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Cotton *PRP5* gene encoding a proline-rich protein is involved in fiber development

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Abstract Proline-rich proteins contribute to cell wall structure of specific cell types and are involved in plant growth and development. In this study, a fiber-specific gene, *GhPRP5*, encoding a proline-rich protein was functionally characterized in cotton. *GhPRP5* promoter directed *GUS* expression only in trichomes of both transgenic *Arabidopsis* and tobacco plants. The transgenic *Arabidopsis* plants with overexpressing *GhPRP5* displayed reduced cell growth, resulting in smaller cell size and consequently plant dwarfs, in comparison with wild type plants. In contrast, knock-down of *GhPRP5* expression by RNA interference in cotton enhanced fiber development. The fiber length of transgenic cotton plants was longer than that of wild type. In addition, some genes involved in fiber elongation and wall biosynthesis of cotton were up-regulated or down-regulated in the transgenic cotton plants owing to suppression of *GhPRP5*. Collectively, these data suggested that GhPRP5 protein as a negative regulator participates in modulating fiber development of cotton.

Keywords Cotton (*Gossypium hirsutum*) · Proline-rich protein · Fiber elongation · Negative regulation

Wen-Liang Xu and De-Jing Zhang contributed equally to this work.

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Introduction

Hydroxyproline-rich glycoproteins (HRGPs) are widespread proteins in higher plants and constitute up to 10 % of the cell wall mass (Varner and Lin 1989). HRGPs are generally categorized into three broad families based on amino acid sequences and glycosylation profiles, namely, lightly glycosylated proline-rich proteins (PRPs), moderately glycosylated extensins (EXTs), and highly glycosylated arabinogalactan proteins (AGPs). HRGPs are critical to the structure and function of both the wall and the plasma membrane (PM)-cytoskeleton continuum (Showalter 1993; Jose-Estanyol and Puigdomenech 2000; Showalter et al. 2010). In addition to the structural component, HRPGs contribute significantly to wall assembly and remodeling during cell growth and development and respond to stress stimuli in diverse organisms ranging from green algae to land plants (Ellis et al. 2010; Doblin et al. 2010). PRP proteins are also classified into three subgroups based on repetitive motifs and domain organization: (1) repetitive PRPs along the whole amino acid sequence; (2) non-repetitive PRPs; (3) multidomain hybrid PRPs (Jose-Estanyol and Puigdomenech 2000). The repeat sequences of PRPs vary considerably with different plant species and among PRPs in an individual species. The unifying feature of this diverse group of proteins is that the proline residues are abundant and they occur in two consecutive repeats.

PRP gene expression profiles indicated that they are temporally and spatially regulated during the development of a particular tissue or cell type (Munoz et al. 1998; Xu et al. 2007). For example, three PRPs in soybean are expressed in diverse cell types. *SbPRP1* mRNA was highly abundant in elongating and mature regions of the seedling hypocotyl epidermal cells, *SbPRP2* mRNA accumulated especially in phloem cells, and *SbPRP3* mRNA was specifically localized

to the endodermoid layer of cells in the hypocotyl elongating region (Wyatt et al. 1992). *Arabidopsis PRP1* and *PRP3* were exclusively expressed in roots, while *PRP2* and *PRP4* transcripts were most abundant in aerial organs of the plant (Fowler et al. 1999; Bernhardt and Tierney 2000). PRPs are proposed to be secreted, where they become insolubilized and incorporated into the wall. The insolubilization of PRPs may lead to the formation of protein–protein or protein–carbohydrate linkages within cell wall and contribute to the stability of the extracellular matrix (Munoz et al. 1998; Fowler et al. 1999; Bernhardt and Tierney 2000; Akiyama and Pillai 2003). This and other interpretations await functional assays to demonstrate the definitive function of PRPs.

Cotton fibers, a premier natural fiber widely used in the textile industry, are derived from epidermal cells of the ovule and, therefore, are also called seed hairs. Approximately 30 % of the ovular epidermal cells elongate and develop into single-celled fibers at anthesis. Each cotton fiber is one of the longest single cells in higher plants. Its elongation rate and the final length are far beyond other plant cells. Fiber development is a highly concerted process involving four overlapping stages: fiber initiation, primary cell wall formation, secondary cell wall thickening, dehydration and maturation. Thus, the cotton fiber represents an excellent model to study plant cell elongation and cell wall biogenesis (Kim and Triplett 2001). Moreover, study of fiber development not only helps the basic understanding of cell differentiation and elongation, but also provides potential avenues to improve cotton fiber via manipulating fiber biosynthesis-related genes.

Although some genes encoding PRP proteins have been isolated from cotton, none were functionally characterized so far (Ji et al. 2003; Feng et al. 2004; Xu et al. 2007; Yuan et al. 2011). As we have shown previously, *GhPRP5* gene encoding a proline-rich protein is specifically expressed in cotton fiber and is fiber-development stage regulated (Xu et al. 2007). Here, we show that overexpression of *GhPRP5* reduces the growth of the transgenic *Arabidopsis* plants, whereas suppression of *GhPRP5* promotes the elongation of cotton fibers.

Materials and methods

Plant materials and growth conditions

Cotton (*Gossypium hirsutum* cv. Coker 312) seeds were surface-sterilized with 75 % (v/v) ethanol for 1 min and 10 % (v/v) H_2O_2 for 2 h, followed by washing with sterile distilled water. The sterilized seeds were germinated on half-strength MS medium under 16 h light/8 h dark cycles at 28 °C. The seedlings were transplanted into soil in a greenhouse for further growth to maturation. Tissues for DNA and RNA extraction were derived from cotton plants grown in a greenhouse.

Seeds of *Arabidopsis thaliana* (ecotype *Columbia*) were surface-sterilized with 75 % ethanol for 1 min followed by 5 % NaClO for 5 min then washed four times with sterile water. The sterilized *Arabidopsis* seeds were plated on germination medium (half-strength MS, 10 g/l sucrose, pH 5.8). After stratified at 4 °C for 3 days, the plates were transferred to LD conditions (16 h light/8 h dark, 22 ± 1 °C) for seed germination. Seedlings were transplanted in soil and grown in a growth room under the conditions of 16 h light/8 h dark cycle, 22 ± 1 °C. Tissues for DNA and RNA extraction were obtained either from plants grown on plates or in a growth room.

DNA and protein sequence analysis

Unless otherwise stated, nucleotide and amino acid sequences were analyzed using DNAstar (DNAstar Inc). Promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict the GhPRP5 transcription initiation site. Putative cis-acting elements were identified by submitting the promoter sequence to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Identification of protein domains and significant sites was performed with Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The hydropathic profile was calculated according to Kyte and Doolittle hydropathy plot (http://fasta.bioch.virginia.edu/fasta_www/grease.htm). SignalP (www.cbs.dtu.dk/services/SignalP/) was used to determine the N-terminal signal sequence.

