1-1-2006

Disruption of cortical microtubules by overexpression of green fluorescent protein-tagged α-tubulin 6 causes a marked reduction in cell wall synthesis

David H. Burk
University of Georgia

Ruiqin Zhong
University of Georgia

W. Herbert Morrison
USDA ARS Russell Research Center (RRC)

Zheng Hua Ye
University of Georgia

Recommended Citation

Burk, D., Zhong, R., Morrison, W., & Ye, Z. (2006). Disruption of cortical microtubules by overexpression of green fluorescent protein-tagged α-tubulin 6 causes a marked reduction in cell wall synthesis. *Journal of Integrative Plant Biology, 48* (1), 85-98. https://doi.org/10.1111/j.1744-7909.2006.00202.x

Journal of Integrative Plant Biology 2006, **48** (1): 85–98 www.blackwell-synergy.com; www.chineseplantscience.com

Disruption of Cortical Microtubules by Overexpression of Green Fluorescent Protein-Tagged α-Tubulin 6 Causes a Marked Reduction in Cell Wall Synthesis

David H. Burk[1*], Ruiqin Zhong[1], W. Herbert Morrison III[2] and Zheng-Hua Ye[1]**

(1. *Department of Plant Biology*, *University of Georgia*, Athens, Georgia 30602, USA;
2. *Richard B. Russell Agriculture Research Center, U.S. Department of Agriculture, Agricultural Research Service*, Athens, Georgia 30604, USA)

Abstract

It has been known that the transverse orientation of cortical microtubules (MTs) along the elongation axis is essential for normal cell morphogenesis, but whether cortical MTs are essential for normal cell wall synthesis is still not clear. In the present study, we have investigated whether cortical MTs affect cell wall synthesis by direct alteration of the cortical MT organization in *Arabidopsis thaliana*. Disruption of the cortical MT organization by expression of an excess amount of green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) in transgenic *Arabidopsis* plants was found to cause a marked reduction in cell wall thickness and a decrease in the cell wall sugars glucose and xylose. Concomitantly, the stem strength of the GFP-TUA6 overexpressors was markedly reduced compared with the wild type. In addition, expression of excess GFP-TUA6 results in an alteration in cell morphogenesis and a severe effect on plant growth and development. Together, these results suggest that the proper organization of cortical MTs is essential for the normal synthesis of plant cell walls.

Key words: *Arabidopsis thaliana*; cell elongation; cell wall synthesis; cortical microtubules; microtubule organization.

In elongating cells of plants, cortical microtubules (MTs) are oriented transversely along the elongation axis. The transverse orientation of cortical MTs is essential for normal cell elongation, as demonstrated in many pharmacological and mutant studies (Baskin 2001; Dixit and Cyr 2004; Lloyd and Chan 2004; Wasteneys 2004). Exactly how cortical MTs regulate the anisotropic cell growth is still not clear. In the past several decades, considerable attention has been focused on the study of the roles of cortical MTs in the oriented deposition of cellulose microfibrils, because the transverse deposition of cellulose microfibrils along the elongation axis is necessary for normal cell elongation (Baskin 2001). However, it is not clear whether cortical MTs affect the synthesis of cell walls in general.

Disruption of cortical MTs by pharmacological drugs has been shown to cause an alteration of the localized deposition of secondary walls in tracheary elements, indicating the importance of cortical MTs in the deposition of patterned secondary walls (Baskin 2001; Roberts et al. 2004; Oda et al. 2005). It has been suggested that the alteration of the localized deposition of secondary walls could be due to a disruption in the localized distribution of cellulose synthase complexes caused by the MT-disrupting drugs (Gardiner et al. 2003). Because quantitative analyses of cellulose content and cell wall composition have not been performed in these studies, it is not known whether disruption of cortical MTs by pharmacological drugs results in a reduction in overall cell wall synthesis.

Recently, a number of *Arabidopsis* mutants with altered cortical MT organization have been isolated (Dixit and Cyr 2004; Lloyd and Chan 2004; Wasteneys 2004) and two of them, namely *fra2* and *mor1*, have been used to investigate the role of cortical MTs in cell wall synthesis. In the

Received 29 Aug. 2005 Accepted 15 Oct. 2005
Supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (2002-35304-12709).
Publication of this paper is supported by the National Natural Science Foundation of China (30424813) and Science Publication Foundation of the Chinese Academy of Sciences.
*Present address: Socolofsky Microscopy Center, Louisiana State University, Baton Rouge, LA 70803, USA.
**Author for correspondence. Tel: +1 706 542 1832; Fax: +1 706 542 1805; E-mail: <zhye@plantbio.uga.edu>.

fra2 mutant, mutation of the katanin MT-severing protein (abbreviated as AtKTN1/AtKSS) has been shown to cause disorganization of cortical MTs, aberrant deposition of cellulose microfibrils, and abnormal anisotropic cell growth (Burk et al. 2001; Burk and Ye 2002). The *bot1* mutant, which is allelic to *fra2*, has also been demonstrated to exhibit alterations in cortical MT organization and anisotropic cell expansion (Bichet et al. 2001). Studies of two other mutant alleles of *AtKTN1*, specifically *erh3* and *lue1*, suggest additional roles of AtKTN1 in root hair cell development and hormonal regulation of MT dynamics (Bouquin et al. 2002; Webb et al. 2002). One of the prominent phenotypes of the *fra2* mutant is the marked reduction in fiber cell wall thickness and a concomitant reduction in stem strength (Burk et al. 2001; Burk and Ye 2002). Quantitative analysis of cell wall composition demonstrated that the *fra2* mutation causes a reduction in cellulose and several cell wall sugars, indicating that the normal organization of cortical MTs is important for cell wall synthesis. In contrast, studies of the temperature-sensitive *mor1* mutant indicate that disorganization of cortical MTs does not affect cell wall synthesis (Sugimoto et al. 2003). The cortical MTs in elongating cells of the *mor1* plants grown under restrictive temperature are fragmented or disassembled and, consequently, cell growth becomes isotropic (Whittington et al. 2001). Analysis of cell walls from the *mor1* seedlings after treatment for 5 d at the restrictive temperature revealed no change in cellulose content and it was concluded that alteration of cortical MTs by the *mor1* mutation does not affect cellulose synthesis (Sugimoto et al. 2003). Therefore, whether cortical MTs influence cell wall synthesis remains a controversy.

Because different MT-binding proteins perform distinct functions on MTs, the discrepancy seen in the studies of *fra2* and *mor1* mutants is most likely due to differences in the intrinsic functions of the two proteins, despite the fact that the cortical MTs were disorganized in both mutants. To investigate the intriguing issue of the relationship between cortical MTs and cell wall synthesis, it is ideal to disrupt cortical MTs directly by modifying tubulin subunits. It has been shown that dominant-negative amino acid substitutions in the α-tubulin subunits 6 and 4 (TUA6 and TUA4, respectively) in the *Arabidopsis lefty1* and *lefty2* mutants cause a right-handed helical orientation of cortical MTs in elongating cells (Thitamadee et al. 2002). The *lefty* double mutant results in fragmented and less well aligned cortical MTs in elongating cells compared with the wild type and a concomitant defect in cell morphogenesis (Abe et al. 2004). A reduction in the expression of α-tubulin genes in *Arabidopsis* has been shown to affect root growth and morphogenesis (Bao et al. 2001). In addition, it has been shown that, whereas a moderate level of expression of green fluorescent protein (GFP)-tagged α-tubulin causes right-handed growth of petioles and floral petals without an obvious alteration in MT organization, expression of hemaglutinin epitope-tagged α-tubulin results in the formation of shallow left-handed MT helices and a concomitant right-handed helical growth of several organs (Abe and Hasimoto 2005).

