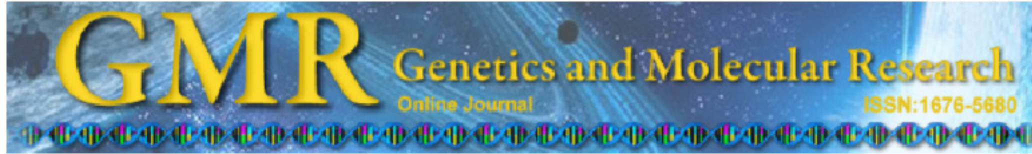

Cloning of a phosphatidylinositol 4-kinase gene based on fiber strength transcriptome QTL mapping in the cotton species *Gossypium barbadense*

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ABSTRACT. Sea Island cotton (*Gossypium barbadense*) is highly valued for its superior fiber qualities, especially fiber strength. Based on a transcript-derived fragment originated from transcriptome QTL mapping, a fiber strength related candidate gene of phosphatidylinositol 4-kinase cDNA, designated as *GbPI4K*, was first cloned, and its expression was characterized in the secondary cell wall thickening stage of *G. barbadense* fibers. The ORF of *GbPI4K* was found to be 1926 bp in length and encoded a predicted protein of 641 amino acid residues. The putative protein contained a clear PI3/4K kinase catalytic domain and fell into the plant type II PI4K cluster in phylogenetic analysis. In this study, the expression of cotton PI4K protein was also induced in *Escherichia coli* BL21 (DE3) as a fused protein. Semi-quantitative RT-PCR analysis showed that the gene expressed in the root, hypocotyl and leaf of the cotton plants. Real-time RT-PCR indicated that this gene in Sea Island cotton fibers expressed 10 days longer than that in Upland cotton fibers, and the main expression difference of *PI4K* between Sea Island cotton and Upland cotton in fibers was located

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in the secondary cell wall thickening stage of the fiber. Further analysis indicated that PI4K is a crucial factor in the ability of Rac proteins to regulate phospholipid signaling pathways.

Key words: cDNA cloning; Fiber strength; *Gossypium barbadense*; Phosphatidylinositol 4-kinase; Real-time PCR

INTRODUCTION

Phosphatidylinositol (PtdIns) constitutes about 10% of total cellular phospholipids in all animal and plant cells, and they can be catalyzed to generate two important second messenger molecules of Ins(1,4,5)P_3 and diacylglycerol in phospholipid signaling pathways. A specific phosphatidylinositol kinase of PI5Ks (PtdIns 5-kinases) (Coburn et al., 2006) and two kinds of PI3/4K-domain-containing proteins, including PI3Ks (PtdIns 3-kinases) (Lingaraj et al., 2008) and PI4Ks (PtdIns 4-kinases) (Carricaburu et al., 2003), play major roles in phosphorylation for three of the five free hydroxyl groups of PtdIns in different combinations.

Recent research has revealed the important roles of phospholipid signaling pathways in multiple development stages in higher plants, including root and pollen growth, vascular development and plant morphogenesis. Evidence in *Arabidopsis thaliana* root (Potikha et al., 1999; Coburn et al., 2006), *Glycine max* cell (Potikha et al., 1999) and *Gossipium hirsutum* fiber (Delmer et al., 1995; Potikha et al., 1999) revealed that phospholipid signaling pathways were regulated by GTPases of RAC/ROP, and their influences were detected in the secondary cell wall thickening process.

In the metabolism pathway of phospholipids, the major precursor of PtdIns(4,5)P_2 is PtdIns4P, which is phosphorylated by PI4Ks from PtdIns, and PI4Ks are considered to be gatekeepers for the production of most phosphoinositides in the phospholipid signaling pathway (Balla and Balla, 2006).

The expression of the two major types of PI4Ks, II and III, has been detected in different plant tissues. The isolation and functional characterization of a full-length plant PI4K cDNA from *A. thaliana* was first reported in 1999 (Xue et al., 1999), but up to the present the majority of information on type II PI4Ks was derived from mammals and yeast, the minority from *A. thaliana*, and little is known about other plants, such as cotton.

It was considered that the biological development characters of cotton fiber had aspects in common with plant root hairs. In *A. thaliana*, PI4Ks were found participating in the polarized secretion of cell wall components in tip-growing root hair cells (Preuss et al., 2006). And the double-mutant of PI4Kβ1/PI4Kβ2 showed unexpected morphogenesis characters in root hair (Preuss et al., 2006). Identification of novel plant specialized development processes involved with PI4Ks are key points to a better understanding of the function of the phospholipid signaling pathway.