RNA gel blot analysis

Tissues such as roots, hypocotyls and cotyledons were collected from 5-day-old cotton seedlings, while anthers, petals, fibers and ovules were from cotton plants grown in greenhouse. On the day of anthesis (flower opening), flower buds were tagged as 0 days post anthesis (dpa) and the corresponding bolls were harvested at 5, 10 and 15 days after that day, immediately frozen in liquid nitrogen, and stored in −80 °C. At each developmental time-point, fibers were isolated from ovules. Total RNA was extracted from different cotton tissues according to our previous method (Li et al. 2002). RNA gel blot analysis was performed with 20 μg per lane from different cotton tissues and were separated on 1.0 % (w/v) agarose-formaldehyde gels and transferred onto Hybond-N nylon membranes (Amersham) by capillary blotting. Gene-specific probe was labeled with α-[^{32}P]-dCTP using Takara random primer labeling kit (Takara). RNA gel blot was performed at 42 °C overnight in RNA hybridization solution (6 × SSC, 1 % SDS, 5 × Denhardts, 100 μg mL^{-1} denatured salmon sperm DNA (Invitrogen) with α-[^{32}P]-labeled gene specific probe. The membranes were washed

three times at 55 °C for 15 min in 0.1 × SSC and 0.5 % SDS. After drying briefly, the membranes were exposed to X-ray film (Eastman Kodak) with two intensifying screens at −75 °C for 1 day.

Real-time RT-PCR analysis

The expression of *GhPRP5* and other fiber-related genes using gene-specific primers (Supplementary Table 2) were determined by real-time reverse transcriptase (RT)-PCR using the fluorescent intercalating dye SYBR-Green (TOYOBO) in a detection system (MJ Research, Opticon 2) according to our previous method (Li et al. 2005).

Promoter isolation and construction of *GUS* reporter cassette

A 2,217 bp segment of promoter was isolated by Genome walking PCR as described previously (Li et al. 2002). For the construction of the GUS reporter cassette, a *Sal* I site and a *Bam*H I site were introduced at the 5′-end and 3′-end of the *GhPRP5* promoter (including the putative promoter fragment and 5′-UTR before translational initiation codon ATG), respectively, by PCR. The *Sal* I/*Bam*H I fragment of *GhPRP5* 5′-upstream region was cloned into the *Sal* I/*Bam*H I sites of the pBI101 vector to generate the chimeric GhPRP5p:GUS construct.

Construction of *GhPRP5* overexpression and RNAi vectors

To construct overexpression vector, the coding sequence of *GhPRP5* was amplified by PCR with *Bam*H I and *Sac* I sites and cloned into pBI121 vector.

To construct GhPRP5 RNAi vector, the first intron (0.2 kb) of the *GhTUB1* gene (Li et al. 2002) was amplified by PCR with *Xba* I and *Not* I, and inserted into a pBluescript SK+ vector at the sites *Xba* I and *Not* I to obtain an intron-containing intermediate construct (pSK-TUBint). The GhPRP5 3′-terminal sequence (377 bp fragment at 296 bp upstream the stop codon to 77 bp downstream the stop codon) was cloned into the 5′ arm with the introduced sites *Bam*H I/*Xba* I and the 3′ arm with the introduced sites *Not* I/*Sac* I of the intron in pSK-TUBint vector for the sequences encoding the inverted repeat RNA. The constructed RNAi of the *GhPRP5* gene was cloned into the pBI121 at *Bam*H I/*Sac* I sites to replace the *GUS* gene.

Transformation and screening of *Arabidopsis thaliana* and tobacco

The *GhPRP5* overexpression and *GhPRP5*:GUS constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, respectively, and transgenic *Arabidopsis* plants were generated by the floral-dip method. Seeds were harvested and stored at 4 °C. For screening, seeds were sterilized in 75 % (v/v) ethanol for 1 min and 5 % NaClO solution for 5 min, followed by washing with sterile water. Kanamycin-resistant plants were selected by incubating plants on MS medium containing 50 μg mL^{-1} of kanamycin.

The transformed *Agrobacterium* strain LBA4404 was used to transform tobacco (*Nicotiana tabacum* L.) with the standard leaf disk method. Transformants were selected on MS medium containing 50 mg/L of kanamycin and 500 mg/L of cefotaxime. The regenerated transgenic tobacco plants were identified by PCR analysis. The homozygous T2 generation of transgenic tobacco was used for further analysis.

Histochemical assay of *GUS* gene expression

Histochemical assays for *GUS* activity in transgenic *Arabidopsis* and tobacco were conducted according to the protocol described previously (Li et al. 2002). The stained tissues were examined and photographed under a Leica stereomicroscope (Leica MZ16f).

Cotton transformation

Hypocotyl explants from cotton (*G. hirsutum*) cv Coker 312 were transformed using *Agrobacterium tumefaciens*–mediated transformation as described previously (Li et al. 2002). Homozygosity of transgenic plants was determined by segregation ratio of the kanamycin selection marker.

Construction of *GhPRP5*:eGFP vector

For GhPRP5 localization assay, the coding region of *GhPRP5* amplified by PCR with *BamH* I and *Xba* I was cloned into the binary vector pBI121. Then, *eGFP* coding region was excised with *Xba* I and *Sac* I and cloned into pBI121 downstream of *GhPRP5*. The resulting construct was introduced into cotton as described previously (Li et al. 2002). The localization pattern of GhPRP5 protein in the transgenic cotton callus cells was observed under a Leica confocal laser scanning fluorescence microscope (Leica TCS SP5).

Yeast two-hybrid analysis

A yeast two-hybrid screen using GhPRP5 as bait was performed to identify interacting partners of GhPRP5 using a method as previously reported (Zhang et al. 2010). For directed yeast two-hybrid assays of protein–protein interaction between cotton GhPRP5 and the identified proteins,

also refer to the procedure as described previously (Zhang et al. 2010).

Bimolecular fluorescence complementation (BiFC) assays

pUC-SPYNE-GhPRP5, pUC-SPYCE-GhPRP5, pUC-SPYCE-1 and pUC-SPYCE-3 vectors were constructed using gene-specific primers, respectively (Supplementary Table 3). The constructs were then introduced into onion epidermal cells by DNA particle bombardment as described previously (Chen et al., 2012).

Results

GhPRP5 encoding a proline-rich protein is fiber-specific

Five genes encoding proline-rich proteins were isolated from cotton cDNA libraries in our previous study. One of which was designated *GhPRP5* and encodes a protein of 182 amino acids. The protein is very rich in proline (13.2 %) and lysine (13.7 %) residues. The deduced protein consists of two parts: the first twenty residues are hydrophobic and represent a putative signal peptide sequence, followed by a proline-rich domain (Xu et al. 2007). Further analysis of the full length genomic sequence of *GhPRP5* gene revealed that the gene contains one intron (Supplemental Fig. 1a). In this study, Northern-blot hybridization further confirmed that *GhPRP5* is fiber-specific, using a gene-specific probe (Supplemental Fig. 1b), consistent with our previous RT-PCR analysis (Xu et al. 2007; Supplemental Fig. 1c and 1d). The results indicated that *GhPRP5* transcripts were the most abundant in 5 dpa fibers and less abundant in 10 dpa fibers, while negligible levels were detected in the other tissues examined.

GhPRP5 promoter displays trichome-specific activity in transgenic *Arabidopsis* and tobacco

To further investigate the tissue-preferential and developmental regulated expression of *GhPRP5*, a 2,217-bp 5′-flanking fragment upstream *GhPRP5* translation start codon (ATG) was isolated by genome walking PCR as described previously (Li et al. 2002). Analysis of the isolated 2,217-bp promoter sequence of *GhPRP5* gene revealed a putative transcription initiation site 91-bp upstream of the ATG translation start codon (http://www.fruitfly.org/seq_tools/promoter.html), and TATA and CAAT boxes were found in this promoter. In addition, several putative cis-acting regulatory elements were identified, such as light-response elements, abscisic acid responsiveness, MeJA-responsiveness, elicitor-responsive elements, gibberellin-responsive elements, salicylic acid responsiveness, wound-responsive elements, and MYB binding sites (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Supplemental Table 1). Since hormone and/or stress response elements are present in the promoter region, it is possible that *GhPRP5* expression may be important for plant development and be involved in stress responses.

