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ORIENTATIONAL VARIABILITY OF PARALLEL ARRAYS OF CORTICAL MICROTUBULES UNDER THE OUTER CELL WALL OF THE *HELIANTHUS* HYPOCOTYL EPIDERMIS

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ABSTRACT

The epidermis of *Helianthus* hypocotyl can be peeled off and, in the form of detached strips can be used as a model system to study the effect on cortical microtubule (cMTs) orientation of these factors, which are difficult to be manipulated in situ, such as apoplastic pH or applied stress. In the first step, however, the orientation and re-orientation of cMTs in the epidermis in situ must be described.

The cMTs under the epidermal wall in hypocotyl epidermis at different positions along the hypocotyl and on its opposite sides were studied by means of immunostaining, using epi-fluorescence microscopy. The angle λ that parallel array of cMTs makes with cell longitudinal axis was measured. The variation of λ in a population of cells was documented by λ -histogram (frequency of cells exhibiting a particular $\lambda \pm \Delta\lambda$ plotted against λ value).

The histograms were of either transverse type (maximum at $\lambda \sim 90^\circ$, denoted as type A) or oblique type (two maxima on both sides of the transverse direction, denoted as type B) in the apical part of the hypocotyl, and were either of B type or of longitudinal type (maximum at $\lambda \sim 0^\circ$ or 180° denoted as type C) in the basal part. The change from A or B to C basipetally may be considered as due to the developmental trend in cMT orientation. The occurrence of B above A in some hypocotyls in their apical part strengthens the hypothesis on the autonomous re-orientation of cMTs. The intermingled occurrence of A and B reorientation in the upper part of hypocotyl is interpreted as a manifestation of a subtle control of cell growth in latitudinal direction. The majority of histograms were asymmetric showing predominance of cMT parallel arrays inclined as the middle part of the letter Z.

KEY WORDS: cortical microtubules, epidermis, *Helianthus annuus*.

INTRODUCTION

A characteristic feature of plant cells during interphase or terminal differentiation is the occurrence of microtubules throughout the cell periphery in close association with the cell membrane. These microtubules, known as cortical microtubules (cMTs), are involved in the control of the orientation of cellulose microfibrils in the cell wall and, hence, of cell growth directions and cell shape (Baskin 2001). The cMT arrays, consisting of bundles, which can be seen in fluorescence microscopy in vivo, are very dynamic (Dixit and Cyr 2004; Lloyd et al. 2000; Shaw et al. 2003).

Many external and internal agents, like gravity, light, hormones, mechanical stress and strain, or electrical field, influence the orientation of cMTs (see review by Shibaoka 1994; Ueda and Matsuyama 2000; Hush and Overall 1991; Fischer and Schopfer 1997, 1998; Hejnowicz et al. 2000). Developmental cMT reorientations occur also when cells change their position or positional value due to organ growth and differentiation (Lang et al. 1982; Granger and

Cyr 2001). However, even in constant conditions a cyclic (rhythmic) reorientation of cMTs may take place in cells, independent of their developmental displacement. This is called an autonomous reorientation (Hejnowicz 2005). In constant conditions the reorientation rhythm may be considered as free running. External and/or internal agents may affect this rhythm, and in this way may influence the observed orientation of cMTs.

Usually the bundles of cMTs are approximately parallel and make an angle (λ) with respect to the longitudinal cell axis. When the cMT array is transverse or longitudinal, $\lambda=90^\circ$ or $\alpha=0^\circ$, respectively. The convention is adopted that λ is measured such that it increases clockwise (λ is negative when measured anticlockwise). Since the cMT bundles are composed of antiparallely overlapping microtubules, and therefore the bundles are not polar (Dixit and Cyr 2004; Tian et al. 2004), $\lambda=0$ is indistinguishable from $\lambda=180^\circ$, i.e. a certain λ (that may be positive or negative) is equivalent to $180+\lambda$, like 150° is equivalent to -30° . The λ of about 45° characterizes a clearly oblique array, which

inclination is as that of the middle part of “Z”; therefore it will be further denoted as Z type. The λ of about 135° gives a clearly oblique inclination of S type.

