

The Cotton Transcription Factor TCP14 Functions in Auxin-Mediated Epidermal Cell Differentiation and Elongation^{1[C][W]}

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Plant-specific TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors play crucial roles in development, but their functional mechanisms remain largely unknown. Here, we characterized the cellular functions of the class I TCP transcription factor GhTCP14 from upland cotton (*Gossypium hirsutum*). GhTCP14 is expressed predominantly in fiber cells, especially at the initiation and elongation stages of development, and its expression increased in response to exogenous auxin. Induced heterologous overexpression of GhTCP14 in *Arabidopsis* (*Arabidopsis thaliana*) enhanced initiation and elongation of trichomes and root hairs. In addition, root gravitropism was severely affected, similar to mutant of the auxin efflux carrier PIN-FORMED2 (PIN2) gene. Examination of auxin distribution in GhTCP14-expressing *Arabidopsis* by observation of auxin-responsive reporters revealed substantial alterations in auxin distribution in sepal trichomes and root cortical regions. Consistent with these changes, expression of the auxin uptake carrier AUXIN1 (AUX1) was up-regulated and PIN2 expression was down-regulated in the GhTCP14-expressing plants. The association of GhTCP14 with auxin responses was also evidenced by the enhanced expression of auxin response gene IAA3, a gene in the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family. Electrophoretic mobility shift assays showed that GhTCP14 bound the promoters of PIN2, IAA3, and AUX1, and transactivation assays indicated that GhTCP14 had transcription activation activity. Taken together, these results demonstrate that GhTCP14 is a dual-function transcription factor able to positively or negatively regulate expression of auxin response and transporter genes, thus potentially acting as a crucial regulator in auxin-mediated differentiation and elongation of cotton fiber cells.

TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCPs) constitute a large group of plant-specific genes found in various species, ranging from lower to higher plants, with 24 TCPs in *Arabidopsis* (*Arabidopsis thaliana*) and more than 20 TCPs in rice (*Oryza sativa*; Cubas, 2002; Navaud et al., 2007; Yao et al., 2007). TCP proteins have a TCP domain that contains a helix-loop-helix (bHLH) motif (Martín-Trillo and Cubas, 2010). Based on sequence

variation in the TCP domains, these proteins can be divided into two subfamilies, class I (TCP-P) and class II (TCP-C) TCPs (Cubas et al., 1999; Kosugi and Ohashi, 2002; Navaud et al., 2007). A number of TCP proteins have been characterized as functional transcription factors, acting either as activators or repressors, and the members of the two TCP subclasses bind to different consensus DNA elements (Cubas et al., 1999; Kosugi and Ohashi, 2002; Trémousaygue et al., 2003).

Accumulating evidence demonstrates that TCP transcription factors play important roles in plant growth and development (Cubas et al., 1999; Li et al., 2005; Hervé et al., 2009). In particular, great progress has been made in functional characterization of the class II TCPs in *Arabidopsis*. These transcription factors play important roles in leaf, flower, shoot morphogenesis, and hormone biosynthesis (Damerval et al., 2007; Koyama et al., 2007; Schommer et al., 2008; Nag et al., 2009; Guo et al., 2010). The cellular functions of class I TCPs are less well explored, but several previous studies indicated that they are involved in a number of cellular or developmental processes. For example, *Arabidopsis* AtTCP14 and AtTCP16 participate in regulation of seed germination and pollen development, respectively (Takeda et al., 2006; Tatematsu et al., 2008a), and

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AtTCP20 and AtTCP15 are involved in the regulation of cell division, expansion, and differentiation (Hervé et al., 2009; Kieffer et al., 2011). In addition, several lines of evidence have demonstrated that the functions of class I TCP proteins are associated with phytohormone signaling, such as auxin, jasmonic acid (JA), and cytokinin (Kosugi and Ohashi, 2002; Danisman et al., 2012; Steiner et al., 2012; Uberti-Manassero et al., 2012). Based on transcriptome analysis, Kosugi and Ohashi (2002) proposed that class I TCP proteins are likely to be involved in up-regulation of auxin-induced genes. This notion is further supported by a recent study of Uberti-Manassero et al. (2012), which showed that AtTCP15 regulates the expression of boundary-specific genes, presumably through alteration of auxin homeostasis. Although these studies represent important achievements in understanding the roles of this family of proteins, elucidation of the functions of class I TCP proteins is still limited (Li et al., 2012).

Dynamic changes in auxin distributions in plant cells or tissues trigger various differentiation and developmental processes (Benjamins and Scheres, 2008; Vanneste and Friml, 2009). Polar auxin transport, which is regulated by auxin efflux carriers such as PIN-FORMED2 (PIN2) and PIN-FORMED3 (PIN3) and auxin influx carriers such as AUXIN1 (AUX1), is essential for dynamic auxin distribution (Leyser, 2005). The functions of the auxin transporters are well characterized in root gravity response of Arabidopsis plant (Sabatini et al., 1999; Sun et al., 2011). Coordinated activities of PINs and AUX1 proteins led to asymmetric auxin distribution in root apex and resulted in alteration of the growth orientation (Bennett et al., 1996; Sabatini et al., 1999; Swarup and Péret, 2012). Auxin transporters also act as key modulators in root hair formation (Ganguly et al., 2010). It has been shown that AUX1 promoted root hair initiation by stimulating auxin influx to specific sites (Rahman et al., 2002; Jones et al., 2009), and overexpression of AUX1 or PIN2 was found to promote or block the root hair elongation (Cho et al., 2007; Ganguly et al., 2010). In addition to regulating the auxin transporter genes, auxin also induces the expression of genes in the AUXIN/INDOLE-3-ACETIC ACID (*Aux/IAA*) family, including auxin response gene IAA3, which plays important roles in auxin-mediated regulation of root growth, gravitropism, and lateral root formation (Tian and Reed, 1999; Tian et al., 2002; Knox et al., 2003).

The cotton (*Gossypium hirsutum*) fiber is a highly elongated seed hair and is considered to be a unique system for studying the mechanisms of plant cell expansion (Kim and Triplett, 2001; Qin and Zhu, 2011). Like Arabidopsis trichomes, cotton fibers are derived from single epidermal cells (Basra and Malik, 1984). Regulation of cotton fiber development are attributed to multiple phytohormone signaling processes, of which the auxin signaling was shown to be important for fiber initiation and elongation (Samuel Yang et al., 2006; Shi et al., 2006; Gou et al., 2007). Several transcriptome analyses revealed the enriched transcripts of

auxin response factors during fiber initiation and fast-elongation stages (Gou et al., 2007; Liu et al., 2012), and external application of IAA promoted fiber initiation (Gialvalis and Seagull, 2001). Recently, Zhang et al. (2011) showed that overexpression of the key IAA biosynthesis gene *iaaM* led to enhanced initiation of fiber cells on the ovule epidermal layer and increased fiber length. All these results pointed out the important roles of auxin signaling in cotton fiber development. However, the upstream factors that control the distribution of auxin signaling in cotton fibers remain unknown.