In the present study, we attempted to disrupt the organization of cortical MTs by expression of an excess amount of GFP-tagged TUA6 in *Arabidopsis* and investigated its effects on cell wall synthesis. We show that expression of excess GFP-TUA6 causes disorientation and fragmentation of cortical MTs in elongating cells. We provide evidence that disruption of the cortical MT organization by GFP-TUA6 results in a marked reduction in cell wall thickness and, concomitantly, a decrease in stem strength. Our results demonstrate that the proper organization of cortical MTs is essential for normal cell wall synthesis.

Results

Expression of excess GFP-TUA6 causes aberrant organization of cortical MTs

In an attempt to disrupt the cortical MT organization directly by modifying tubulin subunits, we overexpressed GFP-TUA6 in *Arabidopsis* plants. Of 65 transgenic plants generated, six exhibited a marked alteration in plant morphology, 19 plants had normal morphology except for the right-handed twisting of petioles and petals, and the remainder displayed wild-type plant morphology. Semiquantitative RT-PCR analysis showed that although the transgenic lines with twisted petioles and petals had a slight increase in TUA6 mRNA levels compared with wild type (data from one representative line, GFP-TUA6-1, are shown in Figure 1A), the transgenic lines with a severe alteration in plant morphology had a substantial elevation in TUA6 mRNA (data from two representative transgenic lines, GFP-TUA-2 and GFP-TUA-3, are shown in Figure 1A). This result indicates a direct correlation between the level of transgene expression and an alteration in transgenic plant morphology. Because we aimed to disrupt cortical MT organization by expression of an excess amount of GFP-TUA6 and the six transgenic lines with a substantial elevation in TUA6 mRNA levels exhibited similar alterations in plant morphology, we selected one representative line (GFP-TUA6-3) for further analyses.

To confirm the accumulation of GFP-TUA6 protein, we used a monoclonal antibody against α-tubulin to detect both GFP-TUA6 and endogenous α-tubulins. It was shown that the GFP-TUA6 overexpressors accumulated a high level of GFP-TUA6 protein, which is similar to the level of endogenous α-tubulins (Figure 1B). The tubulin incorporation assay demonstrated that GFP-TUA6 protein

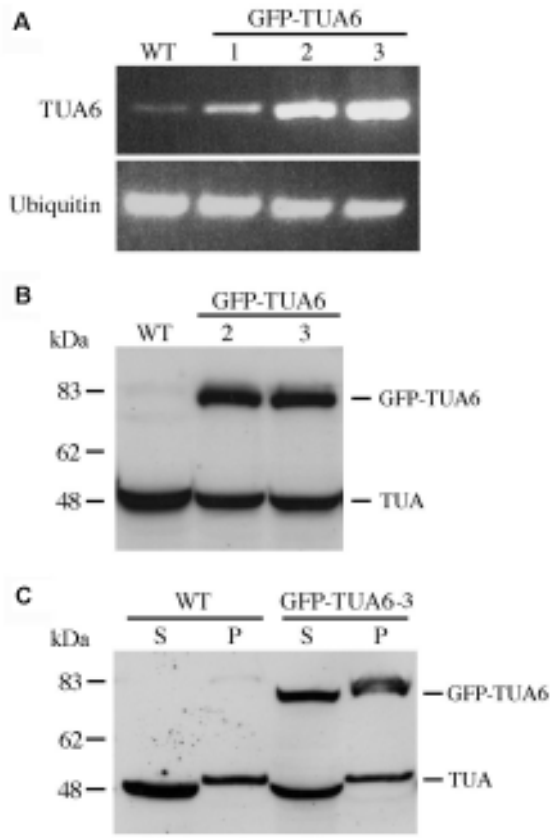

Figure 1. Detection of green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) expression level in transgenic *Arabidopsis* plants. Two-week-old transgenic seedlings were used for isolation of total RNA and proteins.

(A) Semi-quantitative reverse transcription-polymerase chain reaction analysis of endogenous TUA6 and GFP-TUA6 mRNA in the wild type (WT) and in three representative transgenic lines (GFP-TUA-1, -2, and -3). The TUA6-specific primers were used to detect the expression level of both endogenous TUA6 and GFP-TUA6. The expression of a ubiquitin gene was used as an internal control.

(B) Immunoblot detection of endogenous α-tubulins and GFP-TUA6 in the wild type and GFP-TUA6 transgenic plants using a monoclonal antibody against α-tubulin. Note that the level of GFP-TUA6 is equivalent to that of the endogenous tubulins.

(C) The GFP-TUA6 incorporates into microtubules (MTs). Like endogenous tubulins, GFP-TUA6 was detected in the soluble (S) and the MT pellet (P) fractions.

incorporated into MTs at a ratio similar to endogenous α-tubulins (Figure 1C).

We next examined the organization of cortical MTs in elongating cells of the GFP-TUA6 overexpressors (Figure 2). Immunolabeling of MTs revealed that although the cortical MTs in wild-type elongating pith cells of stems (Figure 2A) and elongating parenchyma cells of petioles (Figure 2E) maintained a transverse orientation along the elongation axis, those in the GFP-TUA6 overexpressor line were markedly altered (Figure 2B–D, F). The cortical MTs in elongating cells of the GFP-TUA6 overexpressors became highly disorganized and fragmented, although, in a few cells, the cortical MTs still exhibited a transverse orientation. It was noticed that, in some cells, the cortical MTs were less densely distributed compared with wild type. These results demonstrate that expression of an excess amount of GFP-TUA6 leads to a severe alteration in cortical MT organization.

Effect of expression of excess GFP-TUA6 on cell wall synthesis

To investigate whether the altered MT organization caused by excess GFP-TUA6 had any effect on cell wall synthesis, we examined the wall thickness of interfascicular fiber cells and pith cells in the inflorescence stems of the GFP-TUA6 overexpressors (Figures 3,4). Fiber cells deposit a massive amount of secondary walls, which is ideal for the investigation of cell wall synthesis. Cross-sections of stems showed that the wall thickness of interfascicular fiber cells was markedly reduced in the GFP-TUA6 overexpressors compared with the wild type (Figure 3D, E). Transmission electron microscopy revealed that the wall thickness of fibers in the GFP-TUA6 overexpressors was reduced by 69% compared with the wild type (Figure 4A–D; Table 1). Concomitantly, the stem strength of GFP-TUA6 overexpressors was substantially lower than that of the wild type (Figure 3A). In addition, it was found that the wall thickness of pith cells in the GFP-TUA6 overexpressors was reduced by 42% compared with that of the wild type (Figure 4E, F; Table 1). The decrease in cell wall thickness was further substantiated by cell wall composition analysis, showing that both glucose and xylose, which are the main sugars in cellulose and xylan, respectively, in the secondary walls were reduced in the GFP-TUA6 overexpressors compared with the wild type (Table 2). These results demonstrate that the expression of excess GFP-TUA6 has a marked effect on cell wall synthesis.

