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RESEARCH PAPER

# GbTCP, a cotton TCP transcription factor, confers fibre elongation and root hair development by a complex regulating system

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## Abstract

**As the most important natural raw material for textile industry, cotton fibres are an excellent model for studying single-cell development. Although expression profiling and functional genomics have provided some data, the mechanism of fibre development is still not well known. A class I TCP transcription factor (designated GbTCP), encoding 344 amino acids, was isolated from the normalized cDNA library of sea-island cotton fibre (from –2 to 25 days post anthesis). *GbTCP* was preferentially expressed in the elongating cotton fibre from 5 to 15 days post anthesis. Some expression was also observed in stems, apical buds, and petals. RNAi silencing of *GbTCP* produced shorter fibre, a reduced lint percentage, and a lower fibre quality than the wild-type plants. Overexpression of *GbTCP* enhanced root hair initiation and elongation in *Arabidopsis* and regulated branching. Solexa sequencing and Affymetrix GeneChip analysis indicated that *GbTCP* positively regulates the level of jasmonic acid (JA) and, as a result, activates downstream genes (reactive oxygen species, calcium signalling, ethylene biosynthesis and response, and several NAC and WRKY transcription factors) necessary for elongation of fibres and root hairs. JA content analysis in cotton also confirmed that GbTCP has a profound effect on JA biosynthesis. *In vitro* ovule culture showed that an appropriate concentration of JA promoted fibre elongation. The results suggest that GbTCP is an important transcription factor for fibre and root hair development by regulating JA biosynthesis and response and other pathways, including reactive oxygen species, calcium channel and ethylene signalling.**

**Key words:** Affymetrix GeneChip, *Arabidopsis* root hair, cotton fibre, jasmonic acid, Solexa sequencing, TCP, transcription factor.

## Introduction

Cotton fibres are single-celled trichomes from individual epidermal cells on the outer integument of the ovules and provide the most important natural raw material for the textile industry. Fibre cells in commonly grown cultivars range in length from 22 to 30 mm, which is 1000–3000-times the diameter of the cells. Cotton fibre development consists of four distinctive but overlapping stages: initiation (from –3 to 3 days post anthesis [DPA]), elongation/primary cell-wall synthesis (2–20 DPA), secondary cell-wall synthesis (15–45 DPA), and drying and maturation (45–50 DPA) (Basra and Malik, 1984; Kim and Triplett, 2001). Previous studies indicate that fibre cells elongate via a diffuse-growth mode based on the observations that no organelle zonation and secretory vesicles accumulated in the tips of cotton fibre cells and that the cortical microtubules and newly deposited cellulose microfibrils were transversely oriented with respect to the growth axis in fibre cells (Seagull, 1990; Tiwari and Wilkins, 1995). A recent review suggests that fibre cells may expand via a linear-growth mode, which is the combination of

the tip-growth and diffuse-growth modes (Qin and Zhu, 2011). Experimental evidence supports the common linear cell-growth mode that mainly includes formation of a high $Ca^{2+}$ gradient, expression of vesicle transport protein, and the ethylene pathway. Ethylene, induced by very long chain fatty acids, has an important function in cotton fibre elongation by activating the pectin biosynthesis network (Qin and Zhu, 2011). Elongation of cotton fibres is also affected by other phytohormones. For many years, it was thought that indole-3-acetic acid (IAA) and gibberellin (GA) were required for fibre initiation and elongation in *in vitro* ovule culture (Beasley and Ting, 1973). Overexpression of the IAA biosynthetic gene *iaaM*, driven by an epidermis-specific promoter FBP7, resulted in a >15% increase in lint yield (Zhang *et al*., 2011). Enhanced GA production, by overexpression of *GhGA20ox1*, could promote fibre initiation and elongation in transgenic cotton (Xiao *et al*., 2010). Recent research on the impact of jasmonic acid (JA) on cotton fibre development by *in vitro* ovule culture proved that sustained high concentrations of JA inhibited fibre elongation; this inhibitory effect was apparent in a dose- and development stage-dependent manner (Tan *et al*., 2012).

In addition to the hormones, researchers have also focused on transcription factors, especially their roles in fibre initiation because of their key functions in plant development. Based on expressed sequence tag data, Yang *et al*. (2006) found that a percentage of putative transcription factors, such as MYB, WRKY, AP2/EREBP, C2H2, and bHLH families, might have critical roles in fibre cell initiation. Using cDNA microarray analysis, Wu *et al*. (2006) identified three important transcription factors (GhMYB25, GhMYB25-like, and GhHD1) as regulators of lint fibre initiation. Silencing of *GhMYB25* in cotton led to delayed fibre initiation, shorter fibres, and dramatically reduced trichome numbers on leaves, petioles, and petals, whereas overexpression of *GhMYB25* resulted in an increase of both cotton fibre initiation and leaf trichomes (Machado *et al*., 2009). Suppression of *GhMYB25-like* abolished fibre development on the seed as in the fibreless mutant but did not affect the development of trichomes elsewhere (Walford *et al*., 2011). Reduction in *GhHD1* transcripts delayed the timing of fibre initiation and reduced trichome formation, while overexpression of *GhHD1* increased fibre initiation but had no effect on leaf trichomes (Walford *et al*., 2012). In addition, other transcription factor genes (e.g., *GaMYB2*, *GhMYB109*, *GbML1*, and *GaHOX1*) have been implicated in the regulation of the early stage of cotton fibre development (Wang *et al*., 2004; Guan *et al*., 2008; Pu *et al*., 2008; Zhang *et al*., 2010). However, few transcription factors have been reported as regulating fibre cell elongation except for GhMADS11, a fibre-specific transcription factor that may function in fibre cell elongation based on its promotion of cell elongation of fission yeast (Li *et al*., 2011), although this remains to be verified in cotton. To investigate the transcriptional regulation of *Gossypium barbadense* fibre development, a normalized fibre cDNA library (from –2 to 25 DPA) of *G. barbadense* cv. 3–79 was constructed, in which a putative transcription factor GbBHLH (GbTCP) was identified (Tu *et al*., 2007).

TCP proteins are plant-specific transcription factors involved in multiple developmental pathways. The TCP family contains a conserved non-canonical basic helix-loop-helix (bHLH) domain, which is responsible for DNA binding and dimerization, and can be classified into two subfamilies (class I and II) based on the primary structure of the basic DNA binding domain (Cubas *et al*., 1999). The targeted DNA binding sequences of the two classes are distinct but overlapping: GGNCCCAC for class I and GTGGNCCC for class II (Kosugi and Ohashi, 2002), with an exception that class I TCP16 prefers the class II sequence because of the difference in residue 11 (Viola *et al*., 2012).

Class II TCP proteins regulate several aspects of plant development, such as branching, floral symmetry, and leaf development, mainly by inhibiting cell proliferation (Luo *et al*., 1996; Doebley *et al*., 1997; Nath *et al*., 2003). In contrast, class I TCP transcription factors appear to promote cell proliferation. AtTCP16 plays a crucial role in pollen development, and RNA interference (RNAi) leads to abortion of early pollen development (Takeda *et al*., 2006). Functional and microarray analysis showed that modified AtTCP20 caused severe phenotypic abnormalities by regulating cell expansion, division, and differentiation (Hervé *et al*., 2009). Loss of function of TCP11 resulted in smaller and curly leaves, shorter petioles, pedicels, and siliques, and a higher proportion of abnormal seeds and pollens (Viola *et al*., 2011). AtTCP14 activates embryonic growth during seed germination and influences internode length and leaf shape by promoting cell division (Tatematsu *et al*., 2008). And AtTCP15, which is closely related to AtTCP14, had overlapping functions in the regulation of leaf and inflorescence development (Kieffer *et al*., 2011). Two recent studies have revealed new regulatory pathways of AtTCP15. Li *et al*. (2012) reported that AtTCP15 was expressed in trichomes as well as in rapidly dividing tissues and vascular tissue, and that the protein promoted mitotic cell division but inhibited endo-reduplication by modulating the expression of several key cell-cycle genes. At the same time, a different conclusion was reached, specifically, that TCP15 suppressed the expression of boundary-specific genes, which were regulated by CIN-like class II TCP proteins, by affecting auxin homeostasis (Uberti-Manassero *et al*., 2012). Based on previous studies, the present study group speculated whether the TCP transcription factors regulate plant cell growth and proliferation with class I and class II members acting antagonistically as promoters or inhibitors and whether a certain overlap or redundancy is present in the control mechanism.

However, little is yet known about the roles of TCP proteins in cotton, especially in fibre development. GbTCP, as a class I TCP protein, was preferentially expressed in the fibre elongation stage. β-Glucuronidase (GUS) activity driven by the promoter of *GbTCP* could be detected in cotton fibres and in *Arabidopsis* leaf trichomes and root hairs. RNAi and overexpression strategies were applied to examine its function in cotton and *Arabidopsis* development. GbTCP had a major role in fibre and root hair development and plant architecture by regulating a complex pathway including JA biosynthesis and response.

## Materials and methods

### *Plant materials*

*Gossypium barbadense* cv. 3–79 and *Gossypium hirsutum* cv. YZ1 were used in this study. The cotton plants were cultivated in the field in

Wuhan, China, under normal farming conditions. Ovules and fibres were excised carefully from developing flower buds or bolls on selected days post anthesis and stored at –70 °C before use. Roots, stems, and leaves were collected from 15-day-old seedlings. *Arabidopsis thaliana* ecotype Columbia (Col-0) were grown at 20 °C under 16/8 light/dark conditions. For sampling, 2-week-old seedlings and 8-day-old roots were harvested.

