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Overexpression of a Profilin (GhPFN2) Promotes the Progression of Developmental Phases in Cotton Fibers

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Regular Paper

Cotton fiber development at the stages of elongation and secondary wall synthesis determines the traits of fiber length and strength. To date, the mechanisms controlling the progression of these two phases remain elusive. In this work, the function of a fiber-preferential actin-binding protein (GhPFN2) was characterized by cytological and molecular studies on the fibers of transgenic green-colored cotton (*Gossypium hirsutum*) through three successive generations. Overexpression of *GhPFN2* caused pre-terminated cell elongation, resulting in a marked decrease in the length of mature fibers. Cytoskeleton staining and quantitative assay revealed that thicker and more abundant F-actin bundles formed during the elongation stage in *GhPFN2*-overexpressing fibers. Accompanying this alteration, the developmental reorientation of transverse microtubules to the oblique direction was advanced by 2 d at the period of transition from elongation to secondary wall deposition. Birefringence and reverse transcription–PCR analyses showed that earlier onset of secondary wall synthesis occurred in parallel. These data demonstrate that formation of the higher actin structure plays a determinant role in the progression of developmental phases in cotton fibers, and that GhPFN2 acts as a critical modulator in this process. Such a function of the actin cytoskeleton in cell phase conversion may be common to other secondary wall-containing plant cells.

Keywords: Actin cytoskeleton • Cotton fiber • *Gossypium hirsutum* • Microtubule • Profilin • Secondary wall deposition.

Abbreviations: β-ME, β-mercaptoethanol; CaMV, cauliflower mosaic virus; CD, cytochalasin D; CESA, cellulose synthase; DPA, days post-anthesis; DTT, dithiothreitol; EST, expressed sequence tag; MBS, *m*-maleimidobenzoyl-*N*-hydoxysuccinimide ester; ORF, open reading frame; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; TEM, transmission electron microscopy.

Introduction

Cotton is the most important fiber crop in the world. Development of the single-celled cotton fiber can be divided into four distinct but overlapping stages: initiation, elongation, secondary wall synthesis and maturation (Basra and Malik 1984). Fast growth of fiber cells takes place between 5 and 20 days post-anthesis (DPA), and fibers may reach a final length of up to 6 cm in some cultivars (Kim and Triplett 2001). The development during this stage determines the length of fiber cells. Deposition of the secondary wall cellulose commences before the cessation of fiber elongation and ends about 1 month later (~15–42 DPA) (Wilkins and Jernstedt 1999). The development of fiber cells during this stage determines the fiber strength. During the period of transition of the above two stages, the rate of fiber elongation decreases rapidly whereas that of the secondary wall synthesis increases tremendously (Meinert and Delmer 1977, Wilkins and Jernstedt 1999). The time points for cessation of elongation and initiation of secondary wall synthesis are also important factors influencing the traits of fiber length and strength. To date, very little is known about the molecular mechanisms that instruct the progression of these two phases in developing fibers.

The plant actin cytoskeleton participates in diverse cellular activities such as cell expansion, cytoplasmic streaming and organelle movement (Hussey et al. 2006, Staiger and Blanchoin 2006). In response to developmental and external signals, the actin cytoskeleton undergoes active reorganization both temporally and spatially. A variety of actin-binding proteins take part in the regulation of dynamic rearrangement of actin filaments. Profilin, the main monomer actin-binding protein that plays essential roles in many cellular processes, is considered to be one of the most important modulators (Staiger et al. 1997, McCurdy et al. 2001). Profilins are small actin-binding proteins (14 kDa) that exert dual effects on actin filament assembly. A number of previous studies showed that profilins

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functioned to promote actin polymerization by adding actin monomer to the growing end of the actin filament (Kang et al. 1999, Pollard and Borisy 2003). However, some other studies demonstrated that profilins acted as an actin-sequestering protein and inhibited the formation of actin filaments (Staiger et al. 1994, Bubb et al. 2003). It was also reported that profilin probably accelerated both polymerization and depolymerization at the barbed end of actin filaments (Yarmola and Bubb 2006). More recently, several lines of evidence implicated that profilins are required in the formation of higher actin structures such as actin cables/bundles (Wang and Riechmann 2008, Pujol et al. 2009). In addition to actin, profilins interact with inositol phospholipid, poly-L-proline, proline-rich proteins, formins and multiprotein complex Arp2/3, etc. In higher plants, two classes of profilins are expressed differentially in vegetative and reproductive tissues (Christensen et al. 1996, Kandasamy et al. 2002). Different profilin isoforms were shown to be functionally distinct (Kovar et al. 2000), and actin and profilin isovariants were found to interact in a class-specific manner (Kandasamy et al. 2007). The complex expression patterns and functional diversity of profilin family members have given rise to the hypothesis that specific and distinct profilin isoforms might be needed for specific cellular functions (Kandasamy et al. 2002, Staiger and Blanchoin 2006, Kandasamy et al. 2007).

The developing cotton fiber is also considered as an excellent system for studying the dynamics and functions of the cytoskeleton (Seagull 1990). It has been shown that fiber elongation and secondary wall synthesis are coupled with active changes in the organization of the actin cytoskeleton and microtubules (Quader et al. 1987, Seagull 1990). At the transition from cell elongation to secondary wall formation phases, the cortical microtubules shift from transverse to oblique and helical orientations, and several works indicated that microtubules were involved in directing the deposition of the secondary wall cellulose (Seagull 1990, Seagull 1992). In agreement with the observations in cellular studies, a number of cytoskeletal genes were found to be expressed specifically or preferentially in developing fiber cells (Whittaker and Triplett 1999, Dixon et al. 2000, Ji et al. 2002, Li et al. 2002, Wang et al. 2005), and two functional studies demonstrated that the actin cytoskeleton was essential and critical for fiber elongation (Li et al. 2005a, Wang et al. 2009). Recently, a study by our group found that in elongating fibers of a short-lint fiber mutant (*Ligon lintless*, Li_1), F-actin organization was deformed dramatically and the microtubule organization also appeared significantly different from that in the wild-type fibers (Zhao et al. 2010). These cellular and molecular studies indicated that the cytoskeleton plays a crucial role in fiber development. Hence investigation of the underlying mechanisms by which the dynamic changes of the fiber cytoskeleton and the expression of cytoskeletal genes contribute to fiber development is important both theoretically and practically.

In this study, we characterized the cellular function of a profilin gene *GhPFN2* by its overexpression in transgenic green cotton plants. We found that overexpression of *GhPFN2* resulted in an earlier formation of thick actin bundles and consequently led to an advanced developmental reorientation of microtubules and cellulose deposition in fiber cells. Our results suggest a possible interaction between the actin cytoskeleton and microtubules prior to secondary cellulose deposition, and demonstrated that the dynamic organization of the actin cytoskeleton modulated by the actin-binding proteins such as profilins plays important roles in controlling fiber elongation and the progression of developmental events in cotton fiber cells.

Results

Cloning and expression analysis of GhPFN2

An expressed sequence tag (EST) representing a profilin homolog of cotton was identified via database searching. The full-length cDNA (882 bp) encoding a protein of 131 amino acids was cloned from the cDNA library of 6 DPA cotton fibers via 5′ and 3′ RACE (rapid amplification of cDNA ends) approaches. The corresponding gene was designated *GhPFN2*. Motif analysis indicated that GhPFN2 contained several actin-binding sites, putative phosphatidylinositol 4,5-bisphosphate interaction sites and the highly conserved residues implicated in poly-L-proline binding (**Fig. 1A**).

Semi-quantitative reverse transcription–PCR (RT–PCR) was performed to investigate the expression pattern of *GhPFN2* in wild-type green cotton using gene-specific primers (**Table 1**). **Fig. 1B** shows that *GhPFN2* was expressed preferentially in fiber cells, and the expression was significantly induced at the fast elongation stage (6–9 DPA). Along with the decrease in fiber elongation rate, the amounts of *GhPFN2* transcripts declined and dropped to a low value at the time of transition from the elongation to the secondary wall synthesis phase (around 18 DPA). When active cellulose deposition of the secondary cell wall started (after 18 DPA), *GhPFN2* expression was stimulated again. These results indicate that the expression of *GhPFN2* is controlled by the developmental program in cotton fiber.