In this study, we isolated a fiber-specific TCP gene (designated *GhTCP14*) from upland cotton. *GhTCP14* is expressed primarily in developing fiber cells during the initiation and elongation stages. As Arabidopsis has been employed successfully as a model system for functional characterization of several cotton fiber-specific genes (Wang et al., 2004; Guan et al., 2008, 2011; Shangguan et al., 2008), we therefore investigated the effect of *GhTCP14* on trichome development in Arabidopsis. We show that ectopic expression of *GhTCP14* in Arabidopsis promoted the differentiation and elongation of trichome and root hair cells; moreover, overexpression of *GhTCP14* resulted in alteration of auxin distribution and altered expression levels of auxin-related genes such as *AUX1*, *PIN2*, and *IAA3*. We found that *GhTCP14* bound directly to the promoters of these genes in vitro and in vivo. We also found misregulation of these genes in elongation-deficient fibers of the *Ligon lintless1* (*Li₁*) cotton mutant, in which *GhTCP14* was down-regulated. Our results provide important insights on the function of class I TCP transcription factors in the development of epidermal cells such as Arabidopsis trichomes and cotton fibers.

RESULTS

Identification and Sequence Analysis of *GhTCP14*

A cotton fiber-specific complementary DNA (cDNA) library was constructed by suppression subtractive hybridization using cDNA prepared from 9-DPA fibers ("tester") and cotton leaves ("driver"; Gao et al., 2007). A cDNA clone that encodes a putative TCP-domain protein was found highly expressed in cotton fiber. The full-length cDNA of this clone was obtained by 5' and 3' RACE PCR. It encodes a predicted 395 amino acid protein that contains a bHLH-type DNA-binding domain at its N-terminal region. Phylogenetic analysis of the putative protein with TCP proteins from Arabidopsis, rice, snapdragon (*Antirrhinum majus*), sea-island cotton (*Gossypium barbadense*), and corn (*Zea mays*) showed that it belongs to the class I TCP protein group and has the greatest similarity to AtTCP14 (Supplemental Fig. S1); thus, this gene was designated as *GhTCP14* (accession no. AF165924). Domain analysis showed that the bHLH domain of *GhTCP14* is highly similar to those of other

TCP proteins (Supplemental Fig. S1). Motif analysis revealed a nuclear localization signal at residues 237 to 240 (RKKR) of GhTCP14 protein.

GhTCP14 Protein Localizes to the Nucleus

To examine the subcellular localization of GhTCP14, a GFP reporter gene was fused in frame to the GhTCP14 coding region under the control of the 35S promoter to produce a GhTCP14-GFP fusion protein in transgenic Arabidopsis plants. As seen in Supplemental Figure S2, GhTCP14-GFP fusion proteins were detected predominantly in the nuclei, consistent with sequence-based prediction of the subcellular localization and putative transcription factor activity of GhTCP14.

GhTCP14 Possesses DNA-Binding and Transactivation Activities

The amino acid sequence and subcellular distribution of GhTCP14 pointed to a potential function as a TCP transcription factor. To identify sequences preferentially bound by GhTCP14, we conducted a random binding site selection analysis using recombinant GhTCP14. Binding assays indicated that GhTCP14 can specifically bind to two sequences, TGGGTCCCACAT and TTGTGGGCCCT (Supplemental Fig. S3). This exhibited a perfect match with the consensus TCP DNA-binding specificity of class I (GGNCCCAC) and class II (G[T/C]GGNCCC), indicating that GhTCP14 could bind to two types of TCP binding sites.

Electrophoretic mobility shift assay (EMSA) was conducted to verify the binding of these DNA sequences. Bacterially expressed glutathione *S*-transferase (GST)-GhTCP14 proteins were purified. Double repeats of sequences TGGGTCCCACAT and TTGTGGGCCCT were annealed, labeled, and incubated with purified GST-GhTCP14 fusion proteins. GhTCP14 formed a complex with the labeled probes, and the signal was dramatically decreased by addition of unlabeled DNA probe (competitor; Fig. 1A), indicating that GhTCP14 could bind specifically to the two sequences in vitro.

The ability of GhTCP14 to activate transcription was examined using a dual-luciferase reporter assay system in Arabidopsis protoplasts (Ohta et al., 2001). The coding sequence of *GhTCP14* was fused to the DNA sequence encoding yeast transcriptional activator GAL4 DNA-binding domain to generate the effector plasmid pBD-GhTCP14. GhTCP14 activated the reporter (Fig. 1B), indicating that GhTCP14 has transcription activation activity in vivo.

Phenotypes of Transgenic Arabidopsis Plants Overexpressing *GhTCP14*

To examine GhTCP14 function, we generated transgenic Arabidopsis plants to bypass the difficulties and delays of cotton transformation. The *GhTCP14* cDNA was subcloned into the pZP111 expression vector under the control of the *Cauliflower mosaic virus* 35S promoter, and more than 20 Arabidopsis transgenic lines were obtained. Severe phenotypic alterations were observed with the transgenic plants. For example, nine lines showed strong inhibition of root development; roots were barely developed, and as a result, most of these plants did not survive. The remaining lines had pleiotropic phenotypes. Some plants had highly elongated trichomes and denser trichomes on sepals (Fig. 2, E–H) but displayed dwarf growth (Fig. 2, A and B). In addition, these plants showed delayed flowering, and a large portion of the siliques were aborted with few seeds set (Fig. 2, C and D).

Induced Expression of *GhTCP14* Increases the Density and Length of Root Hairs and Trichomes and Affects Gravitropism

Because constitutive overexpression of *GhTCP14* severely affected the survival and fertility of the transgenic plants, it was difficult to assess the effect of *GhTCP14* overexpression on the plants. As constitutive expression of *GhTCP14* under the 35S promoter is lethal to the transgenic plants, we therefore employed an induced expression strategy to clarify the effect of GhTCP14. To