Because trichomes in *Arabidopsis* and tobacco share some structural and genetic similarities with cotton fibers, researchers use these heterologous model species to monitor the activity of cotton fiber-specific promoters (Hsu et al. 1999; Liu et al. 2000; Delaney et al. 2007; Shangguan et al. 2008). We took advantage of *Arabidopsis* and tobacco for promoter activity analysis. The 2,217-bp fragment of *GhPRP5* promoter was cloned into a pBI101 vector upstream *GUS* gene, and was transferred into *Arabidopsis* and tobacco. In transgenic *Arabidopsis*, *GUS* expression was observed in cotyledons and hypocotyls of 3-day-old germinated seedlings (Fig. 1a), but when the first true leaf emerges and the trichomes are visible, GUS staining was only localized in trichomes (Fig. 1b). With further growth and development of the plants, GUS activity was only detected in trichomes of rosette leaves, but no or very weak staining was observed in other tissues (Fig. 1c–h). Similarly, strong to moderate GUS activities were detected in trichomes of leaves, petioles and stems, but no or weak signals were found in the other tissues of transgenic tobacco plants (Supplemental Fig. 2).

GhPRP5 is mainly localized to the plasma membrane

To gain insight into the localization of GhPRP5 protein in plant cells, GhPRP5- eGFP (enhanced green flurorescence protein) fusion protein was expressed in transgenic cotton cells under the control of 35S CaMV promoter. The eGFP fluorescence was detected under a Leica confocal laser scanning microscope. GhPRP5-eGFP was present on the cell surface of these transgenic cotton callus cells (Fig. 2a–c). Plasmolyzation with 4 % NaCl for 10 min permitted a clear visualization of the plasma membrane labeled with eGFP, but very faint fluorescence in the cell wall, indicating that GhPRP5 is mainly localized at the plasma membrane. (Fig. 2d–f).

Overexpression of *GhPRP5* in *Arabidopsis* hinders plant growth

To evaluate the function of *GhPRP5*, we expressed *GhPRP5* in *Arabidopsis*. The *GhPRP5* ORF was inserted into the expression vector (pBI121) under the control of

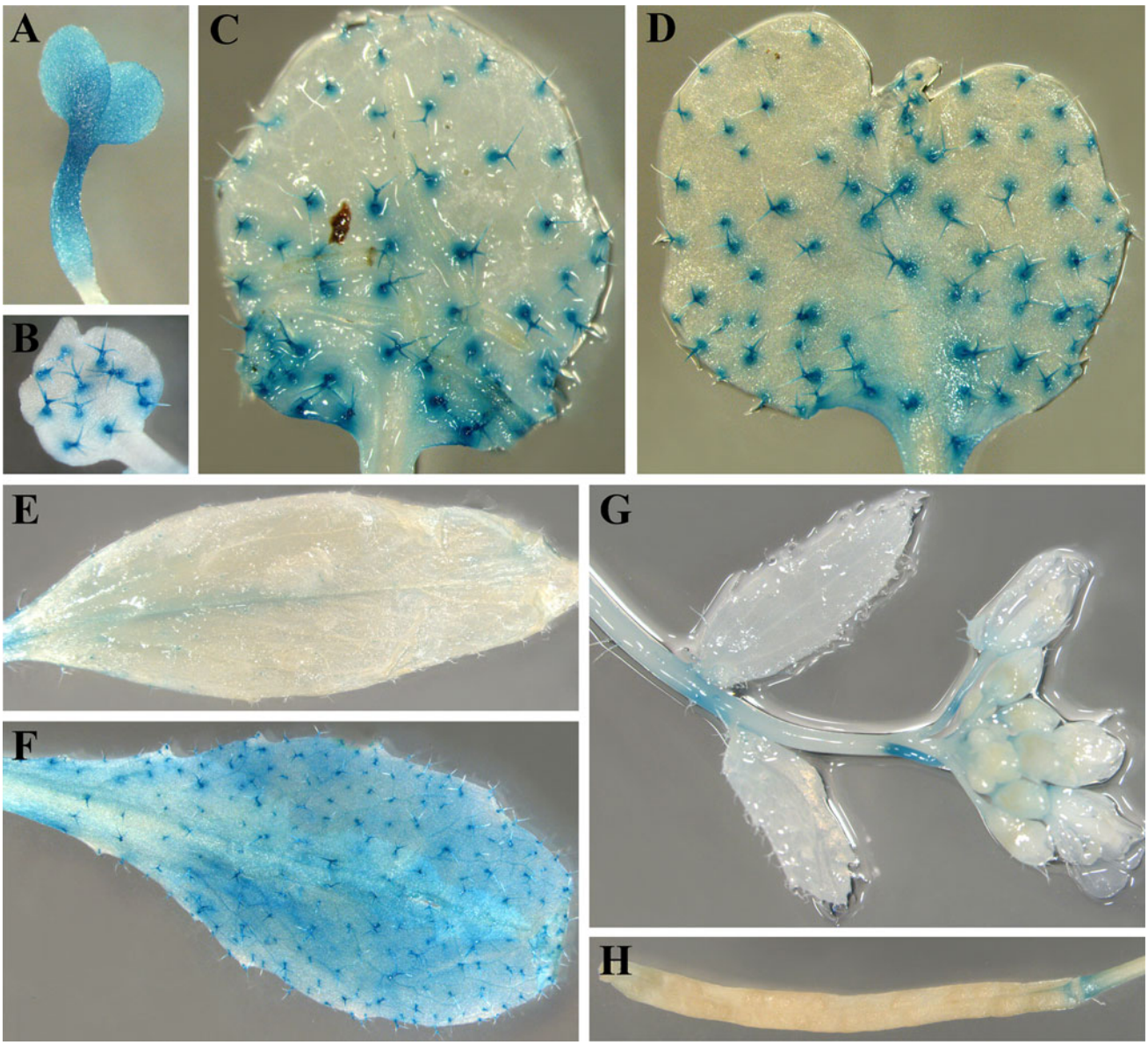

Fig. 1 GUS staining in transgenic *Arabidopsis* plants harboring *GhPRP5*:GUS construct. **a** 3-Day-old seedling. **b** 13-Day-old true leaf. **c** 20-Day-old rosette leaf. **d** 25-Day-old rosette leaf. **e** Seven-week-old cauline leaf. **f** Seven-week-old rosette leaf. **g** Flower. **h** Silique

CaMV 35S promoter and introduced into *Arabidopsis*. RT-PCR analysis showed that *GhPRP5* transcripts were highly abundant in eight homozygous transgenic lines, while undetectable in wild type (Supplemental Fig. 3). All of them exhibited similar phenotypes. Two homozygous transgenic lines (L7 and L11) of the fourth generation (T4) with the highest expression of *GhPRP5* were selected for further phenotypic analysis. Seeds of transgenic lines were germinated in half-strength MS medium supplemented with 1 % sucrose in a plant growth incubator (Sanyo, Osaka, Japan). RT-PCR analysis showed that the *GhPRP5* gene was expressed in the transgenic plants (Fig. 3c). Germination rate, defined by the presence of emerged hypocotyls and green cotyledons, did not show any difference between wild type and transgenic plants (Fig. 3a). However, the root length and leaf size were significantly different at 12 days after sowing. The root length of transgenic lines was significantly shorter and the leaf size of transgenic lines was much smaller than those of wild type (Fig. 3b, d). When the seedlings were transplanted into soil and grown for another 3 weeks, the transgenic plants developed smaller rosettes than wild type (Fig. 3e, f). Epidermal cells were smaller in the leaves of the transgenic plants than those in the leaves of the wild type (Fig. 4a). Numbers of epidermal cells in relative area were identical in the transgenic and wild type plants. Cross-sections of the leaf midvein also demonstrated that cells of transgenic lines were smaller

