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RESEARCH PAPER

Molecular cloning, expression profiling, and yeast complementation of 19 β-tubulin cDNAs from developing cotton ovules

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Abstract

Microtubules are a major structural component of the cytoskeleton and participate in cell division, intracellular transport, and cell morphogenesis. In the present study, 795 cotton tubulin expressed sequence tags were analysed and 19 β-tubulin genes (*TUB*) cloned from a cotton cDNA library. Among the group, 12 cotton *TUB*s (*GhTUB*s) are reported for the first time here. Transcription profiling revealed that nine *GhTUB*s were highly expressed in elongating fibre cells as compared with *fuzzless-lintless* mutant ovules. Treating cultured wild-type cotton ovules with exogenous phytohormones showed that individual genes can be induced by different agents. Gibberellin induced expression of *GhTUB*1 and *GhTUB*3, ethylene induced expression of *GhTUB*5, *GhTUB*9, and *GhTUB*12, brassinosteroids induced expression of *GhTUB*1, *GhTUB*3, *GhTUB*9, and *GhTUB*12, and lignoceric acid induced expression of *GhTUB*1, *GhTUB*3, and *GhTUB*12. When *GhTUB*s were transformed into the *Saccharomyces cerevisiae* inviable mutant, *tub2*, which is deficient in β-tubulin, one ovule-specific and eight of nine fibre-preferential *GhTUB*s rescued this lethality. This study suggests that the proteins encoded by cotton *GhTUB*s are involved during cotton fibre development.

Key words: *fuzzless-lintless* mutant, *Gossypium hirsutum* (cotton) fibre, phytohormone, β-tubulin.

Introduction

Upland cotton (*Gossypium hirsutum*) accounts for most of the world cotton fibre production for the textile industry. Cotton fibres are single-celled trichomes that are similar to *Arabidopsis* trichomes, differentiated from about 15–25% of the ovule epidermal cells (Basra and Malik, 1984; Kim and Triplett, 2001). Fibre cell development occurs through fibre initiation, cell elongation, cell wall deposition, and maturation, and is a highly regulated, fundamental biological process. Fibre cells are initiated on the day of anthesis and elongate up to several centimetres without cell division. This process provides a unique system for studying cell elongation and cell wall and cellulose biosynthesis (Kim and Triplett, 2001). Although microarray transcriptome profiling of cotton unique expressed sequence tags (EST) identified a number of fibre-specific/preferential genes involved in phytohormone biosynthesis, lipid biosynthesis, and cytoskeleton and cell wall structures (Ji *et al*., 2003; Arpat *et al*., 2004; Shi *et al*., 2006; Gou *et al*., 2007; Qin *et al*., 2007*a*), the molecular mechanism of fibre cell elongation is not fully understood.

Microtubules play an important role in plant cell morphology and development (Kopczak *et al*., 1992). For example, microtubule depolymerization by a specific antagonist causes loss of directionality of root hair growth (Bibikova *et al*., 1999). Microtubule reorientation is the key in changing the growth orientation leading to *Arabidopsis* trichome branching (Mathur and Chua, 2000). The principle component of microtubules is a heterodimer of highly conserved α- and β-tubulin, encoded by multigene families (Silflow *et al*., 1987; Goddard *et al*., 1994; Nogales *et al*., 1998). Microtubule nucleation at microtubule-organizing centres is mediated by γ-tubulin (Pastuglia

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et al., 2006), microtubule dynamics, organization, and establishment of polarity controlled by microtubule-associated proteins (Whittington *et al*., 2001; Sedbrook, 2004; Ambrose *et al*., 2007; Korolev *et al*., 2007; Wang *et al*., 2007). *Arabidopsis* has six α-tubulin genes (*TUA*), nine β-tubulin genes (*TUB*), and two γ-tubulin genes (Kopczak *et al*., 1992; Snustad *et al*., 1992; Liu *et al*., 1994). Seven *TUA*s and six *TUB*s are found in *Zea mays* (Montoliu *et al*., 1990; Villemur *et al*., 1994), eight *TUB*s are found in *Oryza sativa* (Yoshikawa *et al*., 2003), and eight *TUA*s and 20 *TUB*s are found in *Populus* (Oakley *et al*., 2007). Most tubulin genes show differential and tissue-specific expression patterns (Snustad *et al*., 1992; Cheng *et al*., 2001; Oakley *et al*., 2007), implying that they are regulated by developmental signals and play roles in specific tissues. In cotton, seven of nine *TUA*s are highly expressed in developing fibres (Dixon *et al*., 1994; Whittaker and Triplett 1999; Li *et al*., 2007). Among the seven *TUB*s previously reported, two accumulate in 20 d post-anthesis (dpa) fibre cells (Dixon *et al*., 1994). *GhTUB*1 and *GhTUB*9 are the only cotton β-tubulins to be characterized (Ji *et al*., 2002; Li *et al*., 2002).

To understand the overall contribution of tubulins in cotton fibre development, 19 tubulin genes were cloned, including 12 *TUB*s newly identified by in-depth analysis of cotton ESTs. The regulation of *TUB*s in response to different phytohormones and by very-long-chain fatty acids, molecules that are important for fibre cell elongation, was studied (Shi *et al*., 2006; Qin *et al*., 2007*a*). A complementation analysis was performed using a non-viable yeast mutant to examine the function of all *TUB*s that are preferentially expressed in fibres as well as certain non-specifically expressed *TUB*s. Several of the tubulins did not complement the yeast mutant, indicating that different tubulins have distinct functions despite the fact that the tubulin gene family is highly conserved.