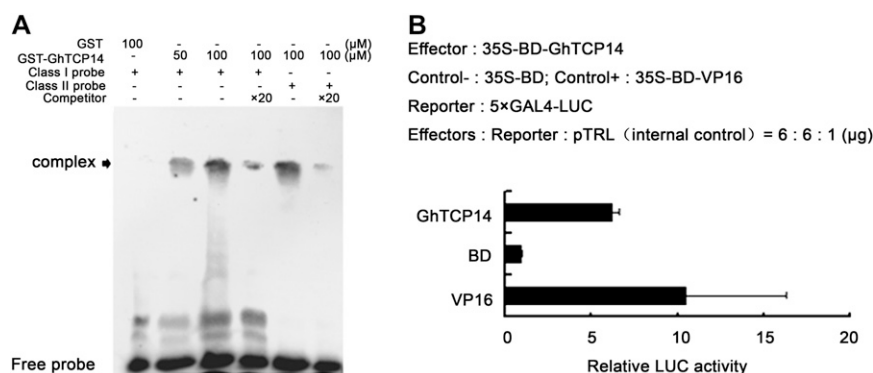
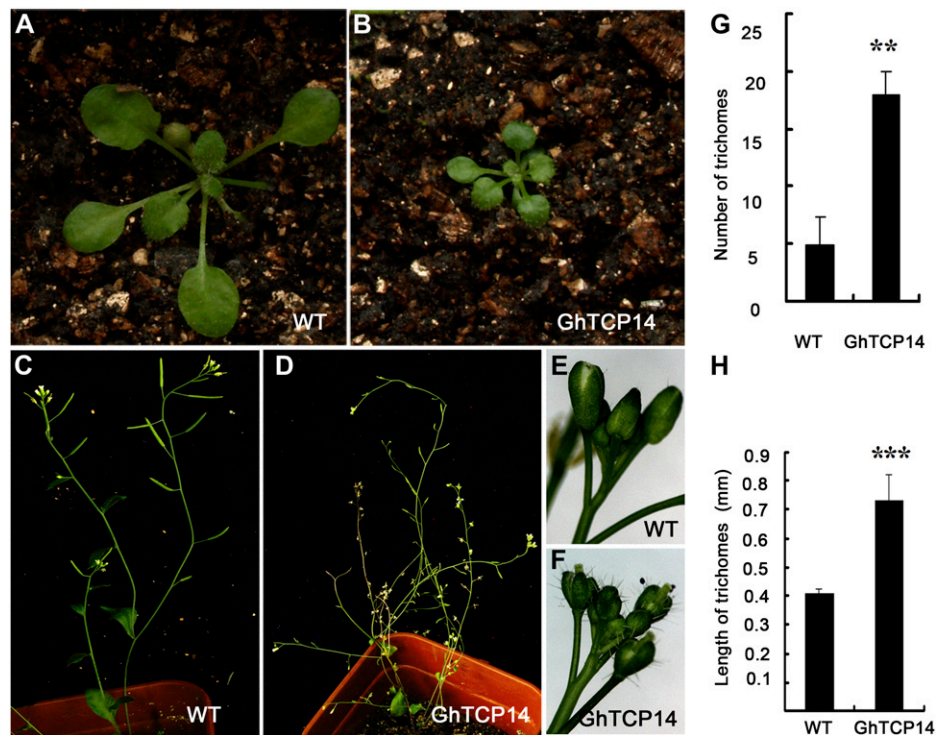


Figure 1. DNA-binding specificity and transcriptional activation activity of GhTCP14. A, Gel-shift assay. GhTCP14 proteins were incubated with biotin-labeled probes (class I probe: 2 \times TGGGTCCCACAT and class II probe: 2 \times TTGTGGGCCCT) in the presence or absence of 20-fold excess of nonlabeled competitor. Arrow indicates the position of protein-DNA complexes. B, Transcriptional activation ability of GhTCP14 in Arabidopsis protoplasts. The GAL4 DNA-binding domain (BD) and BD-VP16 were used as negative or positive control, respectively. Error bars represent the sd of three biological replicates.

Figure 2. Phenotypes of transgenic *Arabidopsis* plants overexpressing *GhTCP14* under 35S promoter. A and B, Three-week-old wild-type (A) and transgenic (B) seedlings. C and D, Six-week-old wild-type (C) and transgenic (D) plants. E and F, Trichomes on sepals of wild-type (E) and transgenic (F) plants. G and H, Quantitative analyses of the number (G) and length (H) of sepal trichomes. Error bars represent the SD of three biological replicates ($n = 30$ each). Asterisks denote values statistically different from the corresponding wild-type controls (** $P < 0.01$, *** $P < 0.001$). WT, Wild type. [See online article for color version of this figure.]



this end, *GhTCP14* was placed under the control of the estrogen-inducible promoter *pER8* (Zuo et al., 2000; Wang et al., 2009), and the construct was transformed into *Arabidopsis* plants. To investigate the effect of *GhTCP14* expression on plant growth and development, T3 homozygote seeds of three lines were germinated on medium with dimethyl sulfoxide (DMSO) as the solvent-only negative control, or with 10 μM β -estradiol, to induce transgene expression. No phenotype was observed in transgenic plants grown on medium containing DMSO. In the presence of β -estradiol, however, all three lines had similar, strong developmental abnormalities. When seeds were germinated and grown on induction medium, the plants were dwarf and lost the gravitropism response (Fig. 3, A and B). The cotyledons were unable to expand and appeared light green (Fig. 3, A and B). Induction was also carried out on 4-d-old plants in which both root and shoot are differentiated. Seeds were grown on Murashige and Skoog (MS) medium for 4 d, and the seedlings were then transferred to media in the presence or absence of β -estradiol and grown for 7 d. A striking phenotypic change was observed with the roots of the transgenic plants. The root appeared as callus-like tissue and turned green when expression of the transgene was induced (Fig. 3, C and D). Also, induced expression of *GhTCP14* significantly increased the density and length of root hairs (Fig. 3, E and F). To find out whether *GhTCP14* can also affect trichomes, we used 10 μM β -estradiol to spray the wild-type and transgenic plants once per day. Following this treatment, the density and length of trichomes on both stem and sepal were increased in transgenic plants compared with wild-type plants (Fig. 3, G–K)

Induced Expression of *GhTCP14* Alters Auxin Levels and Distribution in *Arabidopsis* Roots and Sepal Trichomes

Some phenotypes of the *GhTCP14*-expressing transgenic plants (loss of gravitropism, increased root hair density and length) resembled those of plants in which auxin content or distribution was abnormal. This prompted us to investigate if auxin distribution was affected in the *GhTCP14*-expressing plants. To monitor auxin distribution patterns in the transgenic plants, we introduced the auxin-responsive reporter constructs DR5:GFP and DR5:GUS into the *pER8:GhTCP14* plants. DR5 is a synthetic auxin-responsive promoter with increased auxin responsiveness (Ulmasov et al., 1997). We observed that the activity of the DR5 promoter was obvious in the roots. In the *GhTCP14*-induced root tips, the expression of DR5:GFP (Fig. 4E) and DR5:GUS (Fig. 4G) exhibited dramatic increase compared with that of noninduced plants (Fig. 4, D and F) and plants without *GhTCP14* (Fig. 4, A and B), indicating that auxin levels were substantially increased in lateral root cap cells. In addition, the distribution of signals in the lateral root cap cells was asymmetric. This staining pattern appeared similar to that of wild-type plants treated with exogenous auxin (IAA; Fig. 4C). This may cause the loss of gravitropism observed in the *GhTCP14*-expressing plants (Fig. 3B). Interestingly, GFP could also be visualized in the sepal trichomes and epidermal cells of the DR5:GFP plants when *GhTCP14* expression was induced (Fig. 4I). By contrast, the corresponding cells in the noninduced plants showed no visible fluorescence (Fig. 4H).