It is believed that cotton fiber development of the secondary cell wall thickening stage from 21 days post-anthesis (DPA) imparted fiber strength (Arioli, 2005). In our previous study (Liu et al., 2009), based on QTL mapping with cDNA-AFLP transcriptome makers, *Gossipium barbadense*-originated TDF (transcript-derived fragment) of CCG/TAT-156, targeting a putative PtdIns kinase, was considered as the fiber strength candidate gene for its co-segregating with a major QTL.

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In this study, initiated with the sequence of the TDF of "CCG/TAT-156" that cosegregated with fiber strength QTL in our previous transcriptome study (Liu et al., 2009), the *G. barbadense* gene of PtdIns 4-kinase (*GbPI4K*) was first cloned and characterized for its expression in the fiber development stage of *G. barbadense*. A comparison of the expression profile of *PI4K* in *G. barbadense* (*GbPI4K*) and in *G. hirsutum* (*GhPI4K*) is also presented in this paper. The results provide an insight into the influence of PI4K in the phospholipid signaling pathway on plant cell wall development, and as well as fiber strength.

MATERIAL AND METHODS

Plant materials

Sea Island cotton (*G. barbadense* cv. Pima 90-53) and Upland cotton (*G. hirsutum* cv. CRI 8) were planted in a breeding nursery. Developing stages of cotton bolls were tagged by DPA. Ovules (0 DPA) or fibers (5 DPA and thereafter) were collected from cotton bolls at different developmental stages, frozen in liquid nitrogen, and stored at -80°C before RNA extraction. Roots, hypocotyls and leaves were obtained from cotton plants grown in the field.

Preparation of total RNA and single-strand cDNA

Total RNA was extracted from fibers and other tissues using the PlantRNA reagent (Tiangen, China) and afterwards treated with the RNase Free DNaseI (TaKaRa, China) to avoid genomic DNA contamination, according to the manufacturer protocol, respectively. Quality, purity and concentration of purified RNA were estimated by A260 measurement using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and visualized on 1.0% agarose gel.

The single-strand cDNA, synthesized from total RNA using oligo$(dT)_{18}$ by the PrimeScript™ Reverse Transcriptase (TaKaRa), was used for PCR amplification of *PI4K* open reading frame (ORF), semi-quantitative PCR and real-time quantitative PCR (RT-PCR) analyses.

Isolation and structural characterization of the *GbPI4K* full-length cDNA

Homologous sequences were searched first by the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST) and cotton fiber ESTs of highly homology with the TDF "CCG/TAT-156" were obtained. *In silico* elongation was carried out and a 1840-bp contig sequence was gained. BLASTx then confirmed that the contig sequence targeted a putative PtdIns kinase. RT-PCRs were then performed on the single-strand cDNA synthesized from total RNA of 25 DPA *G. barbadense* "Pima90-53" fibers. A pair of primers, designated as Partial_F and Partial_R (Table 1), was designed and synthesized according to the sequence of the 1840-bp length contig. The PCR amplification was carried out in a volume of 20 µL containing 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 0.20 mM of each dNTP, 0.5 µM of each primer, 50 ng template cDNA and 0.625 U *Pfu* DNA polymerase (MBI Fermentas, Lithuania). The PCR was performed as follows: 94°C for 1 min, then 25 cycles for 45 s at 94°C, 45 s at 60°C, 3 min at 72°C, and finally 72°C for 10 min. The PCR products of the ~1.84 kb expected size

were initially resolved on a 1.0% agarose gel electrophoresis. The purified ~1.84-kb products were inserted into the pGEM-T vector (Tiangen) and five independent clones were completely sequenced from both strands at Sangon (China). Subsequent BLASTx analysis was carried out in order to ensure the reliability and fidelity of the obtained sequence information.

Table 1. Primer oligonucleotide sequences and their applications.

Primer name	Sequence (5'-3')	Description
Partial_F	GTTCAAACTCAGATGGCAGTTG	Partial cDNA cloning primer, forward
Partial_R	ATCCCGGGTAGAGCAACTC	Partial cDNA cloning primer, reverse
3GSP	GAGCAGCTCCCAGGAAATGTGAGCT	3'-RACE-specific primer, outer
3NGSP	TTCCAGGAGTTGCTCTACCCGGGAT	3'-RACE-specific primer, nested
5GSP	CGTTCCGCACGGCACTAAGATCATT	5'-RACE-specific primer, outer
5NGSP	AAAAACCCGTCTCCTCCCAACAGGT	5'-RACE-specific primer, nested
Specific_F	TGAAGCTGGCTGACATGAATG	Specific forward primer
Specific_R	CCTCTGTATCTGCTTCTGACCTA	Specific reverse primer
EF1α_F	GGTGGGATACAACCCTGACA	Forward primer of the internal control
EF1α_R	TTGGGCTCATTGATCTGGTC	Reverse primer of the internal control
ORF_F	GGCGAGCTCATGTCTCGCAAGTTGGA	ORF forward primer
ORF_R	GGCGTCGACCTATCTGGTAACGATAT	ORF reverse primer

RACE = rapid amplification of cDNA end; ORF = open reading frame.

