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Heterologous Expression of GbTCP4, a Class II TCP Transcription Factor, Regulates Trichome Formation and Root Hair Development in Arabidopsis

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Abstract: Two class I family teosinte branched1/cycloidea/proliferating cell factor1 (TCP) proteins from allotetraploid cotton are involved in cotton fiber cell differentiation and elongation and root hair development. However, the biological function of most class II TCP proteins is unclear. This study sought to reveal the characteristics and functions of the sea-island cotton class II TCP gene GbTCP4 by biochemical, genetic, and molecular biology methods. GbTCP4 protein localizes to nuclei, binding two types of TCP-binding cis-acting elements, including the one in its promoter. Expression pattern analysis revealed that GbTCP4 is widely expressed in tissues, with the highest level in flowers. GbTCP4 is expressed at different fiber development stages and has high transcription in fibers beginning at 5 days post anthesis (DPA). GbTCP4 overexpression increases primary root hair length and density and leaf and stem trichomes in transgenic Arabidopsis relative to wild-type plants (WT). GbTCP4 binds directly to the CAPRICE (CPC) promoter, increasing CPC transcript levels in roots and reducing them in leaves. Compared with WT plants, lignin content in the stems of transgenic Arabidopsis overexpressing GbTCP4 increased, and AtCAD5 gene transcript levels increased. These results suggest that GbTCP4 regulates trichome formation and root hair development in Arabidopsis and may be a candidate gene for regulating cotton fiber elongation.

Keywords: sea-island cotton; TCP transcription factor; trichome formation; root hair development

1. Introduction

Cotton is one of the most important fiber crops worldwide. Cotton fiber quality directly affects the market competitiveness of textiles [1]. Cotton fibers are unicellular trichomes derived from ovule epidermal cells. Fiber development can be divided into four distinct yet overlapping stages: initiation, cell elongation, secondary cell wall biosynthesis, and maturation [1,2]. Cotton fiber quality is measured in terms of length and strength, which mainly depend on the cell elongation and secondary cell wall biosynthesis growth stages [3]. Upland cotton (Gossypium hirsutum L.) and sea-island cotton (G. barbadense L.), which are widely grown, are allotetraploid cultivars [4]. Upland cotton has wide adaptability and a high yield, accounting for 99% of the raw cotton produced in China [5]. Sea-island cotton fiber is superior to upland cotton in length, fineness, and strength [3]. Exploring the molecular mechanisms involved in fiber development in sea-island cotton can provide useful information to generate superior fiber quality traits of upland cotton species through molecular breeding [6].
An increasing number of studies have shown that cotton fiber development has a similar transcriptional regulatory mechanism to that of the development of *Arabidopsis* leaf trichomes [7]. Therefore, *Arabidopsis* leaf trichomes can be used as an ideal system to study cotton fiber development genes. Teosinte branched1/cycloidea/proliferating cell factor1 (TCP) is a plant-specific protein family that has a conserved TCP domain and consists of 59 amino acid residues that form a basic helix–loop–helix (bHLH) structure [8]. The TCP domain is required for protein–protein interactions and DNA binding. According to the different TCP domains, the TCP family is divided into two subfamilies: Class I (TCP-P or PCF) and Class II (TCP-C). Class II TCP proteins are further subdivided into two subclasses, ECE (TB1/CYC) and CIN, based on differences in their amino acid domains [8]. In *Arabidopsis*, the TCP family consists of 24 members, some of which are involved in the regulation of trichome development [9,10]. For example, AtTCP4 is a class II TCP protein that inhibits trichome branching in *Arabidopsis* leaves and inflorescence stems by direct transcriptional activation of the trichome branching negative regulator GLABROUS INFLORESCENCE STEMS [9]. AtTCP15 is highly expressed in trichomes, and compared with wild-type (WT) plants, plants in which AtTCP15 expression is inhibited present increased trichome branch numbers [10].

To date, 36 and 38 TCP genes have been investigated in the diploid cotton species *G. arboreum* [11] and *G. raimondii* [12], respectively, and 74 TCPs have been identified in allotetraploid upland cotton [13]. Previous studies have found that two class I family TCP proteins from allotetraploid cotton are involved in cotton fiber cell differentiation and elongation and root hair development [14,15]. For instance, in sea-island cotton, GbTCP, the homologue of the AtTCP15 gene, is a crucial regulator of fiber elongation and root hair development by positively regulating jasmonic acid biosynthesis and the response and other pathways, as determined using overexpression and RNA interference silencing techniques [14]. In upland cotton, GhTCP14 is most similar to AtTCP14. *Arabidopsis* overexpressing GhTCP14 showed enhanced differentiation and elongation of root hairs and trichomes. GhTCP14 regulates the differential expression of several auxin synthesis- and transport-related genes, including IAA3, AUX1, and PIN2 [15]. However, the biological function of most class II TCP proteins in cotton is unclear. Therefore, it is important to identify class II TCP transcription factors in cotton and elucidate their functional mechanism.