A particular value of λ is generally a feature of a cell face (or its part) instead of being a feature of the whole cell, i.e. it is a local feature of a cell (Yuan et al. 1995). In this paper the cMTs under the outer epidermal wall were considered exclusively.

The angle λ (if it is measurable, i.e. if the cMT array is of parallel type), measured at a particular instant, i.e. at the instant of tissue fixation, varies among similar cells. The variation of λ in a population of fixed cells is regarded as a manifestation of a non-synchrony of the autonomous reorientation cycle in the cells (Hejnowicz 2005), and is documented by λ -histogram (frequency of cells exhibiting a given $\lambda \pm \Delta\lambda$ plotted as a function of the λ value). It should be stressed that λ -histogram characterizes a population of cells and not a single cell. The histogram shows instantaneous variation of λ in different cells at the instant of cell fixation, i.e. it shows the spatial aspect of the reorientation cycle or cycles (singular or plural form depending on whether one or more types of the cycle occur in the population) in the considered population of cells. This spatial aspect can be translated into the temporal one, i.e. into the course of reorientation, because the frequency of occurrence is proportional to the relative time which is used for the passing through a particular λ in the reorientation cycle (Hejnowicz 2005).

Previous study indicated that the autonomous reorientation is rotational rather than oscillatory (Hejnowicz 2005). All types of the λ -histograms and also the gradients of λ , which occur often along an individual cell, can be consistently explained on the basis of rotational cycles assuming that rate of λ change (angular velocity, V) is a function of λ ($V \equiv d\lambda/dt$). In particular, the minima on histograms, characteristic for the reorientation type, are interpreted as due to much higher velocity or as a rebuilding stage (see below), when the cycle runs through the angular range corresponding to the minimum; as if the cycle were “jumping” through this range. The higher the V while passing a parti-

cular λ range, the lower the probability of finding this angle of cMT orientation at the instant of fixation.

If the reorientation cycle is rotational, the coordinate for the phase of this cycle is an angle from 0° to 180° . This angle (taken as coordinate) will be denoted as ϕ . The measured angle λ is thus a dimension in this coordinate. So, the more exact expressions for V and for its functional dependence on phase are $V = d\phi/dt$, and $V = V(\phi)$. The difference between λ and ϕ is interpretative only. The former is a measured value while the latter – the coordinate in the rotational cycle. The cycle embraces all angles ϕ from the range $0-180^\circ$, however, not all angles λ from this range must occur in the cycle.

In the reorientation cycle, during the phase interval that corresponds to the empty angular range on the histogram, the cMTs may exist but if so, they are not parallel. This interval may be interpreted as that of rebuilding of the parallel array of one angle λ to another one, or as the interval of “jumping” in the cycle. Depending upon which range is empty, a few basic types of the histograms, and consequently a few types of reorientation cycle were distinguished (Fig. 1): A – with a single maximum at transverse orientation; B – with two maxima, each at one of oblique orientations Z and S; C – a single maximum at longitudinal orientation; D – uniform distribution. In the type A and C, the jumping in the cycle is through the longitudinal and transverse directions, respectively, in the type B it is through both these directions while in the type D there is no jumping at all.

There may be different variants of the histograms, which differ in width, height and shape of the “bell”, or “bells”, and in the degree of histogram asymmetry. The asymmetries can be divided into two groups: (1) an asymmetry of the position of maximum with respect to the morphological directions (longitudinal or transverse, e.g. Fig. 1e), and (2) an asymmetry in the shape of the bell, or in the height of the two bells in the case of the B-type (e.g. Figs 1f, g). In extreme asymmetry of the type B histogram, one type of oblique orientation is missing.