To obtain the full-length sequence of the gene cDNA, rapid amplification of cDNA end (RACE) PCR was performed with a SMART™ RACE cDNA amplification kit (ClonTech, USA) according to the product manual. Two outer-specific primers, designated as 5GSP and 3GSP (Table 1), were designed respectively according to the 3'-end and 5'-end of partial sequences of the *GbPI4K* cDNA obtained. The first-round PCR was performed for five cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 3 min, followed by 25 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 3 min, and finally 72°C for an additional 10 min. Subsequently, using the dilution of the first-step PCR product as templates, the nested PCRs were carried out with nest primers 5NGSP and 3NGSP (Table 1). The nested PCR condition was identical to that of the first-round PCR. The purified amplified fragments were cloned into the pGEM-T vector (Tiangen) and completely sequenced according to methods as mentioned above.

Bioinformatics analysis, sequence alignment and phylogenetic analysis

The DNAstar software was used to assemble a full-length cDNA sequence from 5'-RACE and 3'-RACE overlapping sequences. ORF was analyzed by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequence was searched in the Pfam database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) to find the conserved domains. The molecular weight and isoelectric point (pI) of the encoded protein was predicted by the Expasy tools (http://www.expasy.ch/tools/pi_tool.html). The ProtScale Database (http://expasy.org/tools/protscale.html) was searched for the distribution curve of hydropathy. TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to analyze the trans-membrane region of the protein. The neural network (NN) and hidden Markov model (HMM) analysis were carried out online with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/). Homologous proteins were searched by the BLASTp program in the NCBI database. The multiple sequence alignments with homologous proteins were carried out using the DNAMAN program. The

MEGA 4.0 software was used to perform phylogenetic analysis and construction of a neighbor-joining tree by using the bootstrap method with 1000 bootstrap iterations (Felsenstein, 1985).

Gene expression profile analysis

Organ-specific expression analyses were conducted by semi-quantitative RT-PCR. A set of gene-specific primers (designated as Specific_F and Specific_R) (Table 1) was used for PCRs under the following cycling conditions: 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, with a final extension step at 72°C for 5 min. The cotton *EF1α* gene, used as an internal control for constitutive expression and with the specific primer-pair (designated as EF1α_F and EF1α_R) (Table 1), was amplified (the same cycling conditions as above for 25 cycles) from the same cDNA samples.

Real-time quantitative RT-PCR was employed to reveal the relative changes in the expression of *PI4K* concurrently with cotton fiber development stages. The LightCycler 1.5 Machine (Roche Diagnostics Corporation, Switzerland) was used and PCR products were detected by SYBR Green I fluorescence dye (TaKaRa). A 20-μL reaction mixture contained 10 μL 2X SYBR Premix Ex Taq™, 0.5 μL each of 10 μM Specific_F and Specific_R as primers and a suitable amount of cDNA. The amplification program consisted of 1 cycle of 95°C for 30 s for pre-incubation, followed by 45 cycles of 95°C for 5 s, 56°C for 10 s and 72°C for 10 s. Subsequently, a melting curve analysis was run using the program for one cycle at 95°C for 0 s, 65°C for 15 s and 95°C with 0 s holding in the continuous acquisition mode, followed by a final cooling step at 40°C for 10 s. To normalize the differences in RNA template concentrations in each reaction, a cotton *EF1α* gene was co-amplified as the internal control. A negative control without cDNA template was run with each analysis to evaluate the amplification specificity and three replicates for each sample. Expression values of 0 DPA were used as a calibrator in all the real-time quantitative RT-PCR analyses. All the products generated from the primers in gene expression profile analysis were cloned, sequenced and analyzed to ensure their authenticity and reliability.

Expression of the recombinant protein

The *GbPI4K* ORF fragment was amplified with the primers ORF_F and ORF_R (Table 1), and purified products were cloned into the prokaryotic expression vector of pET-32a(+) (Novagen, Germany), through the restriction sites (*Sac*I and *Sal*I) introduced by PCR. Induced by 1 mM IPTG and 2% glucose for 4.5 h at 28°C, the expression of the recombinant protein in *Escherichia coli* BL21 (DE3) (Tiangen) was analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue R-250.

