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 TUBs (GhTUBs) are reported for the first time here. Transcription profiling revealed that nine GhTUBs 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 GhTUB1 and GhTUB3, ethylene induced expression of GhTUB5, GhTUB9, and GhTUB12, brassinosteroids induced expression of GhTUB1, GhTUB3, GhTUB9, and GhTUB12, and lignoceric acid induced expression of GhTUB1, GhTUB3, and GhTUB12. When GhTUBs were transformed into the Saccharomyces cerevisiae inviable mutant, tub2, which is deficient in β-tubulin, one ovule-specific and eight of nine fibre-preferential GhTUBs rescued this lethality. This study suggests that the proteins encoded by cotton GhTUBs 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., 2007a), 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...
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 and six TUBs are found in Zea mays (Montoliu et al., 1990; Villelmer et al., 2004), eight TUBs 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 are highly expressed in developing fibres (Dixon et al., 1994; Whitaker and Triplett 1999; Li et al., 2007). Among the seven TUBs previously reported, two accumulate in 20 d post-anthesis (dpa) fibre cells (Dixon et al., 1994). GhTUB1 and GhTUB9 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 TUBs newly identified by in-depth analysis of cotton ESTs. The regulation of TUBs 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., 2007a). A complementation analysis was performed using a non-viable yeast mutant to examine the function of all TUBs that are preferentially expressed in fibres as well as certain non-specifically expressed TUBs. 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 (ft) 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 ft 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., 2007b). As a control, S. cerevisiae TUB2 was amplified with forward primer 5′-cccgGGATCAGATGACTAACAC-3′ and reverse primer 5′-ggggGCTCGAGAAGTTAAGTTGTTG-3′ (lower-case letters indicate restriction sites for cloning), and cloned into TRP1-marked pYADE4 behind the same ADH promoter with Smal/ApaI 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 (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 |
|------|--------------------------------------------------------------------------------|
| GhTUB1 | 5'-CAGTGGTGCACTCCCTACAC-3' |
| GhTUB2 | 5'-TTTGTGCACTCCCTACAC-3' |
| GhTUB3 | 5'-AAATGACCAAGAAGAAGT-3' |
| GhTUB4 | 5'-GAGTGGATGAGAAGAAG-3' |
| GhTUB5 | 5'-GAGTGGAGAAGAAGAAG-3' |
| GhTUB6 | 5'-AAGGAGAGAAGAAGAAG-3' |
| GhTUB7 | 5'-ACAGAGAGAAGAAGAAG-3' |
| GhTUB8 | 5'-ACAGAGAGAAGAAGAAG-3' |
| GhTUB9 | 5'-AACTCTCCTCTCCTAC-3' |
| GhTUB10 | 5'-CCAACTCCTCTCCTAC-3' |
| GhTUB11 | 5'-TTGTTTACTGAC-3' |
| GhTUB12 | 5'-TAATCTGAACTTCTAC-3' |
| GhTUB13 | 5'-CACTGAGAAGAAGAAG-3' |
| GhTUB14 | 5'-ACAGGAGAAGAAGAAG-3' |
| GhTUB15 | 5'-AACTCTCACTTCACTAC-3' |
| GhTUB16 | 5'-AACTCTCACTTCACTAC-3' |
| GhTUB17 | 5'-ACTGTGCTTTCACACGC-3' |
| GhTUB18 | 5'-AAAACAAACTCAGTAC-3' |
| GhTUB19 | 5'-AGTGAAGCTCTGACCAAAAC-3' |

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

| Rename | cDNA gene accession no. | Former name |
|--------|------------------------|-------------|
| GhTUB1 | AF484995 | Tubulin β-1 (Tub1) |
| GhTUB2 | AF473151 | β-Tubulin (TUB1) gene |
| GhTUB3 | AF521240 | Xu-142 β-tubulin 1 |
| GhTUB5 | AF546070 | β-Tubulin 5 |
| GhTUB6 | AF546068 | β-Tubulin 6 |
| GhTUB7 | AF546069 | β-Tubulin 7 |
| GhTUB9 | AF546066 | β-Tubulin 3 |
| GhTUB12 | 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 GhTUB1, 3, 5, 6, 7, 9, and 12, have been described previously (Table 2).

All 12 new TUB genes, designated as GhTUB2, 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 GhTUB6, GhTUB8, GhTUB11, and GhTUB13 when aligned with ScTUB2 (Fig. 1A). A phylogenetic tree of full-length GhTUBs is shown in Fig. 1B. The 19 GhTUBs were divided into four groups. GhTUB3 and GhTUB7 occupied a distinct branch that is basal to the clades containing the other GhTUBs. GhTUB9 and GhTUB10 formed one subgroup with the remaining 15 GhTUBs 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 TUBs 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. GhTUB1, GhTUB3, GhTUB5, GhTUB6, GhTUB7, GhTUB9, and GhTUB12 were from GenBank (respective accession numbers: AY345610, AY345606, AY345607, AY345608, AY345609, AF521240, DQ023526). The other GhTUBs were from this study.
The values (mean ±SD; n=3) for expression levels >0.5 relative to that of GhUBQ7 are in bold. In the upper section, GhTUB1, 9, and 8 with expression levels greater than 1.0 relative to that of GhTUB7. ND, Not detected; dpa, days post-anthesis.