A histogram for a population of cells may be a superposition of two basic types when a certain type of the reorien-

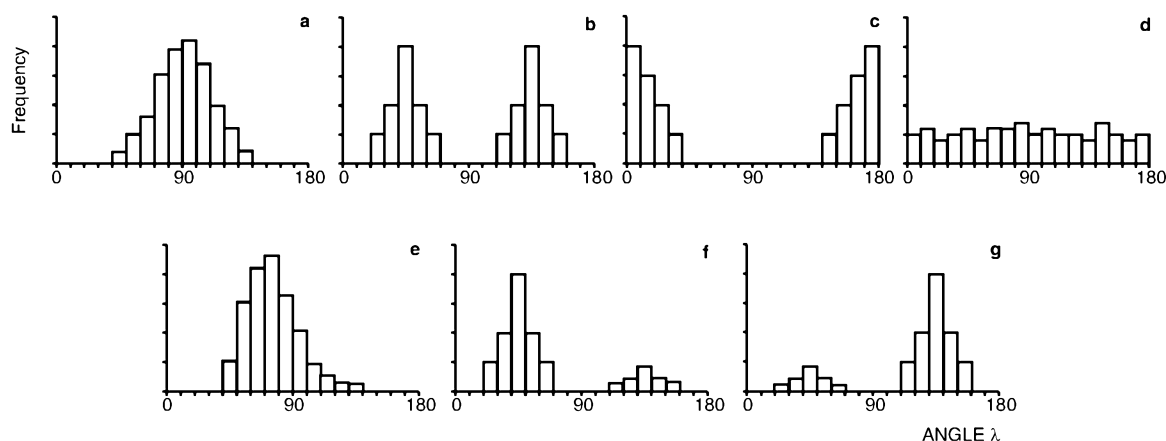


Fig. 1. Schematic representation of basic types of cMT histograms: a – type A histogram with a single maximum at transverse orientation; b – histogram type B with two maxima at oblique orientations; c – histogram type C with a single maximum at longitudinal orientation; d – histogram type D with uniform distribution of cMT orientations; e – asymmetric A type histogram with asymmetry of the position of maximum with respect to the transverse orientation; f, g – asymmetric B-type histograms with asymmetry in the height of the two bells: f – domination of Z type oblique orientation of cMTs; g – domination of S type oblique orientation of cMTs.

tation occurred in some cells of the population and another type in the remaining ones.

Since a histogram represents a certain orientation of cMTs, which existed at the instant of fixation (the spatial aspect of cMT reorientation cycle), the histogram type represents a corresponding type of cMT reorientation cycle, i.e. the type refers either to histogram, or cMT reorientation cycle depending upon the context.

In this paper we studied the distribution of the histogram types in the epidermis of sunflower hypocotyl to see how the growth of the hypocotyl might be regulated by cMTs, and interpreted the results with the assumptions that: (1) there is an autonomous reorientation of cMTs in epidermal cells; (2) the orientation of cellulose microfibrils (MFs) is influenced by the orientation of cMTs; (3) the rate of growth in longitudinal or transverse direction is restricted by MFs oriented in longitudinal or transverse direction, respectively. We were intrigued by the fact that in the elongating part of the hypocotyl the A and B-types of the histograms may be intermingled. This indicated on an existence of a subtle regulation of the elongation and transverse growth.

MATERIAL AND METHODS

Plant material

The sunflower (*Helianthus annuus* L.) achenes were immersed in 2% sodium hypochlorite for 0.5 h, rinsed in the running water for 2 h, planted in moist vermiculite in plastic containers and grown in darkness at room temperature 23-27°C (nearly constant during a particular experiment) for 4 to 5 days. After this time the hypocotyls, 6-7 cm long, were examined.

Visualisation of microtubular bundles

The epidermis was peeled off in form of strips, 55 mm long and 2 mm wide, from the two sides of each hypocotyl, which were opposite in the plane of the apical hook. Each strip extended from 5 mm to 60 mm below apex. Apart from the epidermis the strip contained at least one layer of cortex cells. Usually there were three layers of such cells. The peeling, fixation and preparation for immunostaining was as described in a previous paper (Hejnowicz 2005). It should be mentioned that the fixed strips were incised transversely to facilitate diffusion of antibodies into cells.

