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Interactome analysis of the six cotton 14-3-3s that are preferentially expressed in fibres and involved in cell elongation

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Abstract

Proteins of the 14-3-3 family regulate a divergent set of signalling pathways in all eukaryotic organisms. In this study, several cDNAs encoding 14-3-3 proteins were isolated from a cotton fibre cDNA library. The *Gh14-3-3* genes share high sequence homology at the nucleotide level in the coding region and at the amino acid level. Real-time quantitative RT-PCR analysis indicated that the expression of these *Gh14-3-3* genes is developmentally regulated in fibres, and reached their peak at the stage of rapid cell elongation of fibre development. Furthermore, over-expression of *Gh14-3-3a*, *Gh14-3-3e*, and *Gh14-3-3L* in fission yeast promoted atypical longitudinal growth of the host cells. Yeast two-hybrid analysis revealed that the interaction between cotton 14-3-3 proteins is isoform selective. Through yeast two-hybrid screening, 38 novel interaction partners of the six 14-3-3 proteins (Gh14-3-3a, Gh14-3-3e, Gh14-3-3f, Gh14-3-3g, Gh14-3-3h, and Gh14-3-3L), which are involved in plant development, metabolism, signalling transduction, and other cellular processes, were identified in cotton fibres. Taking these data together, it is proposed that the Gh14-3-3 proteins may participate in regulation of fibre cell elongation. Thus, the results of this study provide novel insights into the 14-3-3 signalling related to fibre development of cotton.

Key words: 14-3-3, cotton (*Gossypium hirsutum*), fibre development, gene expression, protein–protein interaction.

Introduction

Proteins of the 14-3-3 family were originally identified by Moore and Perez (Moore and Perez, 1967). The name 14-3-3 was given to the proteins in brain tissue of mammals on the basis of their migration pattern on starch gel electrophoresis. 14-3-3 proteins are small (27–32 kDa), acidic regulatory proteins that are highly conserved in all eukaryotes (Chaudhri *et al.*, 2003). Highly functional conservation of 14-3-3 proteins was demonstrated by the successful functional complementation of four *Arabidopsis* homologues in yeast mutants (van Heusden *et al.*, 1996). 14-3-3 isoforms are usually made up of homodimers and heterodimers that form a clamp-shaped structure to regulate their target proteins in cells (Yaffe *et al.*, 1997). They participate in various signal transduction and regulatory processes by interacting with their targets in a sequence-specific and phosphorylation-dependent manner. A 14-3-3 dimer can interact with one protein at two different sites or interact with two target proteins at the same time by binding to phosphoserine/phosphothreonine motifs of the type RSXpSXP and RXY/FXpSXP (Braselmann and McCormick, 1995; Muslin *et al.*, 1996). Through these binding reactions, 14-3-3 proteins regulate their target proteins by altering their localization, stability, phosphorylation state, activity, and molecular interactions (Bachman *et al.*, 1996; Muslin and Xing, 2000).

Crystal structure data indicated that 14-3-3 proteins bind to multiple phosphoserine-containing targets through a conserved amphipathic groove (Liu *et al.*, 1995; Muslin *et al.*, 1996). The structural conservation of 14-3-3 isoforms does

not extend to the N- and C-terminal regions, although their sequences are highly conserved (Liu *et al.*, 1995; Petosa *et al.*, 1998). A critical residue Lys49 (K49), which appears to be part of a binding interface with general importance for ligand binding, is identified in the amphipathic groove of mammalian 14-3-3ζ. The K49E mutation disrupts the interaction between 14-3-3ζ and Raf-1 kinase (Zhang *et al.*, 1997). Previous studies indicated that C-terminal differences affected the interactions between 14-3-3s and their targets, and may be related to the isoform-specific function of 14-3-3 proteins (Shen *et al.*, 2003; Visconti *et al.*, 2008). Removal of the last 22 amino acid residues of GF14-6 increases binding to H$^+$-ATPase and stimulation of its activity (Visconti *et al.*, 2008). Another divergent region, the N-terminal region, is responsible for the dimerization of 14-3-3 proteins. An earlier study revealed that dimerization occurs through two N-terminal fragments, but not an N-terminus–C-terminus interaction of two 14-3-3 proteins (Jones *et al.*, 1995).

In the years since their discovery, 14-3-3 proteins have risen to occupy a very important position in cell biology. It was demonstrated that 14-3-3 proteins play crucial roles in the control of many cellular functions (such as metabolism, signal transduction, cell cycle control, apoptosis, protein trafficking, transcription, stress responses, and malignant tumour transformation) (van Hemert *et al.*, 2001). Likewise, plant 14-3-3 proteins participate in various cellular processes through binding to a wide range of transcription factors and signalling proteins. Previous studies revealed that 14-3-3 proteins are involved in the response to biotic and abiotic stress by directly interacting with signalling proteins (reviewed by Roberts *et al.*, 2002). GF14c acts as a negative regulator of flowering in rice by interacting with Hd3a (Purwestri *et al.*, 2009). Barley 14-3-3 proteins specifically interact with the catalytic A-subunit of V-ATPase, and are involved in both blue light perception and the subsequent activation of P-type ATPase (Klychnikov *et al.*, 2007). A previous study on the interaction between H$^+$-ATPase and 14-3-3 protein revealed that the binding of 14-3-3 protein to the autoinhibitory domain released the autoinhibitory action of H$^+$-ATPase (Maudoux *et al.*, 2000). The activation of H$^+$-ATPase by the binding of 14-3-3 proteins in tobacco cells is negatively controlled by phosphorylation of two residues in the H$^+$-ATPase 14-3-3 protein-binding site (Duby *et al.*, 2009). Through interacting with AREB/ABF/ABI5 proteins which act as master signal mediators in the abscisic acid (ABA) signal transduction pathway, 14-3-3 proteins play important roles in controlling ABA signalling during barley seed germination (Schoonheim *et al.*, 2007a). Tobacco 14-3-3 proteins negatively modulated RSG by sequestering it in the cytoplasm to respond to GA (gibberellic acid) signalling. In contrast, the mutant of RSG could not bind to 14-3-3 proteins and, consequently, it exhibited a higher activity than did wild-type RSG (Iqarashi *et al.*, 2001; Ishida *et al.*, 2004). It was believed that 14-3-3 proteins regulate the intracellular localization of the target proteins in plant cells. Previous studies revealed that 14-3-3 proteins mediate brassinosteroid (BR) signal transduction by regulating the nuclear export of a transcriptional factor

BZR1 in *Arabidopsis* (Gampala *et al.*, 2007; Ryu *et al.*, 2007). Similarly, rice 14-3-3 proteins directly regulate OsBZR1 function in BR signalling by reducing its nuclear localization. Mutation of a 14-3-3-binding site in the OsBZR1 protein abolished its interaction with 14-3-3 proteins, resulting in an increased nuclear distribution of the protein (Bai *et al.*, 2007).

Cotton (*Gossypium hirsutum*) is a widely grown textile crop which is cultivated mainly for its fibres. Cotton fibres, which are seed hairs, are single-cell trichomes that arise from the epidermal layer of the seed coat. Approximately 30% of the ovule epidermal cells elongate and develop into single-cell fibres at anthesis. Fibre development is a highly complicated and programmed process that can be divided into four distinct but overlapping phases: initiation [from 2 d before anthesis to 5 d post-anthesis (DPA)], cell elongation (1–20 DPA), secondary wall synthesis (16–40 DPA), and maturation (40–50 DPA) (Basra and Malik, 1984), associated with many functional proteins that are involved in signal transduction and transcriptional regulation. In recent years, many genes that are expressed in fibres have been identified in cotton. Up to now, however, the roles of only a few genes in fibre development have been investigated in detail. It was reported that the genes related to the actin and tubulin cytoskeletons are essential for fibre elongation (Li *et al.*, 2005, 2007). The cotton sucrose synthase gene (*Sus*) plays an important role in fibre initiation and elongation. Delayed or insufficient *Sus* expression leads to delayed initiation and distinctly reduced fibre elongation (Ruan *et al.*, 2005). *GhMYB109*, identified as an important transcription factor in cotton, participates in fibre development. Suppression of its expression results in a substantial reduction in fibre length (Pu *et al.*, 2008). Although the 14-3-3 proteins participating in various signalling pathways have been well characterized in a few plants (such as *Arabidopsis*, rice, and barley), little is known so far about how the cotton 14-3-3 proteins regulate fibre development through interacting with target proteins. Our previous study reported that *Gh14-3-3L* is predominantly expressed in cotton fibre (Shi *et al.*, 2007), but its role, especially how it interacts with other proteins, in fibre development still remains unclear. Here, isolation and characterization of six cotton 14-3-3s and their possible target proteins during fibre development are reported.

Materials and methods

Collection of plant materials

Cotton (*G. hirsutum* cv. Xuzhou142 and Coker312) seeds were surface sterilized with 70% (v/v) ethanol for 60 s and 10% (v/v) H$_2$O$_2$ for 1–2 h, followed by washing with sterile water. The sterilized seeds were germinated on half-strength Murashige and Skoog (MS) medium (pH 5.8) containing 0.8% agar under a 16 h light/8 h dark cycle at 28 °C for 5–6 d. Roots, cotyledons, and hypocotyls were collected from a portion of sterile seedlings. The remaining seedlings were transplanted into soil for further growth to maturation in the field, and the other tissues (such as leaves, stems, petals, anthers, ovules, and fibres) were derived from these cotton plants for RNA isolation.

