cellulose orientation define the direction of anisotropic cell expansion in the epidermis? For 11 of the 12 data points available for the growing epidermis the direction of maximal cell expansion agreed perfectly with the net cellulose orientation (Table 2): cell growth was maximal in the direction perpendicular to the mean microfibril alignment, while isotropic expansion (scale 6 at the interval II–III) was concurrent with a random mean cellulose orientation. The exception was the epidermis of scale 8 that grew predominantly in length between stages III and IV, despite having the net longitudinal microfibril alignment (AR = 1.12).

Cellulose microfibril orientation and degree of growth anisotropy

The involvement of cellulose microfibrils in the control of the degree of growth anisotropy would infer that an increase in the degree of microfibril alignment (seen as a shift in AR value further away from unity) would result in a higher anisotropy in cell expansion. The data obtained do not support such a relationship (Table 2). In the situations when cellulose orientation defines the direction of anisotropic expansion the correlation coefficient between the degree of growth anisotropy (when its value is finite) and the net microfibril alignment is only 0.38. The scales 1 (interval I–II) and 6 (interval III–IV), and 3 and 4 (interval II–III) illustrate well why this link is so weak (Table 2). They have very different degrees of growth anisotropy at the same microfibril alignment whose variability between different cells in each of the above-mentioned scales is similar since the corresponding AR values have statistically the same standard deviations. Cellulose microfibril alignment is thus not the major factor controlling the degree of growth anisotropy.

Mechanisms of cellulose microfibril reorientation

On the one hand cellulose microfibrils define the direction of growth anisotropy and on the other hand their orientation might in turn change as a result of directional growth. This phenomenon was described in the multinet growth theory (Roelofsen, 1958). In a modern interpretation the theory can be read as follows: cellulose synthesis at the plasmalemma is organized in such a way that new microfibrils are deposited on the inside of the wall transverse to the direction of growth anisotropy. As growth proceeds and wall synthesis continues they are displaced deeper into the wall and passively reorient in the direction of growth anisotropy, thus adopting progressively a more ‘longitudinal’
Table 2. Cellulose microfibril orientation and direction and degree of growth anisotropy in the adaxial epidermis of onion bulb scales

The axiality ratio values (mean ± SD, n = 50) are determined at the beginning of the specified intervals of growth measurement (see Fig. 3). Cellulose orientations are shown relative to the long axis of a bulb. The degree of growth anisotropy is calculated on the basis of growth rates given in Fig. 2. The direction of growth anisotropy is shown in bold when it agrees with the net microfibril orientation. Two pairs of data obviously inconsistent with the idea that cellulose microfibril alignment controls the degree of growth anisotropy are underlined and double underlined.

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<tr>
<td>I–II</td>
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<td></td>
<td>CO</td>
<td>Longitudinal</td>
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<td></td>
<td>DRA(DGA)</td>
<td>D(21.8)</td>
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<tr>
<td>II–III</td>
<td>AR</td>
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<td></td>
<td>CO</td>
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<tr>
<td></td>
<td>DRA(DGA)</td>
<td>D(2.12)</td>
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<tr>
<td>III–IV</td>
<td>AR</td>
<td>1.09±0.18</td>
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<tr>
<td></td>
<td>CO</td>
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<td>DRA(DGA)</td>
<td>D(2.18)</td>
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AR, axiality ratio; CO, cellulose orientation; DRA, direction of growth anisotropy; DGA, degree of growth anisotropy; D, cell growth in diameter is higher than cell growth in length; L, cell growth in length is higher than cell growth in diameter; I, equal growth rate in length and in diameter (isotropic growth); –, the direction and the degree of growth anisotropy cannot be determined as there is no significant growth in length or in diameter.

Fig. 3. Cellulose microfibril orientation in outer cell walls of the adaxial epidermis of scales during onion bulb development. The net or mean cellulose orientation is expressed as the axiality ratio. The axiality ratio value of 1.0 marked with the dashed line corresponds to the net random cellulose orientation. Data points located above and below this dashed line indicate, respectively, longitudinal and transverse net cellulose microfibril orientations relative to the long axis of an onion bulb. Numbers I–IV denote successive stages of onion bulb development. Data are means ±SE (n=50).