Generation of GhPFN2 transgenic cotton plants

Overexpression and underexpression approaches were taken to characterize the cellular function of GhPFN2. For overexpression analysis, the cDNA of *GhPFN2* was cloned under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the sense orientation, and the resulting construct was introduced into green cotton via *Agrobacterium*-mediated transformation. In all, 14 independent lines of transgenic cotton plants were generated and three independent homozygous lines (134, 156 and 176) were identified in the T2 generation. PCR analysis using primers for the 35S promoter and *GhPFN2* amplified a band of predicted size in these transgenic lines (**Fig. 2A**). Semi-quantitative RT–PCR was performed to evaluate *GhPFN2* expression in the transgenic plants. Lines 156 and 176 showed a relatively high level expression of *GhPFN2* (**Fig. 2B**) and were used for subsequent experiments.

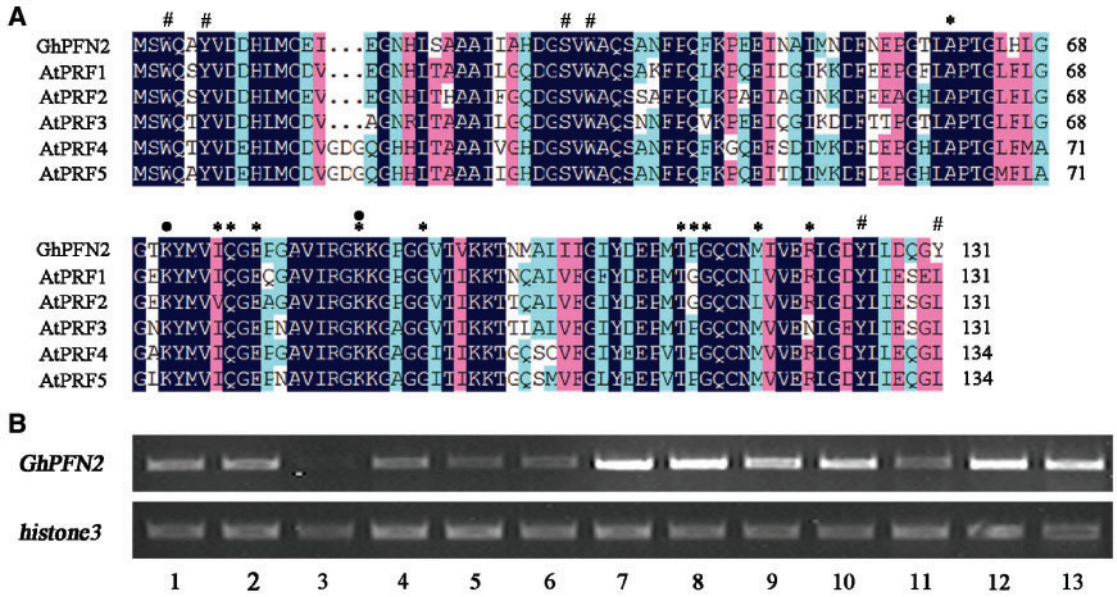

Fig. 1 Alignment of amino acid sequences and expression analysis. (A) The amino acid sequence of GhPFN2 was aligned with those of the Arabidopsis profilin family (GhPFN2, GU237487; AtPRF1, NP_179566; AtPRF2, NP_194664; AtPRF3, AAG10089; AtPRF4, NP_194663; and AtPRF5, NP_179567). The poly-L-proline-binding sites (#), putative phosphatidylinositol 4,5-bisphosphate interaction sites (filled circles) and the actin-binding sites (*) are shown in the graph. (B) RT–PCR analysis of *GhPFN2* expression in wild-type cotton roots (lane 1), hypocotyls (lane 2), leaves (lane 3), flowers (lane 4), 0 DPA ovules (lane 5), 3 DPA ovules (lane 6) and 6, 9, 12, 15, 18, 21 and 24 DPA fibers (lanes 7–13). The *histone3* gene was used as an internal control.

Table 1 Primers used in gene-specific RT–PCR

No.	Gene	Primer pairs
1	*GhPFN2*	F 5′-CATGAATGACTTTAATGAACCGGGTACGCT-3′
		R 5′-GTAAATACGAGTAATACGGAGACGCTTCG-3′
2	*GhPFN2*	F 5′-CATGAATGACTTTAATGAACCGGGTACGCT-3′
		R 5′-AACCCTGATCGAGGAGATAATCGCCG-3′
3	*endo-1,3-β-glucanase*	F 5′-ATATGGGTTTTAAATCTCAGCAATGG-3′
		R 5′-AGGGACGATGTTGGTGTTAACCC-3′
4	*GhRLK1*	F 5′-TGGTAGGATCCCGTATTCG-3′
		R 5′-GGAGAAGCAGCAGCAAATC-3′
5	*GhCelA1*	F 5′-TTGTGGCTTGCCATGAATGTAAT-3′
		R 5′-CTGTCAATGTATGTTTCCCTGTTA-3′
6	*histone3*	F 5′-GCCAAGCGTGTCACAATTATGC-3′
		R 5′-ACATCACATTGAACCTACCACTACC-3′

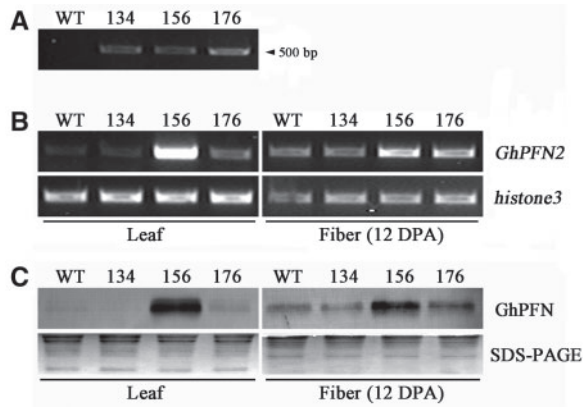

Fig. 2 Molecular characterization of *GhPFN2*-overexpressing cotton plants. (A) PCR analysis of *GhPFN2* integration in the genome of transgenic cotton plants. (B) RT–PCR analysis of *GhPFN2* expression in leaves and 12 DPA fibers of wild-type and transgenic plants. The *histone3* gene was used as an internal control. (C) Western blot analysis of total GhPFN levels in leaves and 12 DPA fibers of wild-type and transgenic plants. The lower panel in C shows the SDS–polycrylamide gels which indicate the equal loading of protein samples. WT, wild-type control; 134, 156, 176, three independent transgenic lines.

Western blot analysis was conducted to verify the overexpression of GhPFN2 at the protein level. The obvious increase in the abundance of total profilin proteins confirmed the overexpression of GhPFN2 in the transgenic plants (**Fig. 2C**).

Overexpression of GhPFN2 resulted in pre-termination of fiber elongation

As the main objective of this study was to understand the role of *GhPFN2* in fiber cells, we focused our investigations on the effects of its overexpression in fiber development in subsequent experiments. Due to the difficulty in using soil-grown fiber cells that form entangled and compact fiber-locks and are encased in the cotton bolls during development, an in vitro cotton ovule culture, which is considered as a good tool for basic fiber

biology study (Triplett 2000), was used in the following experiments unless stated otherwise.

To examine the effects of *GhPFN2* overexpression on fiber development in transgenic cotton plants, the fiber length and strength, the two key determinants of fiber quality, were examined and compared with those of the wild-type control. As shown in **Fig. 3A**, *GhPFN2*-overexpressing fibers were obviously shorter than the wild-type fibers. Quantitative analysis indicated that the fiber length of lines 156 and 176 decreased 14.3 and 14.8%, respectively (**Fig. 3B**). To test the cause of this shorter fiber phenotype, the duration of fiber elongation of the two types of fibers was examined. As can be seen in **Fig. 3C**, elongation of wild-type fiber stopped at around 21 DPA, whereas the transgenic fibers arrested elongation at about 17 DPA. These results demonstrated that the decrease in the length of transgenic fibers was due to the pre-terminated elongation. Apart from fiber length, we also measured the fiber strength of the two types of fibers. Compared with the fiber length, the change in fiber strength was minor; only a slight increase in fiber strength was observed with the transgenic fibers. The fiber length and strength of the transgenic cotton plants were measured continuously for three generations (T3–T5) to confirm this phenotype. The changes in fiber length were observed in each of the generations tested, whereas the changes in fiber strength were variable between years, indicating that the fiber length trait of the transgenic plants could be inherited by the next generations. In addition to fiber quality traits, the *GhPFN2*-overexpressing cotton plants also exhibited delayed flowering and smaller size, indicating that the overexpression of *GhPFN2* also had some impacts on the growth and development of the whole cotton plants.