### *Gene cloning, vector construction, and transformation*

The expressed sequence tag of *GbTCP* was isolated from a normalized cDNA library of *G. barbadense* 3–79 fibre at the elongation stage (Tu *et al*., 2007). The full-length sequence was obtained through the 5'-rapid amplification of cDNA end (5'-RACE) following the SMART RACE cDNA amplification kit user manual (Clontech, Terra Bella Ave. Mountain View, CA, USA) by using 10 DPA cotton fibre cDNA of *G. barbadense* 3–79 as the template. The gene-specific primers are listed in Supplementary Table S1 (available at *JXB* online). The open reading frame, molecular weight, isoelectric point, and conserved domain were predicted with ORF Finder at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the Compute pI/Mw tool (http://web.expasy.org/compute_pi/), and the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Phylogenetic analysis was performed with CLUSTAL X version 1.83 (Thompson *et al*., 1997) and MEGA4 (Tamura *et al*., 2007) by the neighbour-joining method. To isolate the *GbTCP* promoter, BD Genome Walker technology (Clontech) was performed according to the manufacturer's instructions. Gene-specific primers were designed for genome walking (Supplementary Table S1), and promoter prediction software TSSP (http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter) was used to predict the *GbTCP* transcription initiation site. The putative *cis*-elements were analysed using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo *et al*., 1999).

To construct the RNAi vector, a pair of primers with attB1 and attB2 adaptors was designed at the 300th and 400th nucleotides after the ATG of the *GbTCP* sequence (Supplementary Table S1). The PCR product was cloned into pHellsgate4 (Helliwell *et al*., 2002) according to the manufacturer's recommendations. The full length gene was amplified using primers OETCPF and OETCPR and cloned into pK2GW7,0 (Ghent University). The promoter of *GbTCP* was fused with the GUS reporter gene in pGWB433 (Research Institute of Molecular Genetics, Shimane University, Matsue, Japan). The expression vectors were introduced into *G. hirsutum* YZ1 plants and *Arabidopsis* ecotype Col-0 by *Agrobacterium tumefaciens* using strains EHA105 and GV3101, respectively (Clough and Bent, 1998; Jin *et al*., 2006).

### *Nucleic acid extraction and expression analysis*

Genomic DNA was extracted from the young leaves from both transgenic and wild-type cotton plants by the CTAB method with a plant genomic DNA kit DP305 (Tiangen Biotech, Beijing). The presence of the transgene was verified by amplification of *NPTII*. Total RNA was isolated as previously described (Liu *et al*., 2006), and was reverse transcribed to cDNA with the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time (qRT) PCR was performed using the ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA). The primers are listed in Supplementary Table S1. *GhUB7* and *AtACT2* were used as the housekeeping genes. Northern blotting analysis was performed to further confirm the differential expression, a total of 20 μg RNA was size separated by denaturing agarose gel electrophoresis and transferred onto nylon membrane (Millipore, Billerica, MA, USA). A 500-bp probe was prepared using the Prime-a-Gene labelling system (Promega, Madison, WI, USA). Procedures for hybridization and washing the membrane were as previously reported (Tu *et al*., 2007).

### *Histochemical assay of GUS activity*

Fresh tissue was collected from cotton and *Arabidopsis* plants and incubated into staining solution immediately at 37 °C for 12 hours and then washed with 75% ethanol successively. The staining solution was composed of 0.9 g $l^{-1}$ 5-bromo-4-chloro-3-indolylglucuronide, 50 mM sodium phosphate buffer (pH 7.0), 20% (v/v) methanol, and 100 mg $l^{-1}$ chloromycetin. The samples were examined and photographed with a stereomicroscope (Leica Microsystems, Germany) or a Nikon D40 camera (Japan).

### *Scanning electron microscopy and fibre quality measurement*

Ovules at –1, 0, and 1 DPA were collected from the same positions of cotton plants simultaneously and fixed in 2.5% (v/v) glutaraldehyde at 4 °C. The ovules were dehydrated in a series of ethanol dilutions from 30% to 100% at 15-minute intervals. The ethanol was replaced with isoamyl acetate/ethanol (1:1, v/v) and isoamyl acetate separately in 10 minutes at each step. After critical point drying and ion sputtering coating, the samples were viewed and photographed with a JSM-6390/LV scanning electron microscope (Jeol, Japan).

All mature fibres for quality measurement were collected from the bolls at the same positions on the plant at the same time. Initial fibre length measurements were obtained by the hand-combing method. Additionally, fibres and seeds were weighed after ginning, and the lint percentage (fibre weight/seed cotton weight) was determined. Mature fibre samples (>6 g each sample) were then sent to the Center of Cotton Fibre Quality Inspection and Testing, Chinese Ministry of Agriculture (Anyang, Henan province, China) for detailed quality measurement. Data were analysed by the Student t-test.

### *Observation and measurements of root hairs*

For root hair analysis, 8-day-old seedlings of *Arabidopsis* were grown upright on half-strength MS medium. Root hair length from the mature zone of root was measured. Five hairs per root and 20 roots per line (for a total of 100 root hairs) were scored. Digital images were taken with a differential interference contrast microscope (Zeiss, Japan) and analysed with ImageJ software (http://rsbweb.nih.gov/ij/). Data were analysed by the Student t-test.

### *Solexa sequencing and Affymetrix GeneChip analysis*

The 10 DPA fibre RNA from wild type (WT1 and WT2) and the RNAi-silenced cotton plants (ITCP3 and ITCP9) were used for Solexa sequencing. The RNA sequencing and data analysis were performed by Beijing Genomics Institute (Shenzhen, China) using the Illumina Genome Analyzer (Solexa). A sequence dataset collected from cotton unigenes from NCBI (http://www.ncbi.nlm.nih.gov/unigene/?term=txid3633[Organism:exp]) was used as the reference database. The method to identify and analyse the differentially expressed genes was the same as described previously by Xu *et al*. (2011).

Total RNA was isolated with the Spectrum plant total RNA kit (Sigma, St. Louis, MO, USA) from three independent biological replicates for each of *Arabidopsis* 8-day-old roots of the transgenic line 6–9 and wild-type control grown under identical conditions on half-strength MS medium. These samples were used to generate probes for hybridization to the Affymetrix ATH1 GeneChip (http://www.affymetrix.com). The analysis data were provided by Gene Tech Company (Shanghai, China). Gene ontology analysis was applied to predict gene function and calculate the functional category distribution frequency (http://bioinfo.cau.edu.cn/agriGO/analysis.php).

### *In vitro culture of cotton ovules*

Bolls were collected from cotton plants at 0 DPA (about 6:00 p.m.). The bolls were sterilized in 0.1% (w/v) $HgCl_2$ for 15 min and washed three times with sterile distilled water. Ovules were then removed from the bolls under sterile conditions and floated on liquid BT medium (0.5 μM GA3, 5 μM IAA) in the flask. For JA treatments, JA was added to the liquid BT medium at concentrations of 0.05, 0.1, 0.5, and 2.5 μM, respectively. The ovules were cultured in the dark at 30 °C (Beasley and Ting, 1973).

*Extraction and quantification of JA level*

JA was extracted according to the method described previously (Shindy and Smith, 1975). Samples (100–200 mg) were ground into powder and extracted twice with 80% cold methanol (v/v) overnight at 4 °C. The combined extract was evaporated to the aqueous phase with $N_2$, and then dissolved in 0.4 ml methanol and stored at –20 °C before measurement. JA was quantified using an Applied Biosystems 4000Q-TRAR high-performance liquid chromatography-mass spectometry system, with JA (Sigma) as the external standard. Three biological replicates were performed.

## Results

### GbTCP *was preferentially expressed in the elongating cotton fibres*

A plant-specific transcription factor *GbTCP* with a 1278-bp 5'-incomplete cDNA was isolated from a normalized cDNA library of *G. barbadense* 3–79 fibre (GenBank accession no. DQ912941). A putative full-length cDNA sequence of 1366 bp was cloned using 5'-RACE. This gene encoded a predicted polypeptide of 344 amino acids with a calculated molecular weight of 37.6 kDa and isoelectric point of 8.85. Genomic DNA sequences were cloned from *G. barbadense* 3–79 and *G. hirsutum* YZ1. Sequence alignment analysis confirmed that *GbTCP* did not contain any introns, and the sequences from *G. barbadense* 3–79 and *G. hirsutum* YZ1 showed nearly 100% homology. The deduced protein sequence had a conserved TCP domain composed of a 57-amino acid non-canonical bHLH motif is involved in DNA binding and protein–protein interactions. Phylogenetic analysis was used to investigate the evolutionary relationship among the GbTCP and other reported TCP proteins from eudicot *A. thaliana*, monocot *Oryza sativa*, *Antirrhinum majus*, and *Zea mays*. GbTCP was found to belong to class I TCP proteins and had the greatest similarity with AtTCP15 (Fig. 1A). Within the TCP