Effect of expression of excess GFP-TUA6 on plant morphology

It was observed that expression of excess GFP-TUA6 markedly affected plant growth and development (Figure 5; Table 1). The rosette leaves of the GFP-TUA6 overexpressors (Figure 5B, C) were often curled and much smaller in size compared with the wild type (Figure 5A, C; Table 1), which

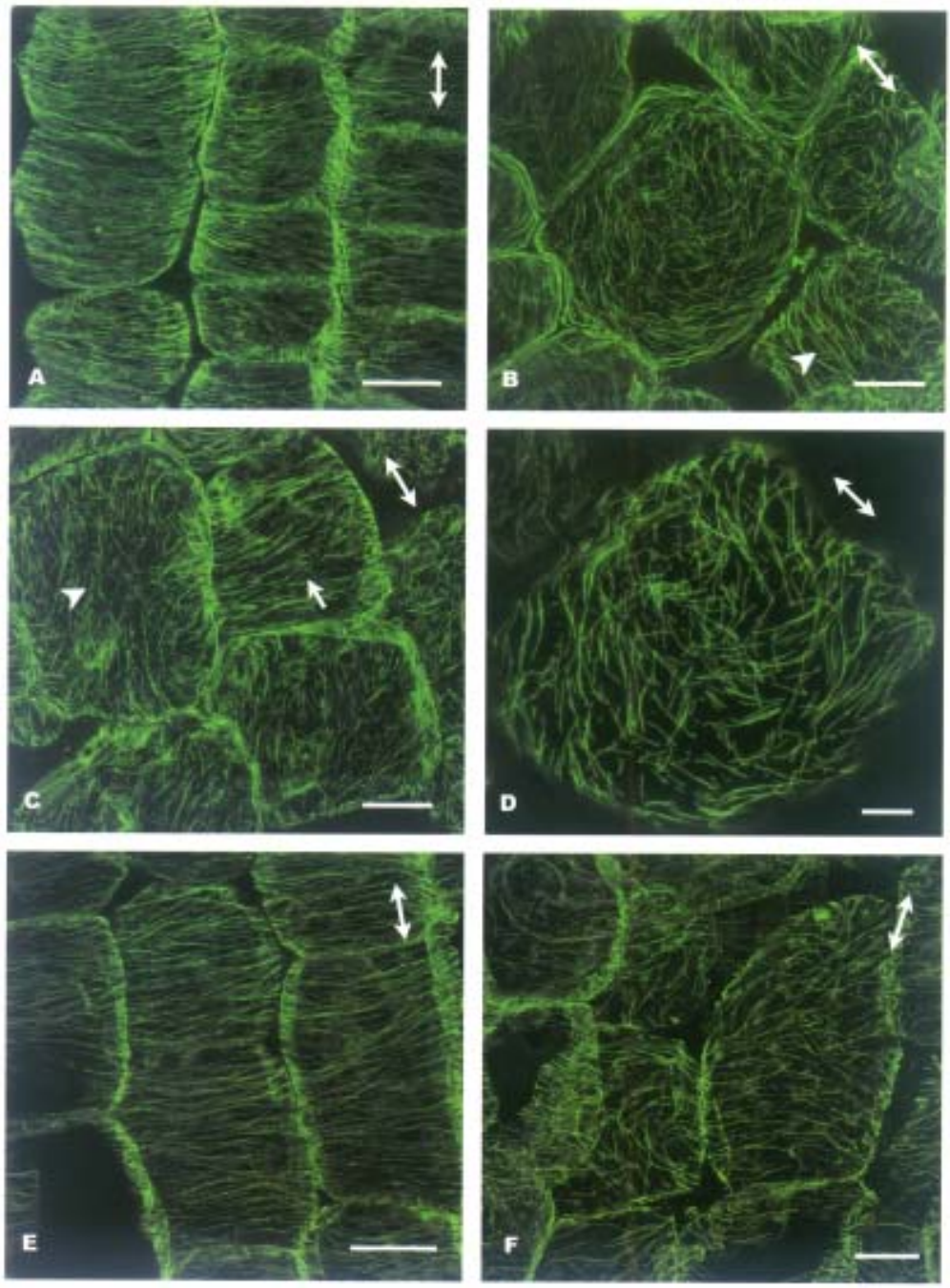

Figure 2. Expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) causes an aberrant organization of cortical microtubules (MTs). Longitudinal sections of elongating stems and petioles were immunolabeled for microtubules with a monoclonal antibody against α-tubulin and fluorescein isothiocyanate-conjugated secondary antibodies. Fluorescent-labeled cortical MTs were viewed under a confocal laser microscope. Double-headed arrows indicate the elongation axis. Bars, 20 μm **(A–C, E, F)**; and 8 μm **(D)**.

(A) Wild-type pith cells showing cortical MTs transversely oriented along the elongation axis.
(B–D) Pith cells from the GFP-TUA6 overexpressors showing disorganized and fragmented cortical MTs. It was noted that, occasionally, in a few cells, the cortical MTs remained in a parallel orientation.
(E) Wild-type petiole cells showing cortical MTs transversely oriented along the elongation axis.
(F) Petiole cells from the GFP-TUA6 overexpressors showing an alteration in cortical MT organization.

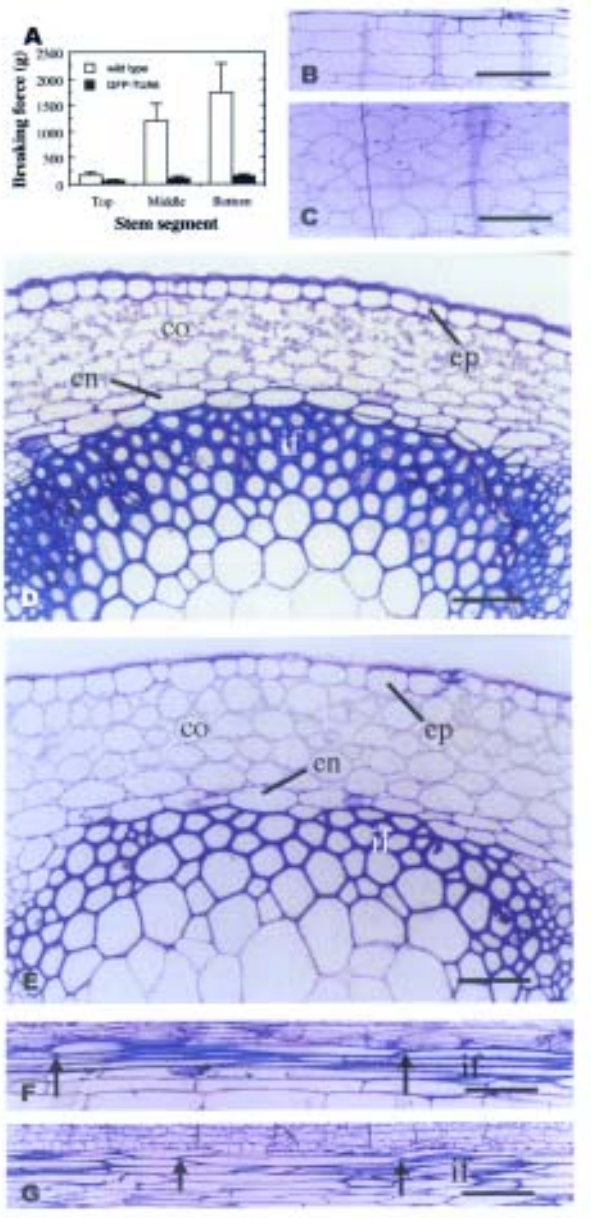

Figure 3. Expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) causes a reduction in stem strength and an alteration in the morphology of fiber cells and pith cells. Inflorescence stems of 8-week-old plants were measured for their breaking strength and the bottom internodes were examined for cell morphology. co, cortex; en, endodermis; ep, epidermis; if, interfascicular fiber. Bars, 130 μm **(B, C)**; 62 μm **(D, E)**; 165 μm **(F, G)**.

(A) Breaking force measurement showing that the force to break stem segments apart was approximately eightfold lower in GFP-TUA6 than in the wild type. Data are the mean ± *SE* of 15 plants.
(B,C) Longitudinal sections of the pith region of the stems showing short pith cells with irregular shapes in GFP-TUA6 **(C)** compared with the wild type **(B)**.
(D,E) Cross-sections of interfascicular regions of stems showing fiber cells with thin walls in GFP-TUA6 **(E)** compared with the wild type **(D)**.
(F,G) Longitudinal sections of interfascicular regions of stems showing shorter fiber cells in GFP-TUA6 **(G)** compared with the wild type **(F)**. Two arrows mark both ends of one fiber cell.