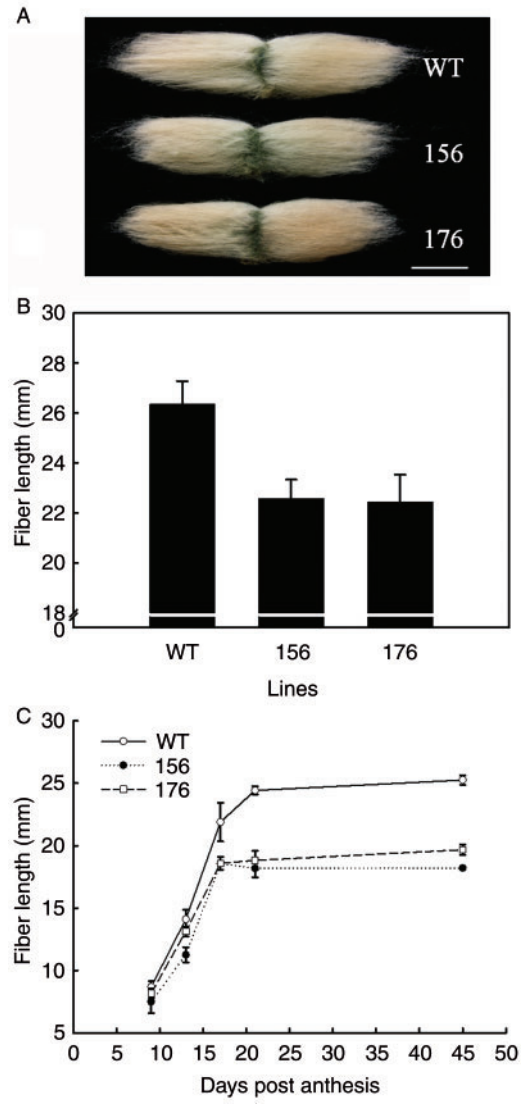

Fig. 3 Measurements of fiber length. (A) Photograph of matured fibers of wild-type and transgenic cotton plants. Bar = 1 cm. (B) Average lengths of mature fibers of wild-type and transgenic plants measured for three continuous generations (T3–T5). Values are the mean ± SE. (C) Lengths of cultured wild-type (open circles) and transgenic (156, filled circle; 176, open square) fibers at 9, 13, 17, 21 and 44 DPA. Ten to fifteen ovules were measured for each sample and three independent measurements were conducted. Values are the mean ± SE. WT, wild-type control; 156, 176, two independent transgenic lines.

F-actin structures in wild-type and transgenic fiber cells

As profilin is involved in the regulation of actin organization, we analyzed the F-actin structure in the transgenic fibers by phalloidin fluorescent staining and compared it with that of the wild-type fibers. As shown in **Fig. 4**, in wild-type green fibers actin filaments formed elaborate organizations consisting of large actin arrays or cables that were running either longitudinally or obliquely, and smaller actin bundles branched from larger ones in the cortical region during 6–18 DPA (**Fig. 4A–E**). At the late stage of fiber elongation at around 14 DPA when fiber cells were entering the transition period from elongation into the secondary wall synthesis stage (see below), the formation of thick actin cables and bundles was evident (**Fig. 4C**). At the active secondary wall synthesis stage, actin filaments changed orientation from axial to helical (after 18 DPA; **Fig. 4F**).

Compared with the wild-type fibers, the F-actin structures appeared different in the transgenic fibers, in which actin filaments formed more and thicker bundles during 10–12 DPA. This incident took place earlier as compared with the wild-type fibers (**Fig. 5A**). As lines 156 and 176 showed very similar changes in F-actin organization, only the results of line 176 are shown. To quantify this change in actin bundle abundance, we tried to assay the F-actin and total actin levels in the wild-type and transgenic fiber cells. However, due to technical difficulties in calculating the volumes and numbers of fiber cells, constant data were not obtained. Instead we conducted a quantitative evaluation by measuring the gray values of the confocal images. It is evident that the amount of F-actin in transgenic fibers was higher than that in the wild-type fibers (**Fig. 5B**).

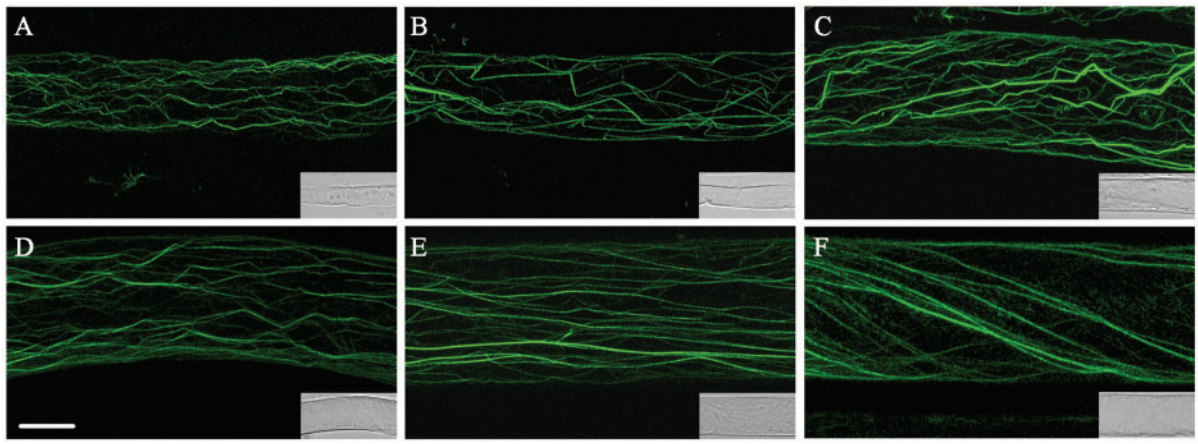

Fig. 4 F-actin structures in wild-type fibers. (A–F) Confocal images of F-actin organization in wild-type fibers at 6, 10, 14, 15, 18 and 22 DPA, respectively. Thick actin cables and bundles formed at around 14 DPA, a time point before developmental transition from the elongation to the secondary wall synthesis stage of the cultured fibers. Corresponding bright field images are shown at a reduced size. Bar = 10 μm.

To assess further the structural difference in the actin cytoskeleton in wild-type and transgenic fibers, the in vitro cultured fibers (10 DPA) were treated with the F-actin-disrupting agent cytochalasin D (CD). Arrays of actin filaments were disrupted upon exposure to CD for 2 d (**Fig. 5C**). Compared with the wild-type fibers in which actin cables/bundles were broken into amorphous and short fragments, longer and thicker fragments of actin bundles could be viewed under the same experimental conditions in the transgenic fibers. These results suggest that actin structures in the transgenic fibers were more stable than those in the wild-type fibers. This may result from the presence of more and thicker F-actin bundles that gave rise to a relatively higher tolerance against CD disruption.

It should be noticed that at the elongation stage (**Fig. 4A–C**), F-actin filaments appeared as jagged cables in fiber cells, in both the absence and the presence of the F-actin-stabilizing reagent, *m*-maleimidobenzoyl-*N*-hydoxysuccinimide ester (MBS) (**Supplementary Fig. S1**). Despite the fact that this type of F-actin staining pattern is rarely seen in other eukaryotic cells, it was observed in fibers (Li et al. 2005a, Xu et al. 2009), implying that fiber cells may possess unique F-actin architecture at the fast-elongating stage.