Quantification of cMT orientation

The strips were observed under an epi-fluorescence microscope with a digital camera (Olympus, Japan) using 100× objective, excitation filter – 470-490 nm and the barrier filter – 515 nm. The outer epidermal wall was the first on the light route, so the fluorescent bundles in the cortical cytoplasm layer under the outer wall were the best visible and could be easily distinguished from the layer adjoining the inner tangential wall. The fluorescent bundles representing cMTs were seen only in the incised cells. The sites with parallel bundles were photographed. Each strip of epidermis was divided into 6 segments (approximately 9 mm long). The examined incised cells exhibiting sites with parallel bundles were chosen from the middle part (approximately 5 mm long) of each segment. A single area under examination was approximately 5 mm². Images were ana-

lysed with the AnalySIS software (AnalySIS Image Processing; Soft Imaging System, GmbH).

Next, the angle between the bundles and the longitudinal direction defined by the longitudinal walls, further denoted as λ , was measured. The number (n) of investigated cells differed depending on the effectiveness of immunostaining (n is given at each histogram). The measured angles were grouped into 18 classes (at 10 degrees interval) and λ frequency histogram for each segment was obtained.

RESULTS

The histograms, each representing an area of 1×5 mm (transverse × longitudinal) of epidermis, were obtained for 336 samples from 28 hypocotyls. Figure 2 shows distribution of histograms for 3 hypocotyls. The presented variation of histograms can be considered as representative for the sunflower hypocotyls studied.

Most histograms observed in the studied material were superposition of the basic types. The superposition involved such combinations of the basic types as A+B and C+B. The combination A+C, i.e. with two minima at oblique orientations has never been observed.

The type D in pure form has never been observed either. There occurred, however, a flattened type A or A+B, or flattened C+B.

The histogram types, which occurred in the apical and middle part of the hypocotyls were of A and B type and their superpositions. An important fact is that in some hypocotyls B occurred apically with respect to A. Type C occurred in the basal part of hypocotyls.

Majority of histograms were asymmetric and among the asymmetric ones those in which the cMTs orientation of Z type were represented in higher quantities, were prevailing (i.e. more cMTs were inclined to the right). The histogram type, which appeared to be most asymmetric, was the type B occurring either alone or in superposition with another type. Though the general prevalence of Z orientation was obvious, there were also considerable variations between hypocotyls. Some hypocotyls even showed a prevalence of S orientation (at the instant of their fixation).

DISCUSSION

Variation of orientation type along hypocotyl

When interpreting a single histogram, it is inferred that at a particular location along the hypocotyl there are cells which: (1) differ in timing of the reorientation cycles so that they show different λ at fixation instant (i.e. the cycles are not synchronous), and (2) may differ also in the cycle type (A versus B, or B versus C).

The change from A or B type of histograms to C when moving basipetally along the hypocotyls may be considered as due to the developmental trend in cMT reorientation. The basipetal trend from transverse to longitudinal is well known (Traas et al. 1984; Sylvester et al. 1989; Liang et al. 1996; Granger and Cyr 2001). However, the occurrence of B above A type strengthens the hypothesis that there is an autonomous reorientation of cMTs in cells, because it seems rather improbable that the B type could occur permanently above the type A in a particular hypocotyl. Then the