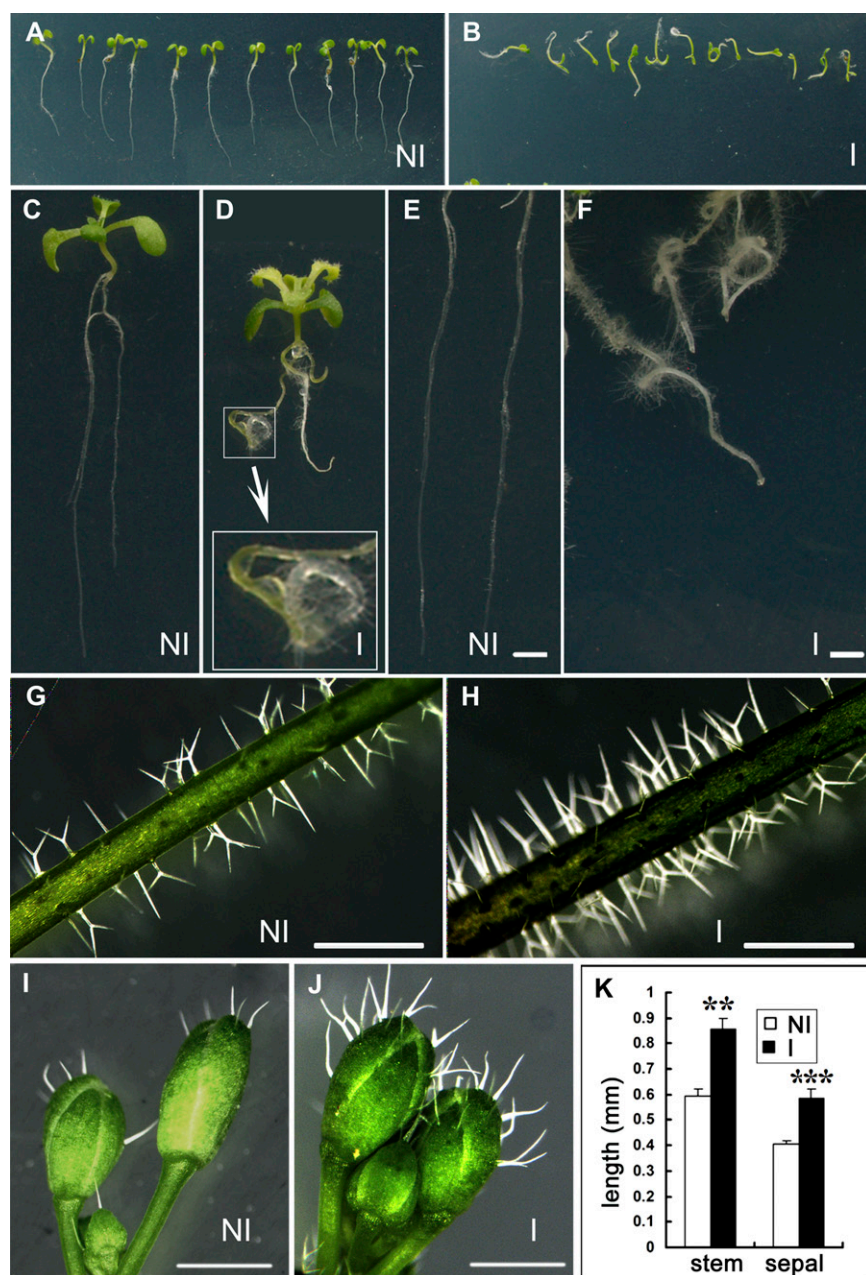


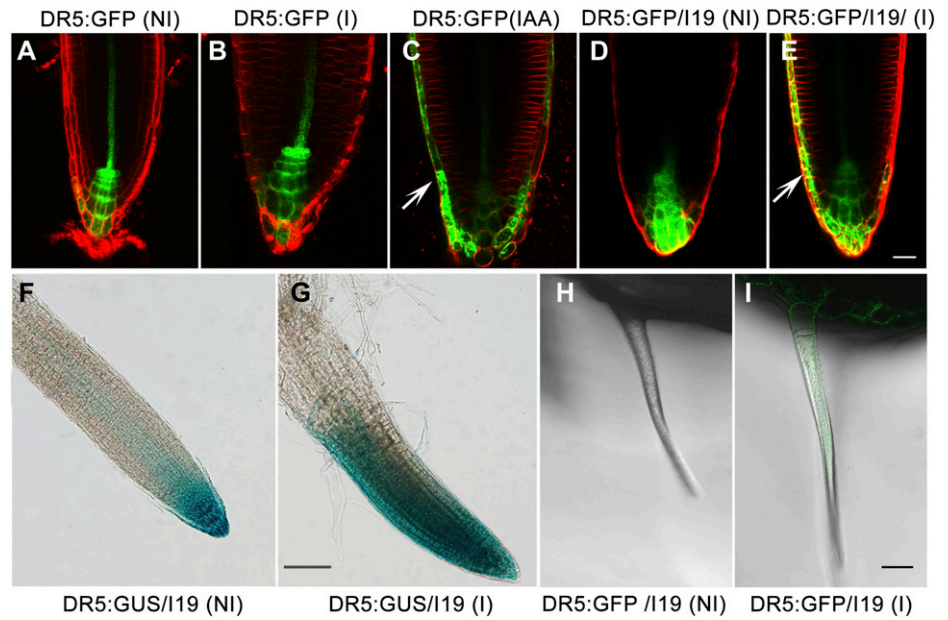
Figure 3. Phenotypes of *pER8:GhTCP14* transgenic Arabidopsis plants. A and B, Seeds directly sown on noninducing (A) or inducing (B) medium with $10 \mu\text{M}$ estradiol and grown for 5 d. C and D, Four-day-old seedlings transferred to noninducing (C) or inducing (D) medium and grown for 7 d. E and F, Root hairs of plants grown under noninducing (E) or inducing (F) conditions. G to J, Trichomes on stems (G and H) and sepals (I and J) of plants grown under noninducing (G and I) or inducing (H and J) conditions. K, Quantitative analysis of trichome length. Error bars represent the SD of three biological repeats ($n = 30$ each). Asterisks denote values statistically different from the corresponding wild-type controls (** $P < 0.01$, *** $P < 0.001$). NI, Noninduced; I, induced. Bars = 1 mm. [See online article for color version of this figure.]

Induced Expression of *GhTCP14* Affects Transcription of Genes Involved in Auxin Responses

The changed auxin content and distribution in *pER8:GhTCP14* transgenic plants indicated that overexpression of the *GhTCP14* transcription factor may change the expression of auxin-related genes. Thus, we next used qualitative reverse transcription (RT)-PCR to measure the expression of genes involved in auxin homeostasis, including auxin synthesis, auxin-mediated gene expression, and auxin distribution. In the induced transgenic plants the expression of *AUX1*, which encodes an auxin uptake carrier, was increased (Fig. 5, B and C). By contrast, expression of *PIN2*, which encodes an auxin

efflux transporter, was down-regulated, but *PIN1*, *PIN3*, *PIN4*, *PIN5*, *PIN6*, *PIN7*, and *PIN8* were not significantly changed (Fig. 5, B and C). In addition, expression of *IAA3*, which encodes an auxin-responsive protein, increased significantly (Fig. 5, B and C). These results indicated that ectopic expression of *GhTCP14* (Fig. 5A) alters the expression of both auxin transporter and auxin-responsive genes (Fig. 5, B and C). To verify this effect, we examined GFP expression from *pPIN2:PIN2-GFP* in *pER8:GhTCP14* transgenic plants (Fig. 5D). In the *GhTCP14*-induced root tip, the brightness of *PIN2-GFP* signal was dramatically lowered compared with that of noninduced plants (Fig. 5D), indicating the reduced activity of *PIN2* promoter.