Isolation of 14-3-3 *cDNAs and their corresponding genes*

To identify the genes that are functionally expressed in cotton fibres, >10 000 cDNA clones were randomly selected from a cotton fibre cDNA library constructed by Li *et al.* (2002) for sequencing. Some cotton *14-3-3* cDNAs with partial or full-length sequences were identified from these clones. Subsequently, a 400 bp fragment of each *Gh14-3-3* partial sequence (including the partial coding region and 3'-untranslated region) was labelled with [α-^{32}P]dCTP and used as probe to screen the cotton fibre cDNA library according to standard procedures. A total of 2×10^5 cDNA clones were screened, and >50 clones were identified. Among them, the full-length clones were sequenced and analysed. In total, six unique clones with complete *Gh14-3-3* cDNA sequences were obtained.

The corresponding *Gh14-3-3* genes were amplified from the genomic DNA of cotton by PCR, using *Pfu* DNA polymerase and gene-specific primers that were designed according to the sequence of each *Gh14-3-3* cDNA (Supplementary Table S1 available at *JXB* online). In total, four *Gh14-3-3* genes were obtained.

RNA isolation and real-time quantitative RT-PCR analysis

Total RNA was isolated from roots, hypocotyls, cotyledons, leaves, petals, anthers, 10 DPA ovules, and developing fibres (3–20 DPA) of cotton by the method described previously (Li *et al.*, 2002). A 2–4 g aliquot of each cotton tissue was randomly collected from 3–10 plants (see 'Collection of plant materials') for RNA isolation. The concentration and purity of total RNA were identified by NanoDrop spectrophotometry and agarose gel electrophoresis. RNA samples were stored at – 80 °C until use.

Expression profiling of the *Gh14-3-3* genes in different cotton tissues (such as roots, hypocotyls, cotyledons, leaves, petals, anthers, 10 DPA ovules, and 10 DPA fibres) and during different stages of fibre development (including 3, 5, 6, 9, 10, 12, 15, 18, and 20 DPA fibres) was carried out by real-time quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green in the detection system (Option 2, MJ Research, Waltham, MA, USA). A cotton polyubiquitin gene (*GhUBI1*, GenBank accession no. EU604080) was used as a standard control in the RT-PCRs. A two-step RT-PCR procedure was performed in all experiments using a previously described method (Li *et al.*, 2005). In brief, total RNA was reverse transcribed into cDNA and used as a template in real-time PCRs with gene-specific primers (Table 1). The real-time PCR was performed using SYBR-Green Real-time PCR Master Mix according to the manufacturer's instructions (Toyoba Co. Ltd, Osaka, Japan). The Ct (cycle threshold), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure of the starting copy number of the target gene. The relative quantity of the target *Gh14-3-3* expression level was determined using the comparative Ct method. The relative value for the expression level of each *Gh14-3-3* gene was calculated by the equation $Y = 10^{\Delta Ct/3.75} \times 100\%$ (ΔCt is the differences in Ct between the control *GhUBI1* products and the target *Gh14-3-3* products, i.e. $\Delta Ct = Ct_{GhUBI1} - Ct_{Gh14-3-3}$, and 3.75 is a parameter for the cycle number which represents a 10-fold expression difference between the target gene and control gene). To achieve optimal amplification, PCR conditions for every primer combination were optimized for annealing temperature and Mg^{2+} concentration. PCR products were confirmed on an agarose gel. The efficiency of each primer pair was detected by using *Gh14-3-3* cDNA as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

DNA sequencing and protein analysis

The sequences of the isolated cotton *14-3-3* genes (cDNAs) and their deduced proteins were analysed using DNAstar software (DNAstar Inc., Madison, WI, USA), and protein sequence homology analysis was performed with ClustalW (http://www.e-bi.ac.uk/clustalw/). Phylogenetic analysis was employed to investigate the evolutionary relationships among the Gh14-3-3 proteins and *Arabidopsis* 14-3-3 proteins. A minimum evolution tree was generated in MEGA3.1 (Kumar *et al.*, 2004). A bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology.

Overexpression of Gh14-3-3 *genes in fission yeast*

The open reading frame (ORF) of each *Gh14-3-3* gene was amplified by PCR using *Pfu* DNA polymerase with gene-specific primers (Supplementary Table S2 at *JXB* online), and was cloned into the yeast vector pREP5N (Alfa *et al.*, 1993) with *Xho*I/*Bam*HI sites. Then the construct was transferred into yeast (*Schizosaccharomyces pombe*) cells by electroporation (Bio-Rad MicroPulser Electroporation Apparatus, USA) according to the manufacturer's instructions. Overexpression of *Gh14-3-3* genes in the transformed yeast cells was induced by the method described previously (Li *et al.*, 2007). The transformants were selected on plates containing minimal medium (MM) with 2 μM thiamine at 30 °C. Then 10 colonies for each construct were picked out to incubate in liquid MM with 2 μM thiamine, which represses the *nmt-1* promoter activity, until mid-log phase in a shaker (220 rpm, 30 °C). Subsequently, the yeast cells were harvested and washed three times with MM without thiamine to de-repress the promoter, and then incubated in the same thiamine-free MM for 20 h (220 rpm, 30 °C). The yeast cells were observed and photographed under a Nikon microscope (Japan), and the lengths of 50 cells per transformant (single colony) were measured for statistical evaluation of the cell elongation, using empty pREP-5N transformants as controls.

Yeast two-hybrid analysis

For directed yeast two-hybrid assays of protein–protein interaction between cotton 14-3-3s, the coding sequences of *Gh14-3-3* genes amplified by PCR using *Pfu* DNA polymerase and gene-specific primers (Supplementary Table S3 at *JXB* online) were cloned into the *Nde*I/*Bam*HI restriction sites of yeast two-hybrid vectors pGBKT7 (bait vector) and pGADT7 (prey vector), creating fusions to the binding domain and activation domain of the yeast transcriptional activator *GAL4*, respectively. Each pGBKT7-Gh14-3-3 construct was introduced singly into the yeast strain Y187 using the high-efficiency lithium acetate transformation procedure (Gietz *et al.*, 1992), and each pGADT7-Gh14-3-3 construct was transferred into the yeast strain AH109. Mating reactions were performed between the two haploid strains containing pGBKT7-Gh14-3-3 and pGADT7-Gh14-3-3 constructs, respectively, and

Table 1. Primer pairs used in gene-specific RT-PCR of the six *Gh14-3-3* genes

Gene no.	Primers
Gh14-3-3L	5′-GCAGATGAACCTCAGGCTGAGAG-3′
	5′-GTCATAAAGACTATAAAGTGTAAC-3′
Gh14-3-3a	5′-GGATTTGTGACCTATGGCTTG-3′
	5′-CCACACTCTAGGGAGCTGCAT-3′
Gh14-3-3e	5′-CAACTTCTCCGGGACAACCTG-3′
	5′-CGCACTATTTTAGTACTAGCATG-3′
Gh14-3-3f	5′-GCTTGACACGCTGGGAGAAGAG-3′
	5′-TAGAGTATAGCATCATGCCATG-3′
Gh14-3-3g	5′-ATTGGGTGAGGAATCCTACAAG-3′
	5′-ACCCAAATTCATGTCCGAATGG-3′
Gh14-3-3h	5′-GCTTTTAAGGGACAACCTTAC-3′
	5′-TTGATCACAGAGGAAGGATg-3′
GhUBI1	5′-CTGAATCTTCGCTTTCACGTTATC-3′
	5′-GGGATGCAAATCTTCGTGAAAAC-3′

plated on double dropout medium (DDO medium, SD/–Leu/–Trp) (BD Biosciences Clontech, Palo Alto, CA, USA). The transformants were further streaked on quadruple dropout medium (QDO medium, SD/–Trp/–Leu/–His/–Ade) and confirmed with colour change on a β-galactosidase filter paper using the flash-freezing filter assay (James *et al.*, 1996).

A yeast two-hybrid library of cotton fibre cDNAs from mRNAs of 10 DPA fibres was constructed, using the Clontech BD Matchmaker Library Construction and Screening Kits according to the manufacturer's instruction (BD Biosciences Clontech). The first-strand cDNAs were reversely synthesized by reverse transcriptase and amplified into double-stranded cDNAs using long-distance PCR. pGADT7-Rec vector (prey vector) and cotton fibre cDNAs were co-transferred into yeast strain AH109, and then the yeast cells were cultivated on SD/–Leu medium at 30 °C for 5 d for selection of transformants. Finally, the transformed cells as the prey library were suspended in fresh SD/–Leu medium and stored as glycerol stock at –80 °C.

For screening target proteins of cotton 14-3-3s, each Gh14-3-3 bait clone was mated with the prey library by incubation for 24 h at 30 °C, and positive clones were selected on QDO medium (SD/–Trp/–Leu/–His/–Ade) at 3 °C. After further cultivation for 7 d, the independent positive clones were restreaked on QDO 2–3 times and LacZ activity was further tested by the flash-freezing filter assay (James *et al.*, 1996).