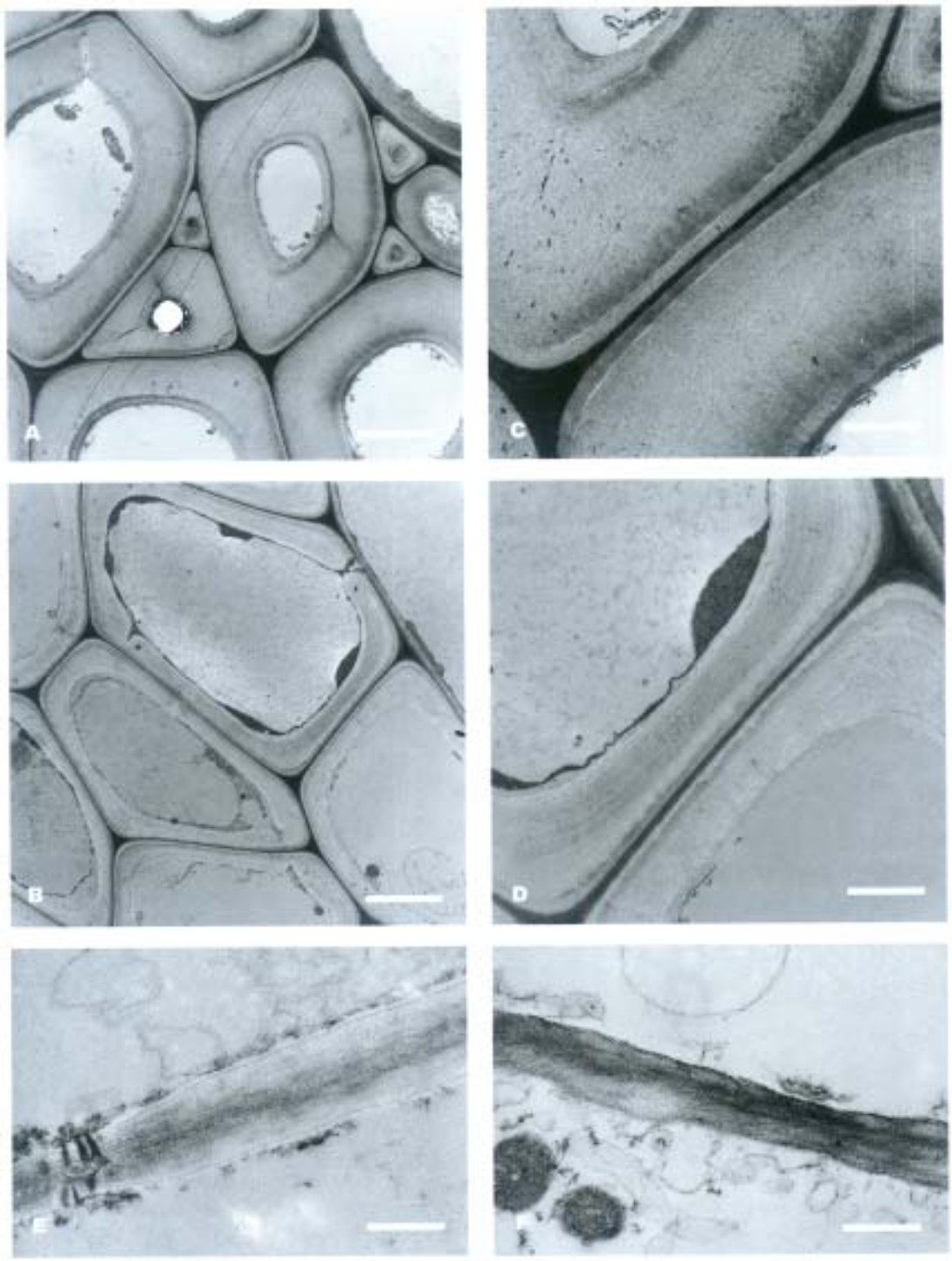

Figure 4. Expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) causes a marked decrease in wall thickness. Fiber cells and pith cells from the bottom internodes of 8-week-old plants were examined for their wall thickness under a transmission electron microscope. Bars, 5.00 μm **(A,B)**; 1.38 μm **(C,D)**; 0.63 μm **(E,F)**.

(A,B) Interfascicular fiber cells showing thin walls in GFP-TUA6 **(B)** compared with the wild type **(A)**.
(C,D) High magnification of fiber walls showing thin secondary walls in GFP-TUA6 **(D)** compared with the wild type **(C)**.
(E,F) Pith cell walls showing that they are thinner in GFP-TUA6 **(F)** than in the wild type **(E)**.

Table 1. Effects of expression of excess green fluorescent protein-tagged α-tubulin 6 on cell wall thickness, cell length, and plant morphology

Sample	Wild type	GFP-TUA6
Fiber cell wall thickness[a] (μm)	3.56 ± 0.72	1.11 ± 0.22
Pith cell wall thickness[a] (μm)	0.36 ± 0.06	0.21 ± 0.04
Pith cell length[b] (μm)	182 ± 25	85 ± 16
Interfascicular fiber cell length[b] (μm)	827 ± 133	532 ± 74
Inflorescence stem length[c] (cm)	25.9 ± 4.9	6.3 ± 0.9
Leaf blade length[d] (mm)	16.5 ± 2.3	6.6 ± 1.3
Leaf blade width[d] (mm)	10.9 ± 1.4	6.3 ± 1.1
Petiole length[d] (mm)	16.3 ± 1.9	4.8 ± 1.1
Root length[e] (mm)	13.6 ± 2.0	14.4 ± 2.3
Hypocotyl length[f] (mm)	16.1 ± 1.1	10.6 ± 2.0

Data are the mean ± *SE* of 25 samples. GFP-TUA6, green fluorescent protein-tagged α-tubulin 6.

[a]Wall thickness was measured from transmission electron micrographs of fibers and pith cells.

[b]Pith and fiber cells were from the basal parts of the inflorescence stems of 8-week-old plants.

[c]Stems from 8-week-old plants were measured for their height.

[d]Fifth rosette leaves from 6-week-old plants were used for measurement of leaf blade width and length, as well as petiole length.

[e]Roots of 4-day-old light-grown seedlings were measured for their length.

[f]Hypocotyls of 4-day-old dark-grown seedlings were measured for their length.

is probably due to a reduced and uncoordinated expansion of adaxial and abaxial epidermal cells. The inflorescence stems of the GFP-TUA6 overexpressors were much shorter than those of the wild type (Figure 5F; Table 1). Interestingly, an aerial view of the GFP-TUA6 overexpressing plants showed that the cauline branches were arranged in a right-handed helical pattern (Figure 5G). Unlike the wild type (Figure 5H), the flowers of the GFP-TUA6 overexpressors were sterile owing to a marked reduction in the length of stamen filaments (Figure 5I), which evidently prevented self-pollination. In addition, it was found that the dark-grown hypocotyls of the GFP-TUA6 overexpressors were shorter than those of the wild type (Figure 5D; Table 1). However, no abnormal growth and morphology were observed in the roots of young seedlings of the GFP-TUA6 overexpressors (Figure 5E; Table 1), indicating that excess GFP-TUA6 did not affect root growth. These results demonstrate that the expression of excess GFP-TUA6 has a severe effect on plant growth and development.