Figure 4. Effects of GhTCP14 on the expression of auxin-responsive reporter genes and auxin distribution. A to C, Root tips of *DR5:GFP* plants grown on noninducing medium (A), inducing medium (B), or medium containing 1 μM IAA (C). D and E, Root tips of *DR5:GFP* \times *pER8:GhTCP14* transgenic plants grown on noninducing (D) or inducing (E) medium. F and G, Root tips of *DR5:GUS* \times *pER8:GhTCP14* plants grown on noninducing (F) or inducing (G) medium. H and I, Sepal trichomes of *DR5:GFP* \times *pER8:GhTCP14* plants grown on non-inducing (H) or inducing (I) medium. Arrows show fluorescent signals in the lateral root cap cells. NI, Noninduced; I, induced; I19, *pER8:GhTCP14*. Bars = 25 μm (A–E), 30 μm (F and G), and 100 μm (H and I). [See online article for color version of this figure.]



The GhTCP14 Transcription Factor Binds Directly to a Specific Motif in Target Promoters

The up- or down-regulated expression of auxin-related genes implied that GhTCP14 may regulate their expression by acting as a transcription factor. The

IAA3 promoter contains TCP binding sites (Koyama et al., 2010). Also, sequence analysis indicated that *PIN2* and *AUX1* promoters contain putative TCP binding elements. To determine whether the transcription of these genes could be affected by GhTCP14 binding directly to their promoters, we conducted

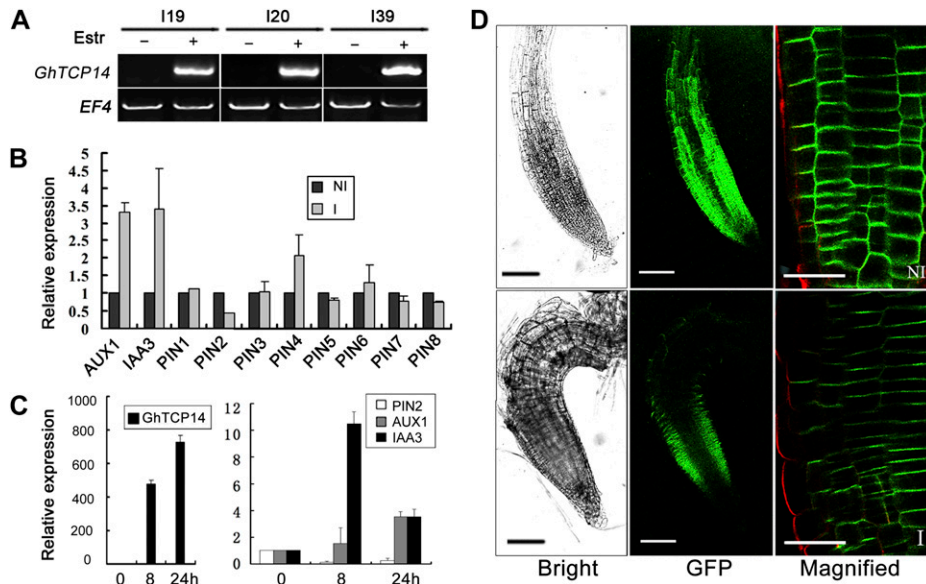


Figure 5. Effects of GhTCP14 on the expression of auxin-related genes. A, Induction of *GhTCP14* expression in *pER8:GhTCP14* transgenic lines (I19, I20, and I39) analyzed by RT-PCR. The elongation factor gene (*EF4*) was used as an internal control. B, Quantitative RT-PCR analysis of the expression of genes related to auxin transport or auxin response in *pER8:GhTCP14* plants grown on noninducing (NI) or inducing (I) medium. C, Time course expressions of *GhTCP14* (left) and some auxin-related genes (right). Line I19 plants were grown in liquid medium and harvested at 8 or 24 h after supplementation with 10 μM estradiol. For B and C, *Actin2* was used as an internal control. Error bars represent the sd of three biological replicates, and value of the noninduced plants was set to 1. D, Expression of *PIN2-GFP* under the *PIN2* promoter in root tips of *pER8:GhTCP14* plants grown on noninducing (top) or inducing (bottom) medium. Bars = 100 μm (left and middle) and 20 μm (right). [See online article for color version of this figure.]

EMSA with *AUX1*, *IAA3*, and *PIN2* promoter sequences and found that GhTCP14 can bind to the promoters of the three genes (Fig. 6).

Expression of *GhTCP14* in Cotton

To gain insights into the native functions of GhTCP14 in cotton, we analyzed its expression pattern. RT-PCR analysis showed that *GhTCP14* was expressed most strongly in developing fiber cells. *GhTCP14* transcript levels were high in the rapid fiber elongation stage (6–15 DPA), and the expression level was rather low in all other tissues examined (Fig. 7A). Because it is technically unfeasible to separate the fiber initials from the ovules at early developmental stages, in situ hybridization was conducted to examine the expression of *GhTCP14* in 0-DPA ovules when cotton fibers initiate from the ovule epidermal cells. *GhTCP14* transcripts were also abundant in the fiber initials (Fig. 7B). These results showed that *GhTCP14* expression is associated with fiber initiation and elongation.

The fiber-specific expression feature and the effect of GhTCP14 on auxin signaling observed in the transgenic Arabidopsis plants likely reflects a native role of GhTCP14 in cotton fiber differentiation and elongation through auxin-mediated signaling. To assess this role of GhTCP14, we analyzed the impact of IAA on its expression. As shown in Figure 7C, when 1-DPA cotton

ovules were treated with 1 μ M IAA for 3, 6, and 24 h, *GhTCP14* expression was induced shortly in about 3 h, and its transcription was increased 3-fold, showing that the expression of *GhTCP14* was up-regulated by auxin at an early stage of fiber development.

GhAUX1, *GhPIN2*, and *GhIAA3* May Be Direct Targets of GhTCP14 during Cotton Fiber Development

To confirm the association of *GhTCP14* expression with fiber elongation, we analyzed its expression in the fibers of *Li₁* cotton mutant. *Li₁* was originally discovered by Griffiee and Ligon (1929), and subsequent studies demonstrated that it is a dominant monogenic mutant with a defect in fiber elongation and plant growth. *GhTCP14* transcript levels were much lower in the short fiber cells compared with the wild-type control during the fast elongation stage of fiber development at 6 to 15 DPA (Fig. 8). Furthermore, we conducted quantitative RT-PCR analysis to see if the expression of the auxin-related genes *AUX1*, *PIN2*, and *IAA3* was coregulated with down-regulation of *GhTCP14* in *Li₁* fibers. Due to the lack of complete genome sequence of *G. hirsutum*, Arabidopsis *AUX1*, *PIN2*, and *IAA3* protein sequences were used to search against the *Gossypium* spp. database (tblasn), and an *AUX1*-like gene (*GhAUX1*, CD486543.1), a *PIN2*-like gene (*GhPIN2*, AY148428), and an *IAA3*-like gene (*GhIAA3*, HQ452481.1) were subsequently cloned. Then, we examined the expression of these auxin-related genes in *Li₁* and wild-type cotton fibers. Consistent with our hypothesis, the expression of *GhAUX1* and *GhIAA3* was reduced along with the down-regulation of *GhTCP14* in *Li₁* fibers. By contrast, the expression of *GhPIN2* increased with the repression of *GhTCP14* in these fiber cells (Fig. 8). These results suggested that GhTCP14 might regulate cotton fiber development by direct modulation of auxin-related genes, including *GhAUX1*, *GhPIN2*, and *GhIAA3*.