The different actin structure in the transgenic fibers indicated that elevation in GhPFN2 amounts led to an increase in the abundance of F-actin bundles. To see if this was true in the wild-type fibers, overall profilin levels in the developing fibers were examined. Western blot analysis was conducted with proteins from the 6–24 DPA soil-grown fibers using the polyclonal antibody raised against GhPFN2. The results shown in **Fig. 5D** indicate that the profilin proteins reached a peak at 15 DPA before the transition from elongation into the secondary wall synthesis stages (see below), a time point when the highest accumulation of F-actin bundles was exhibited. Thus, expression levels of profilin proteins and the amounts of F-actin bundles were correlated in both wild-type and transgenic fiber cells.

Advanced reorientation of transverse cortical microtubules to oblique and helical directions in transgenic fiber cells

Increasing evidence indicates that the interplay between the actin cytoskeleton and microtubules might play a crucial role in various cellular processes, including cell elongation (Anderhag et al. 2000, Preuss et al. 2004, Collings et al. 2006). Therefore, the organization of microtubules in the wild-type and transgenic fibers was analyzed to see if altered F-actin organization in the transgenic fibers exerted an effect on the microtubule cytoskeleton. Although no obvious alteration in the microtubule structure was observed in the transgenic fibers at the elongation and secondary wall synthesis stages, a shift in the time of the rearrangement of microtubules was found during the transition period between the two phases (**Fig. 6A**). In the wild-type fibers, microtubule reorientation from the transverse to the oblique and helical directions occurred at around 15 DPA in the majority of the cells. In contrast this event took place at 13 DPA in more than half of the transgenic fibers, indicating that the time of microtubule reorientation was advanced by 2 d in the transgenic fibers. This promoted reorientation of microtubules can be seen clearly in **Fig. 6B**.

Earlier deposition of the secondary wall cellulose in transgenic fibers

One of the striking features of cotton fiber development is the synthesis of large amounts of cellulose. As secondary wall cellulose synthesis is often indicated by the development of wall birefringence which reflects the presence of crystalline cellulose (Seagull et al. 2000), we further detected the birefringence in wild-type and transgenic fibers under polarized light. The patterns in **Fig. 6C** show that in the wild-type fibers secondary wall synthesis started around 15 DPA as indicated by the significant increase in wall birefringence. Consistent with the advanced rearrangement of microtubules, the onset of

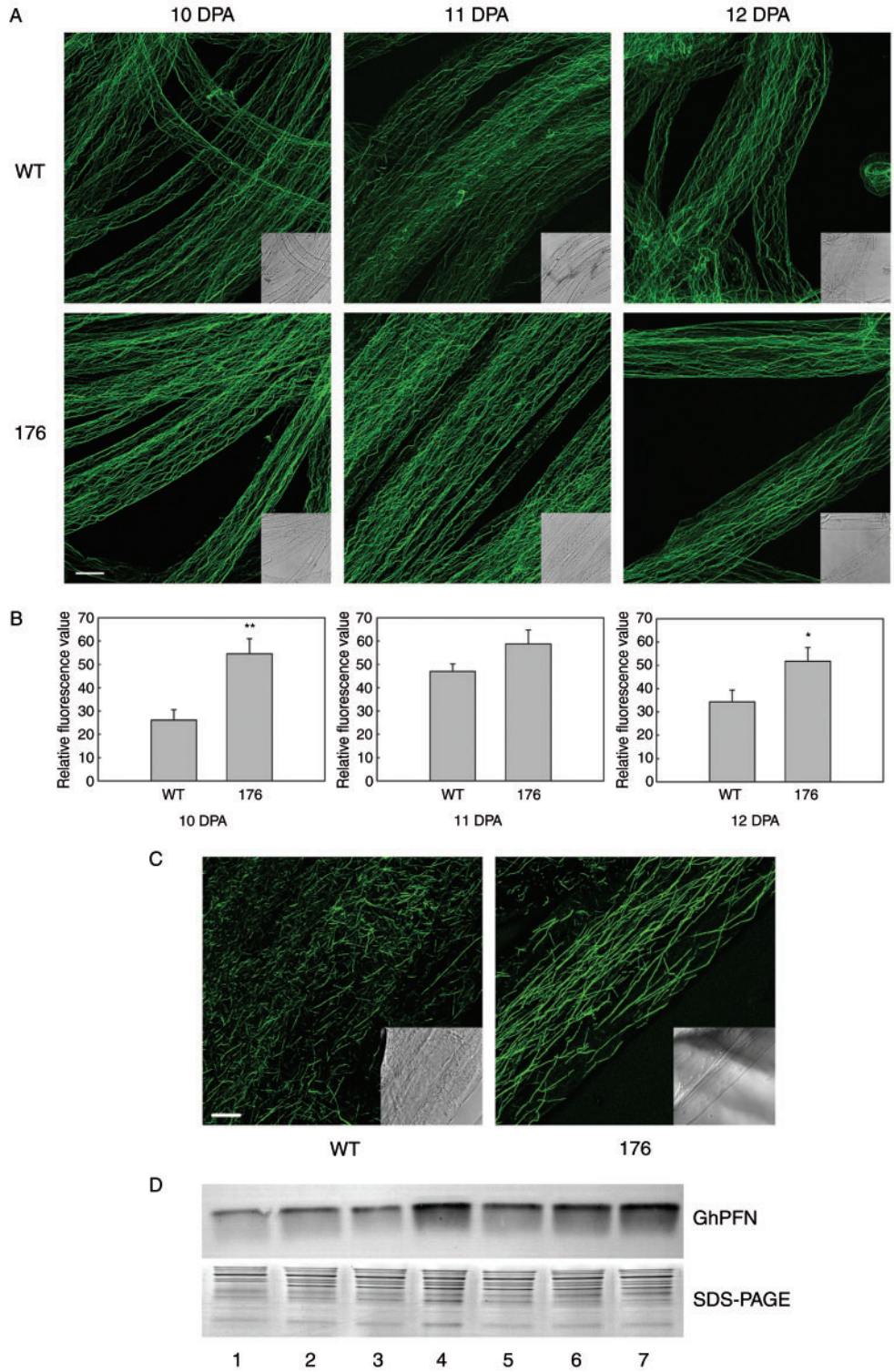

Fig. 5 Correlation of F-actin bundle amounts and profilin levels in cotton fibers. (A) Confocal images of F-actin structures in cultured wild-type and transgenic fibers at 10, 11 and 12 DPA. Note that more abundant and thicker actin bundles were formed in transgenic fibers. Corresponding bright field images are shown at a reduced size. Bar = 25 μm. (B) Relative fluorescence values of F-actin in wild-type and transgenic fibers. The quantifications were conducted in 9–12 individual fiber cells for each sample, and the measurements in each fiber were repeated three times. Mean values (± SE) of the 10 and 12 DPA wild-type and transgenic fibers are significantly different (*$P < 0.05$; **$P < 0.01$). (C) Confocal images of F-actin structures in CD-treated wild-type and transgenic fibers. Note the thicker and longer fragments of actin bundles in the transgenic fibers. Bar = 10 μm. WT, wild-type control; 176, the transgenic line. (D) Western blot analysis of total GhPFN levels in 6, 9, 12, 15, 18, 21 and 24 DPA soil-grown wild-type fibers (lanes 1–7). The lower panel in D shows the SDS–polyacrylamide gel which indicates the equal loading of protein samples.