RESULTS

Isolation and characterization of the *GbPI4K* full-length cDNA

Initiated with the sequence of the TDF of "CCG/TAT-156", and combined with *in silico* elongation and RACE technique, a full-length cDNA of a 2817-bp sequence from *G. barbadense* "Pima90-53" fibers was obtained. The 1926 bp predicted ORF was defined by an

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initiation codon ATG and an in-frame stop codon TGA. The ORF was flanked by a 5'-untranslated region (5'-UTR) and a 3'-untranslated region (3'-UTR) of 546 and 345 bp, respectively. The 3'-UTR contained a potential poly(A) signal, AATAA (2756-2760 nucleotides), followed by an evident poly(A) tail. Nucleotide sequence data were available in the NCBI database with the accession No. HM008674. From the deduced ORF, it was predicted that the cDNA encoded 641-amino acid residues with a predicted molecular mass of 72.36 kDa and a theoretical estimated pI of 5.72, starting from the first methionine according to universal codon usage. The full-sequence of *GbPI4K* cDNA and the deduced amino acid sequences of their products are shown in Figure 1.

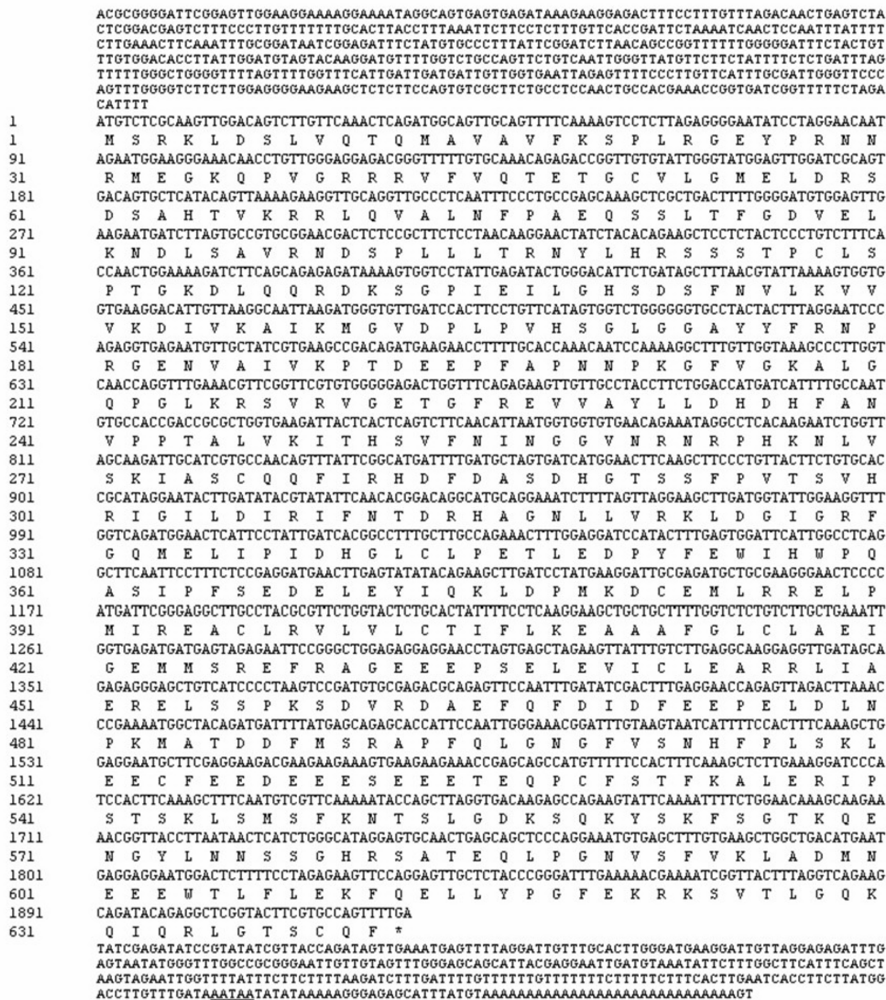

Figure 1. Nucleotide sequence and deduced amino acid sequence of the *GbPI4K*. The predicted amino acid is represented by the one letter code designation below the nucleotide sequence. The potential poly(A) signal is indicated by underlining in the last line.