To analyse interactions of each Gh14-3-3 and its targets, the selected cotton cDNA clones that showed activation of both reporter genes were then inoculated in DDO liquid medium for plasmid DNA isolation by using the Yeast Plasmid Isolation kit (Tiangen Biotech Co. Ltd, Beijing, China). The isolated plasmid DNAs (pGADT7 vector containing cotton cDNA fragments) were then transferred into bacteria (*Escherichia coli*) strain DH5α on LB medium with 100 mg l^{-1} ampicillin. The cDNA clones were sequenced by Sunny Biotech. Co. Ltd (Shanghai, China). Similarities of cDNA sequences were analysed by blasting against the NCBI public databases using the BLASTX program and the DFCI Cotton Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=cotton) using the blastn program. Expressed sequence tags (ESTs) of each gene were collected and classified.

Results

Isolation and characterization of Gh14-3-3 *cDNAs*

To isolate the genes that might be involved in the regulation of fibre development, >10 000 cDNA clones from the cotton fibre cDNA library constructed by Li *et al.* (2002) were randomly sequenced. More than 30 *14-3-3* cDNA clones were identified from these clones. Among them, three clones contain full-length *14-3-3* cDNA sequences, and the remainder have partial *14-3-3* sequences. Subsequently, another three complete sequences were obtained by screening the fibre cDNA library using 400 bp fragments (including the partial coding region and 3'-untranslated region) of each *Gh14-3-3* cDNA as probes (see Materials and methods). In total, six unique full-length cDNAs encoding putative 14-3-3 proteins were identified, including one cDNA (*Gh14-3-3L*) that was previously reported (Shi *et al.*, 2007) and the remainder designated as *Gh14-3-3a*, *Gh14-3-3e*, *Gh14-3-3f*, *Gh14-3-3g*, and *Gh14-3-3h* (GenBank accession nos: EU189220, EU189224, GU451708, GU451709, and GU451710). As shown in Fig. 1, sequence analysis predicted that two *Gh14-3-3* genes (*Gh14-3-3e* and *Gh14-3-3g*) encode a 262 amino acid polypeptide, whereas the other four (*Gh14-3-3L*, *Gh14-3-3a*, *Gh14-3-3f*, and *Gh14-3-3h*)
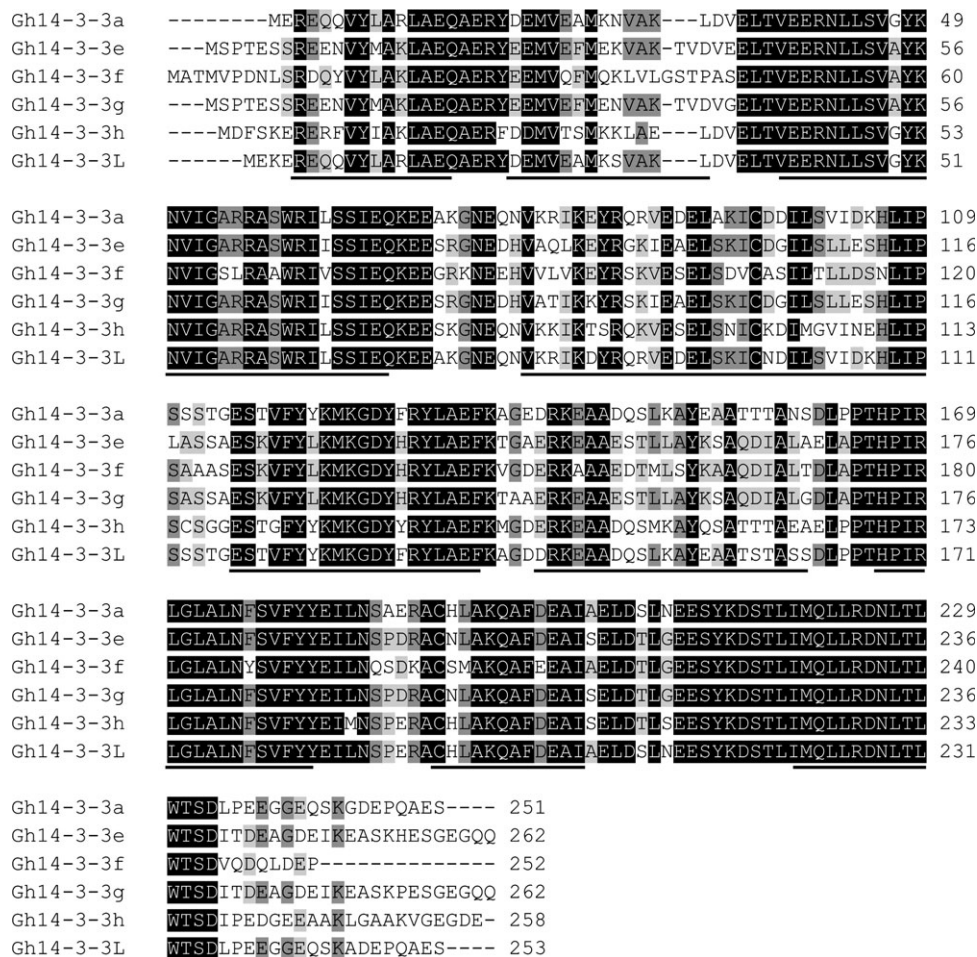
encode polypeptides consisting of 253, 251, 252, and 258 amino acids, respectively. The *Gh14-3-3* genes share high sequence homology at the nucleotide level (62–91% identity) in the coding region and at the amino acid level (62–96% identity). Sequence alignment revealed that all of the predicted proteins encoded by these genes contain nine highly conserved antiparallel α-helices (Fig. 1). In contrast, both the N- and the C-terminal region of Gh14-3-3 proteins display relatively high variability (Fig. 1).

Phylogenetic relationship of Gh14-3-3 proteins

To investigate the evolutionary relationships of the cotton 14-3-3 proteins with other 14-3-3 proteins, all of the known *Arabidopsis* 14-3-3s and cotton 14-3-3s were selected from GenBank for phylogenetic analysis. Plant 14-3-3 proteins can be divided into the ε-like group and the non-ε group (Wu *et al.*, 1997; Sehnke *et al.*, 2002a, b). As shown in Fig. 2, these proteins obviously split into two subgroups. Gh14-3-3L, Gh14-3-3a, and Gh14-3-3h together with the *Arabidopsis* ε-like isoforms form one subgroup, while Gh14-3-3b, Gh14-3-3c, Gh14-3-3d, Gh14-3-3e, Gh14-3-3f, and Gh14-3-3g are located in the non-ε branch of the tree. These results suggest that cotton contains both ε-like and non-ε 14-3-3 proteins, and the divergence in the ε-like and non-ε group could have occurred before the differentiation of the two species. One protein pair (Gh14-3-3e and Gh14-3-3g) share high sequence homology, resulting in an independent branch which is basal to the AtGF14ν/μ clade, indicating that the divergence between Gh14-3-3e and Gh14-3-3g occurred relatively late during evolution. Likewise, Gh14-3-3a and Gh14-3-3L form a distinct clade which is basal to the AtGF14° clade, suggesting that the two isoforms have a close evolutionary relationship. On the other hand, Gh14-3-3f occupies a distinct branch that is basal to the AtGFλ/κ clade, implying that 14-3-3f diverged earlier from the other cotton 14-3-3s. In addition, cotton 14-3-3b, 14-3-3c (Wei *et al.*, 2009), and 14-3-3d (unpublished data), which are preferentially expressed in roots, form another independent branch basal to *Arabidopsis* GF14ω, GF14φ, and GF14χ in the non-ε group.

Isolation and characterization of Gh14-3-3 *genes*

Four *Gh14-3-3* genes (*14-3-3L/a/e/f*) were amplified from the cotton (*G. hirsutum*) genome by PCR, using gene-specific primers. Sequence comparison between the cDNAs and their corresponding genes revealed that ε-like and non-ε *14-3-3* genes share highly conserved structures (as shown in Fig. 3). *Gh14-3-3a* and *Gh14-3-3L* which belong to the ε-like group contain five introns splitting their ORF into six exons. The intron positions are identical (within glutamate, within methionine, between glutamate and alanine, within arginine, and within glycine, respectively) in the two genes, although the length of each intron is slightly variable in both genes. In contrast, *Gh14-3-3e* and *Gh14-3-3f* belonging to the non-ε group contain three introns splitting their ORF into four exons. The positions of all three introns are

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Gh14-3-3a   --------MEREQQVYLARLAEQAERYDEMVEAMKNVAK---LDVELTVEERNLLSVGYK  49
Gh14-3-3e   ---MSPTESSREENVYMAKLAEQAERYEEMVEFMEKVAK-TVDVEELTVEERNLLSVAYK  56
Gh14-3-3f   MATMVPDNLSRDQYVYLAKLAEQAERYEEMVQFMQKLVLGSTPASELTVEERNLLSVAYK  60
Gh14-3-3g   ---MSPTESSREENVYMAKLAEQAERYEEMVEFMENVAK-TVDVGELTVEERNLLSVAYK  56
Gh14-3-3h   ----MDFSKERERFVYIAKLAEQAERFDDMVTSMKKLAE---LDVELTVEERNLLSVGYK  53
Gh14-3-3L   ------MEKEREQQVYLARLAEQAERYDEMVEAMKSVAK---LDVELTVEERNLLSVGYK  51