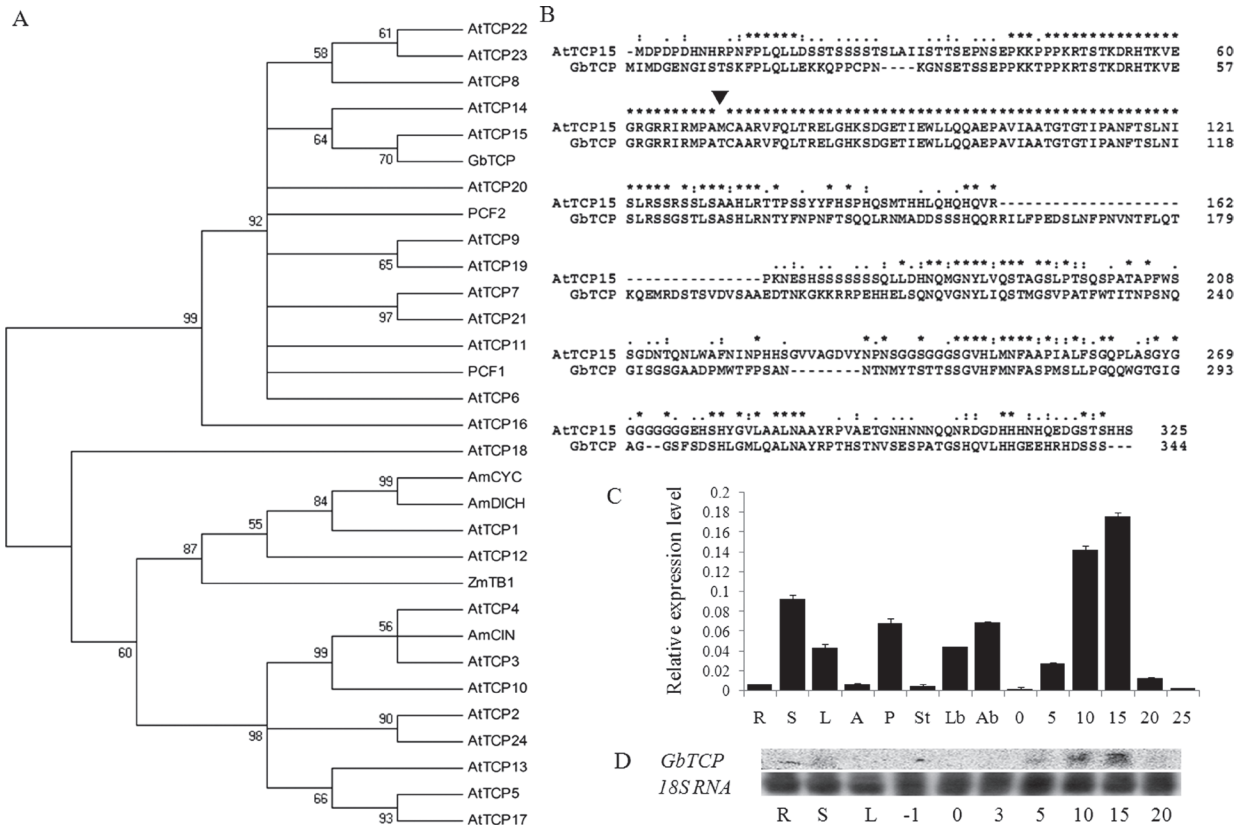


**Fig. 1.** Phylogenetic analysis of GbTCP and expression analysis. (A) Phylogenetic relationships between GbTCP and other reported TCP proteins, from the eudicot *Arabidopsis thaliana* (AtTCP1, At1g67260; AtTCP2, At4g18390; AtTCP3, At1g53230; AtTCP4, At3g15030; AtTCP5, At5g60970; AtTCP6, At5g41030; AtTCP7, At5g23280; AtTCP8, At1g58100; AtTCP9, At2g45680; AtTCP10, At2g31070; AtTCP11, At2g37000; AtTCP12, At1g68800; AtTCP13, At3g02150; AtTCP14, At3g47620; AtTCP15, At1g69690; AtTCP16, At3g45150; AtTCP17, At5g08070; AtTCP18, At3g18550; AtTCP19, At5g51910; AtTCP20, At3g27010; AtTCP21, At5g08330; AtTCP22, At1g72010; AtTCP23, At1g72010; AtTCP24, At1g30210) and some representative members of *Oryza sativa* (PCF1, BAA23142; PCF2, BAA23143), *Antirrhinum majus* (AmCYC, CAA76176; AmDICH, AAF12817; AmCIN, AAO43102), and *Zea mays* (ZmTB1, AAL66761). (B) Alignment of the amino acid sequences of GbTCP and AtTCP15; solid arrow points to the different amino acid in the conserved TCP domain. (C) Quantitative real-time PCR analysis of *GbTCP* in various cotton tissues, including root (R), stem (S), leaf (L), anther (A), petal (P), stigma (St), lateral bud (Lb), apical bud (Ab), and in fibres from 0 to 25 DPA (at 0 and 5 DPA, ovules with fibres; at 10, 15, 20, and 25 DPA fibres only); expression of *GbTCP* was calculated relative to *GhUB7* expression activity; error bars represent the standard deviation of three technical replicates. (D) Northern blotting analysis of *GbTCP* in cotton root (R), stem (S), leaf (L), and fibres from –1 to 20 DPA. 18S RNA was used as an endogenous standard.

domain, only one amino acid difference was apparent between the GbTCP and AtTCP15 proteins, at the junction of the basic region and the first helix. However, the sequences outside the TCP domain differed greatly (Fig. 1B).

The expression pattern was confirmed by qRT-PCR and Northern blotting. qRT-PCR showed that *GbTCP* was preferentially expressed in cotton fibre from 5 to 15 DPA. Moderate expression was seen in stems, apical buds, and petals. A low-level expression was detected in leaf and lateral bud but was undetectable in roots, anthers, and stigmas (Fig. 1C). The result of Northern blotting was consistent with qRT-PCR, and expression was undetectable in initiating fibres but increased and remained high during fibre elongation stage and decreased at the late elongation stage. Some expression was also observed in stems, but not in roots or leaves (Fig. 1D).

To further elucidate the expression profile of *GbTCP*, a 2437-bp promoter region was isolated from *G. barbadense* 3–79. A putative transcription initiation site at 441 bp upstream of the translation start codon was predicted; typical TATA and CAAT boxes were present within the promoter fragment. In addition, several putative *cis*-acting regulatory elements were predicted, including ATHB recognition site (CAATSATTG), MYC responsive element (CANNTG), MYB binding site (YAACKG), AuxRR-core *cis*-acting regulatory element involved in auxin responsiveness (GGTCCAT), ethylene responsive element (AWTTCAAA), and many circadian *cis*-acting regulatory elements involved in circadian control (CAANNNNATC) (Supplementary Table S2), implying that the *GbTCP* promoter may function under complex regulation.

The 2437-bp promoter was fused to the GUS reporter gene and transformed into cotton and *Arabidopsis*. The promoter activity was analysed by histochemical staining and GUS activity measurements. The *PGbTCP::GUS* construct in transgenic cotton was expressed predominantly in elongating fibres from 2 to 25 DPA and peaked at 15 DPA (Fig. 2A). No expression was detected in the ovules without fibres, but a moderate expression was detected in stems. GUS activity could also be detected in cotyledons and roots of 2-day-old seedlings. No expression was seen in leaves, apical buds, petals, and flower organs. The wild-type control did not show GUS staining in all tissues (data not shown). Quantitative results of GUS protein were consistent with the GUS staining (Fig. 2B).

In transgenic *Arabidopsis*, strong GUS activity was detected in trichomes of rosette leaves and roots. No expression was seen

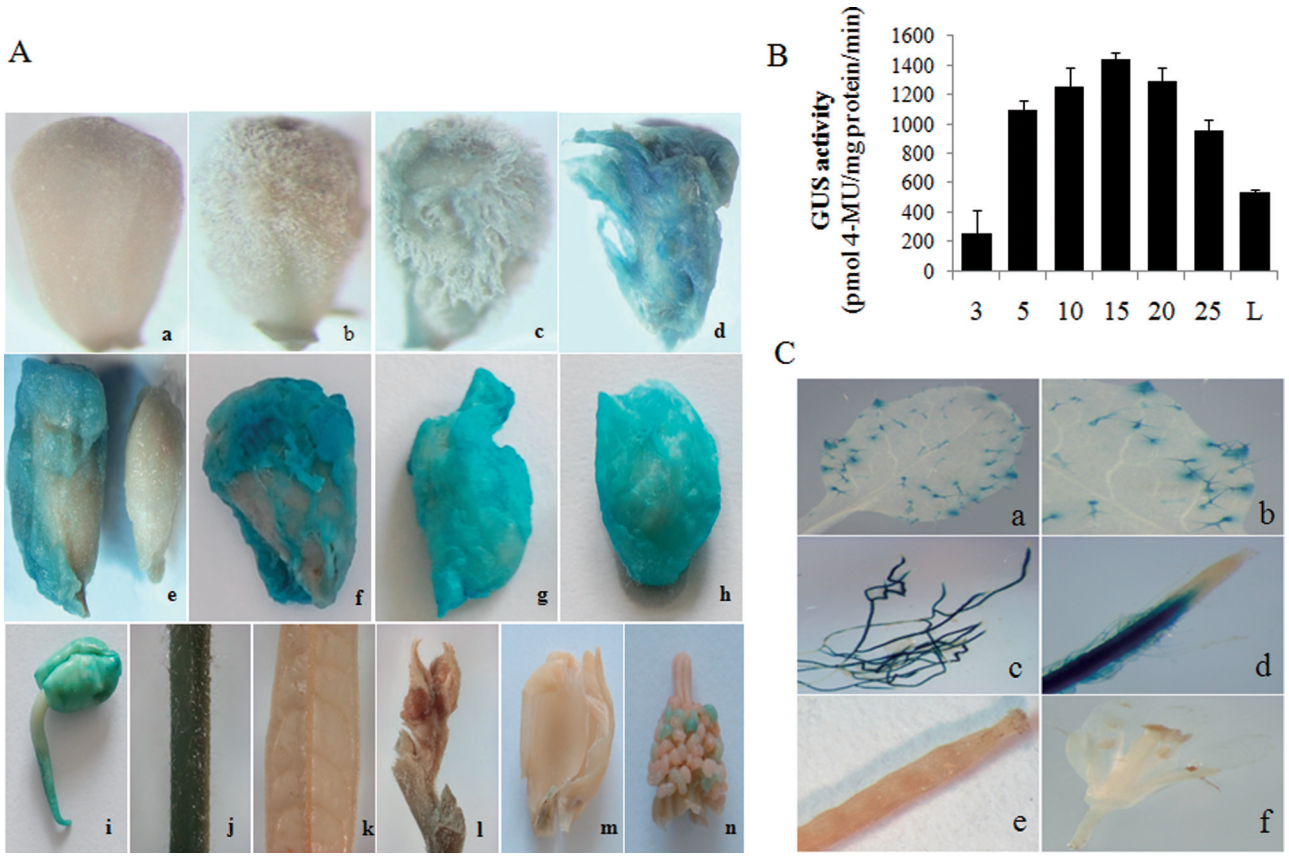


**Fig. 2.** The GUS analysis driven by *GbTCP* promoter in cotton and *Arabidopsis*. (A) Histochemical analysis of GUS activity in transgenic cotton plants: (a–h) ovules with fibres at 0, 1, 2, 5, 10, 15, 20, and 25 DPA, respectively, except for (e) (left, with fibres; right, without fibres); (i–n) 2-day-old seedlings, stems, leaves, apical buds, petals, and pistils and stamens, respectively. (B) Quantitative analysis of GUS activity during fibre elongation and in the cotton leaf: 3 and 5 represent ovules with fibres at 3 and 5 DPA; 10–25 represent fibres alone collected from 10–25 DPA ovules; L is cotton leaf; error bars represent standard deviation of three technical replicates. (C) Histochemical analysis of GUS activity in transgenic *Arabidopsis*: (a) rosette leaf; (b) magnification of a; (c) roots; (d) magnification of c; (e) silique; (f) flower.

in the root meristem zone, but strong expression was observed in other parts of the roots including vascular tissues, epidermal cells, and root hairs in both primary and lateral roots. GUS activity was not detected in siliques and flowers (Fig. 2C), and these results indicated that *GbTCP* is a specific gene expressed in single-celled trichomes and root hairs in different species.