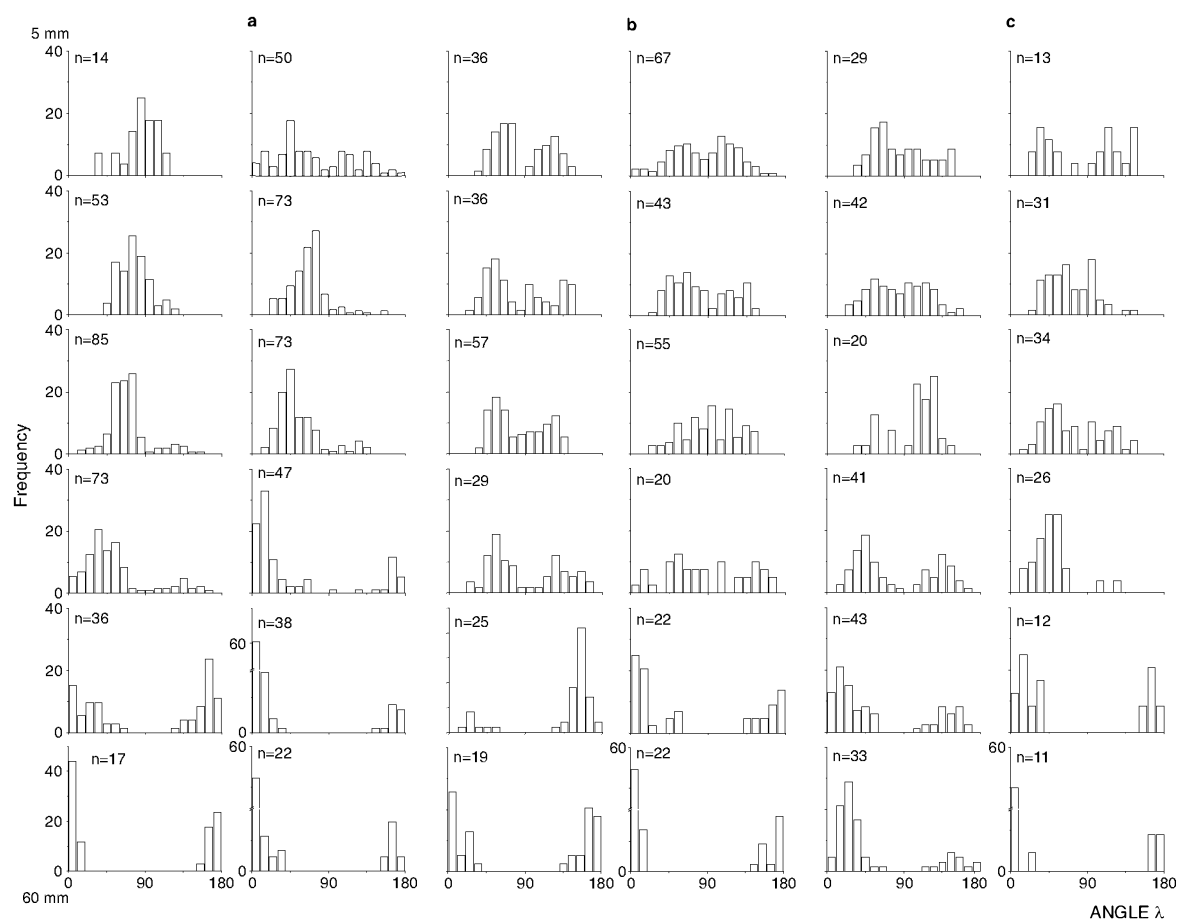


Fig. 2. Histograms of the cMT orientation frequency observed in situ under the outer wall in epidermis of three sunflower hypocotyls (a, b, c). Histograms in one column refer to one side of the hypocotyl. Two opposite sides in the plane of the apical hook were examined in each hypocotyl. Numbers on the left give the distance from the cotyledon's node in mm. The angle λ was measured with respect to the longitudinal cell axis. It is assumed that when $\lambda=180^\circ$ or 0° the cMT array is longitudinal, when $\lambda=90^\circ$ it is transverse. When λ is intermediate between 10° - 90° the cMT array is oblique of Z type (right-handed) and when λ is intermediate between 90° - 170° it is oblique of S type (left-handed). n is the number of cMT areas counted.

cyclic reorientation at a particular location would occur with a change of the type of reorientation cycle. The occurrence of the B type above the A type in some hypocotyls should be interpreted as rotational reorientation with jumping always through the longitudinal direction (A-type) but sometimes also through transverse direction (B-type).