Gh14-3-3a   NVIGARRASWRILSSIEQKEEAKGNEQNVKRIKEYRQRVEDELAKICDDILSVIDKHLIP  109
Gh14-3-3e   NVIGARRASWRIISSIEQKEESRGNEDHVAQLKEYRGKIEAELSKICDGILSLLESHLIP  116
Gh14-3-3f   NVIGSLRAAWRIVSSIEQKEEGRKNEEHVVLVKEYRSKVESELSDVCASILTLLDSNLIP  120
Gh14-3-3g   NVIGARRASWRIISSIEQKEESRGNEDHVATIKKYRSKIEAELSKICDGILSLLESHLIP  116
Gh14-3-3h   NVIGARRASWRILSSIEQKEESKGNEQNVKKIKTSRQKVESELSNICKDIMGVINEHLIP  113
Gh14-3-3L   NVIGARRASWRILSSIEQKEEAKGNEQNVKRIKDYRQRVEDELSKICNDILSVIDKHLIP  111

Gh14-3-3a   SSSTGESTVFYYKMKGDYFRYLAEFKAGEDRKEAADQSLKAYEAATTTANSDLPPTHPIR  169
Gh14-3-3e   LASSAESKVFYLKMKGDYHRYLAEFKTGAERKEAAESTLLAYKSAQDIALAELAPTHPIR  176
Gh14-3-3f   SAAASESKVFYLKMKGDYHRYLAEFKVGDERKAAAEDTMLSYKAAQDIALTDLAPTHPIR  180
Gh14-3-3g   SASSAESKVFYLKMKGDYHRYLAEFKTAAERKEAAESTLLAYKSAQDIALGDLAPTHPIR  176
Gh14-3-3h   SCSGGESTGFYYKMKGDYYRYLAEFKMGDERKEAADQSMKAYQSATTTAEAELPPTHPIR  173
Gh14-3-3L   SSSTGESTVFYYKMKGDYFRYLAEFKAGDDRKEAADQSLKAYEAATSTASSDLPPTHPIR  171

Gh14-3-3a   LGLALNFSVFYYEILNSAERACHLAKQAFDEAIAELDSLNEESYKDSTLIMQLLRDNLTL  229
Gh14-3-3e   LGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  236
Gh14-3-3f   LGLALNYSVFYYEILNQSDKACSMAKQAFEEAIAELDTLGEESYKDSTLIMQLLRDNLTL  240
Gh14-3-3g   LGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  236
Gh14-3-3h   LGLALNFSVFYYEIMNSPERACHLAKQAFDEAISELDTLSEESYKDSTLIMQLLRDNLTL  233
Gh14-3-3L   LGLALNFSVFYYEILNSPERACHLAKQAFDEAIAELDSLNEESYKDSTLIMQLLRDNLTL  231

Gh14-3-3a   WTSDLPEEGGEQSKGDEPQAES----  251
Gh14-3-3e   WTSDITDEAGDEIKEASKHESGEGQQ  262
Gh14-3-3f   WTSDVQDQLDEP--------------  252
Gh14-3-3g   WTSDITDEAGDEIKEASKPESGEGQQ  262
Gh14-3-3h   WTSDIPEDGEEAAKLGAAKVGEGDE-  258
Gh14-3-3L   WTSDLPEEGGEQSKADEPQAES----  253
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Fig. 1. Sequence alignment among the six Gh14-3-3 proteins. Sequences of Gh14-3-3s were aligned. The amino acid residues identical among the sequences are indicated in black, while similar residues are shown in grey, and the regions of the nine conserved antiparallel α-helices (α1–α9) are underlined.

conserved. The first intron is located between codons encoding glutamine and aspartate, and the second intron is positioned between codons encoding glutamine and alanine in the two genes. However, the third intron is inserted between codons encoding theonine and aspartate in *Gh14-3-3e*, but between codons encoding glutamine and aspartate in *Gh14-3-3f*.

Expression of Gh14-3-3 genes is developmentally regulated in fibres

To analyse the expression patterns of the isolated *Gh14-3-3* genes, mRNA levels of these genes in cotton tissues were quantified by real-time quantitative SYBR-Green RT-PCR using gene-specific primers (Table 1). As shown in Fig. 4, four genes (*Gh14-3-3a*, *Gh14-3-3e*, *Gh14-3-3f*, and *Gh14-3-3h*) showed fibre-preferential expression patterns, while the other one (*Gh14-3-3g*) exhibited its relatively high expression level in fibres. *Gh14-3-3a* was preferentially expressed in both fibres and hypocotyls, and showed relatively high expression levels in anthers, whereas its expression activity was relatively lower in other tissues. Strong expression of the *Gh14-3-3e* gene was detected in fibres, and moderate expression was

found in hypocotyls, but relatively weak signals were detected in other tissues such as roots, cotyledons, leaves, anthers, petals, and ovules. *Gh14-3-3h* also displayed a similar expression pattern to *Gh14-3-3e*, but its peak value (10.91) in fibres is much lower than that (44.35) of *Gh14-3-3e*. *Gh14-3-3f* was expressed at the highest level in fibres, and at relatively high levels in leaves and petals, but moderate to weak expression was detected in other tissues. On the other hand, *Gh14-3-3g* was preferentially expressed in leaves, and showed a relatively high expression level in fibres, but its activity was very low in other tissues. In addition, a previous study revealed that the *Gh14-3-3L* gene was expressed at high levels in fibres, ovules, and petals, but at low levels in the other tissues (Shi *et al.*, 2007).

To investigate further the expression profiling of these *14-3-3* genes in fibre development, mRNA accumulation of the *Gh14-3-3* genes during fibre development was determined by quantitative RT-PCR (Fig. 5). The experimental results revealed that *Gh14-3-3a*, *Gh14-3-3e*, *Gh14-3-3f*, and *Gh14-3-3g* were expressed at relatively high levels in the early stage (3 DPA) of fibre development, and their transcripts reached the highest levels in 9–10 DPA fibre cells. As fibre developed further, the expression levels of these genes
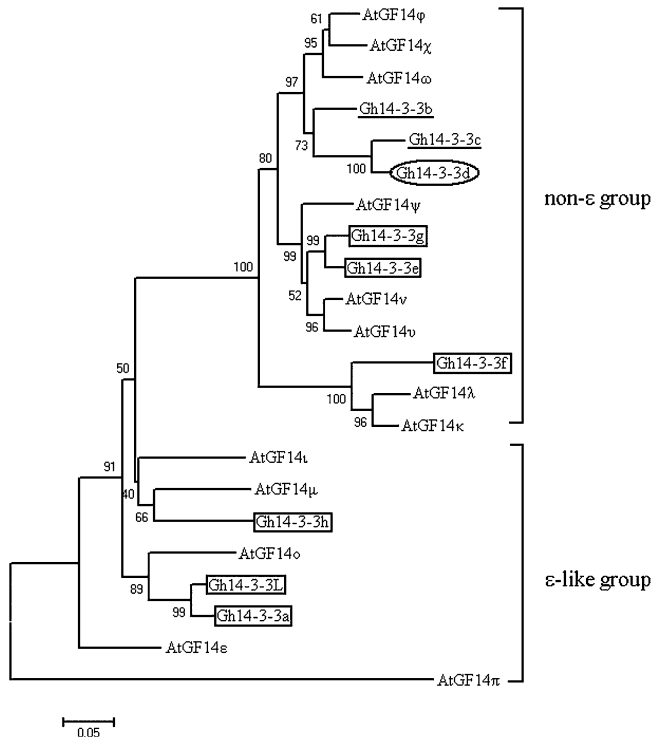
Fig. 2. Phylogenetic relationship of cotton 14-3-3 proteins to *Arabidopsis* 14-3-3 proteins. The minimum evolution tree was constructed in MEGA3.1 from 1000 bootstrap replicates. The accession numbers of cotton and *Arabidopsis* 14-3-3 proteins in GenBank are: Gh14-3-3a (EU189220), Gh14-3-3b (EU189221), Gh14-3-3c (EU189222), Gh14-3-3d (EU189223), Gh14-3-3e (EU189224), Gh14-3-3f (GU451708), Gh14-3-3g (GU451709), Gh14-3-3h (GU451710), Gh14-3-3L (DQ402076), AtGF14κ (AAD51783), AtGF14λ (AAD51781), AtGF14χ (AAA96254), AtGF14φ (AAB62224), AtGF14ω (AAA96253), AtGF14ν (AAD51782), AtGF14υ (AAB62225), AtGF14ψ (AAA96252), AtGF14μ (AAD51784), AtGF14ι (AAK11271), AtGF14° (AAG47840), AtGF14ε (AAD51785), and AtGF14π (NP-565174).

declined dramatically. Similarly, *Gh14-3-3L* and *Gh14-3-3h* genes were also expressed at high levels in early stages (3–10 DPA) of fibre development, but the expression activities of both genes gradually declined to relatively low levels with further development of fibre cells (12–20 DPA). The results suggested that the expression of these *14-3-3* genes was developmentally regulated in fibre cells of cotton.