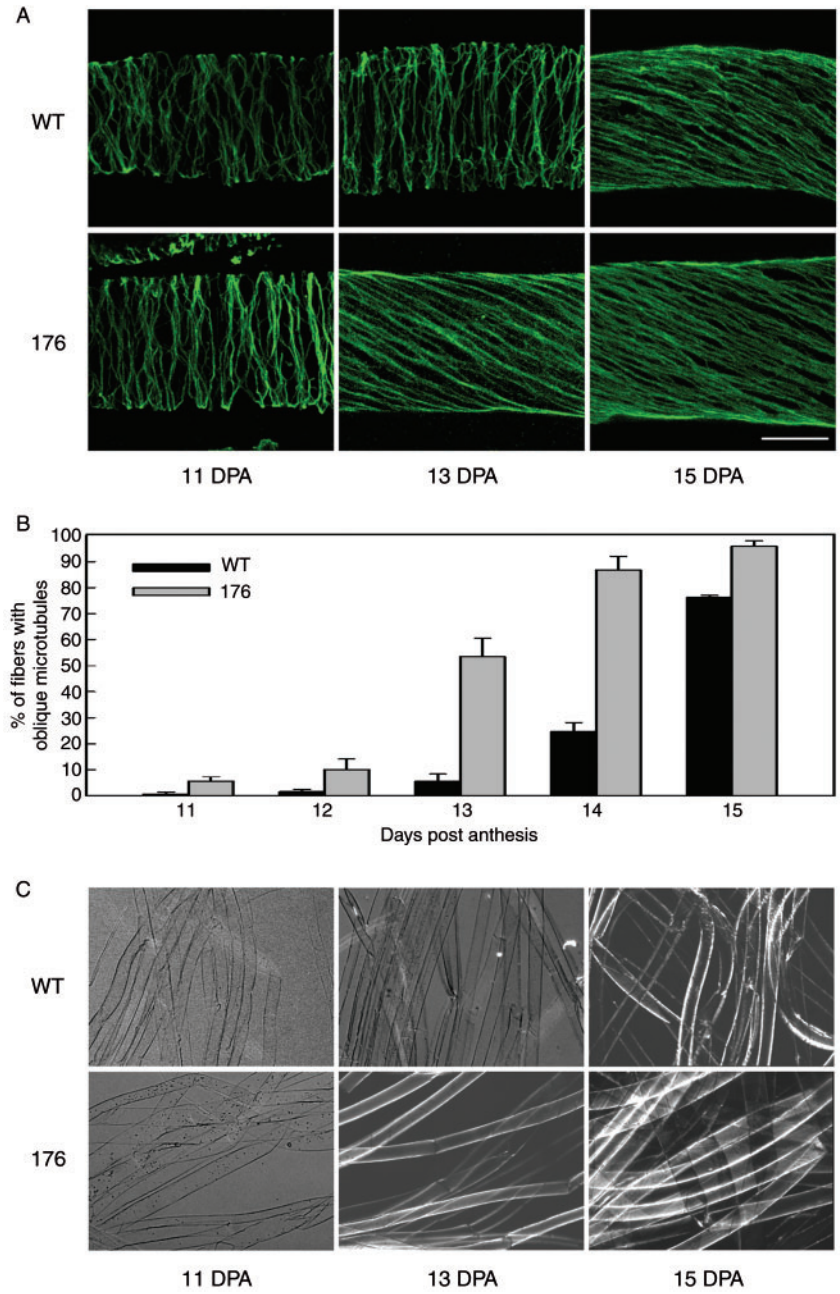

Fig. 6 Earlier microtubule reorientation and advanced onset of secondary wall deposition in transgenic fibers. (A) Confocal images of the arrangement of microtubules in cultured wild-type and transgenic fibers at 11, 13 and 15 DPA, respectively. Transgenic fibers showed advanced reorientation of the microtubules from the transverse to the oblique direction. Bar = 10 μm. (B) Percentage of wild-type and transgenic fibers with oblique microtubules at 11–15 DPA. The mean values (± SE) are 0.67±0.67, 1.53±0.90, 5.57±2.81, 24.63±3.56, 76.13±0.94 for wild-type fibers, and 5.67±1.62, 9.97±4.08, 53.53±6.97, 86.70±5.30, 95.83±2.19 for the transgenic line. (C) Birefringence of cultured wild-type and transgenic fibers at 11, 13 and 15 DPA viewed at ×100 magnification. WT, wild-type control; 176, the transgenic line.

secondary wall deposition was 2 d earlier (13 DPA) in the transgenic fibers.

It should be noted that the developmental progression of the in vitro cultured fibers differs from that of the field-grown fibers in white cotton cultivars. In particular, the time for the onset of secondary wall synthesis is several days earlier in cultured fiber cells than in the fibers grown in planta (Triplett 2000, Singh et al. 2009). On the basis of our experimental results, this is also true for the green-colored fibers. The deposition of secondary cell wall started at 16–18 DPA for the soil-grown fibers (see below) and at 14–15 DPA in the in vitro cultured fibers.

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Molecular and ultrastructural evidence for the shift in the developmental stages of the transgenic fibers

Semi-quantitative RT–PCR was performed using soil-grown fibers to detect the expression of several fiber development-related factors that could be regarded as markers of the transition period, such as *endo-β-1,3-glucanase*, *GhCelA1* and *GhRLK1* (Pear et al. 1996, Shimizu et al. 1997, Li et al. 2005b), to verify the advanced secondary wall synthesis in transgenic fibers at the molecular level. Compared with the low levels of all three genes expressed at 12–18 DPA in the control fibers, those in the fibers of both transgenic lines were augmented a few days earlier (**Fig. 7A**), confirming that the entry into the secondary wall synthesis phase was earlier in the transgenic fibers.

Previous studies have shown that the cell walls of the fibers of green cotton cultivars are suberized. The osmiophilic lipid biopolymer of suberin is distributed within the concentric polylamellate layers in the secondary cell walls and might function as a physiological barrier to moisture exchange (Ryser et al. 1983, Yatsu et al. 1983). Ryser et al. (1983) found that the number of osmiophilic suberin layers was roughly correlated with the number of days of secondary wall formation. To determine whether the suberization status was different in the wild-type and transgenic fibers, cell walls of the wild-type and transgenic fibers were examined by transmission electron microscopy (TEM). The images showed that the transgenic fibers (30 DPA) contained a greater number of suberin layers than the wild-type control. This was particularly evident in the line 156 fibers (**Fig. 7B**). This higher level of suberization of the transgenic fibers provided an additional line of evidence for the advanced secondary wall deposition.

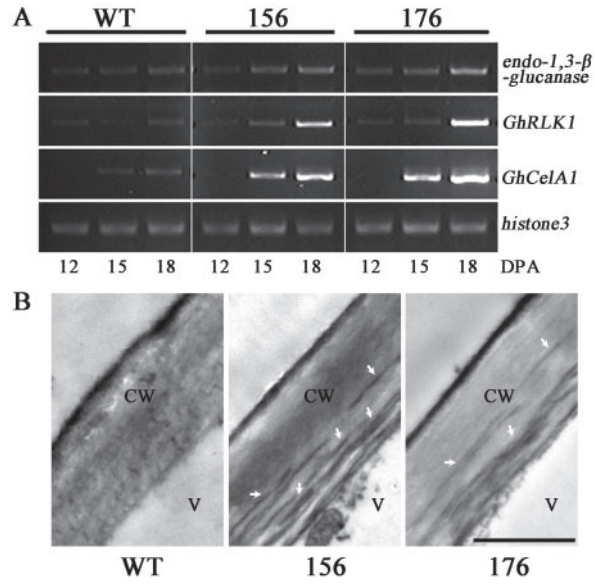

Fig. 7 Molecular and ultrastructural analyses for advanced onset of secondary wall synthesis in transgenic fibers. (A) RT–PCR analysis of *endo-β-1,3-glucanase*, *GhRLK1* and *GhCelA1* expression in soil-grown wild-type and transgenic fibers at 12, 15 and 18 DPA, respectively. Expression of these genes in transgenic fibers was activated a few days earlier than in the wild-type fibers. The *histone3* gene was used as an internal control. (B) TEM analysis of wild-type and transgenic fibers at 30 DPA. CW, cell wall; V, vacuole; Bar = 1 μm. The osmiophilic layers (indicated by arrows) in cell walls of 156 and 176 fibers indicate where the suberins distributed. WT, wild-type control; 156, 176, two independent transgenic lines.

GhPFN2 promoted actin bundling in the presence of AtFH1 in vitro

Complex roles, such as stimulation of actin polymerization, depolymerization and bundling, have been reported for profilin proteins (Sagot et al. 2002, Bubb et al. 2003, Wang and Riechmann 2008). The increase in F-actin bundles that resulted from GhPFN2 overexpression in transgenic fibers suggested that it might act to promote actin polymerization, and, as a result, favor the formation of actin filament bundles. To assess this possibility, we examined the effect of GhPFN2 on the kinetics of actin polymerization in vitro. However, we found that GhPFN2 did not promote actin polymerization under our experimental conditions (data not shown). As it is known that profilin interacts with formin and is required for the bundling activity of formin protein in yeast (Evangelista et al. 2002), we assumed that GhPFN2 might have such a function to aid the formation of actin bundles in the presence of formin proteins. We tested this possibility in vitro, using AtFH1, which was the only biochemically demonstrated formin to possess actin-bundling activity among the Arabidopsis formin family (Michelot et al. 2005). A low-speed co-sedimentation assay was conducted to assess the bundle formation of AtFH1 in the presence of GhPFN2. The amount of bundled actins in the pellets was determined by scanning the densities of the actin bands. Compared with the amount of actin bundles formed in the AtFH1 reaction without GhPFN2, the relative actin amounts in pellets increased by 1.06- to 1.22-fold in the presence of 0.04, 0.1, 0.4 and 1 μM GhPFN2 (**Fig. 8**), indicating that GhPFN2 was able to enhance the actin bundling activity of AtFH1 to some extent.