### *RNAi silencing of* GbTCP *resulted in shorter fibres*

A 35S promoter-driven RNAi construct was introduced into cotton, and 40 independent transgenic lines ($T_0$) were produced. PCR and Southern blotting analysis were performed to select the positive transgenic lines and detect their inserted copy numbers. The transcript level was examined in 10 DPA cotton fibre by qRT-PCR and Northern blotting. A total of five positive transgenic lines (ITCP2, ITCP3, ITCP9, ITCP10, ITCP18) with low copy number (Supplementary Fig. S1A) were selected for further analysis. The expression level of *GbTCP* was significantly decreased in these RNAi-silenced lines compared with the wild-type plants (Fig. 3A and 3B). Detailed phenotype and molecular analysis was performed on the $T_2$ and $T_3$ transgenic lines. Scanning electron microscopy of ovules at –1, 0, and 1 DPA

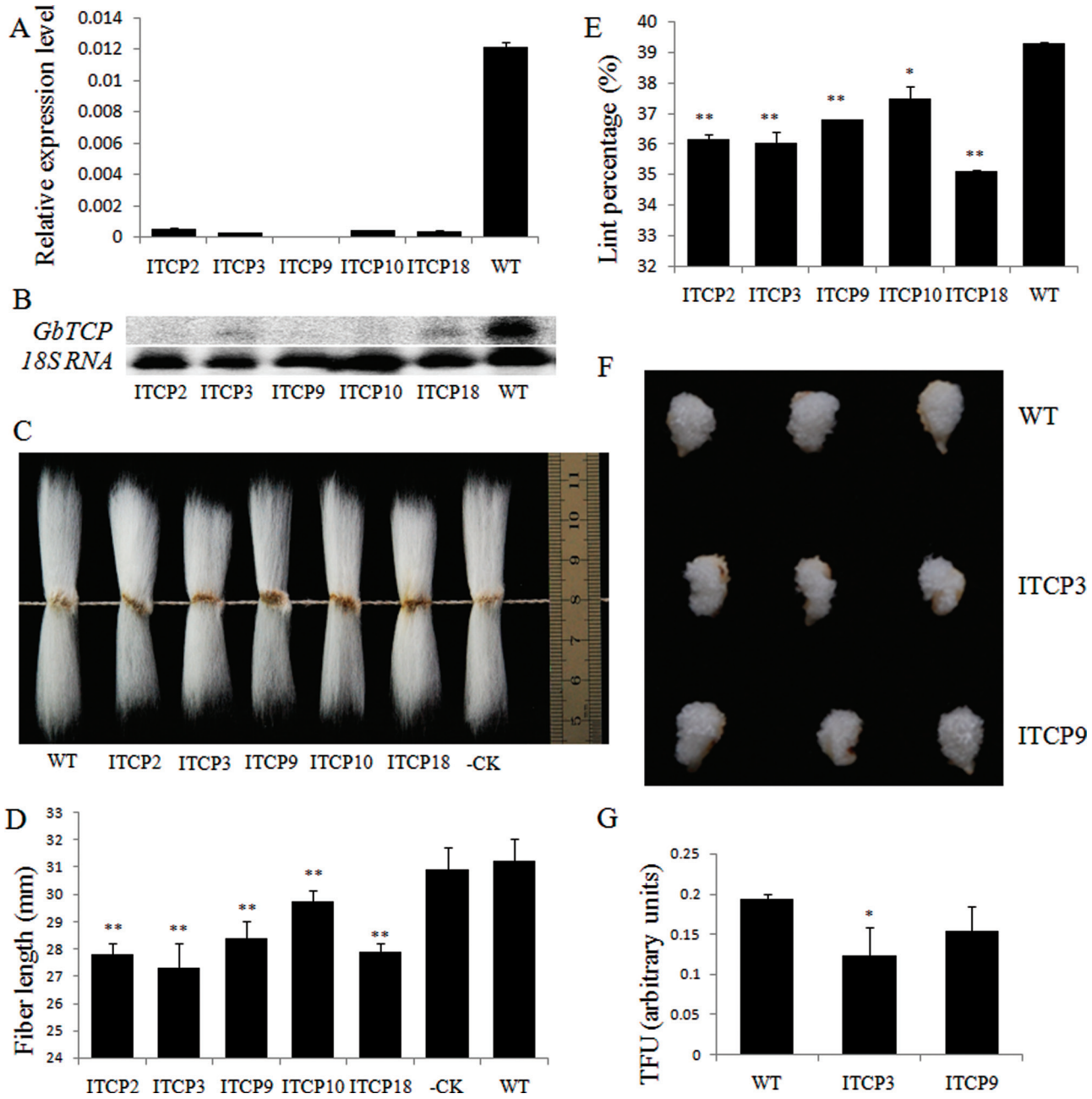


**Fig. 3.** Downregulation of *GbTCP* affects fibre elongation and lint percentage. (A) Quantitative real-time PCR analysis showed that the expression of *GbTCP* was downregulated in the RNAi lines; expression of *GbTCP* was calculated relative to *GhUB7* expression activity; error bars represent the standard deviation of three technical replicates. (B) Northern blotting analysis of *GbTCP* in RNAi lines and wild-type cotton plants; 18S RNA was used as an endogenous standard. (C) RNAi lines had shorter fibre than wild-type cotton plants.

showed no visible difference at the fibre initial stage between the silenced and wild-type plants (Supplementary Fig. S1C). Silencing of *GbTCP* led to significantly shorter mature fibres (27.3±0.90 mm to 29.8±0.41 mm) compared with the control (31.3±0.78 mm) (Fig. 3C and 3D). Lint percentages were 36.2, 36.0, 36.8, 36.5, and 35.1% for transgenic lines ITCP2, ITCP3, ITCP9, ITCP10, and ITCP18, respectively, which were much lower than that for the wild-type plants (39.3%) (Fig. 3E) and a 6.4–10.7% decrease in lint yield. The studies were repeated for 2 years with two generations and the same results were obtained (data not provided). More detailed fibre qualities (Table 1) were measured at the Center of Cotton Fibre Quality Inspection and Testing, Chinese Ministry of Agriculture (Anyang, Henan province, China). In the RNAi lines, the fibre length was notably decreased (28.95±1.11, 28.81±0.59, and 28.65±0.29 mm for ITCP3, ITCP9, and ITCP18, respectively), which was consistent with the initial measurements. Fibre fineness was worse in silenced lines, the micronaire values of the fibres were considerably higher in the transgenic lines (5.70±0.14, 6.03±0.11, and 5.87±0.06 for ITCP3, ITCP9, and ITCP18, respectively) than the wild-type (5.13±0.12) or null segregant plants (5.20±0.42). Fibre elongation was lower in RNAi-silenced lines (5.95±0.07, 6.03±0.06, and 6.00±0.00 for ITCP3, ITCP9, and ITCP18, respectively) than in null segregant (6.15±0.07) and wild-type plants (6.13±0.06). However, no significant alterations in strength and fibre uniformity were observed in the transgenic lines. To further confirm the effect of GbTCP on cotton fibre development, the changes of fibre growth during the fibre elongation stage by *in vitro* ovule culture were observed. The total fibre unit was measured (Beasley and Ting, 1973) after being cultured at 30 °C in darkness for 10 days. As shown in Fig. 3F and 3G, fibre growth of the RNAi-silenced lines (ITCP3 and ITCP9) was significantly reduced compared with the wild-type plants, which is consistent with the shorter mature fibres in these lines. All the silenced lines did not show changes on the trichomes on stems (Supplementary Fig. S1B), vegetative growth, and flower development (data not shown). In summary, suppression of *GbTCP* expression resulted in shorter fibres and lower fibre quality possibly by affecting fibre elongation at the rapid elongation stage.