Overexpression of Gh14-3-3 *genes in yeast promotes cell elongation*

To investigate whether the Gh14-3-3 isoforms play a role in cell elongation, three cotton *14-3-3* genes were heterologously expressed in yeast (*S. pombe*) cells. Two genes (*Gh14-3-3a* and *Gh14-3-3L*) represent the ε-like group, while the other one (*Gh14-3-3e*) represents the non-ε group of the cotton *14-3-3* family. The coding sequences of the three *Gh14-3-3* genes were cloned separately into the yeast vector pREP5N and transferred into yeast cells. As shown in Fig. 6,

morphological studies indicated that there were significant differences in cell length between the transformed cell lines and controls under induction conditions. Transformed cells expressing *Gh14-3-3* genes grown in induction medium (Fig. 6E–G) were remarkably longer than those harbouring the same vector but grown in uninduced conditions (Fig. 6A–C). In contrast, control cells with the empty pREP5N vector displayed normal length in both induced (Fig. 6H) and uninduced conditions (Fig. 6D). Measurement of cell length of the control and 10 randomly selected transformed yeast cell lines revealed that the length of the *Gh14-3-3a*-transformed cells was 1.6- to 2.0-fold greater than that of the control cells, while the length of *Gh14-3-3e*- and *Gh14-3-3L*-transformed cells was 1.2- to 1.5-fold longer than that of the control cells. Statistical analysis indicated that there were significant differences in cell length between the transformed lines and control (*P*-value <0.05 or <0.01 by *t*-test) (Fig. 7). On the other hand, statistical analysis demonstrated that there was no significant difference in the percentage of cell division between the transformed lines and controls (data not shown). The results suggested that overexpression of the cotton *14-3-3* genes stimulates the longitudinal growth of the host cells.

### Gh14-3-3s *display isoform selectivity in protein–protein interaction*

To understand how cotton 14-3-3 proteins interact with each other, yeast two-hybrid technology was employed to analyse the interactions among these Gh14-3-3 proteins. The ORF of each *Gh14-3-3* gene was cloned into pGBKT7 which contains the GAL4-binding domain, and into pGADT7 which contains the GAL4-activation domain. Empty vectors containing the activation domain or binding domain were used as negative controls. Gh14-3-3 proteins showed no self-activation of transcription. As shown in Fig. 8, Gh14-3-3a interacted with both Gh14-3-3e and Gh14-3-3g, and Gh14-3-3L also interacted with these two proteins, while Gh14-3-3g could interact with three proteins (Gh14-3-3a, Gh14-3-3h, and Gh14-3-3L). However, Gh14-3-3f did not interact with any of the other five Gh14-3-3 proteins. In addition, interaction of the same 14-3-3 proteins was not detected in the yeast two-hybrid assays (Fig. 8). These results suggest that each of the six cotton 14-3-3s may display isoform selectivity in formation of heterodimers, and may not combine into its homodimer in cells during fibre development of cotton.

### Interaction partners of Gh14-3-3 *proteins identified in a yeast two-hybrid screen*

To identify interaction partners of cotton 14-3-3 proteins, yeast two-hybrid analysis was performed using the six isolated Gh14-3-3 proteins as bait to screen the two-hybrid library of cotton fibre cDNAs constructed on the prey vector. Each of the six 14-3-3 proteins was used in a separate screen. As shown in Table 2, 38 unique proteins (the sequences are provided in Supplementary Table S4 at *JXB*
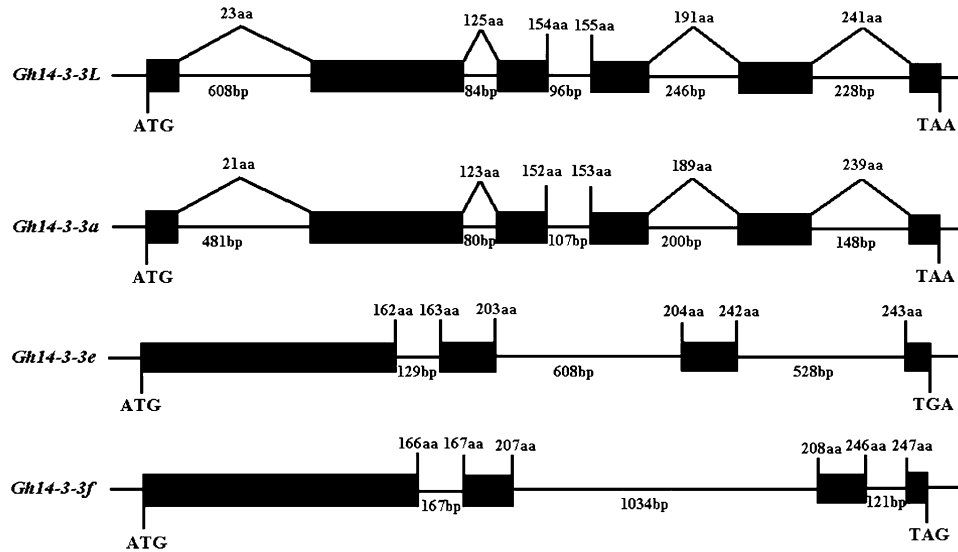
Fig. 3. The structures of four *Gh14-3-3* genes. Exons are denoted by black boxes. Introns, 5′-flanking regions, and 3′-UTRs are denoted by lines. The length of the intron in base pairs is indicated. The position of substitution is denoted by a diagonal line.
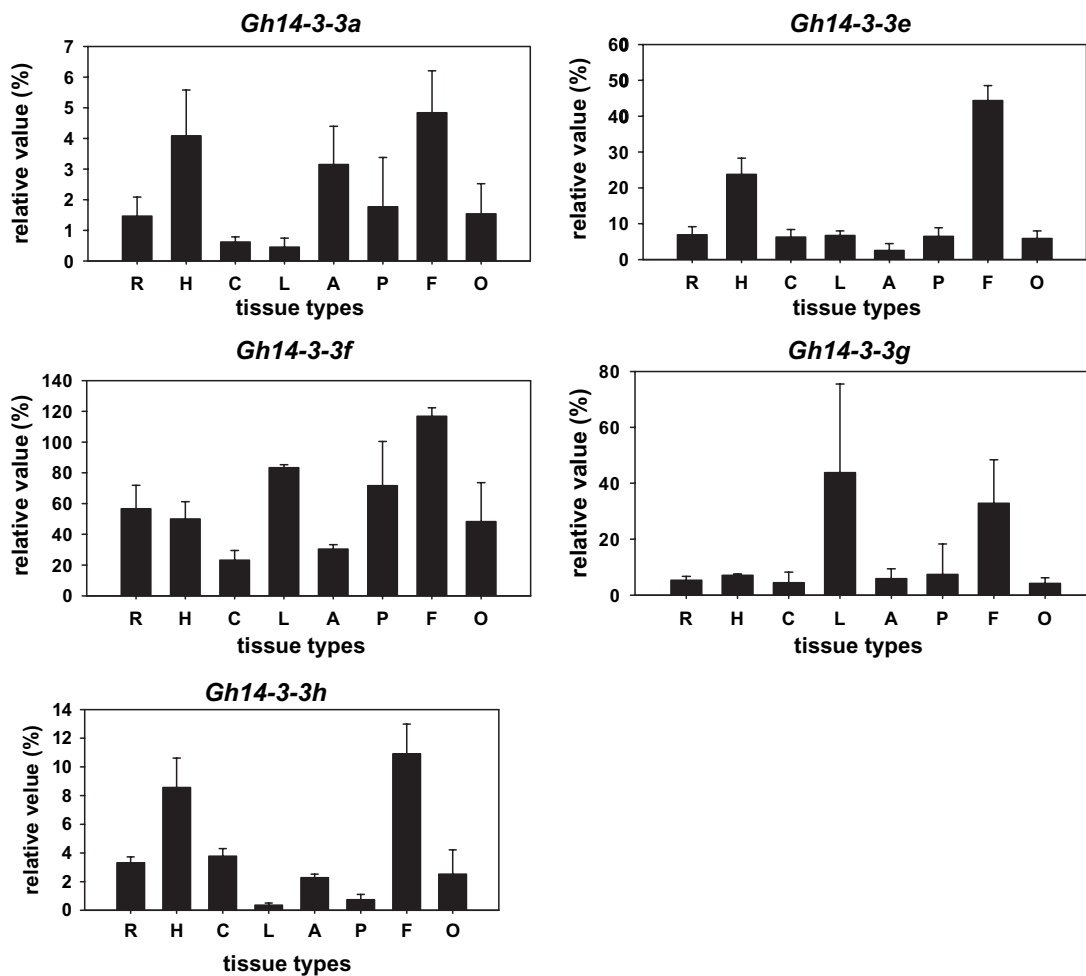
Fig. 4. Real-time quantitative RT-PCR analysis of expression of *Gh14-3-3* genes in cotton tissues. Relative values of *Gh14-3-3* gene expression in cotton tissues are shown as a percentage of *GhUBI1* expression activity. R, roots; H, hypocotyls; C, cotyledons; L, leaves; A, anthers; P, petals; F, 10 DPA (days post-anthesis) fibres; O, 10 DPA ovules.
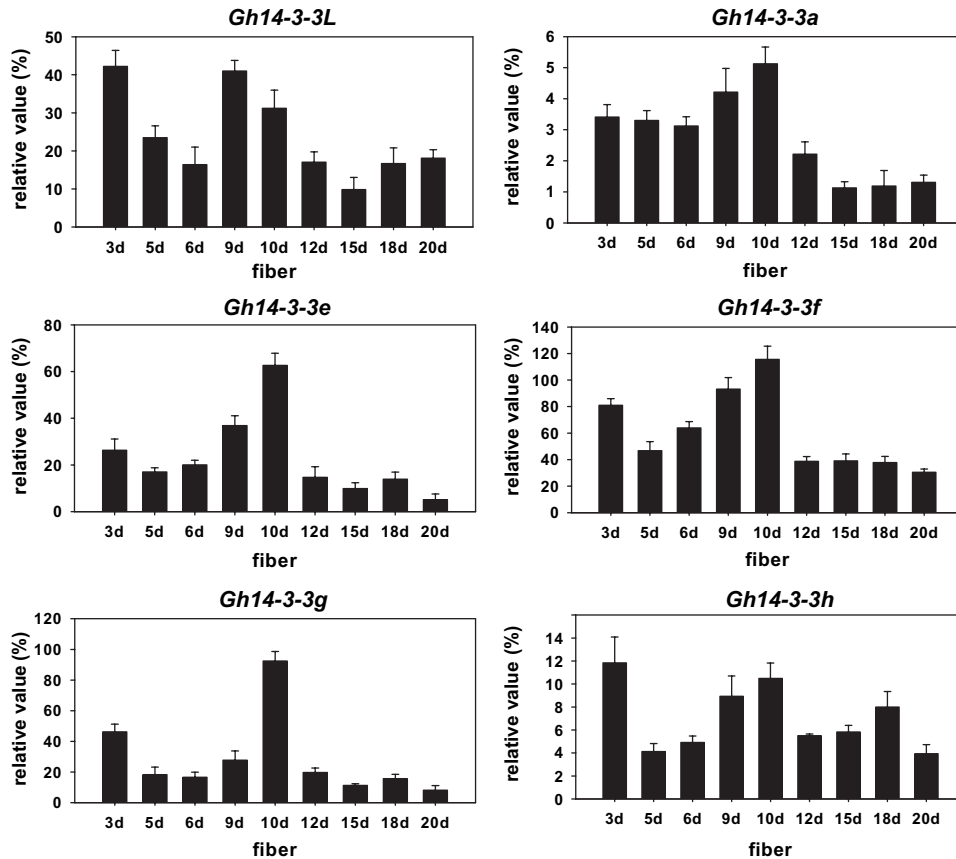
Fig. 5. Expression of *Gh14-3-3* genes during fibre development. Relative values of *Gh14-3-3* gene expression in developing fibres of cotton are shown as a percentage of *GhUBI1* expression activity. 3d, 5d, 6d, 9d, 10d, 12d, 15d, 18d, and 20d refer to 3, 5, 6, 9, 10, 12, 15, 18, and 20 DPA fibres.
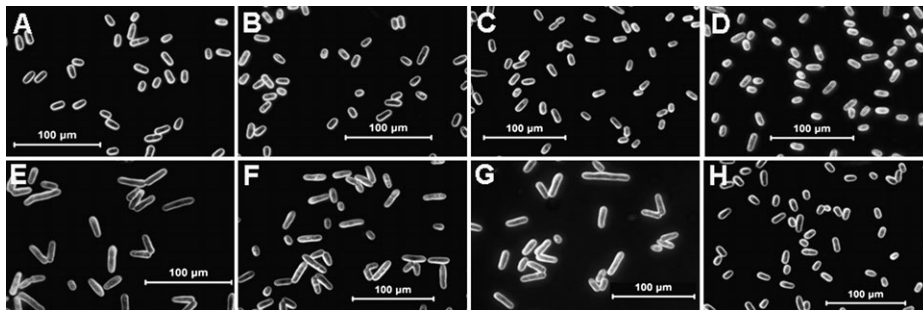