Discussion

Dynamic cytoskeletal reorganization directs the progression of fiber development

In developing cotton fibers, elongation and secondary wall deposition are two independent but overlapping stages. Although previous studies have revealed that different mechanisms such as hormone signaling and cytoskeleton reorganization are involved in these two respective processes (Seagull 1990, Seagull 1992, Shi et al. 2006, Singh et al. 2009), very little is known about the molecular and cellular basis responsible for the switch of these two developmental phases. In this study, we showed that both cortical actin filaments and microtubules underwent active reorganization during fiber development in the wild-type green fibers, especially during the period of transition

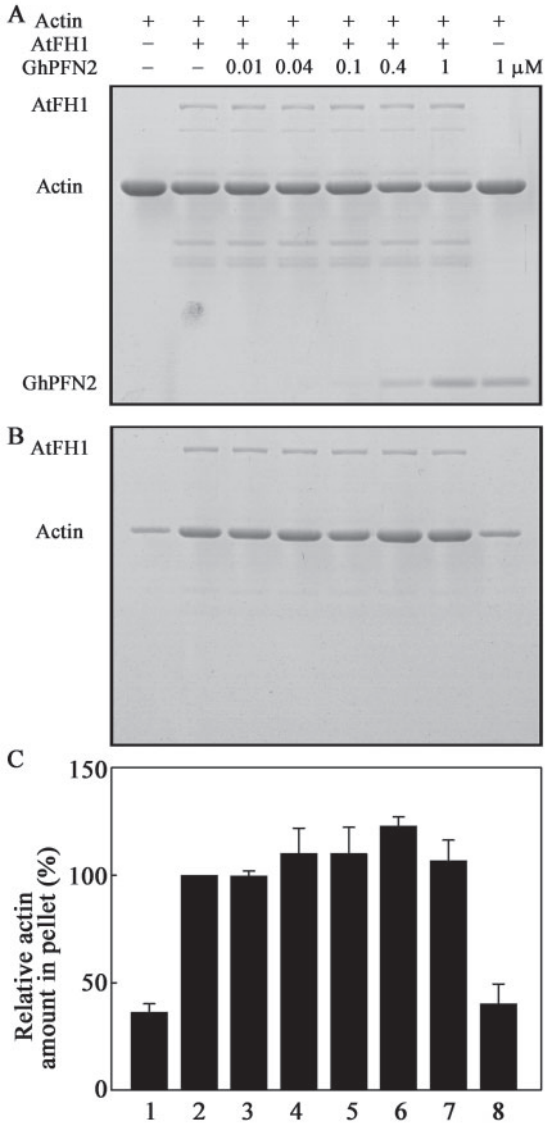

Fig. 8 Low-speed co-sedimentation analysis of actin bundling in vitro. Lane 1, actin alone (2 μM); lanes 2–7, actin (2 μM) together with 0.4 μM AtFH1 in the absence or presence of different concentrations (0.01, 0.04, 0.1, 0.4 and 1 μM) of GhPFN2; lane 8, actin (2 μM) with 1 μM GhPFN2 without AtFH1. The supernatants and pellets of centrifuged samples were separated by SDS–PAGE. (A) Proteins in supernatants. The positions of AtFH1, actin and GhPFN2 are indicated on the left. (B) Proteins in pellets. The positions of AtFH1 and actin are indicated on the left. (C) The gel in B was scanned and the intensity of the actin bands was determined by Glyko Bandscan 5.0. Experiments were repeated five times. The relative actin amount in lane 2 was set to 100%, and the mean percentages (± SE) of other lanes were 36.4±3.7 (lane 1), 99.6±2.3 (lane 3), 110.2±11.6 (lane 4), 110.0±12.1 (lane 5), 122.8±4.3 (lane 6), 106.6±9.6 (lane 7) and 40.3±9.2 (lane 8), respectively.

from elongation into the secondary wall deposition stage. In the *GhPFN2*-overexpressing fibers, along with an earlier formation of abundant thick actin cables/bundles, both the developmental reorientation of microtubules and the onset of secondary wall deposition were advanced by 2 d. These coupled variations between actin cytoskeleton architecture and microtubules and secondary wall cellulose deposition demonstrate that the dynamic cytoskeletal reorganization plays a pivotal role in the progression of fiber development from the elongation stage to the secondary wall synthesis stage during fiber development.

The development at the elongation and secondary wall synthesis stages determines the traits of length and strength of cotton fibers. It is known that the time point initiating the secondary wall synthesis influences the fiber length proportionally as formation of the more rigid secondary cell wall usually signals the cessation of cell expansion (Naithani et al. 1982, Seagull et al. 2000, Ruan et al. 2001). We found that together with the advanced onset of secondary wall synthesis, the fiber elongation of transgenic cotton plants stopped ahead of schedule. This shorter fiber phenotype could be due to the earlier secondary wall deposition that might obstruct the expansion of the primary cell wall, the determinant of the fiber length.

Actin and microtubule cytoskeletons have hierarchical roles in the onset of secondary wall deposition in developing fiber

Several drug studies have suggested that the actin cytoskeleton might participate in the regulation of microtubule organization. It was found that the cyclic reorientation of the cortical microtubules was modified after microfilament disruption in the epidermal cells of azuki bean (*Vigna angularis*) epicotyls (Takesue and Shibaoka 1998, Collings and Allen 2000), and the actin stabilization by jasplakinolide delayed microtubule depolymerization in *Papaver rhoeas* pollen (Poulter et al. 2008). Likewise, Seagull (1990) showed that the microtubules altered their orientations after exposure of young white cotton fibers (<16 DPA) to cytochalasin. In this study, we found that in wild-type fibers, a large amount of F-actin cables/bundles formed before the stage of transition from elongation to secondary wall synthesis, and microtubule reorientation followed with this event. Accordingly, formation of more abundant actin filament bundles in the earlier elongation stage in *GhPFN2*-overexpressing fibers was accompanied by prematurely induced microtubule reorientation. Our results implied that enhancement in F-actin bundling before microtubule reorientation is an essential process for the switch between the two developmental stages.

It has been proposed that microtubule arrays guide the cellulose synthase (CESA) complexes and cellulose deposition, and act to position delivery of CESA complexes to the plasma membrane in Arabidopsis (Paredez et al. 2006, Gutierrez et al. 2009). In cotton fibers, orientation of microtubule networks changed abruptly from the transverse to the helical direction during the period of transition from elongation to secondary wall synthesis (Seagull 1992). In this study, we found that advanced reorientation of microtubules was accompanied by advanced cellulose deposition. Thus, our results from transgenic research have added a line of substantial evidence for

the functional association between microtubule alignment and cellulose deposition in plant cells.

Based on the above results that we obtained reproducibly in the transgenic fibers of three successive generations, it is reasonable to believe that actin and microtubule cytoskeletons have hierarchical roles in developing fiber cells. The formation of a higher structure of actin filaments activates developmental reorientation of microtubules that, in turn, guides the onset of secondary wall deposition. Apart from cotton fibers, other plant cells such as sclerenchyma cells including fibers, sclereids and tracheary elements also possess the secondary walls that are sedimented after cessation of cell expansion (Zhong and Ye 2007). The actin cytoskeleton-dependent cell phase conversion observed in cotton fibers may be common to these secondary wall-containing cells.