Materials and methods

Plant materials

Upland cotton (*G. hirsutum* L. cv. Xuzhou142) and a *fuzzless-lintless* (*fl*) mutant (Zhang and Pan, 1992) were planted in the field. Ovules were excised from cotton bolls, and fibre cells were scraped from the epidermis of the ovule, frozen in liquid nitrogen, and stored at –80 °C before RNA extraction. Other tissues were obtained from cotton plants grown in the field or a fully automated greenhouse as described (Ji *et al*., 2003).

Semi-quantitative reverse transcription (RT)-PCR and real-time quantitative (QRT)-PCR analysis of GhTUB expression in cotton tissues

The wild-type (wt) ovules were collected from –3, 0, 5, 10, 15, and 20 dpa cotton flowers. The fibres were stripped from ovules at different developmental stages. Total RNA was extracted from wt cotton roots, leaves, stems, ovules, fibres, and *fl* mutant ovules. The wt ovules collected from 0 dpa were used for *in vitro* cultures supplemented with different phytohormones or very-long-chain fatty acids. Cotton cDNA was reverse transcribed from 5 μg total RNA. Gene-specific primers were designed (Table 1), and QRT-PCR was carried out using the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection System (MJ Research). The cotton ubiquitin gene, *UBQ7* (accession no. AY189972), was used as an internal control in each reaction. Samples were analysed in triplicate using independent RNA samples and were quantified by the comparative cycle threshold method (Wittwer *et al*., 1997).

Identification and clustering analysis of cotton tubulin ESTs

The tubulin ESTs were identified from 110 812 *G. hirsutum* ESTs (Shi *et al*., 2006; Gou *et al*., 2007; Y X Zhu *et al*., unpublished data) with an expectation value (*E*) of <0.01 and an identity score >50%. They were clustered into contigs using the stackPACK™v2.1 program (http://stackpack2.cbi.pku.edu.cn/). One cDNA clone from a cotton cDNA library (Shi *et al*., 2006) was chosen to represent a *GhTUB* EST contig.

In vitro ovule culture and treatments with exogenous phytohormones or very-long-chain fatty acids

Cotton ovules were collected at 1 dpa. They were sterilized and cultured in medium with or without the following compounds: 5 μM brassinosteroid (BR), 5 μM gibberellin (GA), and 5 μM lignoceric acid (C24:0). Supplementation with ethylene (final concentration: 0.1 μM) was carried out as described (Shi *et al*., 2006). C24:0 was dissolved in methyl tert-butyl ether to a stock concentration of 5 mM.

Functional complementation of the yeast tub2Δ strain by cotton GhTUBs

Saccharomyces cerevisiae diploid strain W1536 *TUB2*/*tub2Δ* was generated by amplifying the *tub2*::kanMX4 cassette by PCR using template genomic DNA extracted from BY4743 *TUB2*/*tub2Δ* (Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0;*tub2*::kanMX4/TUB2, EUROSCARF). The PCR product was transformed into W1536, and selection of W1536 *TUB2*/*tub2Δ* followed the described protocol (Qin *et al*., 2007*b*). As a control, *S. cerevisiae TUB*2 was amplified with forward primer, 5′ cccgggTGGAGTGACATAGCAGCTACTACAAC-3′ and reverse primer 5′ gggcccGCTCGGAAGGTTAAAGGTTGT-3′ (lower-case letters indicate restrictions sites for cloning), and cloned into TRP1-marked pYADE4 behind the same *ADH* promoter with *Sma*I/*Apa*I sites, resulting in pYADE4-ScTUB2, which was transformed into W1536 *TUB2*/*tub2Δ*. The transformants were selected on Sc-Trp (synthetic complete medium lacking tryptophan) plates, and sporulated on plates containing 0.25% (wt/v) yeast extract, 1.5% (wt/v) potassium acetate, and 0.05% (wt/v) D-glucose supplemented with amino acids. After sporulation, ascospores were digested with zymolyase (Seikagaku), and the tetrads were dissected using a Singer MSM manual dissection microscope (Singer Instruments). Separated ascospores were grown on YPD [1% (wt/v) yeast extract, 2% (wt/v) peptone, and 2% (wt/v) D-glucose] for 5 d. The mutant complemented by *ScTUB2* was selected by replica plating on YPD-G418 (YPD supplemented with 300 μg ml^{-1} of geneticin) plates and 2-amino-5-flurobenzoic acid (FAA) plates [synthetic complete medium containing 2% (wt/v) D-glucose and 0.05% (wt/v) FAA] simultaneously. Positive candidates, *tub2Δ* carrying pYADE4-ScTUB2, grew on YPD-G418 plates but not on FAA plates. YCplac-*GhTUB* was transformed into haploid *tub2Δ* carrying pYADE4-ScTUB2 and was selected on Sc-Trp or Sc-Ura plus FAA plates.