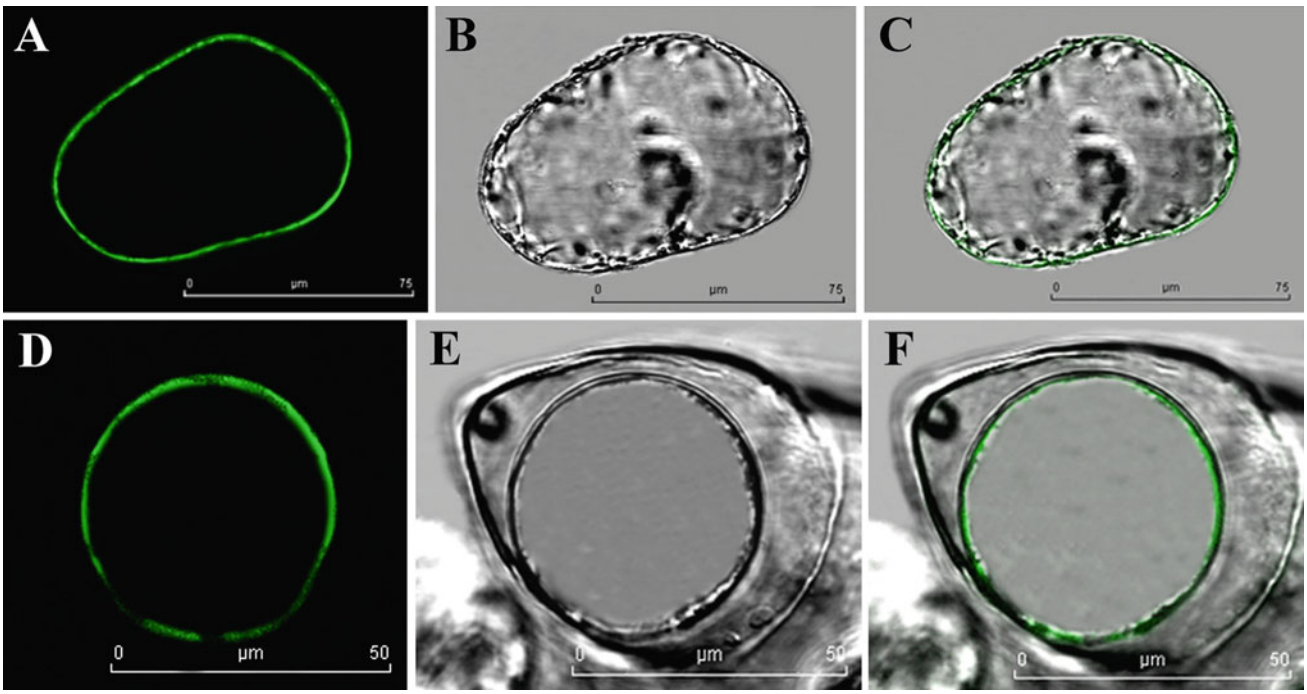

Fig. 2 Subcellular localization assay of GhPRP5 in cotton cells. **a** GFP fluorescence of the cell. **b** The bright field image of the same cell shown in (**a**). **c** Image (**a**) was merged with image (**b**). **d** GFP fluorescence of the cell plasmolyzed with 4 % NaCl for 10 min. **e** The bright field image of the same cell shown in (**d**). **f** Image (**d**) was merged with image (**e**). Scale bars = 50 μm

than those of wild type, whereas the number of cells is similar in leaves of both transgenic plants and wild type (Fig. 4b, c). All of the above results showed that overexpression of *GhPRP5* in *Arabidopsis* hindered cell expansion, but did not influence cell division, leading to the reduced cell size.

In plant cells, alterations in cell wall polysaccharides can regulate cell expansion during development (Cosgrove, 1997). In order to investigate whether overexpression of *GhPRP5* in *Arabidopsis* changes the transcription of genes involved in cell wall biosynthesis, expressions of the genes involved in the synthesis of different cell wall fractions were examined. Semi-quantitative RT-PCR results showed that two genes involved in lignin biosynthesis seems up-regulated, whereas expression of the other genes involved in hemicellulose, pectin and cellulose biosynthesis did not show obvious changes in the transgenic plants (Supplemental Fig. 4). Quantitative RT-PCR further confirmed that *4CL* and *CCoAoMT* transcripts were remarkably enhanced in the transgenic plants (Supplemental Fig. 5). Moreover, the phloroglucinol-HCl staining was used to visualize lignin depositions and distribution in the cell walls. The experimental results revealed that wild type stem sections showed weak staining in both the vessels and interfascicular fibers. In contrast, GhPRP5-overexpression transgenic plants displayed very high levels of lignification in both the xylem vessels and interfascicular fibers (Supplemental Fig. 6).

Suppression of *GhPRP5* expression in cotton enhanced fiber elongation

To validate the functional importance of *GhPRP5* in fiber development, an RNA interference vector was constructed and introduced into cotton for suppression of *GhPRP5* expression. Twelve original (T0) transgenic cotton lines were generated. The transgenic plants were grown to maturation for the collection of the first generation (T1) seeds. The T1 seeds were germinated and positive plants, screened by PCR for the presence of the transgene, grew to maturation for producing second generation (T2) seeds and determining the phenotypes of transgenic fibers. *GhPRP5* expression levels in 5 dpa transgenic fibers from six lines in T1 ranged from 10 to 40 % of that in wild type (Supplemental Fig. 7), and this reduced expression was inherited in the second generation in six lines tested (Fig. 5a). We selected three representative transgenic lines (lines 1, 4, and 8) that exhibited the decreased levels of *GhPRP5* transcripts for further phenotypic analysis. The number and morphology of fiber initials was observed via cross-sections of 0 and 1 dpa ovules. None of the three lines showed obvious differences in fiber initial number and morphology, compared with wild type (data not shown). After fiber maturation in the transgenic progeny plants (T2 and T3 generations), cotton bolls at the similar position and opened at the same time were harvested from transgenic and wild type plants grown alongside each other in field