DISCUSSION

A complex group of transcription factors functions in either promoting or inhibiting leaf trichome initiation and development in Arabidopsis. These include the MYB transcription factors, the bHLH transcription factors, WD repeat proteins, and homeodomain proteins (Tominaga-Wada et al., 2011). Likewise, transcription factors participating in cotton fiber development have been reported by several studies. For example, a few MYB transcription factors were found to play important roles in cotton fiber initiation (Cedroni et al., 2003; Suo et al., 2003; Wang et al., 2004; Pu et al., 2008; Machado et al., 2009; Guan et al., 2011; Walford et al., 2011). In addition, it was recently reported that GbTCP (from sea-island cotton), which shares 47% protein sequence identity with GhTCP14, is involved in cotton fiber development, possibly through JA signaling (Hao et al., 2012). Here, our data indicated that GhTCP14 may

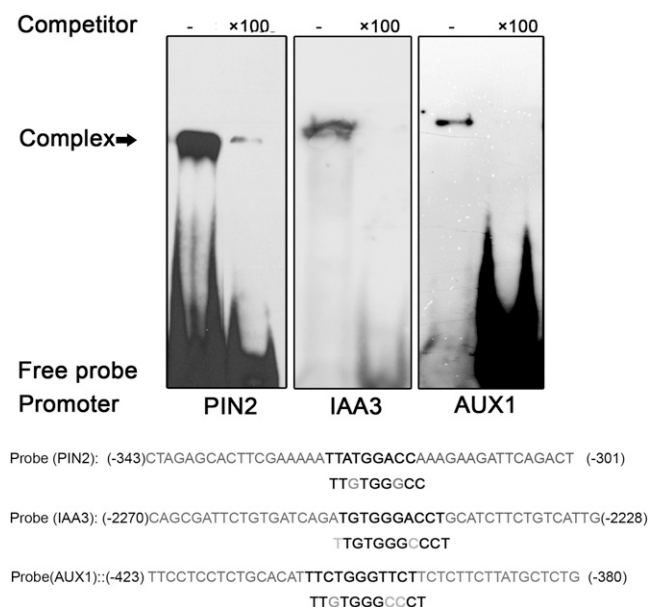
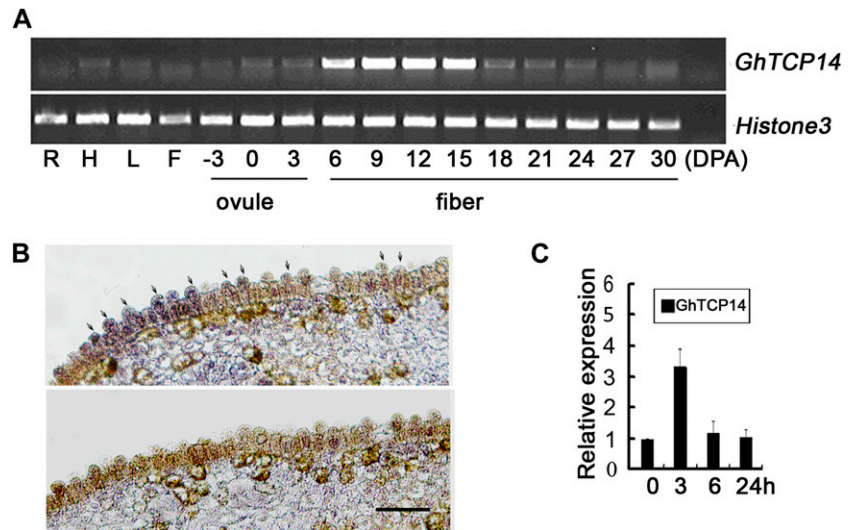


Figure 6. Binding of GhTCP14 to *AUX1*, *PIN2*, and *IAA3* promoter sequences. EMSA analysis was conducted with the recombinant GhTCP14 proteins and DNA sequences containing the promoter region of *PIN2*, *IAA3*, and *AUX1* genes, respectively. The promoter sequences used in EMSA are shown in the bottom portion of the figure. Numbers indicate positions relative to the translation start site of the corresponding gene. Putative TCP binding sequences are in bold.

Figure 7. Expression of *GhTCP14* in cotton. A, Transcript profile of *GhTCP14* in cotton. R, Root; H, hypocotyl; L, leaf; F, flower. The developmental stages of ovule and fiber are indicated as DPA. The *Histone3* gene was used as an internal control. B, In situ hybridization of *GhTCP14* transcripts in the ovule (0 DPA) of cotton with antisense (top) and sense (bottom) probes. Arrows show hybridization signals in fiber initials. Bar = 100 μ m. C, Expression of *GhTCP14* induced by IAA. Quantitative RT-PCR analysis was conducted with the total RNAs extracted from 1-DPA cotton ovules with fiber initials. The value of untreated ovules was set to 1. Error bars represent the sd of three biological repeats. [See online article for color version of this figure.]



be another important TCP transcription factor acting in the regulation of epidermal cell differentiation and development, and more importantly, we found that it may function through auxin pathways.

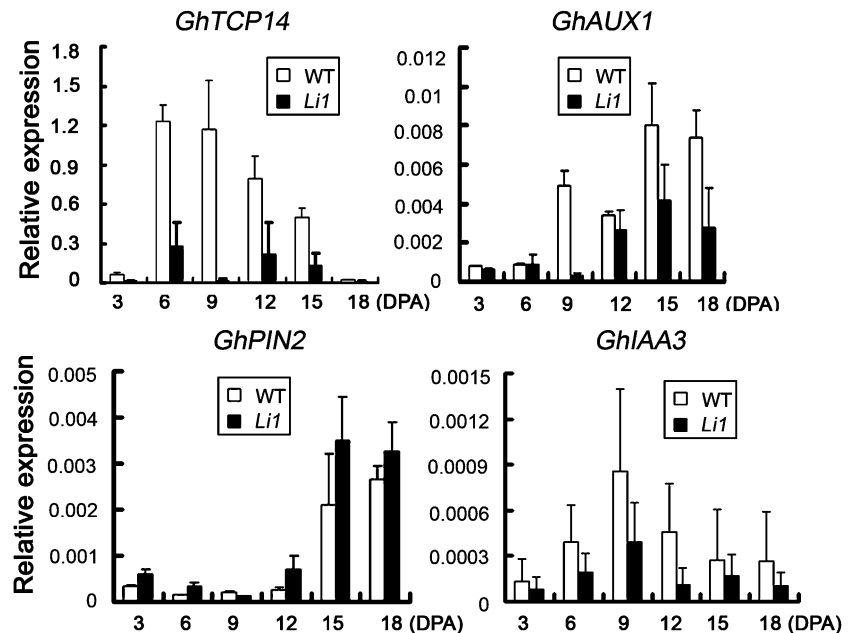
GhTCP14 Is a Dual-Functional Transcription Factor

It has been suggested that TCP proteins may function either as transcriptional activators or repressors, depending on their interactions with other proteins (Hervé et al., 2009). As the *GhTCP14*-overexpressing plants phenocopied auxin distribution mutants such as *pin2*, we analyzed the expression of *PIN2* and other auxin-related genes in the transgenic plants and found that the expression of several auxin-related genes was either increased or decreased. Sequence analyses

indicated that the promoters of these genes contain putative TCP-binding element to which GhTCP14 was able to bind, and dual-luciferase reporter assays showed that GhTCP14 possesses transcription factor activity. These results demonstrated that GhTCP14 acts as a dual functional transcription factor in the regulation of the expression of certain auxin-related genes. Although transcriptional activator or repressor activity has been reported for TCP proteins, to our best knowledge, this is the first report of the dual function of a TCP protein. The distinctive activities of GhTCP14 may depend on its interaction with other proteins (Hervé et al., 2009).