Table 1. *Primers used in the current study*

Gene	Primers used for RT-PCR or QRT-PCR amplification and expression profiling
*GhTUB*1	5′-CACTGTTTGTGACATCCCTC-3′ 5′-TCTCATCCATCCCTTCTCC-3′
*GhTUB*2	5′-TTTGTGACATCCCACCCACT-3′ 5′-ATGGCAATAACTTTCCTTCTCC-3′
*GhTUB*3	5′-AAATGAGCACCAAGGAAGTT-3′ 5′-AATCCCAGCACAAATGAAAA-3′
*GhTUB*4	5′-GAGATGGAGTTTACGGAGGCTGAG-3′ 5′-TCTAACACCCAAACAAGGTATTCAG-3′
*GhTUB*5	5′-GAGTACCAGCAATACCAGG-3′ 5′-AAGAAATCAATCCATCAAA-3′
*GhTUB*6	5′-GAGGAAGAGTACGAGGGGG-3′ 5′-AAAGGAACGAACAGAGCAG-3′
*GhTUB*7	5′-ACAGAAGCGGAAAGTAACA-3′ 5′-AATAAACAAGCCAAAGTGA-3′
*GhTUB*8	5′-ATCAGCAATACCAGGACGC-3′ 5′-AAACACACCAATAGCCACA-3′
*GhTUB*9	5′-AACTCTTCCTACTTCGTCG-3′ 5′-CCAAATCTTCCTCATCCTC-3′
*GhTUB*10	5′-GCTTTCTTGCACTGGTACAC-3′ 5′-TAATCGCCAAACTTCGCTCT-3′
*GhTUB*11	5′-TCACAGAAGCAGAGAGCAAC-3′ 5′-CAAAGGAACGAACAGAGCAG-3′
*GhTUB*12	5′-AAACCTTATTCCATTCCCTCG-3′ 5′-TCACCCTCCTGAACATCTCTTG-3′
*GhTUB*13	5′-CACAGAAGCAGAAAGCAACA-3′ 5′-ATACGAGCAAACCAGAAAAG-3′
*GhTUB*14	5′-ACGAGTATGAGGAAGGAGAG-3′ 5′-AATGCAGAGACACAAGAAAT-3′
*GhTUB*15	5′-TACAAAGCCTTAACTGTCC-3′ 5′-AACTCCATTTCATCCATCC-3′
*GhTUB*16	5′-GAATCTCATTCCCTTCCCC-3′ 5′-ACTGTTCACTCACACGCCT-3′
*GhTUB*17	5′-AAAACAAGAACTCATCCTAC-3′ 5′-ATACACATCAAAATAACTAA-3′
*GhTUB*18	5′-GAGGATTAGGAAGGAAAAAC-3′ 5′-ATGAAAAGCTCAAGAAAAAA-3′
*GhTUB*19	5′-CAGTTTGTGACATTCCACCCA-3′ 5′-TCCTAATCCTCGTACTCCGCT-3′
Gene	**Primers used for construction of all YCplac33-GhTUBs**
*GhTUB*1	5′-CCG*tctaga*ATGAGAGAAATCCTTCACATCCAA-3′ 5′-CCG*ctcgag*TTAAGCCTCTGCCTCGTATTCC-3′
*GhTUB*3	5′-CCG*tctaga*ATGAGAGAAATCCTCCATGTTCAA GCC-3′ 5′-CCG*ctcgag*TCAATTTTCCTCAGCTTCATCTTCA TAC-3′
*GhTUB*4	5′-CCG*tctaga*ATGCGTGAGATTCTTCATATTCAGGC-3′ 5′-CCG*ggtacc*CTATTCCTGGAGTTCCTCCTCCTCA-3′
*GhTUB*5	5′-CCG*tctaga*ATGAGAGAAATCTTGCACATCCAA GGT-3′ 5′-CCG*ctcgag*TCAAGCAGCTTCCTCTTCTTCTTCCT-3′
*GhTUB*7	5′-CCG*tctaga*ATGAGAGAAATCCTCCACGTTCA AGC-3′ 5′-CCG*ctcgag*TTAATTTTCCATTGCCTCATCTTC ATA-3′
*GhTUB*8	5′-CCG*tctaga*ATGAGAGAAATCCTTCACGTTCAAG-3′ 5′-CCG*ctcgag*CTAGTTCTCTTCCACACCTTCCTCC-3′
*GhTUB*9	5′-CCG*tctaga*ATGAGAGAGATCCTTCATGTTCA AGG-3′ 5′-CCG*ctcgag*TCACATGTGTTCTTCATCCAAATCT-3′
*GhTUB*10	5′-CCG*tctaga*ATGAGGGAAATCCTTCACGTACAAG-3′ 5′-CCG*ctcgag*TTACATCTCATGAACAGCTTCCTCG-3′
*GhTUB*12	5′-CCG*tctagag*ATGAGAGAAATCCTTCACATCCAA-3′ 5′-CCG*ctcgag*TTAAGCCTCTGCCTCGTATTCCT-3′
*GhTUB*16	5′-CCG*gctagc*ATGCGTGAAATCCTTCACATCC-3′ 5′-CCG*cccggg*TTAGTCTTGATACTCCTCCTCTTC CTC-3′

Sequence analysis

All cotton β-tubulin sequences were aligned by ClustalW (http://www.ebi.ac.uk). A neighbor-joining tree was constructed in MEGA3.1 (Kumar *et al*., 2004). A neighbor-joining bootstrap tree was constructed from the alignments of *GhTUB*s using Molecular Evolutionary Genetics Analysis (MEGA) software 3.1 with 1000 bootstrap replicates.

Results

Identification and cloning of 19 cotton TUB *genes*

A total of 795 putative ESTs containing cotton α-, β-, and γ-tubulin sequences were identified by aligning *Arabidopsis* tubulin cDNAs with 110 812 *G. hirsutum* ESTs. In accordance with their highly fibre-preferential expression pattern (Ji *et al*., 2003; Arpat *et al*., 2004; Shi *et al*., 2006; Gou *et al*., 2007), 1540 *UBQ* ESTs, 674 *E6* ESTs, 414 expansin ESTs, and 501 ESTs encoding lipid transfer proteins were identified. The tubulin ESTs were further clustered into 46 contigs. All 17 previously reported tubulin genes (Table 2; Dixon *et al*., 1994; Li *et al*., 2007) were represented in 26 contigs, implying that the assembly used in this study should reflect a relatively complete *G. hirsutum* tubulin family.