The meaning of the intermingled occurrence of A and B reorientation in the upper portion of hypocotyl

Let us consider the intermingled occurrence of reorientation types with regard to the relationship between longitudinal and transverse (latitudinal) growth, assuming that cortical microtubules (cMTs) define the orientation of cellulose microfibrils (reviewed by Baskin 2001). It is believed that transverse orientation of microfibrils (MFs) underlies longitudinal growth (elongation) while longitudinal MF orientation hinders the elongation, or, in general, the growth along MFs is hindered. If so, transverse or longitudinal MFs orientation hinders or favours transverse growth, respectively. It is also usually assumed that transverse cMTs characterize elongating cells, which fits the assumption that cMTs control the orientation of MFs. However, the terms "transverse", "longitudinal" with respect to cMT

and/or microfibril orientation are taken in quite a wide sense so that they embrace also oblique arrays. Surely, the obliquely oriented arrays do not prevent either longitudinal nor transverse growth. If so, the cMT arrays of the B type reorientation should allow growth in both directions. So A and B types do not differ significantly with respect to the effects on longitudinal growth, but they differ with respect to the latitudinal growth (thickening of the hypocotyl), because the transverse (A type reorientation) cMTs limits the transverse (latitudinal) growth. Thus both types of the autonomous reorientation of cMTs, A and B, allow the upper portion of the hypocotyl to elongate. The "blending" of the two types may be considered as a subtle way of the control of the latitudinal growth in the hypocotyl. Appearance of the C-type of cMT reorientation in the lower part of the hypocotyl should be considered as linked with a tendency to limit the elongation while allowing for thickening of the hypocotyl. Probably in the case of an organ of bulbous type the blending of C and B reorientation types occurs (Mita and Shibaoka 1984).

However, the relationship between the ability of a cell wall to grow in a particular direction, and the orientation of cMTs relative to this direction is complex. There are two

levels of the complexity: (i) temporal change of cMT orientation due to rotational cycling, which results in the variability in MF orientation in successive layers of cell wall, and (ii) the self assembly of MFs in the formation of cell wall texture.

(i) One reason of the variability of MF orientation in successive layers of cell wall may be the autonomous reorientation of cMTs. The variability gives a certain resultant (net) orientation of the MFs, and it is this resultant orientation which must be considered in the relationship between MF orientation and the direction of cell wall growth. We may assume that the growth rate of the cell wall at a particular locus is the lower, the higher is the resultant orientation of microfibrils in this direction. The resultant orientation is a complicated function of the orientation of MFs in successive layers. For instance, net longitudinal MF orientation may occur in a situation when there are no longitudinal cMTs at all, but there are steep oblique cMTs of Z and S type. Of course, such an orientation (resultant net longitudinal but without longitudinal microfibrils) does not exclude elongation because the inextensible microfibrils can reorient in this direction allowing for an elongation of the wall as a whole. Elongation may indeed occur in cell walls with a net longitudinal orientation of microfibrils (Paolillo 2000). Furthermore, in the *radially swollen* mutant of *Arabidopsis* anisotropic expansion of roots is disrupted and cells become swollen despite transverse MF orientation (Wiedemeier et al. 2002). Theoretically, even if the MFs were oriented longitudinally but were shorter than the cell length (i.e. they overlap) the elongation is still possible by weakening the bonds between overlapping MFs by expansins or xyloglucan endotransglucosylase.

As it has already been mentioned we expect that if the orientation of MFs conforms to the orientation of cMTs of the reorientation type B (MFs oblique, no MFs in longitudinal and transverse orientations) both longitudinal and latitudinal growth is possible. However, type A would be inhibitory for transverse growth, while type C – for longitudinal growth.

The outer wall of the epidermis is thought to be crucial in the control of organ growth (Lang et al. 1982; Kutschera 1989; Bret-Harte and Talbot 1993; Paolillo 2000). This wall controls the surface growth in different directions depending on: (a) its anisotropy due to microfibril orientation; (b) the stress in the wall dependent on the cell shape; (c) the tissue stresses exerted on the epidermis by the inner tissues (Hejnowicz and Sievers 1995). Different external and internal factors affecting growth via microfibril orientation in the outer epidermal wall probably act on the course of cMT reorientation by changing either the dependence of angular rotational reorientation upon phase (angle), or the ranges of angular phases at which the cMT array is rebuilt, or finally the stability of cMT arrays in different phases.