### *Upregulation of* GbTCP *in cotton affected plant architecture, fibre, and trichome development*

To identify the role of GbTCP involved in cotton fibre elongation, the full-length *GbTCP* coding region driven by 35S promoter was introduced into *G. hirsutum* YZ1. Fifteen independent overexpression lines were produced, but only two lines were able to produce flowers. These two lines (OTCP7 and OTCP11) showed an increased expression level compared with the wild-type plant, with one and two copies, respectively (Supplementary Fig. S2A and B). Upregulated expression of *GbTCP* caused abnormal morphology on plant height and branching and a low rate of fruit setting. The bolls from OTCP11 with higher *GbTCP* expression levels withered and did not produce seeds (Supplementary Fig. S2C and D). Line OTCP7 was screened through subsequent generations to produce homozygous $T_2$ plants for analysis. The *GbTCP* transcript level in the OTCP7 line was more than double that in the wild type in 10 DPA fibres (Fig. 4D). Overexpression of *GbTCP* had a significant impact on apical dominance in cotton plants. For example, the plant height was reduced by 36.7% in OTCP7 line (70.9±5.80 cm) compared with wild-type plants (112.0±6.55 cm; Fig. 4A and 4E), whereas the lateral shoots increased by 70% from 10±1.58 to 17±1.51 (Fig. 4F). *GbTCP* overexpression also led to fewer trichomes on the stems, smaller flowers, a longer pedicel, and more buds (Fig. 4B, 4C, and 4G). The transgenic cotton also had significantly smaller bolls and shorter mature fibres compared with the wild type (Fig. 4H and 4I). Scanning electron microscopy of the epidermal surface of

**Table 1.** Comparison of cotton fibre quality parameters between RNAi lines and wild-type plants

| Plant line | Fibre length (mm) | Fibre strength (cN/tex) | Micronaire value | Fibre uniformity (%) | Fibre elongation (%) |
|---|---|---|---|---|---|
| ITCP3 | 28.95±1.11 | 27.43±1.42 | 5.70±0.14* | 85.60±0.36 | 5.95±0.07* |
| ITCP9 | 28.81±0.59* | 26.67±0.58 | 6.03±0.11** | 86.33±0.15 | 6.03±0.06 |
| ITCP18 | 28.65±0.29** | 26.67±0.51 | 5.87±0.06** | 85.83±0.51 | 6.00±0.00* |
| –CK | 30.70±1.15 | 27.20±0.20 | 5.20±0.42 | 85.37±1.27 | 6.15±0.07 |
| WT | 30.60±0.50 | 27.50±0.26 | 5.13±0.12 | 85.97±0.64 | 6.13±0.06 |

Fibre samples were harvested from field-grown $T_3$ RNAi-silenced transgenic cotton plants, corresponding segregating null and wild-type cotton plants for measurement. Values are mean ± standard deviation of assays for samples of three individual plants from each line. –CK, null segregant; WT, wild type. Significant differences between silenced or null control and wild-type plants were determined by Student t-test analysis: *$P < 0.05$; **$P < 0.01$.

(D) Measurement of mature fibre length in $T_3$ RNAi lines and wild-type plants; error bars represent standard deviation of 10 measurements; asterisks indicate statistically significant differences between transgenic lines and wild-type plants, as determined by the Student t-test (*$P < 0.05$; **$P < 0.01$). (E) Lint percentage of $T_3$ RNAi lines and wild-type plants; error bars represent standard deviation of three or more samples (100 seeds per sample). (F) Photograph of ovules cultured for 10 days from wild type and two RNAi lines (ITCP3 and ITCP9). (G) Measurement of fibre surface area by dye binding in total fibre units (TFU) from wild type and two RNAi lines (ITCP3 and ITCP9); error bars represent the standard deviation of three replicates; asterisks indicate statistically significant differences between transgenic lines and wild-type plants, as determined by the Student t-test (*$P < 0.05$; **$P < 0.01$).

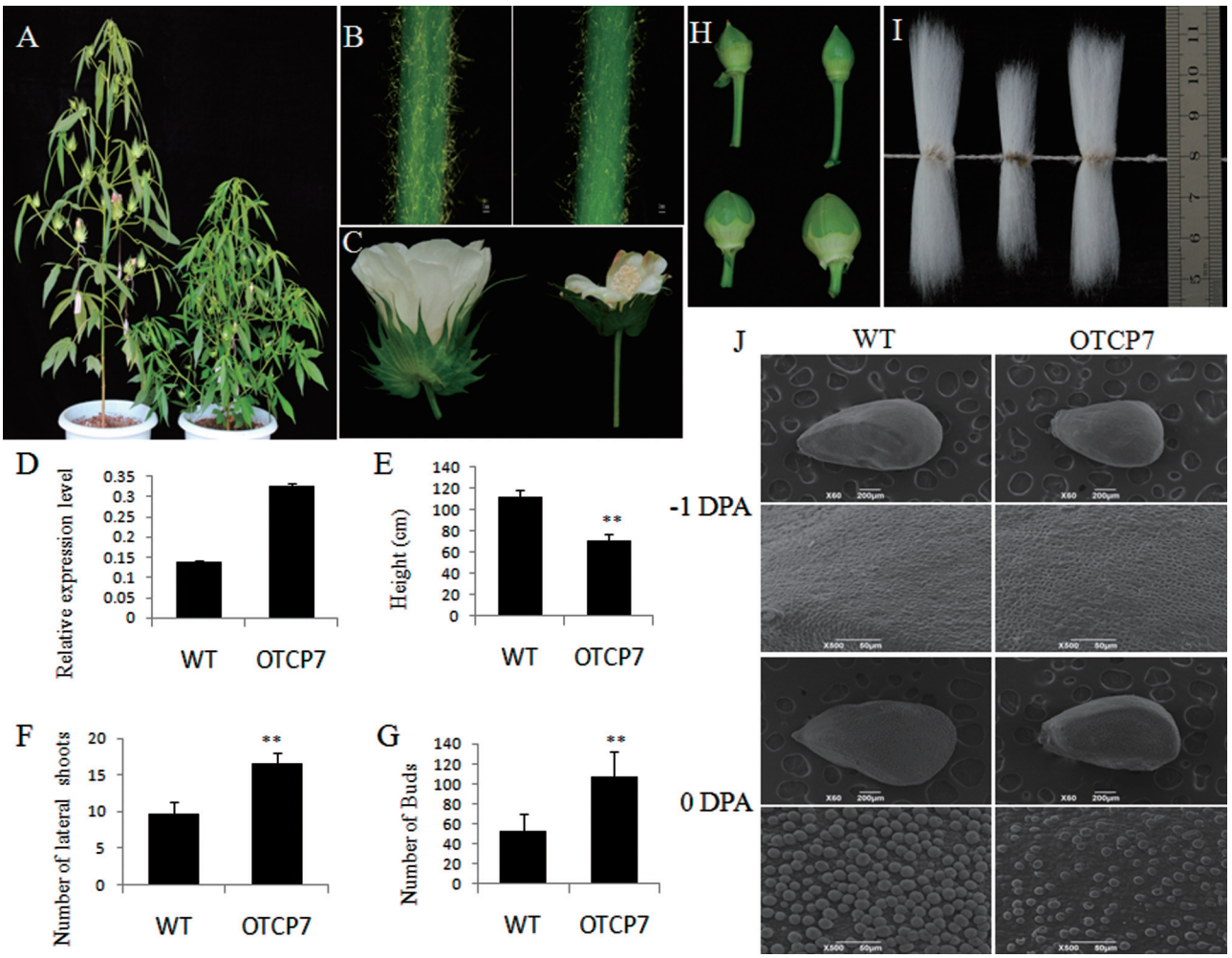


**Fig. 4.** Morphological alterations as a result of *GbTCP* upregulation in cotton. (A) Upregulation of *GbTCP* results in reduced plant height, more lateral shoots, and reduced apical dominance in OTCP7 (right) compared to wild type (left). (B) Upregulation of *GbTCP* leads to fewer trichomes on the stems of OTCP7 (right) compared to wild type (left). (C) Upregulation of *GbTCP* results in smaller flowers on the TCP7 transgenic line (right) compared to wild type (left). (D) Quantitative real-time PCR measurements of *GbTCP* transcripts in 10 DPA fibres from OTCP7 transgenic cotton line and wild-type plants relative to *GhUB7* expression level; error bars represent the standard deviation of three technical replicates. (E–G) Statistical analysis of plant height, lateral shoots, and flower buds, respectively of 3-month-old plants; error bars represent the standard deviation of 10 replicates. (H) Photograph of 5 DPA bolls from OTCP7 plants (top) and wild type (bottom). (I) Photograph of mature fibres from wild type (left), OTCP7 (middle), and null transgenic plant (–CK) (right). (J) Scanning electron micrographs of –1 DPA and 0 DPA ovules from OTCP7 and wild-type plants; the images were taken at a similar position in the middle of ovules; rows 2 and 4 are magnified frames of rows 1 and 3; bars: 200 μm (rows 1 and 3) and 50 μm (rows 2 and 4).

randomly selected ovules showed a delay and lower numbers of fibre initiation in transgenic line than in wild-type control ovules at –1 and 0 DPA (Fig. 4J).

### *Overexpression of* GbTCP *in* Arabidopsis *promoted root hair initiation and elongation*

Overexpression of *GbTCP* in cotton led to abnormal morphology, which made it difficult to obtain progeny for further analysis. Therefore, this study generated transgenic *Arabidopsis* overexpressing *GbTCP*. Six independent homozygous lines with a single copy insertion were obtained (Fig. 5A). Homozygous $T_3$ plants of OETCP6-9 (high expression level), OETCP24-10 (intermediate expression level), and OETCP21-1 (transgenic line without expression change) were used for subsequent analysis. Overexpression of *GbTCP* resulted in more branches, late ripening, and narrower leaves as observed in cotton (Fig. 5B–D). The length of siliques were significantly shorter in line 6-9 (10.2±1.49 mm) and 24-10 (13.1±0.35 mm) than the siliques in line 21-1 (14.5±0.35 mm) and wild-type plants (14.4±0.86 mm) (Fig. 5E and 5G). Ectopic overexpression of *GbTCP* in *Arabidopsis* also significantly enhanced root hair initiation and elongation. The length of root hairs was increased by 69.7 and 44.9% in transgenic lines 6-9 (866.5±51.41 μm) and 24-10 (739.9±30.26 μm), respectively, compared with the wild-type plants (510.7±47.03 μm) (Fig. 5F and 5H). The development of lateral roots was significantly reduced with the increase of expression of *GbTCP* (3.4±0.49, 8±1.02, 13±0.63,