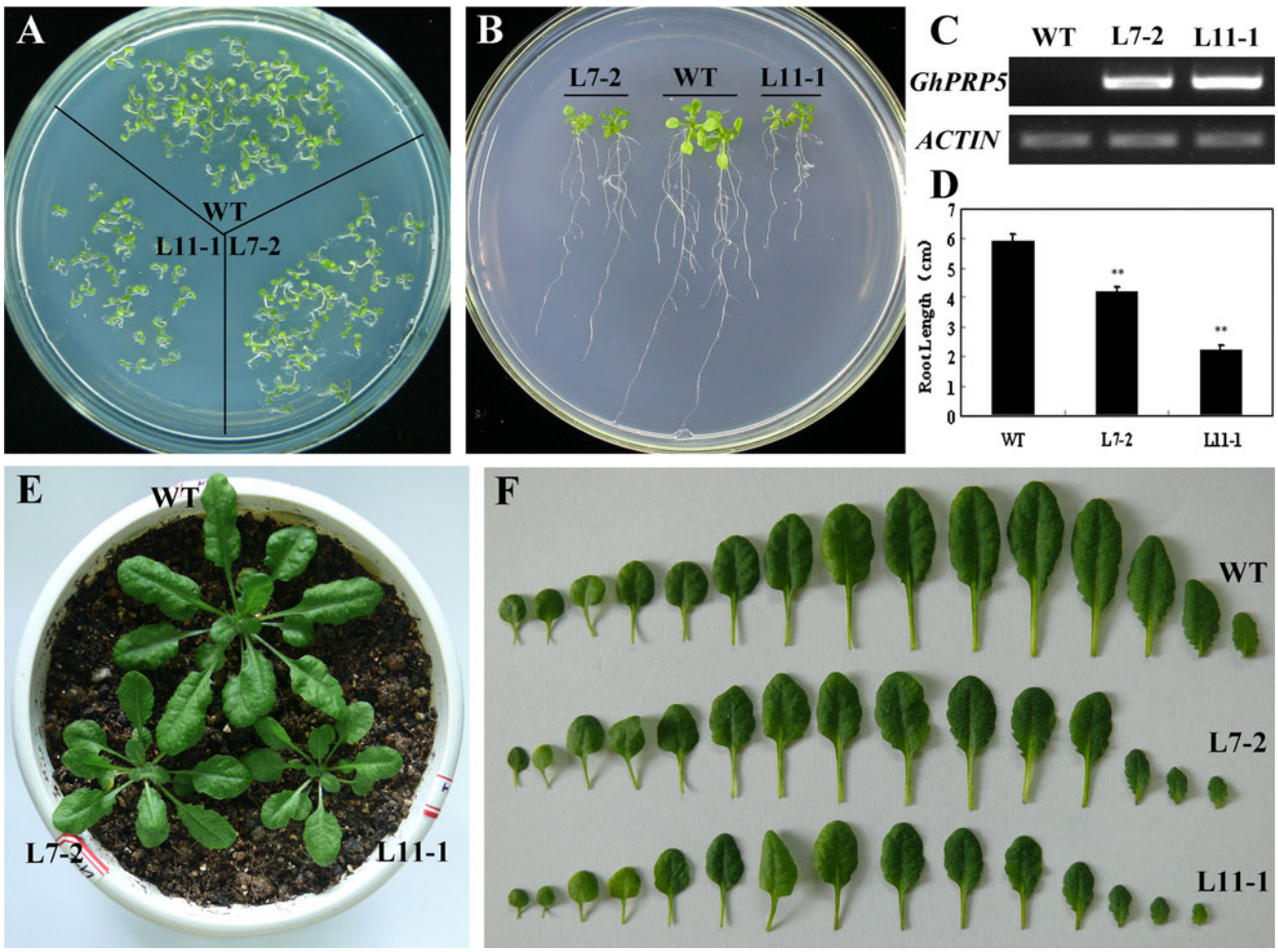

Fig. 3 Comparison of growth between the transgenic Arabidopsis plants overexpressing *GhPRP5* and wild type. **a** 9-Day-old Arabidopsis seedlings. **b** 2-Week-old *Arabidopsis* seedlings. **c** *GhPRP5* transcript levels were measured by RT-PCR, *ACTIN* was used as a control. **d** Values of root length are means of at least 30 plants, error bars represent SD, LSD values were calculated at the probability of 1 % (** $p < 0.01$). There were three replications for each line with 30–50 plants per replication. **e** Growth of soil-grown transgenic plants and the wild type. The seeds were germinated on agar plates for 7 days, then transferred to soil in pot, after 4 weeks, photos were taken. **f** Alignment of rosette leaves of the transgenic plants and wild type shown in (**e**)

simultaneously, the lint fiber length was measured. Experimental results revealed that all transgenic lines examined had longer fibers than those of wild type (controls) (Fig. 5b, c). The fiber length of the transgenic lines is 29.1 ± 1.69 mm, while that of wild type is 27.27 ± 0.87 mm (Fig. 5c). Interestingly, when the lint fibers were torn off, we found that the length of fuzz fibers was also longer in transgenic lines, although this is difficult to measure quantitatively (Fig. 5d). Moreover, when the cotton seeds with fuzz were delinted using concentrated sulphuric acid, we found that the size of naked seed was similar between transgenic lines and wild type (Fig. 5e). All of the transgenic lines examined showed little difference in their gross above-ground vegetative growth and flowering as expected.

Identification of GhPRP5-interacting proteins

To identify proteins that interact with GhPRP5, we performed a yeast two-hybrid assay using GhPRP5 as bait to screen a two-hybrid library of cotton 10 dpa fiber cDNAs constructed on the prey vector (Zhang et al. 2010). Empty vectors containing the activation domain or binding domain were used as negative controls. GhPRP5 showed no self-activation of transcription. Unique proteins were identified as positive clones for both of the reporter genes ADE and LacZ. As shown in Supplemental Table 2, the identified eight proteins were as follows: one is a GCN5-related N-acetyltransferase, one belongs to proline-rich proteins, one is an auxin-responsive family protein and the resting five represent unknown proteins.