Fig. 6. Overexpression of *Gh14-3-3* genes stimulated the longitudinal growth of fission yeast cells. Micrographs were taken using dark-field microscopy. Yeast cells harbouring the constructed vectors pREP5N-*Gh14-3-3L* (A and E), pREP5N-*Gh14-3-3a* (B and F), and pREP5N-*Gh14-3-3e* (C and G), and empty plasmid (pREP5N; D and H) were cultured in non-induction medium (A–D) or induction medium (E–H). Bar=100 μm.

online) were identified as positive clones in these six screens for both of the reporter genes *ADE* and *LacZ*. All positive clones were checked for the presence of a cDNA–AD fusion and further confirmed in the one-to-one interaction analysis.

The targets of Gh14-3-3 proteins were related to various aspects of plant development, metabolism, and signal transduction. Some of the identified proteins, such as BRASSINAZOLE-RESISTANT protein (BZR, spot 3), H$^+$-ATPase (spot 20), ABA responsive element-binding protein (spot 5), and 40S ribosomal protein (spots 8 and 9),

were characterized as 14-3-3 targets in previous studies (Gampala *et al.*, 2007; Ryu *et al.*, 2007; Schoonheim *et al.*, 2007*b*), but the majority were identified as novel interaction partners for cotton 14-3-3s. As shown in Fig. 9, the 38 identified proteins were divided into seven classes. Of the 38 identified targets, nine (spots 30–38) represented unknown proteins. Two 14-3-3 proteins (5%) were identified in the yeast two-hybrid screens as Gh14-3-3 interactors, of which one (spot 7) is Gh14-3-3a and another (spot 6) is Gh14-3-3h identified in this study. Growth and metabolism-
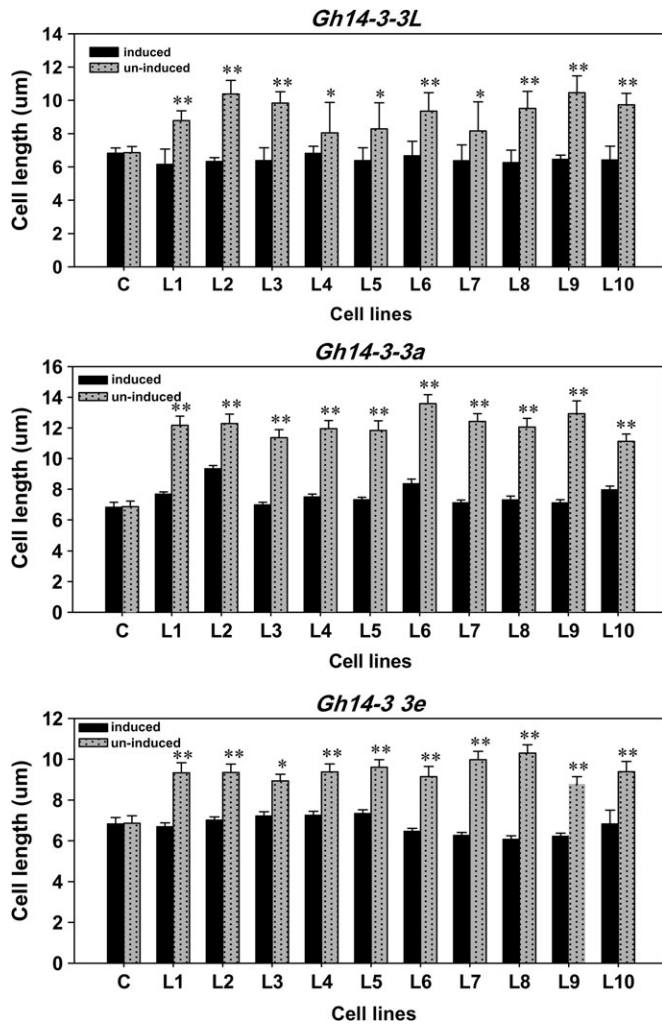
Fig. 7. Statistical analysis of the length of various fission yeast cell lines. C, controls (yeast cells with empty vectors); L1–L10, transformed cell lines (yeast cells harbouring *Gh14-3-3* genes). A single asterisk represents a significant difference (*P*-value <0.05), and double asterisks represent very significant differences (*P*-value <0.01) between the transformed cell lines and the control by *t*-test. The experiments were repeated three times; means represent average values of cell length (*n*=100 cells each line), and bars show standard errors.

related proteins (32%), such as sulphite oxidase (spot 14), carbonic anhydrase (spot 18), neutral/alkaline invertase (spots 19), and photosystem I P700 apoprotein A2 (spot 27), were found to be the major class of 14-3-3 protein inter-actors, and 13% of the 38 interactors were signalling-related proteins (spots 21–25). Furthermore, five proteins (spots 8–12) related to protein metabolism were identified, while three proteins (spots 3–5) involved in hormone response were detected in interaction with Gh14-3-3 proteins.