Table 3. Cellulose microfibril reorientation in the course of anisotropic cell growth in the adaxial epidermis of onion bulb scales

Cellulose microfibril orientation is expressed as the axiality ratio (mean, n=50, Fig. 3). Its values above and below the arrows refer, respectively, to the beginning and to the end of the specified intervals of growth measurement. The data for the direction of growth anisotropy are taken from Table 2. Changes in the axiality ratio are shown in bold if microfibril reorientation takes place in the direction of growth anisotropy. The two data points which are consistent with the active mechanism of cellulose reorientation are underlined.

<table>
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<th>Interval</th>
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<tr>
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a No significant cellulose reorientation. For all the remaining data cellulose reorientation was significant at P <0.05 (t-test). Abbreviations are the same as in Table 2.

orientation. Consequently a change in mean microfibril orientation in the wall may result from the active process (microfibril synthesis and deposition along a predetermined direction) and the passive process (microfibril reorientation in the direction of growth anisotropy). What is the role of these processes in the changes of mean microfibril orientation described in Fig. 3? The data of Table 3 clearly demonstrate that in nine of 11 cases, when statistically significant microfibril reorientations occurred, they took place in the direction of maximal growth, suggesting the involvement of the passive mechanism of reorientation. This assumption was confirmed when epidermal strips with a mean longitudinal
It is a generally accepted idea that cellulose microfibril orientation regulates growth anisotropy by influencing cell wall extensibility, the key parameter defining the rate of plant cell extension (Richmond et al., 1980; Suslov and Verbelen, 2006). The link between the net cellulose microfibril orientation and wall extensibility was studied in the course of onion bulb development. Extensibility of the epidermis walls was estimated in vitro using a constant load (creep) test at stages III and IV, when the majority of scales were large enough to isolate sufficiently long epidermis strips for extensometry (Fig. 4).

The wall extension in vitro correlates rather well with the net microfibril orientation. At stage III scales 6 and 7 have longitudinally oriented microfibrils in the epidermis walls and their mechanical properties are strongly anisotropic. Their extension in vitro is significantly higher in the direction transverse to the mean cellulose orientation than parallel to it (Fig. 4A). At the same time the walls of the epidermis of the mature scales 3 and 4 have randomly oriented microfibrils and isotropic mechanical properties (Fig. 4A). The link between the net cellulose orientation and wall extension in vitro is especially clear in the case of scale 7 development between stages III and IV. The net microfibril orientation in this scale changes from longitudinal at stage III (Fig. 4A) to transverse at stage IV (Fig. 4B). The wall extension in vitro changes accordingly: it is maximal transverse to the bulb’s axis at stage III and in the longitudinal direction at stage IV. Thus wall extension in vitro invariably remains maximal in the direction perpendicular to the cellulose orientation, also during the drastic microfibril reorientation occurring in scale 7.

However, this link between the net cellulose orientation and the wall extension in vitro has only a qualitative character. For example, scales 6 and 7 have statistically the same cellulose alignment in the epidermis walls at stage III (AR 1.41±0.05 and 1.33±0.06, respectively) but their extensions in vitro transverse to the net microfibril orientation are practically 2-fold different (Fig. 4A). Clearly factors other than cellulose alignment alone participate in the quantitative control of the wall extensibility.

**Discussion**

The adaxial epidermis of onion scales was chosen for analysis as it may be a key player in the control of onion bulb growth. The thick outer epidermal cell walls have often been considered to limit growth of whole organs (Kutschera, 2008a,b; Kutschera and Niklas, 2007). This might well be the case for the adaxial epidermis of the onion...
scales as its outer periclinal wall is much thicker than the walls of underlying parenchyma cells and much less extensible than the walls of abaxial epidermis (unpublished results). Moreover, the adaxial epidermis has all the essential properties mentioned in the Introduction required for answering the question of the role of cellulose microfibrils in the control of the direction and the degree of growth anisotropy.