BLASTp analysis of this deduced protein revealed an approximate 79% grade of identity with *Ricinus communis* (EEF47688) and *Populus trichocarpa* (EEF02172) and 70% with *A. thaliana*'*s* (AAL06989) corresponding gene product. Conserved domains of the deduced amino acid sequence of GbPI4K were searched on the Pfam database, and we found that this protein contained a specific PI3/4K kinase catalytic domain (170-427 amino acids) of PtdIns 3-kinase (PI3K)-like family with a bit score of 52.88 and E-value of 1e-37.

The distribution curve of hydropathy, found through a search in the ProtScale Database, suggested that GbPI4K was a hydrophilic protein. The trans-membrane region of GbPI4K amino acid sequences was analyzed by TMHMM 2.0 and the result did not predict a possible N-term signal sequence, which was consistent with the online prediction results of the NN and HMM analysis.

Sequence alignment and phylogenetic analysis

In the recent phylogenetic analysis by Galvão et al. (2008), representative PI3/4K domain-containing proteins identified in Humans (*Homo sapiens*), Yeast (*Saccharomyces cerevisiae*) and *A. thaliana* in the Pfam database (PF004540) were grouped into several clusters including PIKKs, Type II PI4Ks, PI3Ks, VPS-like PI3Ks, and Type III PI4Ks. Here we reconstructed a neighbor-joining phylogenetic tree involving 33 different PI3/4K domain-containing proteins (most of the protein sequences are identical to those cited by Galvão et al., 2008), and we found that GbPI4K had a closer relationship to AT2G03890, AT1G13640 and AT1G26270 from *A. thaliana* within the same cluster as Type II PI4Ks according to the output tree (Figure 2).

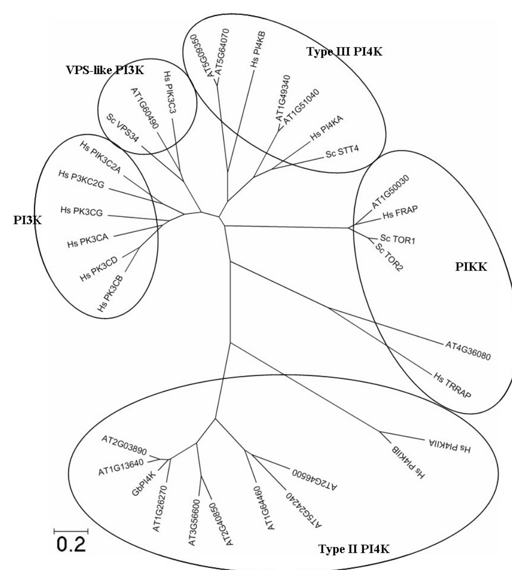

Figure 2. Neighbor-joining tree of PI3/4K kinase domain-containing proteins. The sequence accessions for the identified protein from Humans (Hs) and Yeast (Sc) were as follows: O00329 (Hs PK3CD), P42338 (Hs PK3CB), P42336 (Hs PK3CA), P48736 (Hs PK3CG), O75747 (Hs PK3C2G), O00443 (Hs PIK3C2A), Q8NEB9 (Hs PIK3C3), Q9UBF8 (Hs PI4KB), P42356 (Hs PI4KA), P42345 (Hs FRAP), Q9Y4A5 (Hs TRRAP), Q9BTU6 (Hs PI4KIIA), Q8TCG2 (Hs PI4KIIB), P22543 (Sc VPS34), P37297 (Sc STT4), P35169 (Sc TOR1), and P32600 (Sc TOR2). The sequence accession of *GbPI4K* is HM008674.

In comparison with eight *A. thaliana* PI4K proteins (AtPI4Ks) by the DNAMAN program, the deduced amino acid sequence for the ORF of the GbPI4K cDNA showed a high degree of similarity with previously isolated AtPI4Ks. A clear pattern of high homology of PI3/4K kinase catalytic domain (PI3/4K domain) was demonstrated via sequence alignment (Figure 3).