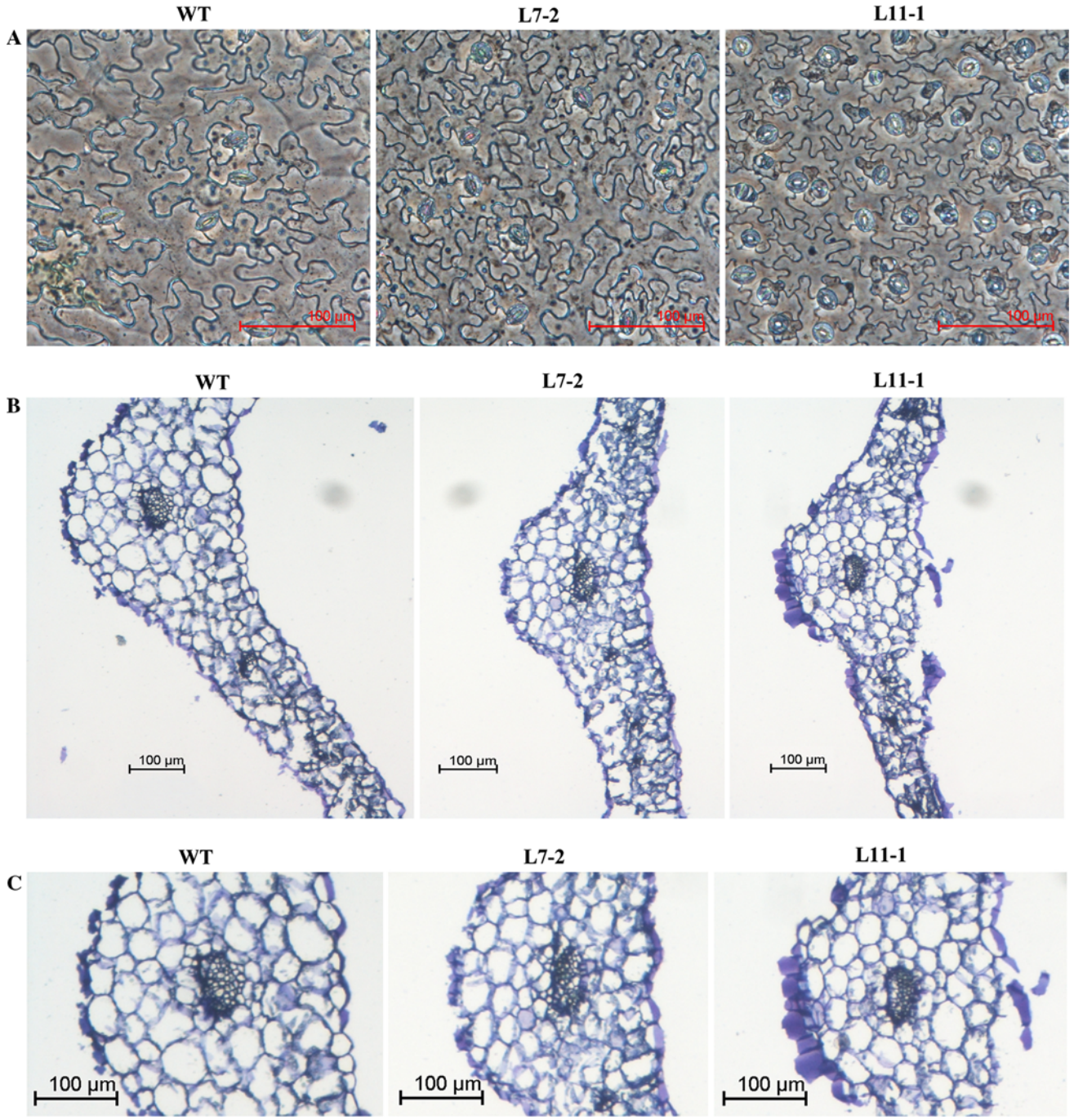

Fig. 4 Comparison of leaf epidermal cells between the transgenic *Arabidopsis* lines and wild type. **a** Comparison of leaf epidermal cells. Bar = 100 μm. **b** Cross section of leaf midvein cells from transgenic plants and the wild type. The sections were observed with toluidine blue staining. **c** Magnified versions of respective cross sections from (**b**)

To investigate the interactions between GhPRP5 and their target proteins, the direct yeast two-hybrid assay was employed. As shown in Fig. 6, three selected proteins (clones no. 1, 2 (the same as GhPRP5) and 3) that we are interested in were checked for the presence of cDNA–AD fusion and confirmed in the one-to-one interaction analysis. Transformants were assayed for growth on QDO nutritional selection medium (Fig. 6a) and confirmed with color change on a β-galactosidase filter paper using the flash freezing filter assay (Fig. 6b). Furthermore, bimolecular fluorescence complementation (BiFC) assay also showed that GhPRP5 could interact with GhPRP5 in vivo (Fig. 6c–h). These results suggested that GhPRP5 may form homodimers to perform its function in cotton fiber cells.

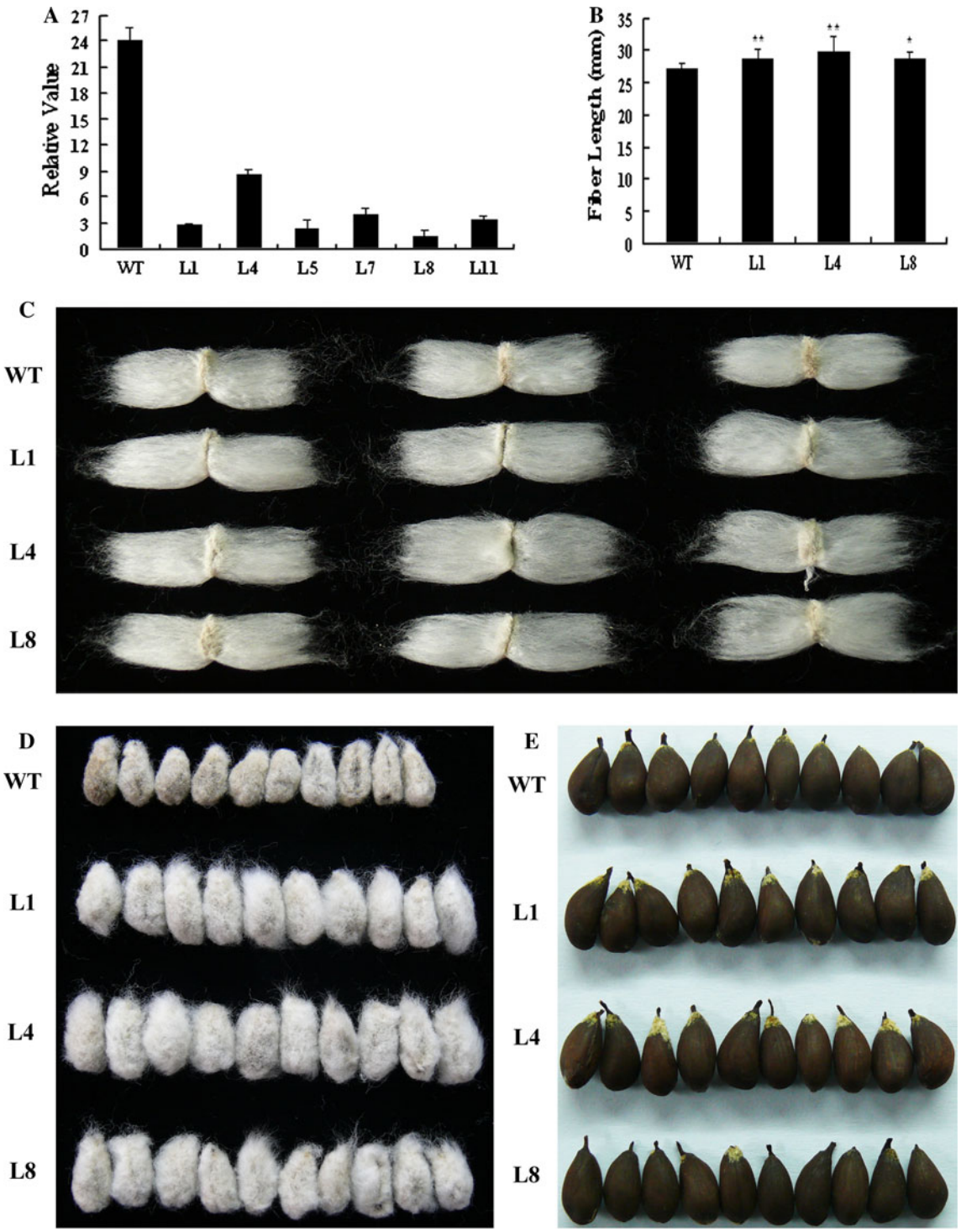

Fig. 5 Comparison of fibers between the transgenic cotton lines and wild type. **a** Quantitative RT-PCR analysis of *GhPRP5* expression in 5 dpa fibers from independent T2 transgenic cotton lines and wild type plants. The cotton *Ubiquitin* was used as an internal control for normalization. The values are mean ± SD of independent triplicate assays. **b** The length of fibers in the transgenic plant is much longer than that in wild type plant. Mature fiber lengths of the T2 cotton transformants and wild type plants. Length of cotton fiber averaged from 50 mature bolls derived from 10 independent plants counted individually. *Error bars* represent SD. LSD values were calculated at the probability of either 5 % (* $p < 0.05$) or 1 % (** $p < 0.01$). **c** Fibers in the transgenic plants were longer than that in the wild type. **d** The length of fuzz fibers was longer in transgenic lines than that in the wild type. **e** The size of naked seed was similar between transgenic lines and wild type