Recently, Koyama et al. (2010) reported that expression of *PIN1*, *PIN5*, and *PIN6* and *PIN3*, *PIN4*, and *PIN7* was either up- or down-regulated, respectively, by overexpression of the class II family

Figure 8. Expression of *GhTCP14*, *GhAUX1*, *GhPIN2*, and *GhIAA3* genes in the wild type and *Li1* mutant. Quantitative RT-PCR analysis was conducted with the total RNAs extracted from ovules (3 DPA) and fiber cells (6–18 DPA). The *Histone3* gene was used as the internal control. Error bars represent the sd of three biological replicates.



member *TCP3 chimeric repressor gene (TCP3SRDX)*, but *PIN2* expression was not affected in the transgenic plants. In our study, we found that *PIN2* expression was down-regulated, while expression levels of other *PIN* genes were not changed in *GhTCP14*-expressing transgenic plants. Combining these data, it is apparent that TCP transcription factors regulate the expression of *PIN* genes. In addition, our results suggested that the class I and II subclasses may have divergent functions in transcriptional regulation of the subgroups of *PINs*.

Overexpression of *GhTCP14* Altered Auxin Patterning

Several TCPs have been shown to function in leaf and flower development or embryogenesis via auxin-signaling pathways (Koyama et al., 2010; Uberti-Manassero et al., 2012). In our study, overexpression of *GhTCP14* altered expression of *PIN2*, *AUX1*, and *IAA3* and also produced phenotypic changes that resembled those of auxin-related mutants (Müller et al., 1998; Marchant et al., 1999; Ganguly et al., 2010). In particular, the numbers and lengths of the root hairs and trichomes were changed. In the root tip, the auxin distribution was substantially altered, and the lateral epidermal cells accumulated higher levels of auxin than other cells. In the trichomes, auxin accumulated more abundantly at the base of transgenic cells, but the signal was almost undetectable in the corresponding cells of the control plants. These results indicate that overexpression of *GhTCP14* affected distribution and homeostasis of auxin, which is required for the differentiation and elongation of the root hair and trichome, both of which differentiate from epidermal cells. Previously, molecular genetic analyses using *Arabidopsis* mutants demonstrated that the differentiation of both types of cells is regulated by similar molecular mechanisms (Tominaga-Wada et al., 2011). Here, our results provide novel evidence to show that the development of root hairs and trichomes might be controlled by a common auxin-mediated mechanism involving TCP transcription factor(s).

In addition to alterations in epidermal cell development, the association of *GhTCP14* function with auxin signaling is further evidenced by the gravitropism defect of the transgenic plants. Under inducing conditions, *GhTCP14*-overexpressing plants showed a severe defect in root gravitropism, which appeared similar to that of plants with elevated IAA levels, observed by Müller et al. (1998), who showed that down-regulation of the auxin transporter gene *PIN2* led to an auxin-hypersensitive phenotype, including a severe gravitropism defect.

Regulation of Auxin-Mediated Trichome Development by TCPs

Compared with the root hairs, the information on auxin-regulated trichome development is limited, although evidence showing the roles of gibberellins, JA,

salicylic acid, and cytokinins in trichome initiation and growth is accumulating (Traw and Bergelson, 2003; Steiner et al., 2012). Recently, it was reported that the sepals of *Arabidopsis tcp14 tcp15* double mutants were glabrous, but several trichomes grow on each sepal of the wild type and the *tcp14* or *tcp15* single mutant (Steiner et al., 2012). Here, we showed that ectopic overexpression of *GhTCP14* affected the differentiation and elongation of sepal and stem trichomes. Also, these phenotypic changes were coupled with a change in the auxin level at the base of the trichomes, implying that the TCP transcription factor participated in trichome development through auxin patterning. Interestingly, although the development of the sepal and stem trichomes and root hairs were both affected by induced *GhTCP14* expression, we did not see an obvious alteration in leaf trichome development in the transgenic plants, suggesting that the leaf trichomes may be under control of distinct mechanism(s). In line with our observation, Steiner et al. (2012) reported that the *Arabidopsis tcp14 tcp15* double mutant lacked sepal trichomes, whereas the leaf trichomes developed normally.

It has been established that auxin and cytokinin interact to control many aspects of plant growth and development (Moubayidin et al., 2009; Müller and Leyser, 2011). Previously, Steiner et al. (2012) showed that *Arabidopsis* TCP14 and TCP15 acted with SPINDLY to promote cell proliferation through cytokinin signaling in leaves and flowers. Here, our results demonstrate that *GhTCP14* regulated epidermal cell initiation and elongation through auxin signaling in roots and trichomes. Together, these results indicated that class I TCP transcription factors are involved in both auxin and cytokinin signaling and perhaps play roles in the cross talk between these two hormones.

The Possible Roles of TCP Transcription Factors in Developing Cotton Fibers

Cotton fibers differentiate from the epidermal layer of the ovule. Usually, only 30% of epidermal cells differentiate into fiber cells. Several transcriptome analyses have indicated that auxin signaling is important for fiber cell initiation and elongation (Gou et al., 2007; Liu et al., 2012). Accordingly, elevated expression of the key auxin synthesis gene *iaaM* was found to promote fiber cell differentiation and elongation, improving fiber yield and quality (Zhang et al., 2011). All these studies pointed out the importance of auxin signaling in fiber development and also raised the question of what mechanism(s) regulate these auxin-related processes. In this work, we found that (1) *GhTCP14* is specifically expressed at the fiber initiation and elongation stages; (2) the expression of *GhTCP14* was up-regulated by auxin at the early stage of fiber development; (3) ectopic expression of *GhTCP14* in *Arabidopsis* led to alterations in auxin levels and distribution, which affected root and trichome epidermal

cell initiation and elongation; and (4) in the *Li₁* short-fiber mutant, expression of *GhTCP14* was down-regulated, accompanied by decreased expression of *GhAUX1* and *GhIAA3* and increased expression of *GhPIN2*. Based on these results, we speculate that GhTCP14 may be an upstream regulator of auxin-mediated fiber initiation and elongation, functioning in transcriptional regulation of these auxin-related genes. Future work should further elucidate the functions of GhTCP14 in cotton plants.