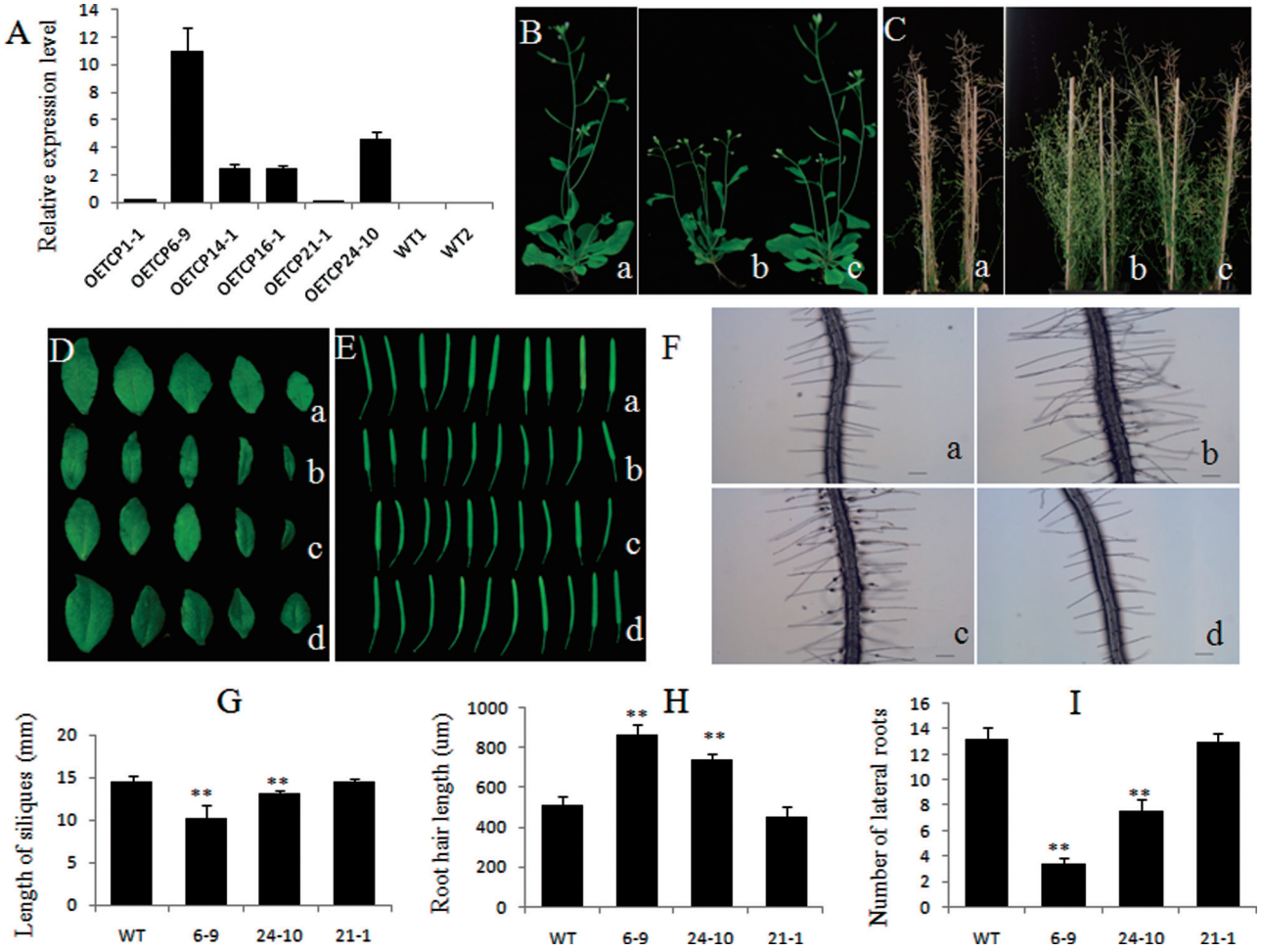


**Fig. 5.** Morphological alterations of the *Arabidopsis* transgenic plants overexpressing *GbTCP*. (A) Quantitative real-time PCR analysis for transgenic lines and wild-type plants; expression of *GbTCP* was calculated relative to *AtACT2* expression activity; error bars represent the standard deviation of three technical replicates. (B) Photographs of 6-week-old wild-type (a) and transgenic plants 6-9 (b) and 24-10 (c); the transgene promoted branching and reduced plant height. (C) Photographs of 3-month-old wild-type (a) and transgenic plants 6-9 (b) and 24-10 (c); the transgene delayed whole-plant senescence. (D) Cauline leaves from different developmental stages; rows a–d are wild type, 6-9, 24-10, and 21-1, respectively. (E) The sixth siliques from the main stem; rows a–d are wild type, 6-9, 24-10, and 21-1, respectively. (F) Root hairs in the primary roots of transgenic lines 6-9 (b) and 24-10 (c) showed an increase of root hair length and amount compared with that of wild type (a) and 21-1 (d); bars: 200 μm. (G) The length of the sixth siliques; data are average of 15–20 siliques; $^{**}P < 0.01$. (H) Root hair lengths were quantified within the mature zone; data are average of 200 root hairs; $^{**}P < 0.01$. (I) The number of lateral roots; error bars represent the standard deviation of replicates; $^{**}P < 0.01$.

and 13±0.98 for line 6-9, 24-10, 21-1, and wild-type plants, respectively) (Fig. 5I and Supplementary Fig. S3A). No obvious difference was seen in the primary root length (Supplementary Fig. S3B). Scanning electron microscopy of *Arabidopsis* leaves showed that the branch number of trichomes decreased more in transgenic line 6-9 than in wild-type plants (data not shown).

### *Solexa sequencing identified many fibre elongation-related genes downregulated in RNAi transgenic cotton lines*

To determine the mechanism by which reduced GbTCP levels results in shorter fibre, Solexa sequencing was used to identify genes that were differentially expressed in 10 DPA fibres between *GbTCP*-silenced (ITCP3 and ITCP9) and wild-type control plants (WT1 and WT2). A total of 18,770 and 18,450 clean tags were obtained from mRNA from wild-type control and transgenic lines, respectively. An unpublished reference database comprised of 20,671 contigs was used for tag signature mapping analysis. A total of 1031 differentially expressed genes were identified in the RNAi lines relative to wild-type control. These included 138 upregulated genes and 893 downregulated genes (Supplementary Table S3). Many of the genes are unknown or were hypothetical or predicted proteins. Of significance, many genes involved in hormone biosynthesis and response, including ethylene, auxin, and jasmonate, are downregulated in the RNAi lines relative to the wild-type control. Some transcription factors (WRKY, MYB, MYC, NAC, and zinc finger protein) and peroxidases or their precursors are also downregulated by silencing *GbTCP*. A number of other genes are also downregulated,

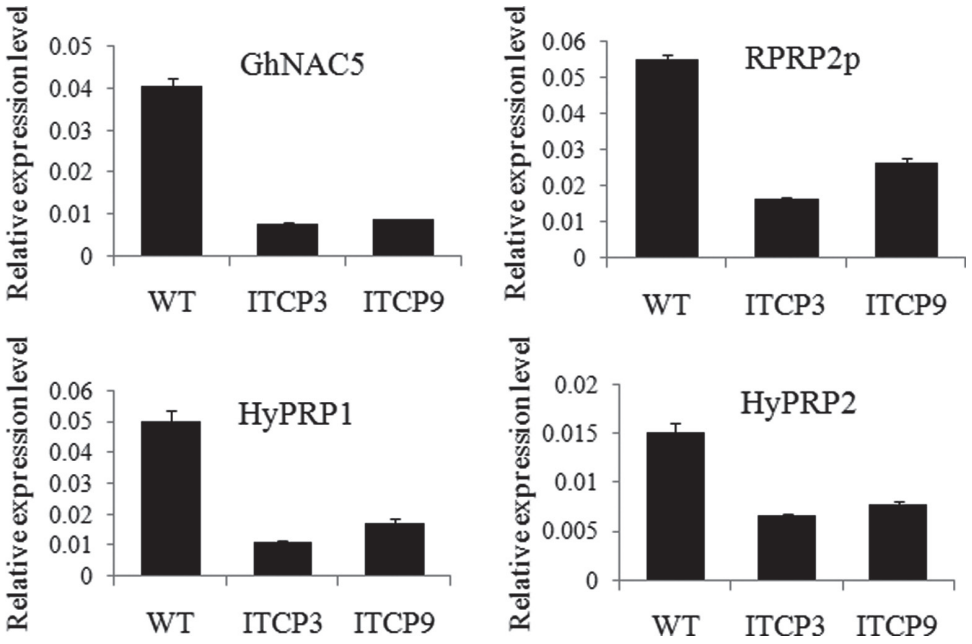


**Fig. 6.** Quantitative real-time PCR analysis of genes differentially expressed between wild type and *GbTCP* RNAi-silenced lines. Gene expression values are relative to *GhUB7* expression activity. Error bars represent the standard deviation of three technical replicates.

including calcium ion-binding protein, UDP-glucosyltransferase, sucrose carrier, and cyclin A. Of particular interest, the expression of many cell-wall proline-rich proteins (PRPs) with a high tag copy number is reduced in the RNAi lines compared with wild-type controls. The most significantly downregulated gene *GhNAC5* and three cell-wall PRP-related genes were validated by qRT-PCR. As shown in Fig. 6, *GhNAC5*, cell-wall repetitive PRP2 precursor (*RPRP2p*), *HyPRP1*, and *HyPRP2* were downregulated significantly in the RNAi lines ITCP3 and ITCP9 relative to the wild type in 10 DPA fibres.