The poly(A) tail of each *GhTUB* was obtained by re-sequencing the cDNA insert in the library, and the 5′-untranslated regions of most *GhTUB* genes were obtained by 5′-RACE using the SMART RACE cDNA amplification kit (Clontech Laboratories, Inc.). Full-length cDNA was obtained by PCR amplification using primers that included 5′- and 3′-untranslated regions to ensure that the 5′-RACE products were not the result of cross-reactions of homologous transcripts derived from different subgenomes. All *GhTUB* cDNAs were cloned into the pGEM-T vector, and the sequences were verified by DNA sequencing from both directions. The open reading frames of *GhTUB*s were further amplified with proofreading *pfu* DNA polymerase using gene-specific primers (Table 1) and were cloned into URA3-marked YCplac33 behind the *ADH* promoter. YCplac33-*GhTUB* was verified by DNA

Table 2. *Renaming cotton TUBs and comparison with their former names*

Rename	cDNA gene accession no.	Former name
*GhTUB*1	AF484959 AF487511 AY345610	Tubulin β-1 (Tub1) β-Tubulin (TUB1) gene β-Tubulin 9
*GhTUB*3	AF521240	Xu-142 β-tubulin 1
*GhTUB*5	AY345607	β-Tubulin 5
*GhTUB*6	AY345608	β-Tubulin 6
*GhTUB*7	AY345609	β-Tubulin 7
*GhTUB*9	AY345606	β-Tubulin 3
*GhTUB*12	DQ023526	Cultivar CIM707 tubulin

sequencing from both directions using the CEQ dye terminator cycle sequencing quick start kit and the CEQ8000 analysis system (Beckman Coulter). Sixteen full-length tubulin cDNAs were obtained from the remaining 20 contigs, such that a total of 33 full-length cDNAs including 12 α-, 19 β-, and two γ-tubulin genes were isolated. Seven of these β-tubulin genes, including *GhTUB*1, 3, 5, 6, 7, 9, and 12, have been described previously (Table 2).

All 12 new *TUB* genes, designated as *GhTUB*2, 4, 8, 10, 11, 13, 14, 15, 16, 17, 18, and 19, were submitted to GenBank with accession numbers EU375992–EU376003. The predicted open reading frames ranged in size from 1335 bp to 1359 bp and shared 74–99% nucleotide identity (Fig. 1A). The putative GhTUB protein sequences varied from 444 to 452 amino acid residues in length and were highly conserved (85–99% overall sequence identity). There were large variations in the C termini, and there was an insertion of two to four residues at position 39 in the N termini of *GhTUB*6, *GhTUB*8, *GhTUB*11, and *GhTUB*13 when aligned with ScTUB2 (Fig. 1A). A phylogenetic tree of full-length *GhTUB*s is shown in Fig. 1B. The 19 *GhTUB*s were divided into four groups. *GhTUB*3 and *GhTUB*7 occupied a distinct branch that is basal to the clades containing the other *GhTUB*s. *GhTUB*9 and *GhTUB*10 formed one subgroup with the remaining 15 *GhTUB*s forming two sister subgroups. No δ-, ε-, ζ-, or τ-tubulins were found in the cotton assembly used in this study.

Nine of 19 GhTUB *genes are preferentially expressed in cotton fibre cells*

To identify *TUB*s differentially expressed in fibres, an *fl* mutant that fails to initiate fibre cells was used as a control