Expression of excess GFP-TUA6 causes an alteration in cell morphogenesis

To investigate whether the aberrant plant morphology of the GFP-TUA6 overexpressors was due to an alteration in cell morphogenesis, we examined cell morphology in various organs of the GFP-TUA6 overexpressors. It was found that the pith and fiber cells in the stems of the GFP-TUA6 overexpressors were much shorter than those of the wild type (Figure 3B,C,F,G; Table 1). The cell files in the pith were not as well aligned as in the wild type, probably due to the altered cell shapes (Figure 3B,C). Examination of leaf epidermal cells revealed that although the ordinary epidermal cells in the wild type were tubular and highly sinuous (Figure 6A,B), those in the GFP-TUA6 overexpressors became much less expanded in both the neck and lobe areas (Figure 6C,D). The marked alteration in leaf epidermal cell morphology is consistent with the proposed role of cortical MTs in the morphogenesis of lobed plant cells (Fu et al. 2005; Panteris and Galatis 2005). The epidermal cells of the stamen filaments in the GFP-TUA6 overexpressors were much shorter and wider (Figure 6F) compared with the wild type (Figure 6E). In addition, it was found that the hook region of dark-grown hypocotyls of the GFP-TUA6 overexpressors was highly swollen and misshapen, and the epidermal cells were considerably enlarged and exhibited a right-handed helical pattern (Figure 6H,J) compared with the wild type (Figure 6G,I). These results demonstrate that the expression of excess GFP-TUA6 causes reduced anisotropic cell growth.

To investigate whether the reduced anisotropic cell growth was associated with any alterations in the oriented deposition of cellulose microfibrils, we examined the orientation of cellulose microfibrils in the innermost layer of the walls of elongating pith cells (Figure 7). It was found that the cellulose microfibrils in the pith cells of the GFP-TUA6 overexpressors were generally oriented transversely along the elongation axis (Figure 7B), a pattern similar to

Table 2. Cellulose and cell wall sugar composition of the wild type and green fluorescent protein-tagged α-tubulin 6 overexpressors

Sample	Cell wall sugar composition (mg/g dry cell wall)						
	Cellulose	Glucose	Xylose	Mannose	Galactose	Arabinose	Rhamnose
Wild type	295 ± 3	334 ± 35	133 ± 4	16.8 ± 2.8	15.1 ± 0.6	10.1 ± 0.7	8.6 ± 3.6
GFP-TUA6	226 ± 21	238 ± 10	101 ± 2	12.8 ± 0.3	22.0 ± 0.1	14.0 ± 0.5	7.3 ± 4.2

Values are the mean ± *SE* of two independent assays. GFP-TUA6, green fluorescent protein-tagged α-tubulin 6.

Cell wall materials used for analysis were from the inflorescence stems of 10-week-old plants.

Figure 5. Alteration of plant morphology by expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6).

(A,B) Four-week-old plants showing a marked reduction in size of the GFP-TUA6 overexpressors **(B)** compared with the wild type **(A)**.
(C) Rosette leaves showing the smaller size and the uneven leaf blade in GFP-TUA6 (right) compared with the wild type (left).
(D) Four-day-old dark-grown seedlings showing the shorter hypocotyls of GFP-TUA6 (right) compared with the wild type (left).
(E) Four-day-old light-grown seedlings showing the similar root length between GFP-TUA6 (right) and the wild type (left).
(F) Eight-week-old plants showing that the inflorescence stems of GFP-TUA6 (right) are much shorter than those of the wild type (left).
(G) An aerial view of the GFP-TUA6 plant showing the cauline branches arranged in a right-handed helical pattern.
(H,I) Flowers showing that unlike in the wild type **(H)**, the stamens in the GFP-TUA6 overpressors are extremely short **(I)**.

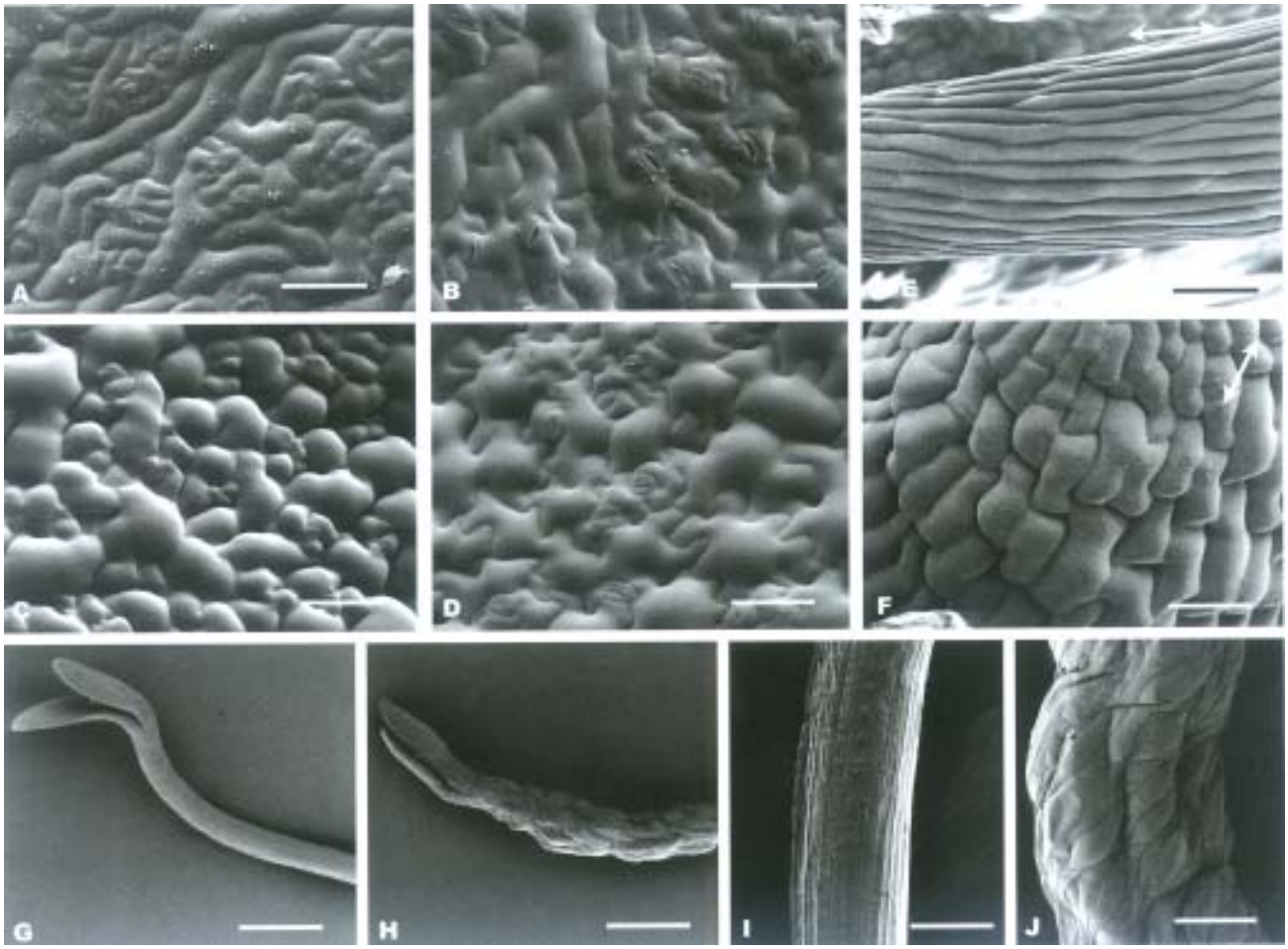

Figure 6. Expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) alters cell morphogenesis. The morphology of epidermal cells was viewed under a scanning electron microscope.