Growth of onion scales includes two phases with different rate and direction of cell expansion

The present work focused on the study of directional cell expansion resulting in onion bulb formation. Accordingly only the lower part of the adaxial epidermis (5–40 mm from the base of a bulb) directly participating in this process was investigated. The anisotropic growth of epidermis cells is characterized by changes in the degree and in the vector of anisotropy (Table 2) and has two phases. First epidermal cells grow predominantly in length, then they extend more in width than in length. Comparable developmental changes in growth anisotropy were described in other flat organs of plants such as fescue leaves (Maurice et al., 1997) and Antirrhinum petals (Rolland-Lagan et al., 2003). The first phase of onion scale growth is a relatively short period of a fast elongation, occurring in newly formed scales. A new scale appears as the base of a leaf (Fig. 11) at the bottom inside the long tube formed by more mature leaf-carrying scales. Rapid elongation during the first phase may thus help the young leaf to reach the sunlight and become active in photosynthesis. This elongation phase of young scales is somehow comparable with the fast elongation occurring in etiolated hypocotyls and mesocotyls in other plant species. The sampling interval used in the present study (once per 2 weeks) does not allow the high growth rate during the first phase to be measured accurately. However, it allows accurate measurements of the slow expansion rate during the second phase when onion scales grow predominantly in width. The maximal growth rate is then only 0.3–0.35%/h which is at least 20 times lower than in leaves (Maurice et al., 1997), etiolated hypocotyls (Refrégier et al., 2004; Kutscher and Niklas, 2007), and coleoptiles (Cosgrove and Li, 1993). However, in contrast to the latter three organs, onion scales grow for a long time, practically for the whole period of vegetation, leading to the formation of large bulbs (~10 cm in diameter in the cultivar studied). According to the literature this slow growth of onion bulbs is not accompanied by cell divisions in the majority of cell types (Heath, 1945; Mita and Shibaoa, 1983). The only exception might be the abaxial (outer) epidermis of onion bulb scales (Mita and Shibaoa, 1983).

The net cellulose orientation determined across the whole wall thickness defines the direction but not the degree of growth anisotropy in onion epidermis

To check if cellulose microfibrils control the anisotropic growth their orientation was studied using polarization confocal microscopy and fluorescent Congo Red staining (Verbelen and Stickens, 1995). In general, polarization methods allow measurement of the net microfibril orientation across the whole wall thickness (Paolillo, 1995; Baskin et al., 1999; MacKinnon et al., 2006). In the present study this would be the case only if Congo Red stains all the layers of the cell wall. The fact that Congo Red, propidium iodide, and toluidine blue stain the same wall thickness, despite their affinity for different cell wall polymers (Fig. 1L–N), indicates that Congo Red fulfills this requirement. Thus the present method gives a quantitative estimate of the net cellulose microfibril orientation across the whole wall thickness.

The net cellulose orientation changed dramatically during development from transverse (to the organ axis) through random to longitudinal, and finally back to transverse (Fig. 3). The first change, from transverse to longitudinal, accompanies early scale development (Table 3). Very similar cellulose reorientations were found during development of other plant organs such as leaves, hypocotyls, and roots (Paolillo, 1995; Kerstens and Verbelen, 2003; Refrégier et al., 2004). The final change from longitudinal to transverse microfibril alignment occurring in older scales (Fig. 3, Table 3) is quite unusual. To our knowledge, it has not been described in the literature before. Overall there is a good agreement between the two phases of scale growth and the two main cellulose reorientations. The preferred scale growth in length occurs at the time when cellulose microfibrils start changing their orientation from transverse to longitudinal, whereas the preponderant scale growth in width takes place when the net microfibril alignment changes from longitudinal to transverse (Table 3).