### *Transcript profiling of* Arabidopsis GbTCP *overexpression transgenic root*

The lack of a good reference database for cotton made it difficult to find key factors involved in the regulatory mechanism of GbTCP in regulating fibre elongation. Cotton fibres and *Arabidopsis* root hairs are single cells that differentiate from single epidermal cells and may, therefore, have developmental regulatory mechanisms in common (Pang *et al*., 2010). Ectopic expression of *GbTCP* in *Arabidopsis* promoted root hair development, and therefore, discovering changes in root hair development regulatory networks may help to clarify the regulatory mechanism of GbTCP in cotton fibre development. Using Affymetrix ATH1 GeneChips, this study performed a comparative transcriptomic analysis of 8-day-old roots of the transgenic line 6-9 and wild-type control. A total of 490 genes were differentially expressed in the overexpression line 6-9 relative to wild-type control (>2-fold higher, $P < 0.05$), with 292 upregulated and 198 downregulated (Supplementary Table S4). Gene ontology annotation showed that genes are involved in molecular functions, including binding (211), catalytic activity (160), transcription regulator activity (59), transporter activity (22), and electron carrier activity (19) (Supplementary Fig. S4). Further analysis revealed that most of the genes that may be associated with root hair development were mainly related to lipid localization, transport, metabolic and biosynthetic processes, JA biosynthesis and response, ethylene biosynthesis and response, auxin response, and calcium ion binding. A number of transcription factors and two cell cycle-related genes were induced by GbTCP. Some of these genes were verified by reverse-transcription PCR (Fig. 7 and Supplementary Table S5). As shown in Fig. 7, most of the JA biosynthesis genes (*LOX4*, *AOS*, *AOC3*, and *OPCL1*) and response genes (*MYC2*, *JAZ1*, and *JAZ2*) were upregulated in the overexpression transgenic *Arabidopsis*, and the increase was positively correlated with the expression level of *GbTCP*. GbTCP could also induce the expression of ethylene biosynthesis and response genes (*ACS6*, *ORA47*, and *ERF13*), calcium ion receptors (*TCH3* and *CML37*), auxin response genes (*GH3.3* and SAUR-like auxin-responsive family), and a gibberellin oxidase (*GA2OX6*). A WRKY transcription factor *WRKY46* and cell cycle-related gene *CYCP3;1* were also regulated by GbTCP. The transcription level of two highly homologous genes, *AtTCP14* and *AtTCP15*, was not affected, indicating that the phenotypic changes in *Arabidopsis* are most likely to be caused by the GbTCP protein directly.

### *Level of JA was affected by GbTCP activity in cotton and low concentration of JA could promote fibre elongation*

In both the Solexa results in cotton and the *Arabidopsis* microarray results, a number of genes related to JA biosynthesis and signalling were all subjected to significant regulatory differences. It is hypothesized that GbTCP may affect the root hair development and fibre development by regulating JA biosynthesis.

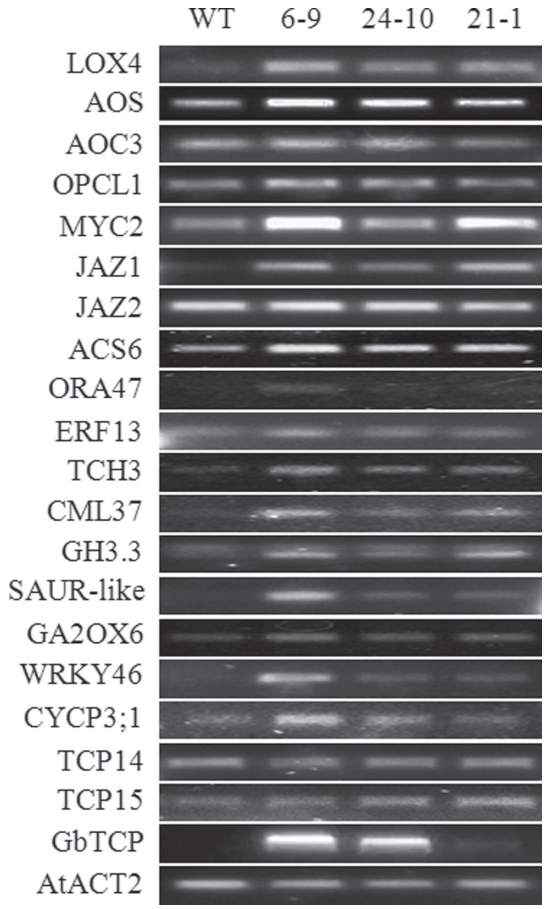


**Fig. 7.** Semi-quantitative reverse-transcription PCR analysis of genes selected from the *Arabidopsis* GeneChip and two *Arabidopsis* genes (*AtTCP14* and *AtTCP15*) in 8-day-old roots from wild type (WT), negative control (21-1), and two overexpression lines (6-9 and 24-10). The gene *AtACT2* was used as the internal control to normalize expression levels.

To further confirm the hypothesis, the plant hormone JA was extracted and quantified in cotton. In both 0 DPA ovules and 10 DPA fibres, the content of JA was increased in the overexpression lines but decreased in RNAi lines compared with wild-type controls, and the changes were more obvious in 10 DPA fibres than in 0 DPA ovules (Fig. 8A and 8B). These results indicated that GbTCP can regulate the biosynthesis of JA.

It has been reported that JA can promote not only the formation of trichomes, but also the initiation and elongation of root hairs. Recently, Tan *et al*. (2012) found that fibre development was inhibited when treated with sustained high concentrations of JA at the early stage (ovules at –1, 0, and 1 DPA) in *in vitro* ovule culture and that the inhibition was dose dependent. However, the role of JA on fibre elongation still requires further validation. In this study, it appears that GbTCP regulates JA biosynthesis in fibre, based on the JA content measurement (Fig. 8A and 8B). To illustrate this phenomenon clearly and reveal the direct role of JA on fibre elongation, different concentrations of JA were used to treat ovules collected from the elongating phase in *in vitro* ovule culture. The 0 DPA ovules of YZ1 were collected for ovule culture with BT medium *in vitro*. After 6 days of culture, the ovules were transferred into BT medium with differing concentrations of JA (0, 0.05, 0.1, 0.5, and 2.5 μM) for an additional 14 days, after which fibre length was measured manually. As shown in Fig. 8C and 8D, a low concentration of JA (0.05 μM) could significantly promote fibre elongation, but the promoting effect decreased when the concentration was up to 0.1 μM. The fibre length of the ovules was significantly reduced when JA concentration was >0.5 μM, suggesting that cotton fibres require an appropriate concentration of JA to elongate. A concentration that is too high or too low is unfavourable to fibre development. GbTCP might regulate fibre elongation via finely tuning JA levels.

## Discussion

### *GbTCP regulated fibre elongation via a complex system*

TCP proteins are a small family of plant-specific transcription factors that are involved in a wide range of growth and developmental regulation. *Arabidopsis* has 24 TCP members and rice has 29 members (Navaud *et al*., 2007). No report has been published on the role of the TCP family in the important economic crop, cotton. In this study, the first cotton TCP transcription factor GbTCP isolated from *G. barbadense* was characterized. It was found to be similar to AtTCP15 (class I TCP) with only one amino acid difference within the TCP domain. In the *GbTCP*-silenced cotton lines, the mature fibres were shorter and of lower quality. Fibre development was monitored and no visible difference at the fibre initial stage between the silenced and wild-type plants was observed, but fibre elongation was seen to be inhibited significantly in the silenced lines. This correlates with the expression pattern of *GbTCP*, with high expression from 5 to 15 DPA. To elucidate the mechanism of GbTCP regulation, Solexa sequencing was performed on silenced lines and wild-type control plants. A total of 893 genes were downregulated, which is about 90% of the total differentially expressed genes (1013). Compared to the wild type, the metabolic activity in the fibre of RNAi line is reduced, which includes the positive factors/pathways that are believed to be important in fibre elongation, such as ethylene and peroxidases (Shi *et al*., 2006; Mei *et al*., 2009). Three ethylene biosynthesis genes (ACC oxidase 1, ACC oxidase 2, and ACC oxidase 3) and many ethylene-responsive element-binding proteins and class III peroxidases were downregulated in the RNAi lines relative to wild-type control. Additionally, *RPRP2p*, *HyPRP1*, and *HyPRP2* were also downregulated. RPRPs are a subgroup of the hydroxyproline-rich glycoproteins, a major class of structural proteins present in the primary cell wall of higher plants that cross-linked to extensins, allowing them to lock the cellulose microfibrils within the three-dimensional network of cell walls (Akiyama and Pillai, 2003). HyPRPs are a subclass of the PRPs and may have varied functions during specific developmental stages and in response to biotic and abiotic stresses (He *et al*., 2002; Yeom *et al*., 2012). An *Arabidopsis* proline-rich cell-wall protein, AtPRP3, is expressed in a cell type-specific manner during root hair development and is strongly enhanced by ethylene and auxin (Bernhardt and Tierney, 2000). GhNAC5, a NAC transcription factor that responds to abiotic stresses (Meng *et al*., 2009), was significantly downregulated in cotton