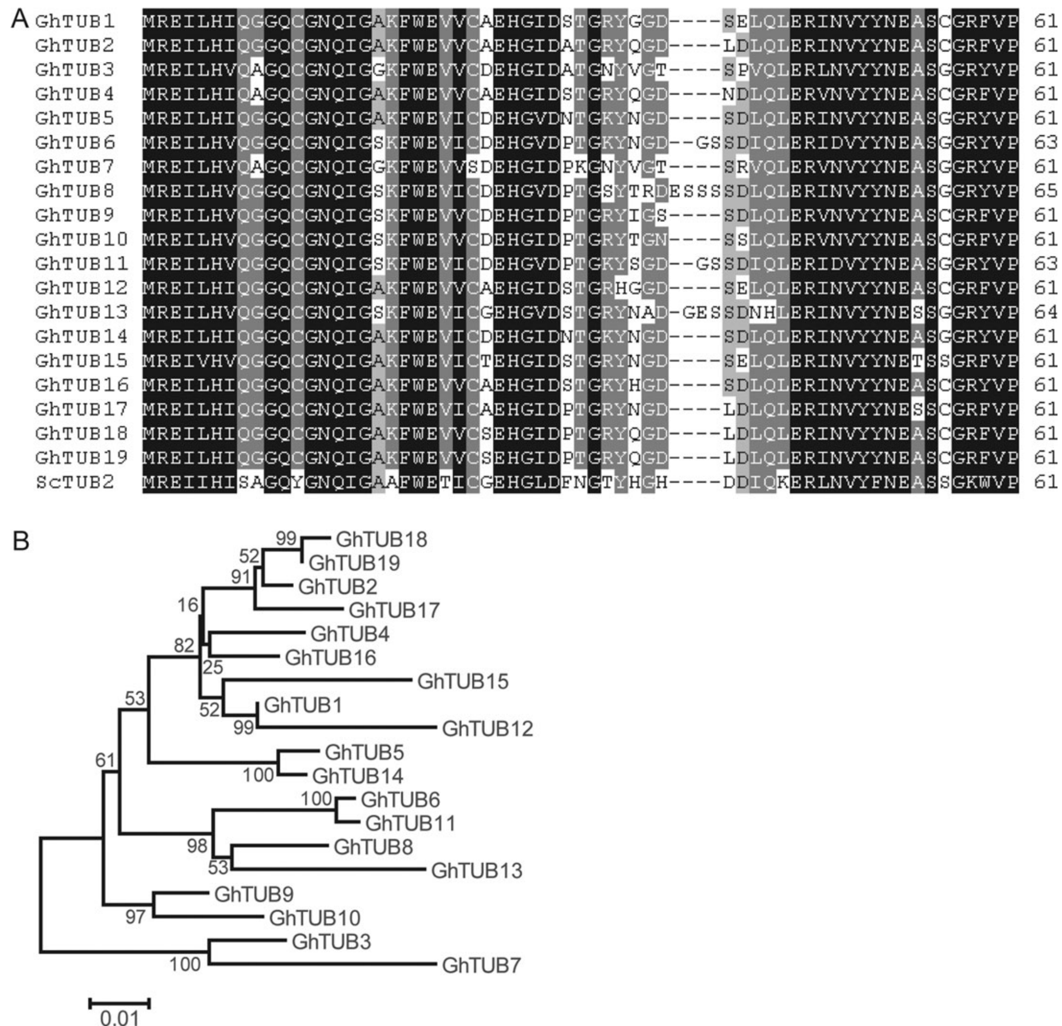

Fig. 1. Alignments and neighbor-joining tree of the predicted amino acid sequences encoded by cotton *GhTUB* genes. (A) Amino acid sequences at the N termini of 19 cotton TUBs are aligned. The conserved residues are shaded in black. (B) A neighbor-joining tree was constructed in MEGA3.1 from 1000 bootstrap replicates. The scale bar corresponds to 0.1 estimated amino acid substitutions per site. *GhTUB*1, *GhTUB*3, *GhTUB*5, *GhTUB*6, *GhTUB*7, *GhTUB*9, and *GhTUB*12 were from GenBank (respective accession numbers: AY345610, AY345606, AY345607, AY345608, AY345609, AF521240, DQ023526). The other *GhTUB*s were from this study.

(Ji *et al*., 2003; Shi *et al*., 2006). The transcript levels of all *GhTUB*s were examined by RT-PCR (Fig. 2). The transcripts of *GhTUB*3, 4, 7, and 9 predominantly accumulated in 10 dpa fibres stripped from ovules (Fig. 2A). The transcripts of *GhTUB*1, 5, 10, and 12 were detected at higher levels in 10 dpa wt fibres than in wt-0 and 10 dpa *fl* mutant ovules (Fig. 2A), whereas *GhTUB*8 transcripts were detected with similar expression levels in both 10 dpa wt fibres and *fl* mutant ovules (Fig. 2A). Low transcript levels were observed in roots, stems, and leaves for these *GhTUB*s, except that *GhTUB*3 was highly expressed in leaves (Fig. 2A). *GhTUB*16 transcripts were not detected in 10 dpa wt fibres but had a higher expression level in roots (Fig. 2A). The transcript levels of other *GhTUB*s did not vary between wt and *fl* mutants (Fig. 2B). These data suggest that *GhTUB*1, 3, 4, 5, 7, 8, 9, 10, and 12 are preferentially expressed in wt fibres, in which *GhTUB*16 is not expressed. The expression patterns of these *GhTUB*s in various fibre developmental stages were further determined by QRT-PCR (Table 3). The *GhTUB*1, 8, 9, and 12 transcripts reached peak expression levels of >1.0 at 10 dpa relative to that of the *GhUBQ*7 (Table 3). The *GhTUB*16 transcripts accumulated predominantly in 0 dpa ovules and rapidly decreased to low levels in later stages (Table 3), indicating that it is not a fibre-specific tubulin gene.

GhTUB *genes are up-regulated by phytohormones or very-long-chain fatty acids*

Plant hormones and lignoceric acid are important regulators of fibre development (Lee *et al*., 2007; Qin *et al*., 2007*a*). Exogenous treatment of wt ovules with ethylene, GA, BR, or C24:0 were performed to identify cotton *TUB*s regulated by phytohormones. QRT-PCR results showed that the expression of *GhTUB*1 or *GhTUB*3 was significantly increased 3-fold in 3 h to 5-fold in 24 h by