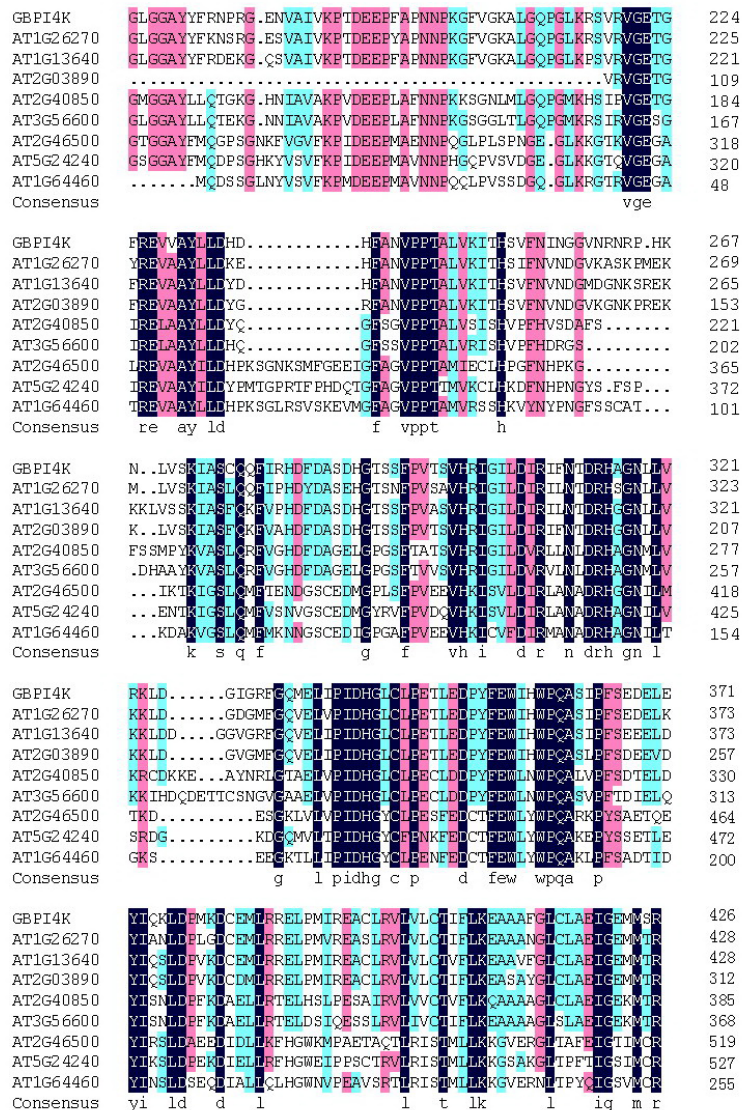

Figure 3. Alignment of the deduced amino acid sequences of PI3/4K domain (170-427 amino acids) of GbPI4K with type II AtPI4Ks of *Arabidopsis thaliana*. Numbers on the right indicate the amino-acid position of different sequences. Identical amino acids are shaded in colors. The PI3/4K domain sequence according to the Pfam (PF004540) database is indicated.

Organ-specific expression of *PI4K*

The expression pattern of *GbPI4K* in the various cotton organs was analyzed by semi-quantitative RT-PCR with *PI4K*-specific primers (Table 1). The housekeeping gene of *EF1α* was used as an internal control in the experiment. As shown in Figure 4, transcripts of *PI4K* were observed in the entire cotton root, hypocotyl and leaf tissues examined. The expression level of *PI4K* in the three types of tissue showed no apparent differences between the two *Gossypium* species, while in the root of cotton, *PI4K* expression level was a little lower than that in hypocotyl and leaf tissues.

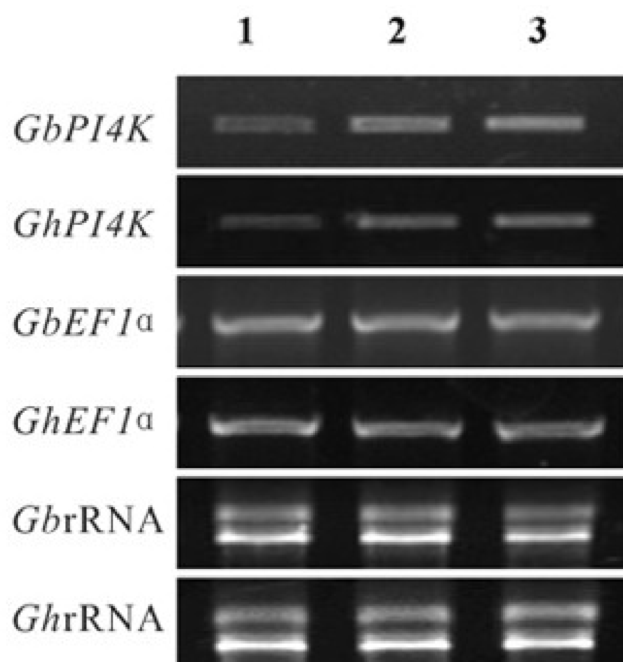

Figure 4. Semi-quantitative RT-PCR analysis of *PI4K* expression in cotton root (*lane 1*), hypocotyl (*lane 2*) and leaf (*lane 3*). *EF1α* was used as the internal control for RT-PCR. Genes in *Gossipium barbadense* and *G. hirsutum* are designed as *Gb* and *Gh*, respectively.