(ii) Self-assembly in cell walls leads to helicoidal structure (Neville 1988; Giraud-Guille 1996). A planar helicoidal structure is a kind of twisted plywood composed of long molecules, further called fibrils, arranged in sheets. Within a sheet the fibrils are mutually parallel, but in consecutive sheets they are rotated, always in the same direction, by a small angle $\Delta\alpha$ relative to the neighbouring sheet beneath, where α is the angle which the fibrils make with respect to a certain distinguished direction. Molecules that

are to be able to self-assemble must exhibit certain properties (Neville 1988). This condition is fulfilled by hemicelluloses but not by other cell wall components, including cellulose. However, cellulose microfibrils associated with hemicelluloses are able to self-assemble helicoidally as demonstrated in vitro (Reis et al. 1991). If hemicelluloses fulfill the qualitative requirements for helicoidal self-assembly, the angle $\Delta\alpha$ depends on the number of hemicellulose backbones between two successive microfibrils, i.e. on the ratio of deposition of hemicellulose and microfibrils (Hejnowicz 2005).

The ideally helicoidal wall as such is principally neutral for the morphogenesis, because as a whole it is isotropic. Thus for the sake of cell morphogenesis the tendency for helicoidal self-assembly must cooperate with other mechanism(s) affecting the amount of microfibrils exhibiting a particular orientation and in consequence changing the isotropy into anisotropy. Such a mechanism may depend on differential rate of microfibril growth which in turn depends on the instantaneous mutually parallel orientation of cMTs and microfibrils (Hejnowicz 2005). It is suggested that when both elements are parallel, microfibrils grow faster, i.e. more microfibrils are formed, than when the parallelism is lost. As a result, the helicoidal self-assembly of the microfibrils is considered as biased by the cMT orientation. Alternatively, cMTs could affect the MF organisation controlling the length of nascent MFs. If cMTs and MFs were parallel, long MTs are produced which ensured growth perpendicular to their orientation (Wastnneys 2004).

Chiral asymmetry in cMT arrays

Majority of λ histograms is asymmetric with respect to the morphological direction (longitudinal, transverse) and usually there is an asymmetry in the positioning of the histogram "bell" (Fig. 1e), or/and in its shape in the case of A and C histograms, or in the height of the two bells in the case of the type B (Fig. 1f). The histograms are constructed by measuring angles with respect to the longitudinal (morphological) direction. It is possible that the "physiological" direction involved in the controlling of cMT orientation is a principal direction of a certain tensor, like mechanical stress, and that this direction makes an angle with the morphological direction, leading to a positional asymmetry in the histogram. However, the asymmetry in the shape of histogram bell(s) cannot be explained in this way; rather an asymmetry in the function $V(\lambda)$ is involved. For instance, if the velocity is lower when the cycle passes the range of Z obliquity ($0^\circ \ll \lambda \ll 90^\circ$) than through the range of S obliquity $90^\circ \ll \lambda \ll 180^\circ$, the Z obliquity of cMTs will prevail.

Asymmetry in the shape of the histogram bell means that one of the two chiral forms (Z, S) predominates in the cMT orientation. The predomination may be either local (spatially and temporally), i.e. it concerns a particular population of cells at a particular instant, or it may be global (spatially) at a certain level of a hypocotyl. The number of the histograms showing predominance of Z orientation was higher than that of S one in all the three series of hypocotyls investigated. However, there was a considerable variation between the series and also between hypocotyls in a series, though no obvious reason could be recognized for this variation. Any further discussion on the asymmetry would need more detailed data about the reorientation cyc-

le. In this paper we wish only to signal the phenomenon of the asymmetry in cMT orientation. This chiral phenomenon calls for further study.

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