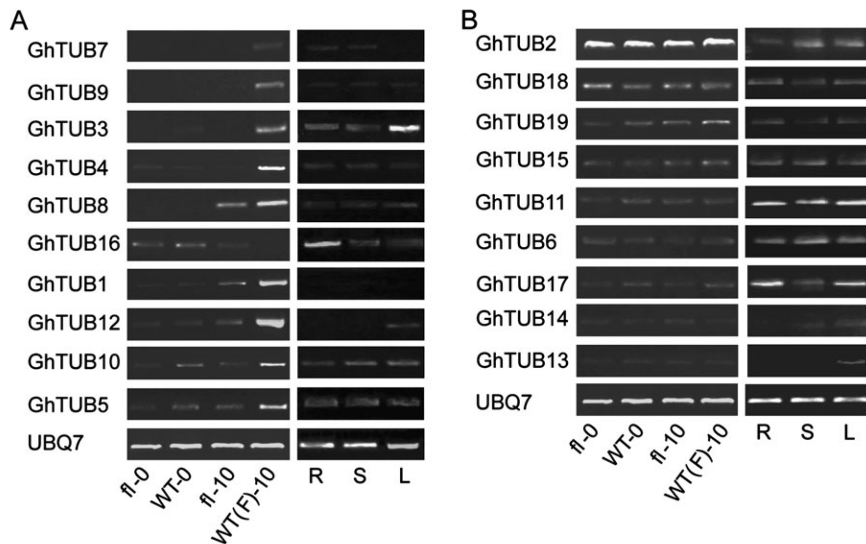

Fig. 2. RT-PCR analysis of 19 *GhTUB* transcripts in wild-type and *fl* mutants. (A) RT-PCR analysis of nine *GhTUB* transcripts expressed preferentially or differentially in fibres. (B) RT-PCR analysis of nine *GhTUB* transcripts that were not preferentially expressed in fibres. Dpa, Days post-anthesis; WT-0, 0 dpa wild-type ovules; WT(F)-10, 10 dpa wild-type fibres stripped from ovules; *fl*-0, 0 dpa *fl* mutant ovules; *fl*-10, 10 dpa *fl* mutant ovules; R, roots; S, stems; L, leaves. UBQ7 (GenBank accession no. AY189972) was included as a control.

Table 3. *Relative expression levels of* GhTUB *compared with* GhUBQ7 *by QRT-PCR during different cotton fibre growth stages*

The values (mean ±SD; n=3) for expression levels >0.5 relative to that of *GhUBQ*7 are in bold. In the upper section, *GhTUB*1, 9, and 8 with expression levels greater than 1.0 relative to that of *GhTUB*7. ND, Not detected; dpa, days post-anthesis.

	–3 dpa	0 dpa	5 dpa	10 dpa	15 dpa	20 dpa
*GhTUB*12	0.05±0.01	0.15±0.03	**1.66±0.11**	**5.54±0.16**	**5.25±0.25**	**1.25±0.25**
*GhTUB*1	0.08±0.01	0.08±0.04	0.32±0.08	**3.71±0.13**	**2.97±0.17**	**0.85±0.13**
*GhTUB*9	0.05±0.01	0.07±0.01	0.43±0.11	**1.89±0.16**	**1.45±0.12**	0.13±0.03
*GhTUB*8	ND	ND	0.25±0.03	**1.52±0.05**	**1.25±0.02**	**1.13±0.03**
*GhTUB*7	ND	ND	0.08±0.02	0.25±0.03	**1.13±0.06**	0.43±0.04
*GhTUB*16	**0.75±0.06**	**1.01±0.09**	0.42±0.04	0.04±0.01	0.03±0.00	ND
*GhTUB*3	0.03±0.01	0.08±0.03	0.25±0.02	**0.87±0.03**	**0.83±0.04**	**0.85±0.03**
*GhTUB*5	0.25±0.01	0.31±0.02	**0.82±0.03**	**0.86±0.03**	**0.76±0.03**	**0.71±0.03**
*GhTUB*10	0.26±0.01	0.46±0.02	**0.52±0.02**	**0.71±0.03**	0.46±0.03	0.37±0.03
*GhTUB*4	0.24±0.01	0.27±0.01	0.46±0.03	0.45±0.02	**0.54±0.02**	**0.56±0.02**

BR, whereas the expression of *GhTUB*9 and *GhTUB*12 transcripts increased in response to BR at 6 h and 24 h, respectively (Fig. 3). C24:0 induced the expression of *GhTUB*1, 3, and 12 with a similar pattern, starting at 3 h (Fig. 3). GA increased *GhTUB*1 and *GhTUB*3 transcript levels at 3 h and 24 h, respectively, and ethylene induced an ~2-fold increase in the expression of *GhTUB*5, 9, and 12 (Fig. 3). Non-fibre-specific *GhTUB*16 was up-regulated after BR treatment (Fig. 3).

Functional characterization of cotton TUB *genes in a yeast mutant*

Saccharomyces cerevisiae ScTUB2 encodes β-tubulin (Thomas *et al.* 1985; Reijo *et al.*, 1994). The yeast *tub2Δ* deletion mutant is lethal to cell growth (Thomas *et al.* 1985). To elucidate the essential biological function of the cotton *TUB* genes, the viability of the *tub2Δ* mutant cells complemented by individual *GhTUB*s was examined. The wt *ScTUB2* was transformed into the diploid W1536 *TUB2/tub2Δ* and two viable spores were dependent on the presence of *TUB2* for survival. The cells were unable to grow on medium containing FAA (Fig. 4A). The open reading frames of *GhTUB*s were separately cloned downstream of the *ADH* promoter in *URA3*-marked YCplac33 vectro, which were subsequently transformed into *tub2Δ* mutant cells carrying *ScTUB*2. The transformants were able to lose the pYADE4-ScTUB2 plasmid, as indicated by their survival on FAA-containing medium, confirming that *GhTUB*s, but not the plasmid backbone, were required for survival (Fig. 4B). The data showed that eight *GhTUB*s, excluding *GhTUB*6, 8, 11, and 13, and *GhTUA*9, encoding a cotton α-tubulin, were able to complement *tub2Δ*. These cotton *TUB*s were transcribed in yeast cells (Fig. 4B), indicating that the genetic complementation correlated with the cellular functions of *GhTUB*s. Amino acid sequence alignment of GhTUBs with ScTUB2 revealed that there are an additional two to four residues in the N termini of *GhTUB*6, 8, 11, and 13 (Fig. 1A), which may explain why they failed to rescue the lethality of *tub2Δ* (Fig. 4B).