(A,B) Adaxial **(A)** and abaxial **(B)** epidermal cells of wild-type leaves. Note the tubular ordinary epidermal cells with many prominent lobes.
(C,D) Adaxial **(C)** and abaxial **(D)** epidermal cells of GFP-TUA6 leaves showing swollen and short ordinary epidermal cells with small lobes.
(E,F) Epidermal cells of the stamen filaments in GFP-TUA6 **(F)** are short and radially enlarged compared with the wild type **(E)**.
(G,H) The hook region of dark-grown hypocotyls in GFP-TUA6 **(H)** is much wider than that of the wild type **(G)**.
(I,J) High magnification of the hook region of dark-grown hypocotyls showing enlarged epidermal cells with right-handed helical orientation in GFP-TUA6 **(J)** compared with the wild type **(I)**.

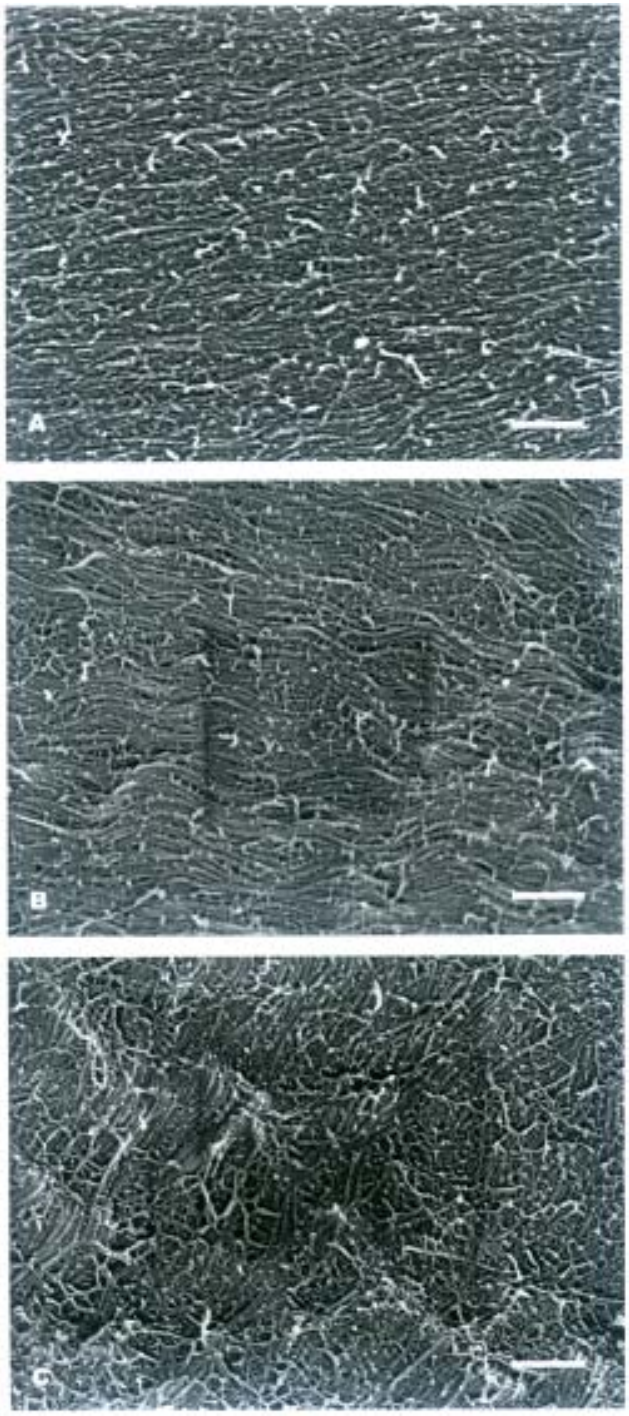

Figure 7. Expression of excess green fluorescent protein-tagged α-tubulin 6 (GFP-TUA6) does not result in randomly oriented cellulose microfibrils. Elongating stems were sectioned longitudinally through pith cells and the cellulose microfibrils in the innermost layer of pith cells walls were visualized under a field emission scanning electron microscope. The vertical direction of the image corresponds to the elongation axis of the cell. Bars, 270 nm.

(A) Cellulose micrifibrils in a wild-type cell showing their parallel alignment in the transverse orientation.
(B,C) Cellulose microfibrils in GFP-TUA6 cells showing their parallel alignment in the transverse orientation **(B)** and, occasionally, a strong deviation from the transverse orientation **(C)**.

that of the wild type (Figure 7A). It was noticed that, occasionally, in some cells, the cellulose microfibrils had an oblique orientation, but were still aligned in a relatively parallel order among themselves (Figure 7C). These results indicate that the reduced anisotropic cell growth in the GFP-TUA6 overexpressors is not caused by an alteration in cellulose microfibril orientation.

Discussion

Green fluorescent protein-tagged α-tubulin has been widely used for visualization of MTs *in vivo*. It has been shown that, when expressed at a moderate level (at 20%–30% of the endogenous tubulin level), GFP-TUA6 incorporates into MTs and has no apparent effect on MT organization (Ueda et al. 1999; Abe and Hashimoto 2005). In the present study, we have demonstrated that expression of an excess amount of GFP-TUA6 causes a severe disorganization of cortical MTs, leading to fragmentation and aberrant orientations. Therefore, the disruption of cortical MTs by expression of excess GFP-TUA6 could be used as a tool to further investigate the importance of cortical MTs in various cellular processes. One intriguing question is how excess GFP-TUA6 could result in such a disorder of cortical MTs. It has been demonstrated recently that expression of modified α-tubulins, such as hemaglutinin epitope-tagged TUA6, leads to suppression of MT dynamicity and promotion of polymerization in *Arabidopsis* plants (Abe and Hashimoto 2005). It was proposed that incorporation of N-terminal-tagged α-tubulin inhibits GTP hydrolysis, thus producing polymerization-prone MTs with an extended GTP cap (Abe and Hashimoto 2005). In *Dictyostelium*, expression of GFP-α-tubulin has been shown to interfere with MT nucleation and/or assembly (Kimble et al. 2000). As discussed by Kimble et al. (2000), the addition of GFP to the amino terminus of α-tubulin could directly hinder MT assembly because the amino terminus of α-tubulin is located between adjacent tubulin dimers, a location that would be important for assembly of the MT polymer. Therefore, the marked alteration of MT organization by expression of excess GFP-TUA6 observed in the present study may be caused by an abnormal MT dynamicity and/or assembly.

We have used the GFP-TUA6 overexpressors to investigate the effect of disordered cortical MTs on cell wall synthesis. Our results demonstrated that the proper organization of cortical MTs is essential for normal cell wall synthesis. Because the reduction in cell wall thickness is much more marked than the decrease in cellulose amount, it is apparent that the altered MT organization affects overall cell wall synthesis. This finding is consistent with the early report in the *fra2* mutant showing that disorganization of cortical MTs results in an overall decrease in cell wall synthesis (Burk et al. 2001). Although how cortical MTs influence cell wall synthesis is not clear, it is possible that cortical MTs may be essential for normal cellulose synthesis. It has been reported that disruption of cortical MTs in developing xylem cells causes improper distribution of cellulose synthase complexes in the plasma membrane (Gardiner et al. 2003). It is conceivable that such an alteration in cellulose synthase distribution may impede the efficiency of cellulose synthesis. Another possibility is that cortical MTs may be required for the local transport and fusion with the plasma membrane of vesicles carrying cellulose synthase complexes and non-cellulosic wall components at the cell cortex. Disruption of cortical MTs may affect this process, thus resulting in defective cell wall synthesis. Although vesicle transport in the interphase cells is thought to be mediated by the actin cytoskeleton, but not MTs (Steinborn et al. 2002), the possibility that cortical MTs play a role in the local transport and fusion of vesicles with the plasma membrane could not be excluded.