A systematic approach was employed to evaluate the pub-licly available cotton resources, and to help in the selection of specific subsets of the target genes. Using this approach, an expression summary of targets of each 14-3-3 protein was obtained from the cotton gene index (Table 2). The results indicated that there are a large number of ESTs for all
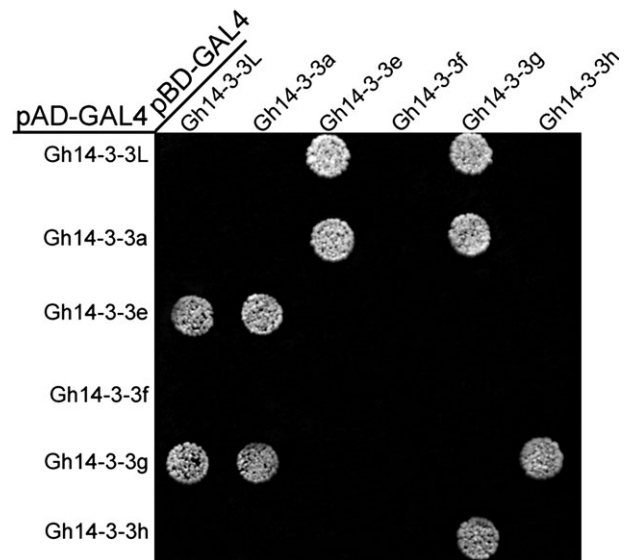
Fig. 8. Isoform-specific interactions among the Gh14-3-3 pro-teins. The coding sequences of the *Gh14-3-3* genes were cloned into the yeast two-hybrid vectors pGADT7 and pGBKT7, and introduced into yeast cells (see Materials and methods). Interac-tions among the Gh14-3-3 proteins were analysed by yeast mating. Transformants were assayed for growth on QDO nutri-tional selection medium.

38 genes encoding the proteins targeted by Gh14-3-3s. Based on the percentage of ESTs detected in EST databases of cotton fibres, it was demonstrated that almost all targets are predominantly expressed in fibres, except 26S proteasome regulatory subunit 7 (28.2%) and carbonic anhydrase (39.4%).

To understand whether the interactions between Gh14-3-3s and their target proteins are isoform selective, the direct yeast two-hybrid assay was employed to analyse the interactions between each Gh14-3-3 and its target proteins. Isoform-specific interactions were observed in this study. As shown in Fig. 10, some proteins (such as root phototropism protein, ubiquitin, ATP-binding protein, H^+-ATPase, and BZR1) showed high affinity with more than one 14-3-3 protein, whereas the others (such as putative R2R3-Myb transcription factor and ABA responsive element-binding protein 2) interacted with only one 14-3-3 isoform.

Discussion

Plant 14-3-3 proteins belong to a large conserved family which can be divided into two groups (ε-like group and non-ε group) based on phylogenetic clustering (Sehnke *et al.*, 2002*a*, *b*). In *Arabidopsis*, there are fifteen *14-3-3* genes, of which 13 are found to be functionally expressed, and the intron positions in the gene family are also conserved (Wu *et al.*, 1997; Rosenqust *et al.*, 2001). Similarly, the present data indicated that both ε-like and non-ε 14-3-3 proteins exist in cotton. Furthermore, the gene structure splicing patterns of the isolated *Gh14-3-3* genes are consistent with phylogenetic analysis of the ε-like and non-ε clustering (Figs 2 and 3), like the *Arabidopsis* 14-3-3 family. *Gh14-3-3a* and

Table 2. Identified cDNA clones from the Gh14-3-3 yeast two-hybrid screen

| Spot no.	Gh14-3-3 proteins used as bait	Homologous protein and species	E-value	Homology	Score	Total ESTs	Percentage of ESTs expressed in fibre
1	L, e, h	Nucleic acid-binding protein (*Ricinus communis*)	6e-52	Id. 85%; Po. 94%	207	57	80.7
2	e	Putative R2R3-Myb transcription factor (*Citrus sinensis*)	9e-38	Id. 61%; Po. 69%	160	98	53.1
3	L, e, f, g, h	BRASSINAZOLE-RESISTANT 1 protein (*Ricinus communis*)	2e-66	Id. 83%; Po. 91%	254	78	73.1
4	h	Auxin-repressed protein (*Gossypium barbadense*)	5e-40	Id. 68%; Po. 81%	167	218	59.6
5	h	Abscisic acid responsive element-binding protein 2 (*Populus suaveolens*)	2e-31	Id. 69%; Po. 80%	138	65	86.2
6	g	14-3-3h protein (*Gossypium hirsutum*)	2e-113	Id. 100%; Po. 100%	412	139	79.1
7	e	14-3-3a protein (*Gossypium hirsutum*)	3e-136	Id. 99%; Po. 99%	488	141	80.1
8	a, f, h	40S Ribosomal protein S20 (*Ricinus communis*)	4e-25	Id. 100%; Po. 100%	117	76	82.9
9	g	40S Ribosomal protein S20 (*Ricinus communis*)	1e-24	Id. 100%; Po. 100%	115	103	85.4
10	L, a, e	26S Proteasome regulatory subunit 7, psd7 (*Ricinus communis*)	1e-99	Id. 91%; Po. 95%	272	103	28.2
11	a, g	Ubiquitin (*Gossypium hirsutum*)	1e-28	Id. 96%; Po. 98%	129	14	78.6
12	h	Aspartic proteinase (*Theobroma cacao*)	1e-58	Id. 82%; Po. 95%	278	321	50.2
13	h	KEU (keule); protein transporter (*Arabidopsis thaliana*)	2e-43	Id. 73%; Po. 83%	178	4	75
14	f, h	Sulphite oxidase (*Hibiscus cannabinus*)	2e-100	Id. 92%; Po. 96%	368	32	71.9
15	f	Multicopper oxidase (*Ricinus communis*)	1e-71	Id. 84%; Po. 90%	272	52	78.8
16	f	Cytosolic phosphoglucomutase (*Solanum tuberosum*)	1e-46	Id. 93%; Po. 96%	189	74	64.9
17	f	Transferase (*Ricinus communis*)	2e-05	Id. 52%; Po. 67%	52.0	44	75
18	g, h	Carbonic anhydrase (*Gossypium hirsutum*)	2e-42	Id.45%; Po. 62%	176	495	39.4
19	h	Neutral/alkaline invertase (*Coffea Arabica*)	2e-13	Id. 94%; Po. 97%	78.6	96	51
20	L, e, f, h	Autoinhibited H^+ ATPase (*Populus trichocarpa*)	1e-11	Id. 98%; Po. 98%	73.2	78	48.7
21	L, e, g, h	ATP-binding protein (*Ricinus communis*)	3e-46	Id. 88%; Po. 92%	187	112	58.9
22	h	Signal transducer (*Ricinus communis*)	2e-47	Id. 75%; Po. 83%	191	41	63.4
23	h	Signal transducer (*Ricinus communis*)	3e-16	Id. 37%; Po. 47%	89.0	211	46.4
24	h	Calmodulin-binding protein (*Ricinus communis*)	2e-42	Id. 56%; Po. 68%	176	102	84.3
25	h	Plant synaptotagmin (*Populus trichocarpa*)	2e-39	Id. 83%; Po. 93%	164	31	74.2
26	L, a, e, f	Root phototropism protein (*Ricinus communis*)	7e-65	Id. 74%; Po. 83%	250	83	54.2
27	h	Photosystem I P700 apoprotein A2 (*Gossypium hirsutum*)	5e-64	Id. 98%; Po. 98%	246	21	61.9
28	h	Harpin-induced 1 (*Medicago truncatula*)	3e-23	Id. 55%; Po. 73%	111	64	76.6
29	h	Clathrin coat assembly protein ap-1 (*Ricinus communis*)	4e-63	Id. 90%; Po. 96%	243	33	66.7
30	h	Hypothetical protein (*Zea mays*)	2e-44	Id. 87%; Po. 88%	182	17	70.6
31	h	Conserved hypothetical protein (*Ricinus communis*)	2e-38	Id. 62%; Po. 73%	161	29	55.2
32	g	Predicted protein (*Populus trichocarpa*)	5e-86	Id. 76%; Po. 85%	322	21	76.2
33	e	Predicted protein (*Populus trichocarpa*)	4e-43	Id. 60%; Po. 75%	179	66	77.3
34	L, a	Predicted protein (*Populus trichocarpa*)	6e-25	Id. 57%; Po. 66%	117	38	84.2
35	f, h	Predicted protein (*Laccaria bicolor S238N-H82*)	8.3	Id. 30%; Po. 43%	33.5	63	74.6
36	L	Hypothetical protein isoform 2 (*Vitis vinifera*)	2e-22	Id. 70%; Po. 81%	108	8	87.5
37	h	Unnamed protein product (*Vitis vinifera*)	1e-35	Id. 59%; Po. 72%	152	21	71.4
38	g	Hypothetical protein (*Vitis vinifera*)	1e-25	Id. 50%; Po. 58%	119	62	58.1

The clones that showed activity of both two reporter genes were identified as positive clones, and DNA was isolated. Sequences were BLAST searched against the NCBI databases.