Discussion

Higher plants have greater numbers of *TUA* and *TUB* genes compared with mammals (Sullivan, 1988). The present current study identified 12 new β-tubulin genes, bringing the total known *GhTUB*s to 19 (Fig. 1), which is comparable with the recently identified 20 *Pupulus TUB*s (Oakley *et al.*, 2007). The present findings suggest that there must be more *TUA*s than reported previously (Li *et al.*, 2007), as plant cells cannot tolerate an imbalance in the ratio of α-tubulin to β-tubulin within the cytoplasm (Anthony and Hussey, 1998).

Comprehensive QRT-PCR analyses showed that nine of 19 *GhTUB*s were highly expressed at 10–15 dpa (Table 3),

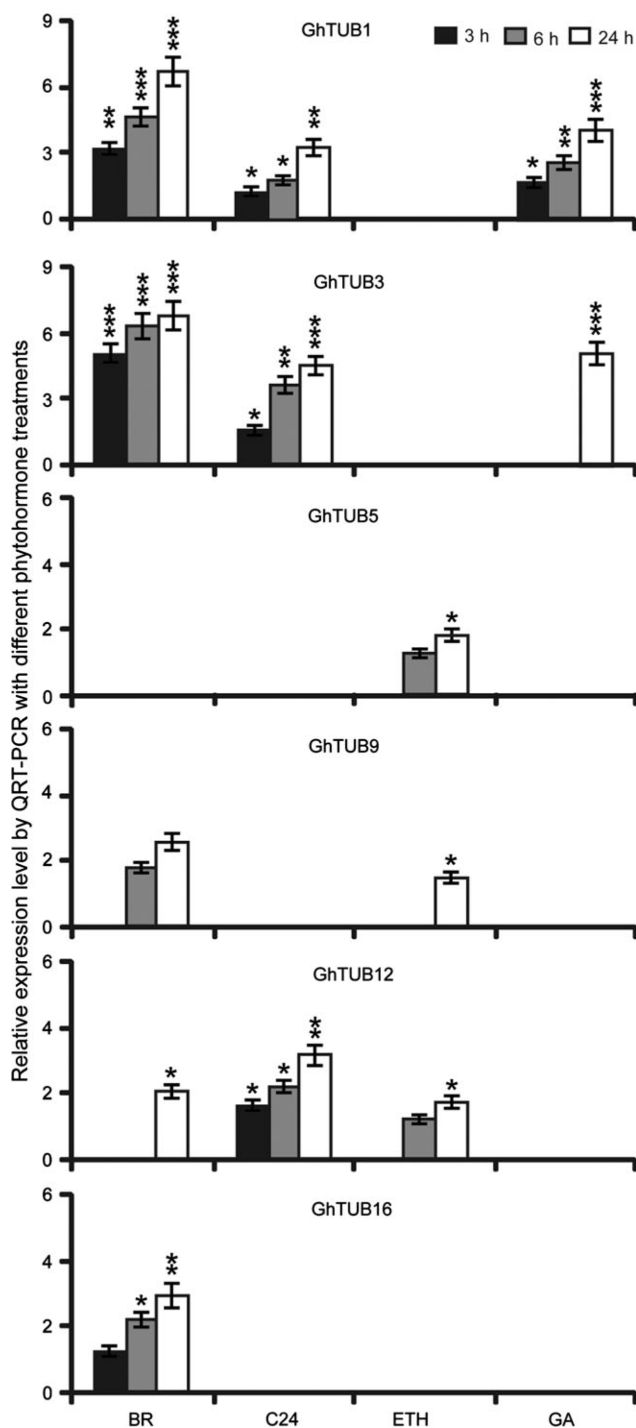

Fig. 3. Exogenous phytohormones and lignoceric acid promote the accumulation of *GhTUB* transcripts. RNA samples were prepared from three independent ovule cultures in the presence or absence of 5 μM brassinosteroid (BR), 0.1 μM ethylene (ETH), 5 μM gibberellin (GA), and 5 μM lignoceric acid (C24:0) for the indicated times. QRT-PCR experiments used the gene-specific primers reported in Table 1. The relative expression level of each *GhTUB* transcript is expressed relative to the level of the same transcript in the medium with or without ~0.8 μM methyl tert-butyl ether for lignoceric acid supplementation at each indicated time. Values <0.1 are not shown. Statistically significant differences were determined using one-way ANOVA combined with Tukey's test. ***, P <0.001; **, P <0.01; *, P <0.05.