It is intriguing to find that although disorganization of cortical MTs in the GFP-TUA6 overexpressors caused a marked reduction in cell wall synthesis, no significant alteration in the orientation of cellulose microfibrils was observed. Occasionally, in a few cells, the cellulose microfibrils were found to have deviated from the transverse orientation, but they remained in a relatively parallel order among themselves. Disruption of MTs without an alteration in cellulose microfibril deposition has been observed in the temperature-sensitive *mor1* mutant, which has fragmented cortical MTs under the restrictive temperature, and in wild-type cells treated with MT-disrupting drugs (Sugimoto et al. 2003; Himmelspach et al. 2003). However, disorganization of cortical MTs has been shown to correlate with altered cellulose microfibril deposition in the *fra2* mutant (Burk and Ye 2002). Disruption of cortical MTs in oryzalin-treated *Arabidopsis* roots has been demonstrated to result in reduced uniformity of cellulose microfibril alignment (Baskin et al. 2004). Therefore, whether cortical MTs influence cellulose microfibril deposition may depend on a variable relationship between the MTs and cellulose synthase complexes (Somerville et al. 2004).

It has been suggested that the ordered deposition of cellulose microfibrils depends on the rate of cellulose synthesis, because a reduction in cellulose synthesis in the temperature-sensitive *rsw1* mutant or by treatment with the cellulose synthesis-inhibiting drug 2,6-dichlorobenzonitrile results in an aberrant orientation of cellulose microfibrils (Wasteneys 2004). However, our finding that a reduction in cellulose synthesis caused by expression of excess GFP-TUA6 did not lead to disordered deposition of cellulose microfibrils indicates that there is no simple correlation between reduced cellulose synthesis and aberrant microfibril deposition.

Cortical MTs have long been known to regulate anisotropic cell growth, but the underlying mechanisms are still not clear (Baskin 2001; Dixit and Cyr 2004; Lloyd and Chan 2004; Wasteneys 2004). Because normal cell wall synthesis is essential for cell morphogenesis, as shown in the *rsw1* mutant (Sugimoto et al. 2001), the altered cell morphogenesis seen in the GFP-TUA6 overexpressors may be partially attributable to defective cell wall synthesis. It is conceivable that normal incorporation of new wall materials into the cell walls is essential for cell elongation and a reduction in cell wall synthesis impairs this process, thus leading to an attenuation of anisotropic cell growth.

In conclusion, we have demonstrated that expression of an excess amount of GFP-TUA6 causes aberrant organization of cortical MTs, thus leading to reduced cell wall synthesis and altered cell morphogenesis. Our findings provide further evidence that cortical MTs are essential for normal cell wall synthesis. Further understanding of how cortical MTs influence cell wall synthesis will likely help us uncover the mechanisms underlying the MT control of the anisotropic cell growth.

Materials and Methods

Generation of transgenic plants overexpressing GFP-TUA6

The *Arabidopsis* α-tubulin 6 (TUA6) cDNA was amplified with the high-fidelity elongase by polymerase chain reaction (PCR; primers 5'-ATGAGAGAGTGCATTTCGATCCAC-3' and 5'-TTAGTATTCCTCTCCTTCATCATC-3') and ligated in a pGEM-T cloning vector (Promega, Madison, WI, USA). After being confirmed by sequencing, the TUA6 cDNA was ligated in-frame at the C-terminus of the GFP cDNA (ABRC, Columbus, OH, USA). The GFP-TUA6 fusion cDNA was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in a binary vector. The GFP-TUA6 construct was introduced into wild-type *Arabidopsis* (ecotype Columbia) plants by *Agrobacterium*-mediated transformation (Bechtold and Bouchez 1994). Transgenic plants were selected on kanamycin and the T_2 progeny were used for molecular and phenotypic characterizations.

Reverse transcription-PCR analysis

Total RNA was isolated from 2-week-old seedlings grown on Murashige-Skoog (MS) medium using a Qiagen RNA isolation kit (Qiagen, Valencia, CA, USA). One microgram of the purified RNA was treated with DNase I to remove any potential genomic DNA contamination and then used for first-strand cDNA synthesis. One-twentieth of the first-strand cDNA was used for PCR amplification of the α-tubulin 6 cDNA with gene-specific primers (5'-GGTCTTCAAGGATTCCTTGTT-3' and 5'-GCAGGTTCAAAGGCACTGTTC-3'). The PCR was performed for variable cycles to determine the logarithmic phase of amplifications for the samples. The reverse transcription (RT)-PCR reactions were repeated three times and identical results were obtained. The expression of a ubiquitin gene was used as an internal control for determining the RT-PCR amplification efficiency among different samples.

Immunoblot analysis

Two-week-old seedlings grown on MS medium were used for total protein extraction. Seedlings were ground in liquid nitrogen and then extracted in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride. Extracts were centrifugated at 12 000*g* for 15 min and the supernatants were saved for immunoblot analysis. Total proteins (10 μg) were separated on a 12.5% sodium dodecyl sulfate (SDS) polyacrylmide gel and transferred onto a nitrocellulose membrane. The membrane was probed with a monoclonal antibody against chicken α-tubulin (Sigma-Aldrich, St Louis, MO, USA) and horseradish peroxide-conjugated secondary antibodies. Signals were detected with the chemiluminescent reaction reagent (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

Tubulin incorporation assay

Two-week-old seedlings grown on MS medium were ground gently at room temperature in MT stabilizing buffer (50 mmol/L HEPES, pH 7.4, 2 mmol/L EDTA, 5 mmol/L Mg acetate, 10% sucrose, 2% NP40, and 20 μmol/L taxol) and centrifugated at 1 500*g* for 5 min to remove cell debris. Extracts were centrifugated at 21 000*g* for 15 min to pellet MTs. Proteins from the supernatants and the pellets were separated on an SDS polyacrylamide gel, transferred onto a nitrocellulose membrane, and detected for α-tubulins with a monoclonal antibody against chicken α-tubulin and horseradish peroxide-conjugated secondary antibodies.

Break force measurement

Inflorescence stems of 8-week-old plants were divided into three equal segments and each segment was measured for their breaking force using a digital force-length tester (model DHT4-50; Larson System, Minneapolis, MN, USA). The breaking force was calculated as the force needed to

break apart a stem segment (Zhong et al. 1997).

Cell wall analysis

Inflorescence stems of 8-week-old plants were collected for cell wall isolation. Stems were ground into fine powder in liquid nitrogen with a mortar and pestle, homogenized with a Polytron homogenizer (Fisher Scientific, Pittsburgh, PA), and extracted in 70% ethanol at 70 °C. The remaining cell wall residues were dried in a vacuum oven at 60 °C and used for analysis of the amount of cellulose and sugar composition. Crystalline cellulose was measured with the acetic-nitric anthrone reagent according to Updegraff (1969). Cell wall sugars (as alditol acetates) were determined following the procedure described by Hoebler et al. (1989). All samples were run in triplicate.

Microscopy

Stem samples were fixed in 2% (v/v) glutaraldehyde in PEMT buffer (50 mmol/L PIPES, 2 mmol/L EGTA, 2 mmol/L $MgSO_4$, 0.05% (v/v) Triton X-100, pH 7.2) at 4 °C overnight. After being washed in phosphate buffer (50 mmol/L, pH 7.2), samples were post-fixed in 2% (v/v) OsO_4 for 2 h and then dehydrated through a gradient series of ethanol, cleared in propylene oxide, and embedded in Araldite/Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Sections (1 μm) were cut, stained with toluidine blue, and viewed under a light microscope. For transmission electron microscopy, 90-nm ultrathin sections were cut, mounted on Formvar (EMS, Fort Washington, PA)-coated gold slot grids, post-stained with uranyl acetate and lead citrate, and observed under a Zeiss EM 902A electron microscope (Carl Zeiss, Thornwood, NY, USA).

For visualization of the epidermis, leaves, hypocotyls, and flowers were cryoprepared, surface coated with gold, and observed under an LEO982 FE scanning electron microscope (LEO, Thornwood, NY, USA).