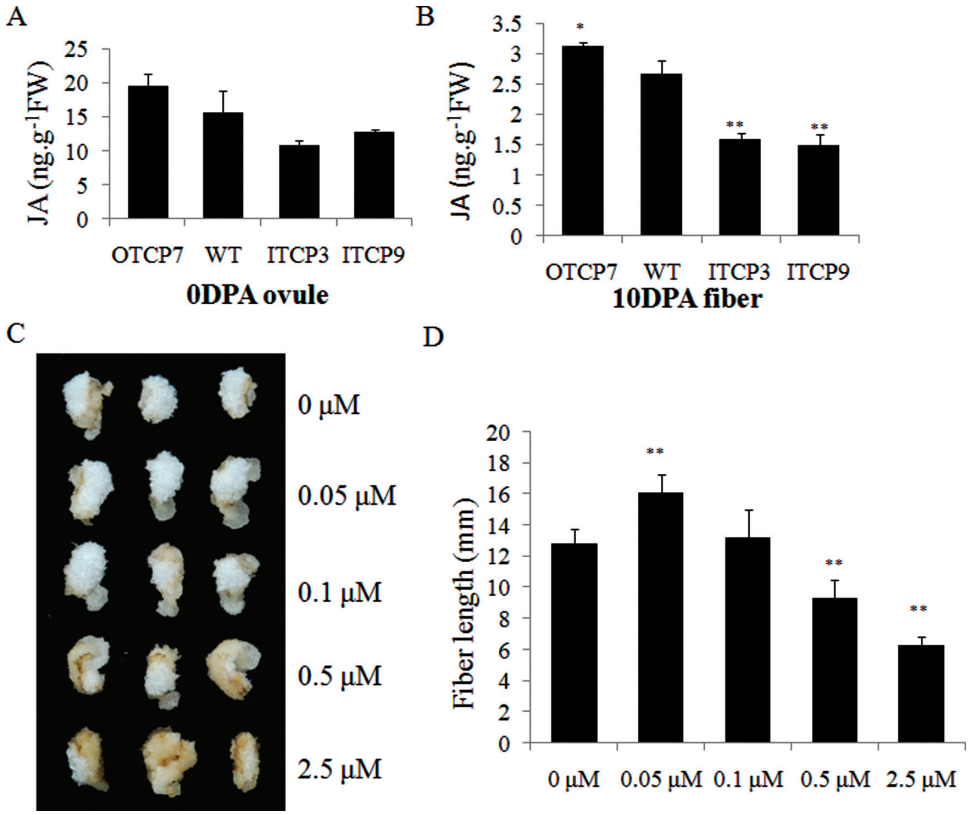


**Fig. 8.** Jasmonic acid (JA) content measurement and exogenous JA treatment in *in vitro* ovule culture. (A, B) Measurement of JA content in cotton 0 DPA ovules and 10 DPA fibres, respectively, from overexpression line OTCP7, wild type, and two RNAi lines (ITCP3 and ITCP9); error bars represent the standard deviation of three replicates; asterisks indicate statistically significant differences between transgenic lines and wild type, as determined by the Student t-test (*$P < 0.05$; **$P < 0.01$). (C) Photograph of ovules cultured for 20 days with different concentrations of JA added (0, 0.05, 0.1, 0.5, and 2.5 μM). (D) Fibre length measurements of cultured cotton ovules; error bars represent the standard deviation of 18 replicates; asterisks indicate statistically significant differences between transgenic lines and wild type, as determined by the Student t-test (**$P < 0.01$).

*GbTCP*-silenced lines compared with the wild-type plants. In *Arabidopsis*, a drought-induced NAC transcription factor NTL4 can activate the production of reactive oxygen species (ROS) under drought conditions; *ntl4* mutants exhibit enhanced drought resistance and delayed leaf senescence (Lee *et al*., 2012). It is possible that GhNAC5 acts upstream of ROS and is positively regulated by GbTCP. In addition, several downregulated WRKY transcription factors were identified in this study. WRKY proteins are mainly involved in regulating plant defence responses and have a diverse role in regulating other developmental processes in plants, including trichome initiation (Johnson *et al*., 2002). GaWRKY1 participates in regulation of sesquiterpene biosynthesis by activating the expression of CAD1-A in cotton, and expression levels of both *GaWRKY1* and *CAD1-A* are strongly induced by a fungal elicitor preparation and methyl jasmonate (Xu *et al*., 2004). The role of WRKY transcription factors in the regulation of cotton fibre development has not yet been reported. The reasons that the response of genes to biotic and abiotic stresses were downregulated in the RNAi lines are unknown and may require further study. A cell-cycle gene, *cyclin A*, was downregulated by more than 2-fold in the silenced lines compared with wild-type plants. Cyclin A protein is synthesized and localized to the nucleus where it has a major role in the control of DNA replication in mammalian fibroblasts (Girard *et al*., 1991). As previously mentioned, AtTCP15 could modulate the expression of several key cell-cycle genes to promote mitotic cell division and inhibit endoreduplication (Li *et al*., 2012).

### *Root hair elongation in* Arabidopsis *by GbTCP confirmed its function in cell elongation*

Overexpression of *GbTCP* in *Arabidopsis* resulted in more branches, smaller leaves, shorter siliques, fewer lateral roots, and delayed senescence compared with wild-type plants. Most of these phenotypes are similar to those observed in transgenic cotton overexpressing *GbTCP* constitutively. Ectopic overexpression of *GbTCP* in *Arabidopsis* significantly enhanced root hair elongation. GUS reporter studies have also shown that its expression is mostly restricted to epidermal cells, being expressed in cotton fibres, *Arabidopsis* root hairs, and trichomes. Therefore, it is speculated that GbTCP has quite conservative functions in different species and may have a general function in regulating development of specialized epidermal cells. To further clarify the regulation mechanism of GbTCP in root hair, the genes activated by GbTCP were identified through Affymetrix GeneChip analysis on RNA from 8-day-old *Arabidopsis* roots from the

transgenic line 6-9 and wild-type control. A total of 292 genes were upregulated in the transgenic line relative to the wild type, which is 60% of the total differentially expressed genes (490). Genes involved in metabolic activity in the root of the overexpression line were increased compared to wild-type plants. These include JA biosynthesis and response, ethylene biosynthesis and response, auxin response, calcium signalling, and cell cycle regulation genes, this agrees with what was observed in cotton. Early studies have reported that auxin and ethylene have important roles in root hair elongation (Pitts *et al*., 1998). Many ethylene biosynthesis (*ACS6*) and response genes (*ERF*) were upregulated in the *Arabidopsis* overexpression line, which suggested that GbTCP may regulate the ethylene signalling pathway in root hairs as well as in cotton fibres. A number of differentially expressed genes encoding calcium ion-binding proteins were induced by *GbTCP* overexpression in *Arabidopsis*. Calcium is required for *Arabidopsis* root hair growth and the rate of growth is positively correlated with $Ca^{2+}$ endogenous polarity at the tip (Bibikova *et al*., 1997). Interestingly, *GbTCP* overexpression also upregulated two cycle genes, *CYCP3;1* and *CYCP3;2*, in *Arabidopsis* root. Considering that a cell cycle gene *cyclin A* was also regulated by GbTCP in cotton fibre, GbTCP may regulate cell cycle-related genes to control cell division and differentiation. The branch number of leaf trichomes was decreased in the transgenic line 6-9 compared to wild-type plants (data not shown); this result is the same as that reported by Li *et al*. (2012). It is possible that overexpression of *GbTCP* inhibits endoreduplication, but the DNA content in *Arabidopsis* root would have to be measured to validate this suggestion. Qin and Zhu (2011) have suggested a signalling pathway in fibre cell elongation: $Ca^{2+}$ channels activated by ROS upregulated ethylene biosynthesis and then stimulated pectin biosynthesis and scaffold establishment to promote fibre elongation. It is proposed that GbTCP may regulate fibre and root hair elongation through the same pathway. Nonetheless, the precise order of these signalling steps is unclear and further experimental verification is needed to clarify the regulatory mechanism of GbTCP.

### *JA would be a crucial factor promoting cotton fibre elongation*

Different methods were used in the two expression profile analysis and the two results were not totally consistent. The reason may be that the reference data for cotton are limited. It is noteworthy, however, that JA biosynthesis and response form the common pathway in fibre and root hair. It was downregulated in *GbTCP* RNAi cotton fibre and upregulated in the overexpression *Arabidopsis* root hair. A JA biosynthesis-limiting enzyme (with high homology to AtLOX3 and AtLOX4) and a direct JA-responsive factor (with high homology to AtMYC2) were identified in cotton. In *Arabidopsis*, more genes related to JA biosynthesis and response, including *AtLOX4* and *AtMYC2*, were induced when *GbTCP* was upregulated. The fatty acid-derived signalling molecule JA has a key role in the regulation of root growth, pollen development, senescence, and defence response. Appropriate concentration of JA can promote trichome patterning and root hair elongation, and a complex cross-talk exists between JA and other hormones (Traw and Bergelson, 2003; Zhu *et al*., 2006). The JA-responsive bHLH transcription factor AtMYC2 extensively cross-talks with JA, ethylene, ABA, and GA signalling pathways (Hong *et al*., 2012). TCP proteins directly participate in the regulation of JA biosynthesis. Class II TCPs (AtTCP2, AtTCP4, and AtTCP10) can induce the expression of *LOX2* and promote JA biosynthesis, thereby inhibiting cell proliferation and promoting leaf senescence (Schommer *et al*., 2008). In contrast, the class I TCP protein AtTCP20 inhibits the expression of *LOX2* antagonistically to regulate JA metabolism and leaf development (Danisman *et al*., 2012). Therefore, it is proposed that JA biosynthesis may be directly downstream of *GbTCP*, and the class I TCP proteins regulate JA biosynthesis in a different way in different tissues. JA might promote the elongation of fibres and root hairs by regulating WRKYs, NAC, ROS, calcium channel, ethylene signalling, and pectin biosynthesis.

As previously reported, JA is a negative regulatory factor in fibre elongation; the development of ovules and fibres is severely affected when they are treated with sustained high concentrations of JA (Tan *et al*., 2012). Overexpression of *GbTCP* in cotton fibre elevated the JA content and caused fewer and shorter fibres, which is consistent with these reported results. In an *in vitro* ovule culture, a low concentration of JA (0.05 μM) significantly promoted fibre elongation, but a higher concentration of JA suppressed fibre development. This result might imply that fibre elongation requires an optimal JA concentration; too high or too low is unfavourable for fibre development. These results also suggest that a similar regulatory mechanism may exist in cotton fibre and *Arabidopsis* root hair elongation.

## Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Southern blotting and trichomes and fibres initiation observations of RNAi lines.

Supplementary Fig. S2. Characteristics of *GbTCP* overexpression transgenic cotton.

Supplementary Fig. S3. The roots of the overexpression transgenic *Arabidopsis*.

Supplementary Fig. S4. Gene ontology analysis of molecular function on 490 differentially expressed genes selected from *Arabidopsis* Affymetrix ATH1 GeneChip.

Supplementary Table S1. Primers used in the research.

Supplementary Table S2. Distribution of *cis*-acting elements in *GbTCP* promoter region.

Supplementary Table S3. Significantly differentially expressed genes in 10 DPA fibre of transgenic RNAi line relative to wild-type control.

Supplementary Table S4. Significantly differentially expressed genes in *Arabidopsis* root of transgenic line 6-9 relative to wild-type control.

Supplementary Table S5. Genes and primers used for reverse-transcription PCR in *Arabidopsis*.

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