Relative expression analysis of *PI4K* in fibers

To further investigate the expression regulations of the *PI4K* associated with the developmental stage of cotton fibers at the transcriptional level, the gene expression patterns were analyzed by real-time quantitative SYBR-Green RT-PCR using gene-specific primers as described in Table 1. There was a five-day interval between two adjacent determinations in the *PI4K* expression activity trial.

As shown in Figure 5, the *GbPI4K* and *GhPI4K* genes were developmentally regulated in fibers and they shared similar expression pattern as "from high to low". They expressed steadily in the fibers before 20 DPA with little undulation except that *GbPI4K* tran-

scription activity fell into a relative low ebb at 10 DPA. They both reached a high level and subsequently down-regulated. *GbPI4K* reached its highest transcript activity at 25 DPA and retained its slight but distinct expression level at 35 DPA, while *GhPI4K* exhibited a peaking at 15 DPA and nearly vanished at 25 DPA. These data indicated that *GbPI4K* expressed about 10 days longer than *GhPI4K* did in their own fibers, and the main expression difference between *GbPI4K* and *GhPI4K* is located in the secondary cell wall thickening stage of the fiber. The result also provided a fundamental reason for the expression difference in 24-25 DPA fibers between *G. barbadense* and *G. hirsutum* in our former transcriptome analysis via cDNA-AFLP (Liu et al., 2009).

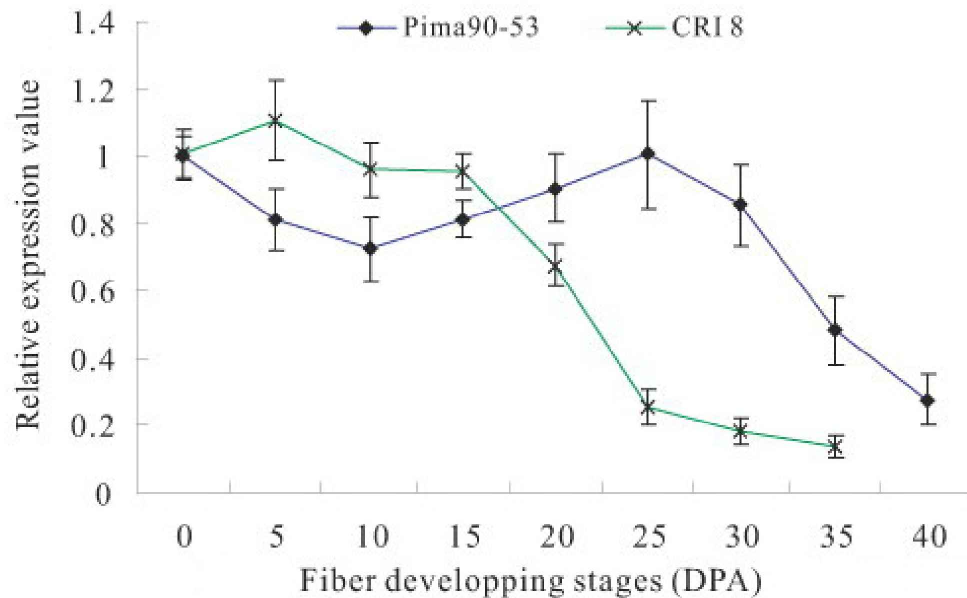

Figure 5. Relative expression analysis of *PI4K* mRNAs in developing fibers by real-time quantitative PCR. *EF1α* was used as the internal control for RT-PCR. Days post-anthesis (DPA) 0, 5, 10, 15, 20, 25, 30, 35, and 40 show the cotton fiber developing stages.

Prokaryotic expression of the recombinant GbPI4K protein

The coding region of *GbPI4K* was subcloned into the prokaryotic expression vector pET-32a(+) and the recombinant pET-GbPI4K plasmid, fused with a His-tag, was used for protein over-expression in the *E. coli* strain BL21 (DE3). To determine the optimal condition for expressing abundant soluble protein, a ladder of temperature, IPTG concentration and induction time were tested for induction (data not shown). Optimal expression was obtained 4.5 h after induction with 1.0 mM IPTG at 28°C. On SDS-PAGE, the subunit molecular mass of recombinant protein was found to be ~92.0 kDa, along with the vector pET32a(+) Tag fusion proteins (20.4 kDa) (Figure 6). This result was consistent with the theoretical molecular mass of 72.36 kDa, based on the deduced 641-amino acid residues.