Immunodetection of microtubules

Elongating inflorescence stems of 6-week-old plants and elongating leaf petioles of 3-week-old plants were used for immunodetection of cortical MTs according to Sugimoto et al. (2000). Stem and petiole segments were fixed in PEMT buffer containing 1.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde. Thin sections (100 μm) were cut longitudinally with a vibratome. Sections were incubated with a monoclonal antibody against chicken α-tubulin and fluorescein isothiocyanate-conjugated secondary antibodies. The fluorescence-labeled microtubules were viewed under a Leica TCs SP2 spectral confocal microscope (Leica Microsystems, Heidelberg, Germany). Images were saved and processed with Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA).

Visualization of cellulose microfibrils

Field emission scanning electron microscopy was applied to visualize cellulose microfibrils in the innermost layer of cell walls according to Sugimoto et al. (2000). Segments of elongating inflorescence stems of 6-week-old plants were sectioned longitudinally through pith cells and then fixed in PEMT buffer containing 4% (v/v) formaldehyde. After dehydration, sections were dried in a semidry critical-point drier (Tousimis, Rockville, MD, USA) and mounted on stubs with carbon paste. Sections were coated with platinum using an Edwards 306 vacuum evaporator (Edwards High Vacuum International, Wilmington, MA, USA) and then examined for cellulose microfibrils using an LEO982 FE scanning electron microscope (LEO).

References

Abe T, Hashimoto T (2005). Altered microtubule dynamics by expression of modified α-tubulin protein causes right-handed helical growth in transgenic *Arabidopsis* plants. *Plant J* **43**, 191–204.

Abe T, Thitamadee S, Hashimoto T (2004). Microtubule defects and cell morphogenesis in the *lefty1lefty2* tubulin mutant of *Arabidopsis thaliana*. *Plant Cell Physiol* **45**, 211–220.

Baskin TI (2001). On the alignment of cellulose microfibrils by cortical microtubules: A review and a model. *Protoplasma* **215**, 150–171.

Baskin TI, Beemster GTS, Judy-March JE, Marga F (2004). Disorganization of cortical microtubules stimulates tangential expansion and reduces the uniformity of cellulose microfibril alignment among cells in the root of *Arabidopsis*. *Plant Physiol* **135**, 2279–2290.

Bechtold N, Bouchez D (1994). *In planta Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. In: Potrykus I, Spangenberg G, eds. *Gene Transfer to Plants*. Springer-Verlag, Berlin. pp. 19–23.

Bichet A, Desnos T, Turner S, Grandjean O, Höfte H (2001). *BOTERO1* is required for normal orientation of cortical microtubules and anisotropic cell expansion in *Arabidopsis*. *Plant J* **25**, 137–148.

Bouquin T, Mattsson O, Nasted H, Foster R, Mundy J (2002). The *Arabidopsis lue1* mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. *J Cell Sci* **116**, 791–801.

Burk DH, Ye ZH (2002). Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule severing protein. *Plant Cell* **14**, 2145–2160.

Burk DH, Liu B, Zhong R, Morrison WH, Ye ZH (2001). A

katanin-like protein regulates normal cell wall biosynthesis and cell elongation. *Plant Cell* **13**, 807–827.

Dixit R, Cyr R (2004). The cortical microtubule array: From dynamics to organization. *Plant Cell* **16**, 2546–2552.

Fu Y, Gu Y, Zheng Z, Wasteneys G, Yang Z (2005). *Arabidopsis* interdigitating cell growth requires two antagonistic pathways with opposing action on cell morphogenesis. *Cell* **120**, 687–700.

Gardiner JC, Taylor NG, Turner SR (2003). Control of cellulose synthase complex localization in developing xylem. *Plant Cell* **15**, 1740–1748.

Himmelspach R, Williamson RE, Wasteneys GO (2003). Cellulose microfibril alignment recovers from DCB-induced disruption despite microtubule disorganization. *Plant J* **36**, 565–575.

Hoebler C, Barry JL, David A, Delort-Laval J (1989). Rapid acid-hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid chromatography. *J Agric Food Chem* **37**, 360–367.

Kimble M, Kuzmiak C, McGovern KN, de Hostos EL (2000). Microtubule organization and the effects of GFP-tubulin expression in *Dictyostelium discoideum*. *Cell Motil Cytoskel* **47**, 48–62.

Lloyd C, Chan J (2004). Microtubules and the shape of plants to come. *Nat Rev Mol Cell Biol* **5**, 13–22.

Oda Y, Mimura T, Hasezawa S (2005). Regulation of secondary cell wall development by cortical microtubules during tracheary element differentiation in *Arabidopsis* cell suspensions. *Plant Physiol* **137**, 1027–1036.

Panteris E, Galatis B (2005). The morphogenesis of lobed plant cells in the mesophyll and epidermis: Organization and distinct roles of cortical microtubules and actin filaments. *New Phytol* **167**, 721–732 .

Roberts AW, Frost AO, Roberts EM, Haigler CH (2004). Roles of microtubules and cellulose microfibril assembly in the localization of secondary-cell-wall deposition in developing tracheary elements. *Protoplasma* **224**, 217–229.

Roudier F, Fernandez AG, Fujita M et al. (2005). COBRA, an *Arabidopsis* extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. *Plant Cell* **17**, 1749–1763.

Somerville C, Bauer S, Brininstool G et al. (2004). Toward a systems approach to understanding plant cell walls. *Science* **306**, 2206–2211.

Steinborn K, Maulbetsch C, Priester B et al. (2002). The *Arabidopsis PILZ* group genes encode tubulin-folding cofactor orthologs required for cell division but not cell growth. *Gene Dev* **16**, 959–971.

Sugimoto K, Williamson RE, Wasteneys GO (2000). New techniques enable comparative analysis of microtubule orientation, wall texture, and growth rate in intact roots of *Arabidopsis*. *Plant Physiol* **124**, 1493–1506.

Sugimoto K, Williamson RE, Wasteneys GO (2001). Wall architecture in the cellulose-deficient *rsw1* mutant of *Arabidopsis thaliana*: Microfibrils but not microtubules lose their transverse alignment before microfibrils become unrecognizable in the mitotic and elongation zones of roots. *Protoplasma* **215**, 172–183.

Sugimoto K, Himmelspach R, Williamson RE, Wasteneys GO (2003). Mutation or drug-dependent microtubule disruption causes radial swelling without altering parallel cellulose microfibril deosition in *Arabidopsis* root cells. *Plant Cell* **15**, 1414–1429.

Thitamadee S, Tuchihara K, Hishimoto T (2002). Microtubule basis for left-handed helical growth in *Arabidopsis*. *Nature* **417**, 193–196.

Ueda K, Matsuyama T, Hashimoto T (1999). Visualization of microtubules in living cells of transgenic *Arabidopsis thaliana*. *Protoplasma* **206**, 201–206.

Updegraff DM (1969). Semimicro determination of cellulose in biological materials. *Anal Biochem* **32**, 420–424.

Wasteneys GO (2004). Progress in understanding the role of microtubules in plant cells. *Curr Opin Plant Biol* **7**, 651–660.

Webb M, Jouannic S, Foreman J, Linstead P, Dolan L (2002). Cell specification in the *Arabidopsis* root epidermis requires the activity of ECTOPIC ROOT HAIR3, a katanin-p60 protein. *Development* **129**, 123–131.

Whittington AT, Vugrek O, Wei KJ et al. (2001). MOR1 is essential for organizing cortical microtubules in plants. *Nature* **411**, 610–613.

Zhong R, Taylor JJ, Ye ZH (1997). Disruption of interfascicular fiber differentiation in an *Arabidopsis* mutant. *Plant Cell* **9**, 2159–2170.

(Managing editor: Wei Wang)