Possible mechanisms for the interplay of the two cytoskeleton elements in developing fibers

Several studies have implied a link between actin and microtubule cytoskeletons in plant cells, although definitive proof showing their interaction is currently lacking. Based on the limited information published so far we postulate two possible mechanisms that may be involved in the functional association between the two cytoskeleton elements in fiber cells. It has been reported that actin bundles act as robust supporters of cytoplasmic streaming and intracellular transport in living cells (Thomas et al. 2009), and the dynamics of microtubule reorientation might be pushed by the force of actomyosin-driven streaming (Collings 2008, Sainsbury et al. 2008, Petrasek and Schwarzerova 2009). It is possible that in *GhPFN2*-overexpressing fibers increased and thicker actin bundles provided more stable longitudinal tracks for the cytoplasmic streaming and resulted in the accelerated reorientation of microtubules to the oblique directions. Alternatively, a number of proteins have been demonstrated to function in the coordination of actin filaments and microtubules in plants, such as a 190 kDa protein in tobacco BY-2 cells, SB401 in *Solanum berthaultii* and AtFH4 in Arabidopsis (Igarashi et al. 2000, Huang et al. 2007, Deeks et al. 2010), and two kinesin isoforms isolated from cotton fibers, GhKCH1 and GhKCH2, were shown to bind to both microtubules and microfilaments (Preuss et al. 2004, Xu et al. 2009). Thus it is also possible that these linkers of the two major cytoskeletons were evoked and played a role in promoting microtubule reorientation in *GhPFN2*-overexpressing fibers. Further studies are needed to provide deeper insight into these speculations.

The cellular role of GhPFN2

Profilin was first discovered as a factor that caused actin depolymerization in vitro, yet it is now frequently described as a promotor of actin polymerization (Yarmola and Bubb 2006). In addition, profilins were shown to be required for the action of formin proteins in actin filament assembly in yeasts (Evangelista et al. 2002), to be essential for proper organization of the F-actin structure required for polarized growth in moss *Physcomitrella patens* (Vidali et al. 2007) and to be involved in actin bundle formation and cortical anchoring of the microtubule minus ends in the *Drosophila* oocyte (Wang and Riechmann 2008). In this study, we found that overexpression of *GhPFN2* in green fibers resulted in the presence of more abundant F-actin bundles. Collectively, these results indicated that profilins have complex roles in the assembly and organization of the actin cytoskeleton. Although our biochemical analysis showed that GhPFN2 could enhance bundle formation in the presence of a formin protein, this might not reflect a sole function of GhPFN2 in fiber cells. In addition to formins, profilins have other ligands such as the Arp2/3 complex and proteins in the WAVE families. Depending on the cellular context of fiber cells, GhPFN2 might interact with these ligands and exert various functions on the actin cytoskeleton, such as, for example, aiding actin bundle formation and promoting actin polymerization.

In an earlier work, we observed that down-regulation of *GhADF1*, an actin depolymerizing factor, resulted in the occurrence of more abundant F-actin and increased fiber length in the transgenic plants producing white fibers as compared with the wild-type control (Wang et al. 2009). Unlike in *GhPFN2*-overexpressing fibers, we did not observe a change in the time of microtubule reorientation in *GhADF1*-underexpressing fibers. This could be due to a difference in the relative amounts of F-actin in the two types of transgenic fibers. The higher F-actin amount was due to the reduced depolymerization of F-actin in *GhADF1*-underexpressing fibers, whereas in the case of this study, the increased F-actin abundance might have resulted from the formation of more actin bundles. Together, these data indicate that the impact of F-actin on microtubule reorganization may be dependent on the status of the F-actin higher structure.

In addition to *GhPFN2*-overexpressing transgenics, we also made great efforts to generate *GhPFN2*-underexpressing plants by an antisense approach. Unfortunately, we failed to obtain any regenerated transgenic seedlings, perhaps due to the indispensability of profilins for cell growth and differentiation during the regeneration process. Additionally, it is worth noting that in wild-type fibers, the *GhPFN2* transcripts level was elevated again at the active secondary wall synthesis stage (**Fig. 1B**). This expression pattern indicated that GhPFN2-related cytoskeleton structuring is also important for the later stage of fiber development.

The regulation of cotton fiber development is an attractive area to study because of its theoretical and practical importance. Further efforts will be made to achieve double labeling of microfilaments and microtubules in cotton fibers in order to understand how an increased amount of F-actin bundles initiates a premature reorientation of the cortical microtubules. Additionally, we are attempting to introduce the overexpression and RNA interference (RNAi) constructs of *GhPFN2* that are under the control of a fiber-specific promoter to generate transgenic cotton plants with an altered program of fiber

development and modified fiber properties, but without affecting other aspects of cotton growth and development.

Materials and Methods

Plant materials and growth conditions

The cultivated variety MY (from the Institute of Cotton Research, Shanxi Academy of Agricultural Sciences) of green-colored cotton (*Gossypium hirsutum* L.) was used in this study. Cotton seeds were treated with H_2SO_4, surface sterilized with 10% H_2O_2 solution for 2–3 h, washed with sterile water 3–4 times and sowed on the medium or into the soil. Cotton plants were grown in the greenhouse under natural and additional artificial light at day/night temperatures of 28–35°C/20–22°C, and seedlings were grown in MS agar medium under a light/dark cycle of 16/8 h at 26°C. Roots, hypocotyls and leaves were collected from the tissue-cultured seedlings grown for about 2 weeks, while flowers and fibers were harvested from soil-grown plants. Flowers were tagged on the day of anthesis, and bolls were harvested at 0, 3, 6, 9, 12, 15, 18, 21 and 24 DPA. Ovules were excised from the bolls and fibers were scraped from the ovules. All the collected materials were immediately frozen in liquid nitrogen and stored at −80°C until use.

In vitro culture of cotton fibers

Cotton ovules were cultured in vitro according to the protocol of Beasley and Ting (1973) with minor modifications. In brief, bolls were harvested at 2 DPA and sterilized by immersing them in 80–95% (v/v) ethanol for about 5 s. After flaming briefly to get rid of the ethanol residue, ovules were dissected from the ovaries under sterile conditions and floated immediately on the basal liquid BT medium containing 5 μM IAA, 0.5 μM GA_3 and 200 mg l^{-1} cefotaxime. Cultures were kept at 32°C in the dark without agitation.

Ovules cultured for a given number of days were fixed in a mixture of methanol and acetic acid (3 : 1). Fixed ovules were placed in plates and fibers were combed carefully using sharp forceps and photographed using a digital camera. Fiber length was measured using the Image-Pro Plus program (Olympus, Tokyo, Japan). About 10–15 ovules were measured for each sample and the measurements were conducted using samples of three biological replicates.

For the drug inhibition experiment, ovules cultured for 8 d (10 DPA) were transferred into basal BT medium supplemented with 5 μM CD (Sigma-Aldrich, St. Louis, MO, USA) and kept at 32°C in the dark until use.

Cloning of GhPFN2 cDNA

A 2 μg aliquot of total RNA from 6 DPA cotton fibers was used in the RACE experiments using a BD SMART RACE kit (Clontech, TAKARA BIO Group, USA). The primers used in 3′ RACE (5′-CATGAATGACTTTAATGAACCGGGTACGCT-3′) and 5′ RACE (5′-AACCCTGATCGAGGAGATAATCGCCG-3′) were designed based on the EST sequence (GenBank accession No. AI728639) obtained from the NCBI. The nucleotide sequence was determined from overlapping clones, and full-length cDNA (GenBank accession No. GU237487) was obtained by PCR. Open reading frame (ORF) and motif analyses were performed with NCBI Blast and DNAMAN version 6. Protein sequence alignment was performed by the DNAMAN program.

Vector construction and plant transformation

The ORF of *GhPFN2* was amplified using forward primer PrfI (5′-GCTCTAGAATGTCGTGGCAAGCTT-3′) and reverse primer PrfII (5′-CGGGATCCTCAATAACCCTGATC-3′). After *Xba*I and *Bam*HI digestion, the fragment was inserted into the plant expression vector pBin438 (Li et al. 1994) under the control of the CaMV 35S promoter. The recombinant plasmid was introduced into cotton by *Agrobacterium*-mediated transformation.