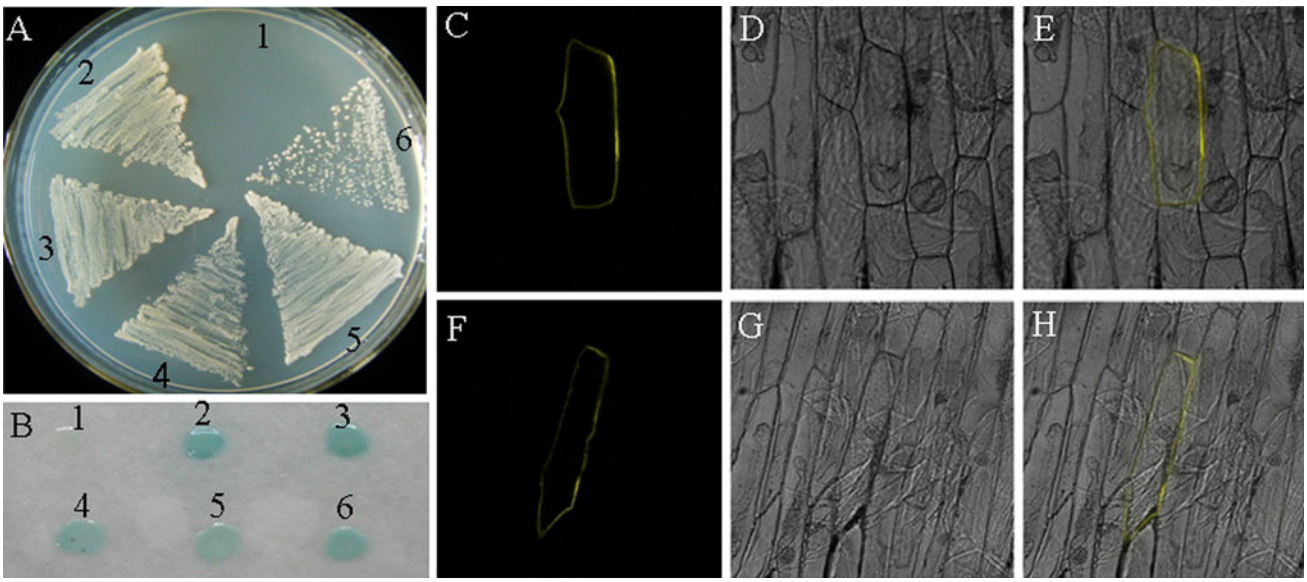

Fig. 6 Interactions between GhPRP5 and its target proteins. **a**, **b** Three interactors were selected to analyze the interaction with GhPRP5 protein by yeast mating. Transformants were assayed for growth on QDO nutritional selection medium (**a**) and confirmed with color change on a β-galactosidase filter paper using the flash freezing filter assay (**b**). **c**–**h** Further confirmation of interactions in vivo by BiFC (bimolecular fluorescence complementation) assay in onion epidermal cells. **c**–**e** BiFC visualization of GhPRP5 interaction with GhPRP5; **f**–**h** BiFC visualization of GhPRP5 interaction with 185 (an auxin-responsive protein). **c**, **f** YFP fluorescent images; **d**, **g** bright field images of images (**c**) and (**f**); **e**, **h** fluorescent images were merged with their bright-field images. *1*. pGADT7-RecT × pGBKT7-Lam (negative control); *2*. pGADT7-RecT × pGBKT7-53 (positive control); *3*. pGADT7-RecT × pGBKT7-53 (positive control); *4*. pGADT7-GhPRP5 × pGBKT7-GhPRP5; *5*. pGADT7-185 × pGBKT7-GhPRP5; *6*. pGADT7-a18 × pGBKT7-GhPRP5

Transcriptional up-regulation or down-regulation of several fiber-related genes in *GhPRP5*-suppressed transgenic cotton

In this study, we analyzed the expression levels of some fiber-development related genes in the transgenic cotton plants (T2 and T3 generations) by quantitative RT-PCR, using gene-specific primers (Supplemental Table 3). The experimental results indicated that *GhACT1* expression was little changed, but *GhTUA9* was up-regulated 20–74 % in transgenic lines, compared with wild type, whereas *GhTUB1* mRNA level was reduced dramatically (by down to 50 %). We further examined the expression of some fiber-related *GhFLA*s in the transgenic lines. The results showed that *GhFLA2* mRNA level in transgenic lines is only about 38–82 % of that in wild type, and *GhFLA14* were markedly decreased (by almost 80 %) in transgenic plants. *GhFLA4* transcript level was increased by up to twofold and *GhFLA15* mRNA level was up-regulated about tenfold in the transgenic plants, compared with that of wild type (Fig. 7).

Liu et al. (2000) proposed that some cotton AGPs (e.g. *GhAGP2* and *GhFLA1*) may interact with the cell wall loosening proteins to initiate fiber elongation. In this study, expression of the gene encoding expansin was analyzed. As shown in Fig. 7, expression level of the expansin gene (*GhEXP*) was higher (with more than twofold increase) in all the transgenic lines than that in wild type, indicating that down-regulation of *GhPRP5* may promote *GhEXP* expression.

The cell wall modifying proteins xyloglucan endo-transglycosylase/hydrolases (XTH) have been reported to be involved in the biosynthesis of the cell wall and cell wall loosening during fiber cell elongation. GhXTH1 is the predominant XTH in elongating fibers (Lee et al. 2010). We analyzed the expression level of *GhXTH1* in transgenic lines and found that the transcript level of this gene increased dramatically by up to threefold (Fig. 7). The above data suggested that suppression of *GhPRP5* may alter the expression levels of some fiber-related genes and consequently promotes fiber development of cotton.

Discussion

GhPRP5 is likely to be an important member of HRGP network and function in determining fiber cell wall structure

Proline-rich proteins were initially identified as wound-responsive gene products in carrot roots. Subsequent studies demonstrated that many genes encoding this kind of proteins were preferentially expressed in specific cell types. In the past decade, however, progress on the roles of PRP

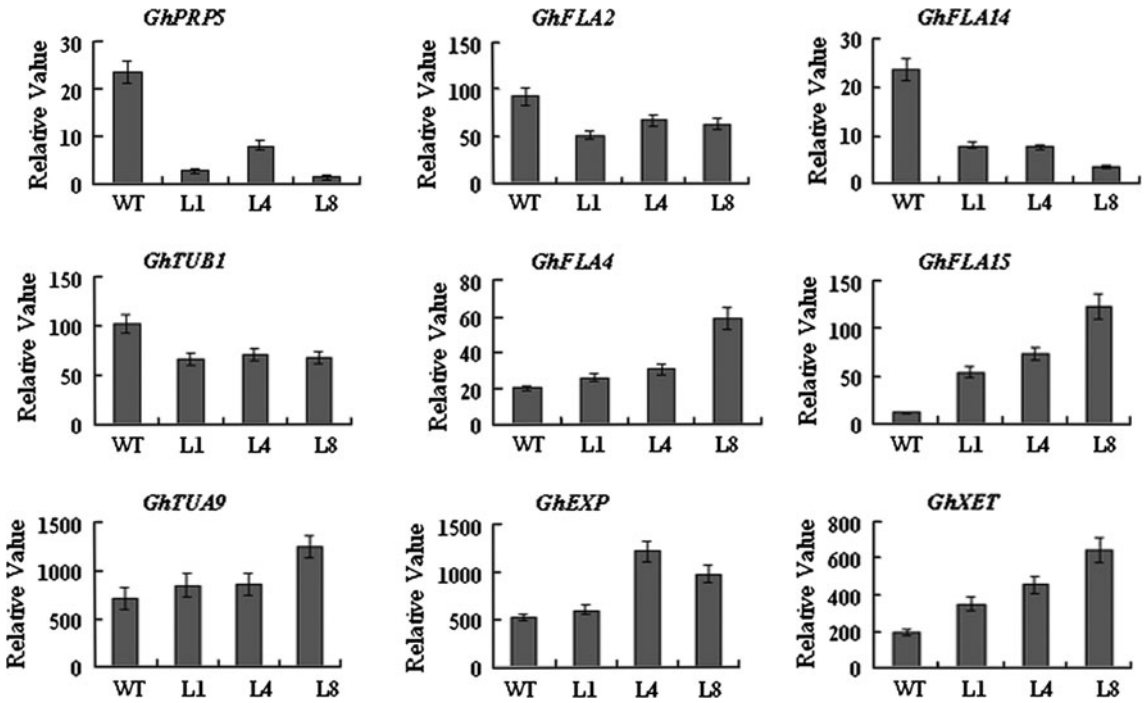

Fig. 7 Quantitative RT-PCR analysis of fiber development-related genes in the transgenic cotton lines and wild type. A cotton ubiquitin gene (*GhUBI1*) was used as an internal control for normalization. The values are mean ± SD of independent triplicate assays