MATERIALS AND METHODS

Plant Materials and Treatments

The cotton variety Texas-Maker-1 (TM-1; the genetic standard of *Gossypium hirsutum*) and *Li₁* mutant were used in this study. Seeds were sown on agar plates containing MS medium (Murashige and Skoog, 1962) and grown under tissue culture conditions for 2 weeks. Roots, hypocotyls, and leaves were collected from the seedlings. Flowers and fibers were harvested from field-grown cotton plants. Fibers were removed from the ovules at 6 to 30 DPA. The collected materials were immediately frozen in liquid nitrogen and stored at -80°C .

Seeds of *Arabidopsis thaliana* ecotype Columbia were germinated on agar plates containing MS salts, 1% Suc (*w/v*), and 0.7% (*w/v*) agar, pH 5.7. After vernalization at 4°C for at least 2 d, seedlings were grown in an illuminated growth chamber at 23°C . The induction of *GhTCP14* gene was carried out by two means: (1) direct germination of seeds on medium supplemented with $10\ \mu\text{M}$ β -estradiol or the solvent DMSO (used for mock treatment) and (2) transplant of the 4-d-old seedlings to medium containing β -estradiol or DMSO with continuing growth for 7 d.

Gene Cloning and RNA Analysis

The cDNAs of cotton genes were amplified by RT-PCR from total RNAs of cotton root, hypocotyl, leaf, flower, ovules, and fiber cells according to the ultracentrifugation method (John and Crow, 1992). The cDNAs of *Arabidopsis* genes were amplified by RT-PCR from total RNA extracted from the seedlings using TRIzol reagent (Invitrogen). In each case, $2\ \mu\text{g}$ of total RNA was used for reverse transcription. Quantitative RT-PCR assays were performed by using SYBR Green Real-Time PCR Master Mix (Toyobo) and the DNA Engine Option 2 Real-Time PCR Detection System (MJ Research). The primers used in RT-PCR and quantitative RT-PCR are listed in Supplemental Table S1.

Sequence Alignment, Domain, and Phylogenetic Analyses

The protein sequences were aligned by DNAMAN 6.0, the conserved domains were predicted at the National Center for Biotechnology Information, and phylogenetic analysis was performed with ClustalX version 1.83 (Thompson et al., 1997) and MEGA4 (Tamura et al., 2007) by the neighbor-joining method. Nuclear localization signal was predicted with WoLF PSORT.

Plasmid Construction and Plant Transformation

Full-length *GhTCP14* cDNA was amplified using the BD SMART RACE cDNA Amplification Kit. An *EcoR1-Sal1* fragment of *GhTCP14* cDNA was cloned into the pGEX-6p-1 vector for production of GST-GhTCP14 proteins. To construct 35S:GhTCP14 and pER8:GhTCP14 plasmids, *GhTCP14* open reading frame was amplified and inserted into plant expression vectors pPZP111 or pER8. Both constructs were introduced into *Arabidopsis* by the *Agrobacterium tumefaciens* floral dip procedure (Clough and Bent, 1998), and transgenic plants were selected using $50\ \text{mg mL}^{-1}$ kanamycin. Homozygous T3 *pER8:GhTCP14* plants were cross fertilized with plants carrying DR5:GFP, DR5:GUS, or PIN2:PIN2-GFP to generate DR5:GFP \times pER8:GhTCP14, DR5:GUS \times pER8:GhTCP14, and PIN2:PIN2-GFP \times pER8:GhTCP14 plants.

Subcellular Localization of GhTCP14-GFP Fusion Proteins

The *GhTCP14* open reading frame was fused to the GFP coding sequence and cloned into pPZP111 vector under the control of the *Cauliflower mosaic*

virus 35S promoter. Transgenic *Arabidopsis* plants were generated by floral dip method and selected using $50\ \text{mg mL}^{-1}$ kanamycin. Leaves of *GhTCP14-GFP* transgenic plants were stained with 4, 6-diamidino-2-phenylindole (Roche), and the signals were visualized with a confocal laser microscope (Leica TCS SP5).

EMSA

GST-GhTCP14 fusion proteins were expressed in *Escherichia coli* strain BL21 and then purified using glutathione-Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). Random binding site selection analysis (Nørby et al., 1992) was conducted using recombinant GhTCP14, and EMSAs were carried out using biotin-labeled probes and the Pierce LightShift Chemiluminescent EMSA kit (Thermo). The binding reaction was carried out in $20\ \mu\text{L}$ reaction mixture containing indicated GST-GhTCP14 proteins and $20\ \text{nmol}$ of synthetic biotin-labeled DNA probe in the presence or absence of nonlabeled competitor. The reaction mixtures were incubated at room temperature for 30 min and then applied onto a 6% native polyacrylamide gel in $0.5\times$ Tris-borate/EDTA buffer. The labeled probes were detected according to the instructions provided with the EMSA kit.

Protoplast Assay of Transcription Factor Activity

The dual-luciferase reporter assay (Ohta et al., 2001) was conducted to assess the transcription factor activity of GhTCP14 in *Arabidopsis* protoplasts. Reporter plasmid and plasmid pPTRL were kindly provided by Masaru Ohme-Takagi. The GAL4 reporter plasmid contains the firefly luciferase gene, driven by the minimal TATA region of the 35S promoter with five GAL4 binding elements upstream. The *Renilla* luciferase gene driven by the 35S promoter was used as an internal control. For construction of effector plasmid, the coding region of *GhTCP14* was cloned into the pRT-BD vector to generate 35S-BD-GhTCP14. The effector, reporter, and internal control plasmids were cotransfected into *Arabidopsis* protoplasts by PEG transformation. After culturing for 16 h, luciferase assays were performed with the Promega dual-luciferase reporter assay system and the GloMax 20-20 luminometer (Promega).

GUS Assay

GUS activity was analyzed by histochemical staining using a solution containing $1\ \text{mM}$ 5-bromo-4-chloro-3-indolyl- β -D-glucuronide; $10\ \text{mM}$ phosphate buffer, pH 7.0; $0.05\ \text{mM}$ potassium ferrocyanide; $0.05\ \text{mM}$ potassium ferricyanide; $2\ \text{mM}$ EDTA; and 0.1% (*v/v*) Triton X-100. *Arabidopsis* seedlings were incubated in staining solution at 37°C for 6 h in the dark, followed by washing with $3:1$ (*v/v*) ethanol:acetic acid solution before observation. Samples were examined under an Olympus BX51 microscope.

In Situ Hybridization

The 3'-untranslated region fragment of *GhTCP14* cDNA was PCR amplified (primers used are listed in Supplemental Table S1) and subcloned into the pMD-18T Simple vector (Takara). The plasmid was used for probe preparation according to the manual of DIG RNA Labeling Kit (SP6/T7; Roche). Cotton ovules were collected at 0 DPA and embedded in paraffin. *GhTCP14* mRNAs were detected with a digoxigenin-labeled riboprobe using the method described by Marrison and Leech (1994). The hybridized probes were detected using the anti-DIG-alkaline phosphatase conjugate and 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium as substrates. Sections were visualized under an Olympus BX51 microscope.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF165924.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic analysis of TCP proteins and bHLH domain comparison.

Supplemental Figure S2. Nuclear localization of GhTCP14.

Supplemental Figure S3. Random binding site selection analysis using recombinant GhTCP14.

Supplemental Table S1. Primers used in this study.

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