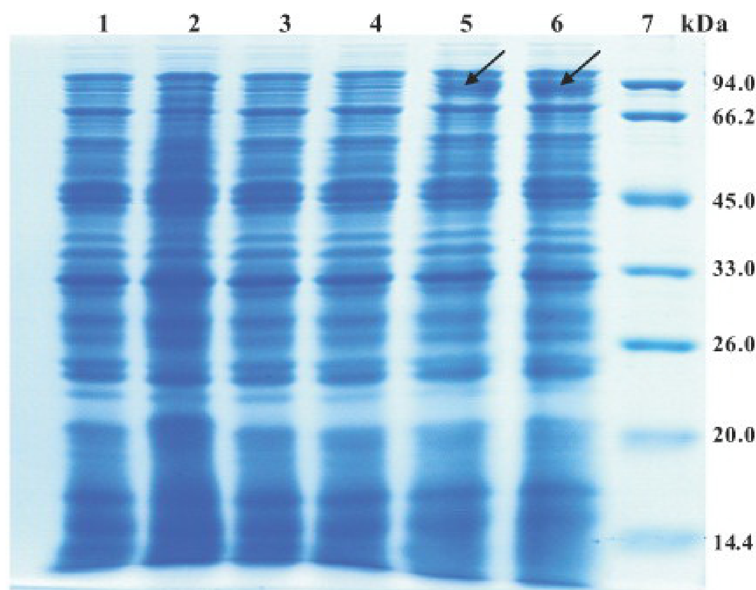

Figure 6. Expression of the recombinant GbPI4K in *Escherichia coli* on SDS-PAGE. *Lane 1* = Induced control *E. coli* cell containing no plasmid; *lane 2* = induced control *E. coli* cell containing pET-32a(+); *lanes 3* and *4* = non-induced control *E. coli* cell containing recombinant pET-GbPI4K; *lanes 5* and *6* = 4.5 h induced control *E. coli* cell containing recombinant pET-GbPI4K. The black arrows indicate the position of the GbPI4K recombinant protein. Total cellular extracts from *E. coli* cell were separated by 10% SDS-PAGE and stained with Coomassie brilliant blue. The *E. coli* strain is *E. coli* BL21 (DE3).

DISCUSSION

Initiated with the sequence of the TDF derived from transcriptome QTL mapping (Liu et al., 2009), the cotton fiber strength-related candidate gene, *GbPI4K*, was first cloned, and its expression was characterized in the secondary cell wall thickening stage of *G. barbadense* fibers. The ORF of *GbPI4K* was 1926 bp in length and encoded a polypeptide with 641 amino acid residues theoretically. The putative protein contained a typical PI3/4K kinase catalytic domain and had a closer relationship with Type II PI4Ks. Semi-quantitative RT-PCR analysis found that the gene expressed in cotton root, hypocotyl and leaf tissues. Real-time RT-PCR indicated *GbPI4K* expressed 10 days longer in Sea Island cotton fibers than *GhPI4K* in Upland cotton fibers. In this study, cotton *GbPI4K* was also successfully induced to express a fused protein in *E. coli*.

Sea Island cotton has higher fiber quality exceeding Upland cotton, especially fiber strength. As a result, Sea Island cotton was widely used in mining fiber strength candidate genes. In this study, real-time RT-PCR analyses in fibers presented the pattern of PI4K associated with the developmental stage of cotton fibers at the transcriptional level, from which we know that *GbPI4K* reached its highest level at 25 DPA while *GhPI4K* activity nearly disappeared. In fibers, expression lasted about 10 days longer in *GbPI4K* than in *GhPI4K*, and the main expression difference between *GbPI4K* and *GhPI4K* was located in the secondary cell

wall thickening stage of the fiber. This result indicates a reasonable association between the higher fiber strength and the longer period of PI4K expression duration in Sea Island cotton.

As mentioned in the introduction of this paper, the plant secondary cell wall thickening process was influenced by Rac13 (a small GTP-binding protein) (Delmer et al., 1995; Potikha et al., 1999; Coburn et al., 2006), while the expression level of Rac13 was found to decline during the stage of maximal secondary wall cellulose synthesis (about 24-28 DPA) (Delmer et al., 1995), which coincided with *GbPI4K*'*s* declining stage in fibers. As Rac proteins are considered to regulate the phospholipid signaling pathway-related enzymes (Oude Weernink et al., 2007), it follows that the gatekeeper of PI4K is a crucial factor in the ability of Rac-proteins to regulate phospholipid signaling pathways.

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