Gh14-3-3L genes encoding ε-like 14-3-3 proteins contain three introns, whereas *Gh14-3-3e* and *Gh14-3-3f* genes which encode non-ε 14-3-3 isoforms have five introns. All the introns in cotton *14-3-3* genes are positioned in conserved codons, compared with the known plant *14-3-3* genes (Rosenqust *et al.*, 2001). In addition, the data presented in Fig. 1 revealed that cotton 14-3-3 proteins contain the expected conserved structure, the nine antiparallel α-helical regions, but show great divergence in their N- and C-terminal regions, like all the other 14-3-3 proteins.
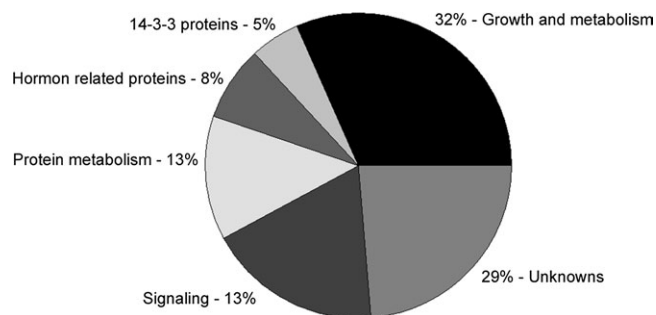
Fig. 9. Venn diagram of classification of the isolated Gh14-3-3 interactors. The proteins identified in cotton fibres by yeast two-hybrid screening were classified into seven classes.
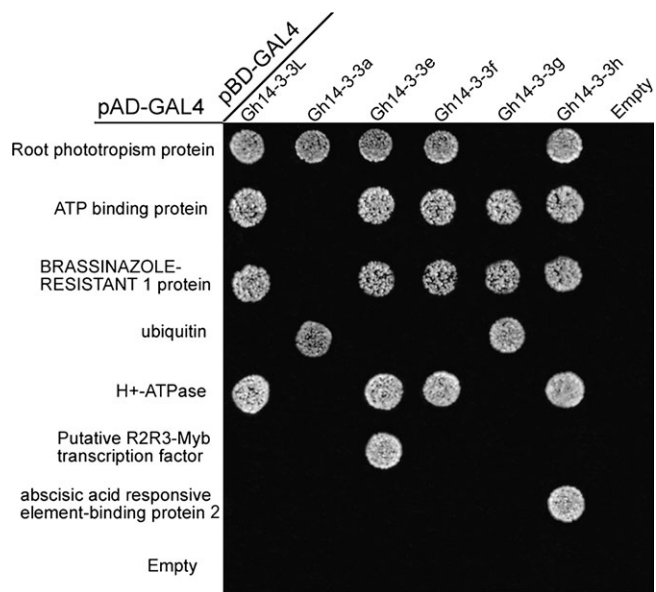
Fig. 10. Isoform-specific interactions between Gh14-3-3s and target proteins. Seven interactors were selected to analyse the interaction with each Gh14-3-3 protein by yeast mating. Transformants were assayed for growth on QDO nutritional selection medium.

A previous study indicated that *Arabidopsis* 14-3-3 gene expression is restricted to specific tissues (Daugherty *et al.*, 1996). Similarly, the present results showed that the mRNAs of all the six *Gh14-3-3* genes are abundant in cotton fibres. The transcripts of these genes reached their peak values in fibres at 9–12 DPA during fibre development, but remarkably declined to lower levels as fibre cells developed further, indicating that the high expression levels of *Gh14-3-3* genes are consistent with rapid elongation of cotton fibres. Furthermore, overexpression of *Gh14-3-3L*, *Gh14-3-3a*, and *Gh14-3-3e* in fission yeast significantly induced the longitudinal growth of the host cells, like the cotton fibre-specific *TUA9* gene (Li *et al.*, 2007). These data suggest that Gh14-3-3 proteins participate in regulation of cell polar growth and may play an important role in fibre development.

Dimerization is important for the function of 14-3-3 proteins. A recent study showed that two aldosterone-induced

14-3-3 isoforms, β and ε, interact with phosphor-Nedd4-2 as an obligatory heterodimer. Knockdown of either 14-3-3β or 14-3-3ε reduced the association of Nedd4-2 with the other isoform (Liang *et al.*, 2008). In this study, interactions were found in five cotton 14-3-3 protein pairs, Gh14-3-3L–Gh14-3-3g, Gh14-3-3L–Gh14-3-3e, Gh14-3-3a–Gh14-3-3e, Gh14-3-3a–Gh14-3-3g, and Gh14-3-3g–Gh14-3-3h, but no homodimer was detected in these proteins. Each pair of the interacting proteins may form a heterodimer involved in the same signal transduction during fibre development of cotton.

From the yeast two-hybrid screening, 38 cotton 14-3-3-interacting proteins which were related to plant growth, development, metabolism, and signal transduction were identified in cotton fibres (Table 2). By searching in the cotton EST index, it was verified that these target proteins are very abundant in cotton fibres. Among them, some targets have been characterized as 14-3-3 target proteins. A previous study revealed that 40S ribosomal protein and 26S protease regulatory subunit 7 are 14-3-3 interactors in barley, and the interaction between ABF and 14-3-3 proteins was involved in the ABA signal transduction pathway in barley (Schoonheim *et al.*, 2007a). The auto-inhibitory action of H^+-ATPase was released by binding of 14-3-3 protein (Maudoux *et al.*, 2000). Likewise, the present data revealed that the above interactions in fibre cells may be important for fibre development of cotton.

Cotton 14-3-3 proteins may regulate fibre development by participating in phytohormone signal transduction. Fibre development of cotton is positively affected by a number of phytohormones, including auxin, GA, brassinosteroid (BR), and ethylene, but negatively impacted by ABA and cytokinin (Lee *et al.*, 2007). SAR5 was first isolated as an auxin-repressed protein (ARP) by differential screening of auxin-deprived strawberry receptacles (Reddy and Poovaiah, 1990). It was reported that auxin levels precede fibre initiation, decline after anthesis (1–3 DPA), and rebound after 4 DPA during fibre development (Guinn and Brummett, 1988). Furthermore, knockdown of tobacco ABP1 expression causes deficient auxin-induced cell elongation (Chen *et al.*, 2001). Previous studies revealed that BZR1 is a key transcription factor in BR signal transduction. Interaction between BZR1 and 14-3-3 protein affects the cytoplasmic translocation of BZR1 in *Arabidopsis* and rice (Bai *et al.*, 2007; Ryu *et al.*, 2007). Treatment of cotton floral buds with a BR inhibitor Brz resulted in the complete absence of fibre differentiation, whereas exogenous BL promoted fibre elongation, suggesting that BRs are essential for cotton fibre initiation and development (Sun *et al.* 2005). In this study, three proteins (ABA responsive element-binding protein, ARP, and BZR1 protein) involved in the response to phytohormone signalling were identified as Gh14-3-3 interactors. These data suggested that the interaction between Gh14-3-3s and the phytohormone-related proteins may be crucial for responding to phytohormone signalling or maintaining an appropriate phytohormone levels for rapid fibre cell elongation during fibre development of cotton.

It was demonstrated that MYB proteins participate in the regulation of sets of cell signalling in plants. Recently, a study by Pu *et al.* (2008) indicated that GhMYB109 is required for fibre development of cotton. Suppression of GhMYB109 activity led to a substantial reduction in fibre length. The fact that cotton 14-3-3 proteins interacted with MYB transcription factors suggests that 14-3-3 proteins may participate in fibre elongation by regulating MYB activity, controlling its intracellular localization or some other ways.

Gh14-3-3 proteins may regulate fibre cell elongation by modulating the pH of the cell wall. The interaction between the plasma membrane (PM) H^+-ATPase and 14-3-3 proteins has been extensively explored. The PM H^+-ATPase is known to play a major role in the control of various cell processes. The enzyme pumps protons from the cytoplasm to the cell exterior by using ATP as energy source and creates an electrochemical gradient across the PM that constitutes the driving force for nutrient uptake, phloem loading, water movements, and stomatal closure and opening (Comparot *et al.*, 2003). The 'Acid Growth Theory' states that cells excrete protons into the wall at an enhanced rate when exposed to auxin, resulting in a decrease in apoplastic pH which activates the wall-loosening processes and causes cell elongation (Rayle *et al.*, 1970, 1992). A study indicated that H^+-ATPase was activated in wheat embryo by auxin, resulting in apoplastic acidification. This process contributes to cell wall loosening and cell elongation (Rober-Kleber *et al.*, 2003). The binding of 14-3-3 proteins to the auto-inhibitory domain of the H^+-ATPase leads to the formation of a complex that activates the pump (Maudoux *et al.*, 2000). Likewise, through the interaction with its target, the Gh14-3-3 protein may activate PM H^+-ATPase, resulting in the loosening of cell walls for fibre cell elongation. On the other hand, the roles of the other target proteins of 14-3-3s in fibre development still need to be investigated in details. Gh14-3-3 proteins may regulate these target proteins by modulating their activities, localizations, or molecular interactions with other proteins for fibre development.

In conclusion, the data presented in this study indicated that the isolated *Gh14-3-3* genes are fibre preferential and developmentally regulated in cotton, and overexpression of these genes in yeast promoted the longitudinal growth of the cells, suggesting that 14-3-3 proteins may participate in regulation of fibre elongation. Furthermore, the study revealed that the interaction between cotton 14-3-3 proteins is isoform selective, and 38 novel interaction partners for cotton 14-3-3 proteins were identified in cotton fibres by yeast two-hybrid screening. Thus, the results of this work provide novel insights into the 14-3-3 signalling related to fibre development of cotton.

Supplementary data

Supplementary data are available at *JXB* online.
Table S1. Primer pairs used in isolation of *Gh14-3-3* genes.
Table S2. Primer pairs used in overexpression of *Gh14-3-3* genes in yeast cells.
Table S3. Primer pairs used in yeast two-hybrid analysis.
Table S4. Sequences of the 38 identified cDNAs encoding target proteins which interact with cotton 14-3-3 proteins.

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