|     | –3 dpa  | 0 dpa      | 5 dpa      | 10 dpa     | 15 dpa     | 20 dpa     |
|-----|---------|------------|------------|------------|------------|------------|
| GhTUB12 | 0.05±0.01 | 0.15±0.03  | 1.66±0.11  | 5.54±0.16  | 5.25±0.25  | 1.25±0.25  |
| GhTUB1  | 0.08±0.01 | 0.08±0.04  | 0.32±0.08  | 3.71±0.13  | 2.97±0.17  | 0.85±0.13  |
| GhTUB7  | 0.03±0.01 | 0.07±0.01  | 0.43±0.11  | 1.89±0.16  | 1.45±0.12  | 0.13±0.03  |
| GhTUB7  | ND       | 0.25±0.03  | 1.52±0.05  | 1.25±0.02  | 1.13±0.03  |            |
| GhTUB16 | 0.75±0.06 | 1.01±0.09  | 0.42±0.04  | 0.04±0.01  | 0.03±0.00  | 0.43±0.04  |
| GhTUB3  | 0.03±0.01 | 0.08±0.03  | 0.25±0.02  | 0.87±0.03  | 0.83±0.04  | 0.85±0.03  |
| GhTUB4  | 0.25±0.01 | 0.31±0.02  | 0.82±0.03  | 0.86±0.03  | 0.76±0.03  | 0.71±0.03  |
| GhTUB4  | 0.26±0.01 | 0.46±0.02  | 0.52±0.02  | 0.71±0.03  | 0.46±0.03  | 0.37±0.03  |
| GhTUB4  | 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 GhTUB9 and GhTUB12 transcripts increased in response to BR at 6 h and 24 h, respectively (Fig. 3). C24:0 induced the expression of GhTUB1, 3, and 12 with a similar pattern, starting at 3 h (Fig. 3). GA increased GhTUB1 and GhTUB3 transcript levels at 3 h and 24 h, respectively, and ethylene induced an ~2-fold increase in the expression of GhTUB5, 9, and 12 (Fig. 3). Non-fibre-specific GhTUB16 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 GhTUBs 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 GhTUBs were separately cloned downstream of the ADH promoter in URA3-marked YCplac33 vector, which were subsequently transformed into tub2Δ mutant cells carrying ScTUB2. The transformants were able to lose the pYADE4-ScTUB2 plasmid, as indicated by their survival on FAA-containing medium, confirming that GhTUBs, but not the plasmid backbone, were required for survival (Fig. 4B). The data showed that eight GhTUBs, excluding GhTUB6, 8, 11, and 13, and GhTUA9, encoding a cotton α-tubulin, were able to complement tub2Δ. These cotton TUBs were transcribed in yeast cells (Fig. 4B), indicating that the genetic complementation correlated with the cellular functions of GhTUBs. Amino acid sequence alignment of GhTUBs with ScTUB2 revealed that there are an additional two to four residues in the N termini of GhTUB6, 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 GhTUBs to 19 (Fig. 1), which is comparable with the recently identified 20 Pupulus TUBs (Oakley et al., 2007). The present findings suggest that there must be more TUA 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 GhTUBs were highly expressed at 10–15 dpa (Table 3),
a period of fast elongation and primary cell wall synthesis, pointing to their roles in fibre development. Among these genes, \textit{GhTUB} 4, 8, and 10 were newly identified here, and the transcript level of \textit{GhTUB} 8 ranked third highest behind that of \textit{GhTUB} 1 and 12 (Table 3). \textit{GhTUB} 1, 5, 7, and 9 were highly expressed in fibre cells, as reported (Dixon \textit{et al.}, 1994; Ji \textit{et al.}, 2002; Li \textit{et al.}, 2002). Interestingly, the phylogenetic location of \textit{GhTUB} 3 and 7 is basal to the clade containing the other 17 \textit{GhTUBs} (Fig. 1B), suggesting that \textit{GhTUB} 3 and 7 may have diverged early from the other \textit{GhTUBs}. The \textit{GhTUB} 3/7, \textit{GhTUB} 9/10, and \textit{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 \textit{GhTUBs} 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 \textit{TUBs} were expressed primarily in rapidly dividing cells or growing tissues such as root tips and elongating stems (Creelman and Mullet, 1991; Joyce \textit{et al.}, 1992).

The need for increased tubulin biosyntheses during the accelerated elongation process was verified by quantitative analysis of various \textit{GhTUB} transcript levels after exogenous application of BR, ethylene, GA, and C24:0 to cultured cotton ovules. Interestingly, many \textit{TUBs}, such as \textit{GhTUB} 1, 3, 9, 12, and 16 responded to BR treatment, whereas \textit{GhTUB} 1, 3, and 12 were also up-regulated by C24:0. Ethylene induced expression of \textit{GhTUB} 5, 9, and 12, and GA was only able to induce expression of \textit{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 \textit{et al.}, 2006). Tubulins are involved in microtubule assembly and function, and disruption of microtubule structure upon a reduction of \(\alpha\)-tubulin expression causes abnormal cell expansion (Bao \textit{et al.}, 2001). Complementation of the \textit{S. cerevisiae} \textit{tub2}\(\Delta\) mutant by cotton \textit{TUB} genes (Fig. 4) provides evidence that these genes, functionally equivalent to \textit{ScTUB2}, are essential for cell growth. Interestingly, \textit{GhTUB} 8 or the phylogenetically related \textit{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 \textit{S. cerevisiae} \textit{tub2}\(\Delta\) mutant (Fig. 4B). This region is important for interactions between the tubulin dimers (Che`ne \textit{et al.}, 1992), and mutations in this region are lethal to cells (Reijo \textit{et al.}, 1994). In \textit{Populus}, additional residues at this position are also present in \textit{TUB} 19 and \textit{TUB} 20, which may be involved in pollen development (Oakley \textit{et al.}, 2007). These data suggest that the insertion region in plant \textit{TUBs} may interfere with the tubulin–tubulin interactions in yeast cells but may perform some plant-specific functions. The existence of \textit{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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