proteins lagged behind those of AGPs and EXTs, and the most of previous studies focused mainly on gene expression. Only recently, by using genetic methods of overexpression, RNAi, and T-DNA mutants, functional insights of several PRPs were provided. A detailed investigation of OsPRP3 showed that its mRNA was mainly present in rice flowers and accumulated abundantly during the late stages of flower development. No significant changes were found in the *OsPRP3*-overexpressed transgenic plants under normal conditions, whereas suppression of *OsPRP3* expression by RNAi led to significant defects in floral organogenesis (Gothandam et al. 2010). Though a number of PRPs have been reported in cotton, none of them were functionally characterized. In this study, our data revealed that the *GhPRP5*-suppressed transgenic cotton exhibited longer fibers than wild type, while transgenic *Arabidopsis* with overexpressing *GhPRP5* grew slowly, resulting in the decreased cell size and smaller plants, compared with the wild type.

Proline rich proteins play an integral role in the extracellular matrix of specific cell types and they form a structure-determining network within the extracellular matrix that adds to the mechanical strength of the wall and assists in proper wall assembly (Varner and Lin 1989). It is also proposed that PRPs are secreted into the wall, where eventually they become insolubilized. The PRPs incorporated into the wall may further lead to the formation of protein/protein or protein/carbohydrate crosslinks within the cell wall and contribute to the stability of the extracellular matrix (Fowler et al. 1999; Bernhardt and Tierney 2000). It has been proposed that OsPRP may crosslink with itself and form a homopolymer network of OsPRP proteins which could also interact with other sugar molecules (Akiyama and Pillai 2003). Showalter et al. (2010) found that many *PRPs* were coexpressed with many *AGPs* and *EXTs* and sets of HRGP genes seem to be co-regulated by a variety of conditions, implying there exists a HRGP gene network. In a more specific screen for genes co-expressed with root-specific or root-enriched *EXTs* (*EXT6*, *EXT7*, *EXT9*, *EXT12*, *EXT13*) in *Arabidopsis*, Velasquez et al. (2011) identified several cell wall-related genes such as *AtPRP1*, *ATEXP7,* and leucine-rich repeat extensin1 (*AtLRX1*). Crosslinking of phenolics and structural proteins, lignins and pectins may contribute to cell wall rigidification. It has been shown that the extent of crosslinking between hydroxyproline-rich proteins may be an important mechanism to limit cell expansion during development (Brownleader et al. 2000). De Cnodder et al. (2005) also suggested that crosslinking of cell wall hydroxyproline-rich proteins is essential in restricting cell elongation. In this study, yeast two-hybrid screen identified GhPRP5 as its interacting partners that provided direct evidence for crosslinking between hydroxyproline-rich proteins and the HRGP network hypothesis. HRGPs crosslinking may be a reason for the reduced cell expansion.

HRGPs may have a capacity to interact or associate with the plasma membrane and to influence the cytoskeleton

(Knox 1995). Predictions of secondary structure using COUDES software (Fuchs and Alix 2005) indicated that the proline-rich repeats of GhPRP5 form β-turn helices which allow the protein backbone to turn back on itself. The subcellular localization also suggested that GhPRP5 might be a wall-membrane linker protein. Moreover, the hydrophilic feature of the central and C-terminal region of the protein implied that GhPRP5 is not locked on the membrane, but suggests an extended structure that may interact with itself and with other cell wall components through ionic interactions. Also, its relatively high tyrosine content makes it a good candidate to use isodityrosine cross-linking for strengthening the cell wall. Our results revealed that expression of genes involved in cell wall synthesis has altered, especially two genes involved in lignin synthesis were up-regulated, and phloroglucinol-HCl staining confirmed that GhPRP5-overexpression *Arabidopsis* plants have very high levels of lignification in both the xylem and interfascicular fibers. The relatively large degree of lignification in the transgenic plants may account for the reduced cell expansion. On the other hand, we found that suppression of *GhPRP5* promoted fiber elongation by coordinated up-regulation or down-regulation of a number of genes (such as tubulins, AGPs, PRPs, XET, and expansin) involved in fiber development. It is possible that the HRGP gene network participates in fiber development by linking cell wall and plasma membrane and even affecting cytoskeleton. Since precise functions of these genes are largely unknown, further investigation is needed to analyze how the cross-linkage of the HRGP network within the wall determines the extracellular matrix structure of fibers and contributes to fiber development.

GhPRP5 promoter can be used for genetically improving cotton fiber quality

With the isolation and characterization of a fiber-specific promoter, we may express target genes in the developing fiber through genetic engineering. Because generating transgenic cotton is time-consuming and labor-intensive, the ease and speed of *Arabidopsis* and tobacco transformation has been utilized to examine the expression patterns of cotton fiber-specific/preferential promoters. Many studies have shown that cotton fibers and *Arabidopsis* or tobacco trichomes may share some common regulatory elements for tissue-specific expression. For example, a cotton gene for lipid transfer protein *Ltp6* was specifically expressed in fiber cells and its promoter directed *GUS* expression only in trichomes of transgenic tobacco (Hsu et al. 1999). *Ltp3* promoter could drive the *GUS* expression in the trichomes, but *GUS* expression was also observed in the leaf epidermis and vascular veins of the transgenic plants (Liu et al. 2000). The promoter of another fiber-specific gene, *FSltp4,* was strongly active in all types of tobacco trichomes (Delaney et al. 2007). Promoter of the fiber-specific *MYB2* gene directs expression specifically to *Arabidopsis* trichomes and tobacco glandular trichomes (Shangguan et al. 2008). More direct evidence comes from cotton. When *FSltp4* promoter:*GUS* or *MYB2* promoter:*GUS* chimeric genes are transferred into cotton, GUS activity was mainly localized in fibers (Delaney et al. 2007; Shangguan et al. 2008). These results strongly suggest that some general parallels may regulate trichome morphogenesis in *Arabidopsis*, tobacco, and cotton. In this study, The *GhPRP5* promoter drove *GUS* expression only in trichomes of transgenic *Arabidopsis* and tobacco. The high specificity of the *GhPRP5* promoter makes it a valuable tool for genetic improvement of fiber, though cis-elements and the related transcription factors conferring fiber expression need further identification.

In summary, our results indicated that GhPRP5 protein as a negative regulator participates in fiber development of cotton. The data presented in this study enable us to further understand the biochemical function of PRP proteins in fiber development, and thereby to improve the fiber quality and yield of cotton by genetic manipulation.

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