Genomic DNA extraction and PCR analysis

Genomic DNA was extracted from leaves of cotton plants using the method described by Zhang and Stewart (2000). PCR analysis of integration of the *GhPFN2* ORF into the cotton genome was carried out using the forward primer for the CaMV 35S promoter (5′-AAGGAAGTTCATTTCATTTGGAG-3′) and the reverse primer PrfII for the transgene.

RNA isolation and semi-quantitative RT–PCR

Total RNA from cotton roots, hypocotyls, leaves, flowers, ovules and fibers was extracted according to the ultracentrifugation method described by John and Crow (1992). A 4 μg aliquot of total RNAs was used for the reverse transcription for first-strand cDNA synthesis. Aliquots (1 μl) of the reverse transcription products were then used for PCR with the gene-specific primers listed in **Table 1**. Primer pair No. 1 was used for the analysis of *GhPFN2* expression in various organs and fibers of the wild-type cotton plants. Primer pair No. 2 was used for the analysis of *GhPFN2* overexpression in the transgenic plants. The cotton *histone3* gene was used as an internal control.

Antibody preparation and Western blot analysis

The ORF of *GhPFN2* cDNA was cloned into the pET28a expression vector (Novagen/Merck, Darmstadt, Germany) and the plasmid construct was introduced into the BL21 (DE3) strain of *Escherichia coli* by electroporation. The recombinant proteins were purified according to the protocol provided by the supplier and used subsequently to immunize rabbits to raise antibody against GhPFN2.

Proteins of soil-grown cotton fibers were prepared by extraction with a buffer containing 50 mM $NaPO_4$ (pH 7.4), 0.5 M NaCl, 1 mM EDTA, 1% (v/v) β-mercaptoethanol (β-ME), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% (w/w) polyvinylpolypyrrolidone (PVPP). The protein concentration in the supernatant was determined according to the method of Bradford (1976). Proteins of leaves were extracted in buffer containing 30% (w/v) sucrose, 0.1 M Tris–HCl (pH 8.8),

2% (w/v) SDS and 0.07% (v/v) β-ME. An equal volume of phenol saturated with Tris–HCl (pH 7.9) was added to the extract and the mixture was vortexed for 5 min and then centrifuged at 12,000×*g* for 10 min at room temperature. The upper phenolic phase was removed to a new tube and the proteins were precipitated with 5 vols. of 0.1 M ammonium acetate in methanol at −20°C overnight. After centrifugation at 12,000×*g* for 15 min at 4°C, the pellet was rinsed with ice-cold 0.1 M ammonium acetate in methanol and then with ice-cold 80% acetone. After air drying, the pellet was resuspended in the extraction buffer without sucrose. Due to the high concentration of SDS in the extraction buffer, the protein concentration was determined with the 2-D Quant kit (GE Healthcare Life Sciences, USA).

A 5 μg aliquot of proteins was separated by 15% SDS–PAGE and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked overnight with 5% (w/v) milk powder in 20 mM Tris–HCl (pH 7.5) and 0.9% (w/v) NaCl, and then incubated with the polyclonal antibody raised against GhPFN2 (1:3,000) for 1 h at 37°C. After two washes, the membrane was incubated for 1 h with goat anti-rabbit IgG AP-conjugated antibody (1:7,500) (Jackson, Pennsylvania, USA) and then received three post-incubation washes. Signals were developed using BCIP/NBT Color Development Substrate (Promega, Madison, USA).

Analyses of fiber properties and cell wall structure

The length and strength of matured fibers were measured at the National Center for Evaluation of Fiber Quality (Anyang, China) with HFT9000 (Premier, India) using standard criteria.

The 30 DPA soil-grown cotton fibers were used for TEM according to a previous report (Wang et al. 2009).

Fluorescent staining, microscopic analyses and quantification of the cytoskeleton

Actin microfilament staining was performed mainly according to Seagull (1990). Cultured ovules with fibers attached were incubated for 10 min in phosphate-buffered saline (PBS; pH 7.0) containing 0.066 μM Alexa488–phalloidin (Molecular Probes, Eugene, OR, USA), 0.1 M PIPES (pH 6.9), 0.05% (v/v) Triton X-100, 1 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.3 mM PMSF, 5 mM EGTA and 0.25% glutaraldehyde. After briefly rinsing in PBS, fibers were carefully cut off the ovules and mounted onto glass slides. Fluoro-Guard Antifade Reagent (Bio-Rad, CA, USA) was used to prevent fluorescence quenching.

For microtubule staining, cultured ovules with fibers attached were fixed with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in PME buffer (50 mM PIPES, 2 mM $MgSO_4$ and 5 mM EGTA, pH 6.9) containing 0.1% (v/v) Triton X-100 and 0.3 M mannitol. After three washes in PME buffer, fibers were carefully cut off the ovules and placed onto Poly-Prep™ slides (Sigma-Aldrich, St. Louis, MO, USA), then incubated with 1% (w/v) cellulase R-10 (Yakult Pharmaceutical Industry Co., Japan) and 0.1% (w/v) pectolase Y-23 (Yakult Pharmaceutical Industry Co., Japan) in PME buffer supplemented with 1 mM PMSF at room temperature (4–10 min for wild-type fibers and 8–20 min for transgenic fibers). Fibers were washed again once in PME buffer and twice in PBS (pH 6.9), and then incubated in PBS containing 1% (w/v) bovine serum albumin (BSA) for 15 min. Slides were incubated in primary antibody (anti-α-tubulin, Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight, washed with PBS three times, and then incubated with the secondary antibody [fluorescein isothiocyanate (FITC)-conjugated affinipure goat anti-mouse IgG; Proteintech Group, Inc., Chicago, IL, USA] in the dark for 2 h at 37°C. Slides were washed twice with PBS to remove residual antibodies, mounted with 50% (v/v) glycerol and sealed.

Both actin microfilament and microtubule observations were performed under a confocal laser microscope (Leica TCS SP5, Leica Microsystems, Germany) and the fluor label was excited at 488 nm with emission from 543 nm. The relevant fluorescent images were projections of Z-series sections (at 0.75 μm steps with two-line averaging and one-frame averaging) acquired from the top to the bottom surfaces of the respective fiber cells, while the bright field images were selected from single sections. Wall birefringence was observed under a microscope (Olympus BX51, Tokyo, Japan) with filter combination and polarization optic sets recommended by the manufacturer.

For quantitative analysis, the F-actin abundance was measured by determining the gray values for confocal image projections with the Line Analyzer tool of ImageJ 1.43s (http://rsbweb.nih.gov/ij/). Each pixel where the gray value is >20 is defined as a unit of relative fluorescence value. Statistical evaluations with *t*-test were performed between the values of the wild-type and transgenic fibers at the same DPA. For quantitative analysis of the timing of microtubule reorientation, the numbers of fibers with transverse or oblique microtubules were counted. Up to 50–60 immunostained cells that were observed under 10 randomly selected visual fields were included in the analysis for each sample. Three independent experiments were conducted.

Low-speed co-sedimentation assay

Actin was prepared from acetone powder of rabbit skeletal muscle (Spudich and Watt 1971). Monomeric Ca-ATP-actin was purified by gel filtration chromatography on a Sephacryl S-300 HR column (GE Healthcare, USA) at 4°C in G buffer (5 mM Tris–HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM DTT and 0.2% NaN_3). AtFH1 was purified as described previously (Michelot et al. 2005).

All proteins and related solutions were pre-clarified at 200,000×*g* for 1 h prior to the experiments. Mg-ATP-actin was prepared by incubation of Ca-ATP-actin on ice with 1 mM EGTA and 0.1 mM $MgCl_2$, and used immediately. Actin (2 μM) was incubated at 22°C for 1 h either alone, or with 0.4 μM AtFH1 in the absence or presence of different concentrations (0.01, 0.04, 0.1, 0.4 and 1 μM) of GhPFN2, or with 1 μM GhPFN2 without adding AtFH1 in 1× KMEI buffer (10× stock: 500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, 100 mM imidazole, pH 7.0).

The samples were then centrifuged at 13,500×*g* for 30 min. The supernatants and pellets were separated by SDS–PAGE and protein bands were visualized with Coomassie Brilliant Blue R250. Gels were scanned and the intensity of actin bands was determined with Glyko Bandscan version 5.0.

Supplementary data

Supplementary data are available at PCP online.

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