This link between directional growth and cellulose realignment during development suggests that microfibrils may control anisotropic expansion. To check this, cellulose orientation at the beginning of each interval of sampling was compared with the preferred direction of expansion during this interval. Practically in all cases the anisotropic expansion was directed perpendicular to the net cellulose orientation (Table 2). Thus cellulose microfibrils do define the vector of cell growth in the adaxial epidermis of onion scales, which is in agreement with maize and Arabidopsis root data (Baskin et al., 1999; Sugimoto et al., 2000). The expansion of scale 8 during the interval III–IV was the only exception where the vector of growth was directed parallel to the net cellulose orientation (Table 2). An analogous extension, parallel to the net microfibril orientation, has been described in several plant organs where it took place after the phase of the most rapid elongation (Paolillo, 2000). The unusual behaviour of scale 8 might be caused by active formation of new scales between stages III and IV (Table 1). The predominant elongation of newly formed scales (Fig. 11, J; Table 2) tightly adjoining scale 8 could make the latter grow in length, parallel to the net microfibril orientation in its outer cell wall. Apparently, in this case, forces exerted by growth of adjacent scales can outweigh the effect of cellulose on the direction of growth anisotropy.
On the other hand, the net cellulose orientation shows a poor correlation ($r^2=0.38$) with the degree of anisotropy (Table 2), questioning the involvement of microfibrils in its control in line with the literature data (Baskin et al., 1999; Wiedemeier et al., 2002). The method used here to measure cellulose orientation, like other polarization techniques, involves the averaging over large numbers of microfibrils in a given cell (MacKinnon et al., 2006). Moreover the data on cellulose alignment reported in Fig. 3 result from averaging of the AR values of 50 individual cells. According to the definition of Baskin et al. (2004), these data reflect ‘global alignment’ of cellulose microfibrils in groups of cells. The variability of such global alignment characterized by the standard deviations of the reported AR values was usually similar in the situations when onion scales had the same net cellulose orientation but a different degree of growth anisotropy (Table 2). This fact is inconsistent with the hypothesis that the global alignment of cellulose microfibrils defines the degree of growth anisotropy (Baskin et al., 2004). The hypothesis that microfibril length controls the degree of growth anisotropy (Wasteneys, 2004) can only be checked after reliable methods of cellulose microfibril length determination are developed.

**Two mechanisms of cellulose reorientation in onion epidermis may have different physiological roles**

A change in microfibril alignment may, however, also result from anisotropic expansion (Refrégier et al., 2004). Comparing the changes in the net cellulose orientation with the direction of growth anisotropy, it was found that microfibrils reoriented in the direction of maximal expansion in the majority of cases (Table 3), which is consistent with the multinet or passive mechanism of cellulose reorientation (Roelofsen, 1958; Green, 1960; Preston, 1982). Confirmation came from extensiometry experiments. When epidermis strips were extended *in vitro* under a constant load in the direction of growth anisotropy, the resulting microfibril reorientation was comparable with that found in living scales (Fig. 1E–H, Table 3). As no cellulose deposition occurs in freeze-killed epidermis strips, all changes in microfibril orientation in the walls can only be passive, resulting from the exogenously applied force. Of course, the uniaxial stretching used here does not exactly match the effect of turgor exerting equivalent orthogonal biaxial force on the cell wall. Such uniaxial stretching may cause higher cellulose reorientations compared with those *in vivo* (Chanliaud et al., 2002). On the other hand, the anisotropic growth, the non-random microfibril orientation, and some deviation of onion scale geometry from spherical are all the factors contributing to a passive cellulose reorientation *in vivo* (Matas et al., 2004). Thus the high ability of microfibrils to realign passively in the direction of strain *in vitro* suggests that a similar process is partly responsible for the cellulose reorientations observed *in vivo*.

In two cases the net orientation of microfibrils changed from random to longitudinal in the absence of anisotropic growth (Table 3). These two realignments were thus not mediated by passive reorientation but resulted from active deposition of longitudinally oriented microfibrils.