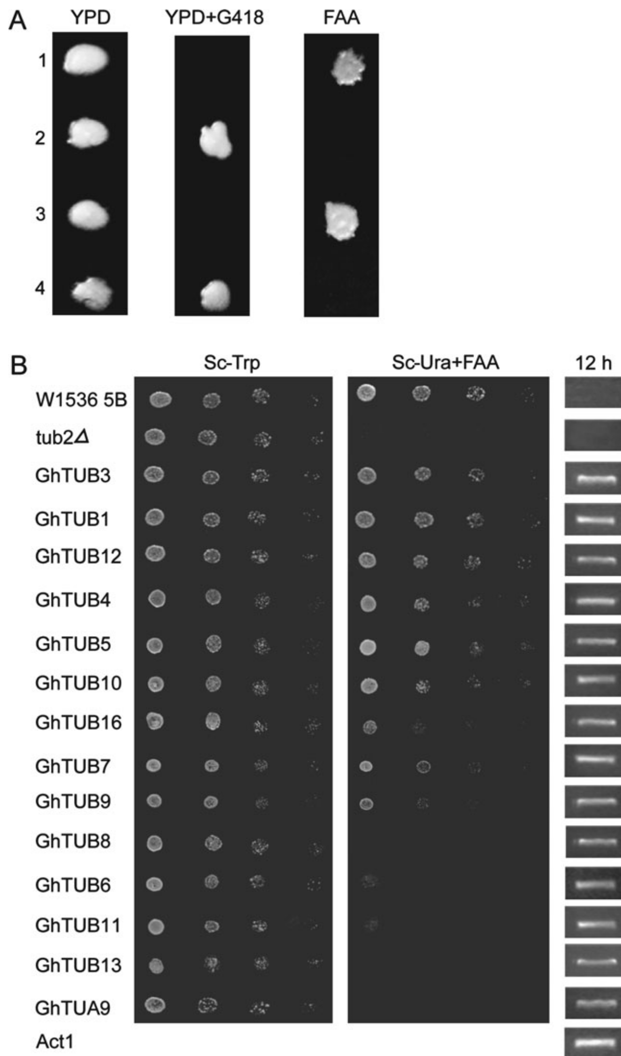

Fig. 4. Certain *GhTUB*s restore the viability of *S. cerevisiae tub2Δ* haploid cells. (A) Tetrad from diploid cells W1536 *TUB2*/*tub2Δ* transformed with pYADE4-ScTUB2. The ascospores grown on the YPD plate were replicated to G418 and FAA plates. (B) Complementation of *tub2Δ* mutant cells by individual *GhTUB*s. 12 h, RT-PCR analysis of yeast mutant cells expressing different *GhTUB*s harvested at 12 h from Sc-Trp culture medium. One *S. cerevisiae* actin gene (*Act*1) was used as the template control of RT-PCR analysis.

a period of fast elongation and primary cell wall synthesis, pointing to their roles in fibre development. Among these genes, *GhTUB*4, 8, and 10 were newly identified here, and the transcript level of *GhTUB*8 ranked third highest behind that of *GhTUB*1 and 12 (Table 3). *GhTUB*1, 5, 7, and 9 were highly expressed in fibre cells, as reported (Dixon *et al*., 1994; Ji *et al*., 2002; Li *et al*., 2002). Interestingly, the phylogenetic location of *GhTUB*3 and 7 is basal to the clade containing the other 17 *GhTUB*s (Fig. 1B), suggesting that *GhTUB*3 and 7 may have diverged early from the other *GhTUB*s. The *GhTUB*3/7, *GhTUB*9/10, and *GhTUB*1/12 pairs reside on distinct branches (Fig. 1B), implying that the genes encoding proteins that are preferentially expressed in fibres diverged during evolution. The expression levels of *GhTUB*s in developing fibre cells are in accordance with the need for fast assembly of microtubule arrays to meet rapid cellular expansion. Previous work also indicated that plant *TUB*s were expressed primarily in rapidly dividing cells or growing tissues such as root tips and elongating stems (Creelman and Mullet, 1991; Joyce *et al*., 1992).

The need for increased tubulin biosyntheses during the accelerated elongation process was verified by quantitative analysis of various *GhTUB* transcript levels after exogenous application of BR, ethylene, GA, and C24:0 to cultured cotton ovules. Interestingly, many *TUB*s, such as *GhTUB*1, 3, 9, 12, and 16 responded to BR treatment, whereas *GhTUB*1, 3, and 12 were also up-regulated by C24:0. Ethylene induced expression of *GhTUB*5, 9, and 12, and GA was only able to induce expression of *GhTUB*1 and 3 (Fig. 3), indicating that different chemicals promote fibre cell elongation via different mechanisms.

Plant cells contain ordered cortical microtubules, which may guide the movement of the cellulose synthase complex in the plasma membrane and regulate deposition of cellulose (Paredez *et al*., 2006). Tubulins are involved in microtubule assembly and function, and disruption of microtubule structure upon a reduction of α-tubulin expression causes abnormal cell expansion (Bao *et al*., 2001). Complementation of the *S. cerevisiae tub2Δ* mutant by cotton *TUB* genes (Fig. 4) provides evidence that these genes, functionally equivalent to *ScTUB2*, are essential for cell growth. Interestingly, *GhTUB*8 or the phylogenetically related *GhTUB*6, 11, and 13, which are poorly expressed in cotton fibres, contain amino acid insertions at position 39 (Fig. 1A) and were unable to complement the *S. cerevisiae tub2Δ* mutant (Fig. 4B). This region is important for interactions between the tubulin dimers (Chène *et al*., 1992), and mutations in this region are lethal to cells (Reijo *et al*., 1994). In *Populus*, additional residues at this position are also present in *TUB*19 and *TUB*20, which may be involved in pollen development (Oakley *et al*., 2007). These data suggest that the insertion region in plant *TUB*s may interfere with the tubulin–tubulin interactions in yeast cells but may perform some plant-specific functions. The existence of *TUB* genes in higher plants supports the notion that specialized tubulins are required for the growth and development of a plant cell and specifically for fibre cells and pollen tubes. Extensive arrays of microtubules are essential for the assembly of transversely oriented cellulose microfibrils to accommodate fast elongation.

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