There is a general agreement that the active synthesis and oriented deposition of new microfibrils on the inside of the cell wall define the direction of growth (Sugimoto et al., 2000; MacKinnon et al., 2006). Because of that concept, cellulose orientation is often determined on the inner face of the cell wall without paying attention to deeper wall layers (Marga et al., 2005). However, there is no consensus about the role of passive microfibril reorientation in growth regulation. According to old data obtained with giant internodal cells of *Nitella*, the inner 25% of the wall thickness defines the majority of its load-bearing properties (Richmond, 1983). These data are usually extrapolated to the walls of higher plants. If this extrapolation is valid, then the passive microfibril reorientation has a rather negligible effect on growth since it mostly occurs in the outer wall layers (Preston, 1982). To our knowledge, the only supporting evidence of the special role of the inner 25% of the wall thickness in higher plants was obtained using the inner epidermis walls of *Arabidopsis* hypocotyls (MacKinnon et al., 2006). However, these walls represent just a tiny fraction of the total wall volume in that organ and, hence, are unable to limit its growth, unlike the thick outer epidermal walls whose fraction in the total wall volume is ~60% (Derbyshire et al., 2007). In addition to that, morphological, biochemical, and physiological data clearly show that the extrapolation of the properties of charophycean algae cell walls to the walls of higher plants may not be valid (Baskin et al., 1999; Cosgrove, 2000; Popper and Fry, 2003; Proses and Boyer, 2006). Moreover, an ultrastructural analysis of outer periclinal walls in the epidermis of several plant species demonstrated that their inner portions transmit the main part of the transverse stress while their outer portions transmit the main part of the longitudinal stress (Hejnowicz and Borowska-Wykrzet, 2005). Thus according to these data all wall thickness is load bearing. If so, then a passive microfibril reorientation in the direction of growth anisotropy may gradually slow down expansion and help to determine final cell size (Burgert and Fratzl, 2007). This mechanism is consistent with the phenomenon of strain hardening observed in tomato fruit epidermis peels (Matas et al., 2004). Repeated extension *in vitro* realigned cellulose microfibrils in the direction of strain, making the peels stiffer. So passive cellulose reorientation may play an accessory role in the regulation of growth anisotropy which is principally controlled by oriented microfibril deposition. Finally, the role of microfibril reorientation in the control of plant cell expansion can be even more important. According to a traditional point of view, cell wall expansion is limited by covalent and non-covalent bonds within the cellulose-xyloglucan network (Cosgrove, 2000). According to a recent alternative model, cell wall mechanics reflect the properties of cellulose microfibrils, with the wall extension being determined by the ability of microfibrils to move
Relative to one another (Thompson, 2005). This hypothesis infers that the role of passive cellulose realignment in the control of anisotropic expansion is at least of the same importance as that of active cellulose synthesis and oriented deposition.

Onion adaxial epidermis as an extensiometric model to study the control of anisotropic growth in higher plants

To reveal how the cellulose microfibril reorientation affects the mechanical properties of the cell wall during scale development, the wall extension in vitro was measured using a constant load (creep) test. These experiments demonstrated an obvious qualitative link between the net cellulose alignment and the epidermis wall extension in vitro. Cell walls were always more extensible in the direction transverse to the net cellulose orientation regardless of the drastic changes in the net orientation from longitudinal to transverse during development (Fig. 4). These data are in line with the results of previous extensiometric studies on the role of cellulose microfibrils in defining the wall mechanical anisotropy (Richmond et al., 1980; Kerstens et al., 2001; Suslov and Verbelen, 2006). A qualitative rather than a quantitative character of this link (Fig. 4) confirms that microfibrils control the direction but not the degree of growth anisotropy. The latter may be regulated by different cell wall loosening proteins stimulating shear or separation between microfibrils. It was demonstrated recently that the enzyme xyloglucan endotransglycosylase/hydrolase (XTH) from Selaginella kraussiana stimulated the wall extension in vitro transverse but not parallel to the net cellulose orientation (Van Sandt et al., 2007). The assumption that some cell wall proteins may control the degree of growth anisotropy is also supported by the fact that different expansins are associated with elongation and with widening in maize leaves (Muller et al., 2007).

The conclusion that the inner 25% of the wall thickness defines the majority of its load-bearing properties was drawn from experiments with Nitella in which the wall extensibility data did not agree with the overall wall cellulose orientation (Richmond et al., 1980). On the other hand, the net microfibril orientation, which is apparently determined across the whole wall thickness (Fig. 1L–N), correlates well with the wall extension in vitro in the onion epidermis (Fig. 4). This could indicate that all wall layers are responsible for its load-bearing properties in epidermis of higher plants, supporting the data of Hejnowicz and Borowska-Wykręt (2005). A study of correspondence between the microfibril orientation in the innermost wall layer and that across the whole wall thickness is needed to check this hypothesis.

To sum up, the present study of onion bulb scale development allows the conclusion that the net cellulose microfibril orientation controls the direction but not the degree of growth anisotropy. This system can be used as a model to study the participation of some protein of interest in the regulation of anisotropic expansion. A ‘map’ of different cellulose orientations (Fig. 3) during onion scale development can be used to choose the epidermis with a suitable microfibril alignment. Strips isolated from that epidermis in the directions perpendicular and transverse to the suitable cellulose orientation can be used to examine an effect of the protein on the wall extensibility in vitro. If the effect depends on the microfibril alignment, this can be considered as a direct extensiometric confirmation of the protein’s role in the regulation of growth anisotropy. Such an approach was successfully used with the XTH from S. kraussiana (Van Sandt et al., 2007).

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References