Our previous study identified 75 GbTCP genes from sea-island cotton [16]. Real-time quantitative PCR (qPCR) results revealed that some GbTCP genes were expressed at different cotton fiber developmental stages, suggesting that these GbTCP genes may be involved in the regulation of cotton fiber development [16]. In this study, a sea-island cotton class II TCP gene, GbTCP4, was cloned and functionally identified. Overexpression of the GbTCP4 gene increased root hair length, root hair and trichome density, and the lignin content in transgenic *Arabidopsis* plants relative to WT plants. CAPRICE (CPC) and AtCAD5 are target genes of GbTCP4. These results indicate that GbTCP4, a class II TCP transcription factor, regulates trichome formation and root hair development in *Arabidopsis* and can be used as a candidate gene for cotton fiber quality improvement.

### 2. Materials and Methods

#### 2.1. Plant Materials

The experimental material of this study was the sea-island cotton cultivar Xinhai 21, which was planted in the experimental field of Alar City, Xinjiang. The ovules (0 d), flowers, calyces and receptacles were collected on the flowering day, as were the cotton fibers at 5 d, 10 d, 15 d, 20 d, 25 d, 30 d, and 35 d after flowering. The seeds of Xinhai 21 were soaked in 70% absolute ethanol, disinfected for 3 min, washed repeatedly with sterile water 3 to 5 times, spread in a germination box containing filter paper and cultured at 28 °C for 4 d. The hypocotyls were collected. The seedlings were transplanted into the nutrient solution to continue to grow, and when the first true leaves emerged, the main roots, fibrous roots, stems and leaves were collected. All samples were stored at −80 °C after liquid nitrogen freezing for RNA extraction.
Plants of the *Arabidopsis thaliana* ecotype Columbia (Col-0) were used to transform the *GbTCP4* gene. *Arabidopsis* was cultured in a greenhouse with a photoperiod of 16/8 h (day/night) and a culture temperature of 23 °C. The first two rosette leaves and roots of the transgenic and WT *Arabidopsis* were collected for analysis of the transcript level of the *CPC* gene. *Arabidopsis* stems grown for 6 weeks in nutrient soil were collected for analysis of lignin content and transcript levels of genes involved in the lignin pathway.

2.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted, and contaminating genomic DNA was removed as previously described [17]. Total RNA (200 ng) was reverse transcribed to cDNA using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania).

2.3. Real-Time Quantitative Polymerase Chain Reaction

qPCR was performed with green qPCR Master Mix (Fermentas, Vilnius, Lithuania) [16]. The *GbUBQ7* and *AtUBQ3* genes were used as internal standards for sea-island cotton and *Arabidopsis*, respectively. Amplification was performed using an ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems Inc., Foster City, CA, USA). The qPCR amplification system and amplification program followed the manufacturer’s instructions. The gene and internal reference gene cycle threshold (Ct) values of each sample were detected. Experimental data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The experiments were performed in three biological replicates. The hypocotyls were used as control samples to analyze the transcript level of the *GbTCP4* gene in different tissues of cotton. Primer sequences are shown in Supplementary Table S1.

2.4. Gene Cloning

According to the transcriptome data from the ovules of the sea-island cotton cultivar Xinhai 21 [16], the *GbTCP4* gene was amplified using the first strand of the ovule cDNA as a template. The amplified *GbTCP4* gene was ligated into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced to obtain the open reading frame (ORF) sequence of *GbTCP4*. Primer sequences are shown in Supplementary Table S1.

2.5. Bioinformatic Analysis

The molecular weight (MW), isoelectric point (pI), and homology analysis of the *GbTCP4* protein were predicted using DNAMAN7 software [16]. Multiple sequence alignments were performed using ClustalX 1.83 software, and phylogenetic trees were constructed using MEGA 4.1 software [16]. The subcellular localization of *GbTCP4* was predicted online using the PSORT program [16]. The 3000 bp promoter sequence upstream of *GbTCP4* and some of the known transcription factor genes involved in root hair and trichome development were obtained from the *G. barbadense* genome (https://cottonfgd.org) and The *Arabidopsis* Information Resource (TAIR, http://arabidopsis.org), respectively. The PLACE database was used for promoter sequence analyses [18].

2.6. Subcellular Localization

For the subcellular localization experiment, the ORF of *GbTCP4* was subcloned into the plant expression vector pCAMBIA1304 containing the GFP reporter gene to generate the 35S:*GbTCP4*-GFP fusion expression vector. Transient transformation of onion epidermal cells was performed by the *Agrobacterium*-mediated method. First, 1 cm² of onion epidermal cells was soaked in *Agrobacterium* solution for 30 min, and the surface of the bacterial solution was filtered, transferred to 1/2 MS solid medium and incubated at 25 °C for 2 d under a 16 h light/8 h dark photoperiod. After the epidermal cells of the inoculated and co-cultured cells were washed, they were placed on a glass slide and observed under a Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany).
2.7. Transcriptional Activation Assay

For the transcriptional activation assay, the ORF sequence of \textit{GbTCP4} was cloned into the pGBK7 vector. The pGBK7 and GbTCP4-pGBK7 plasmids were transformed into AH109 yeast cells using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Orange, CA, USA). The pGBK7 vector carries the TRP1 nutritional marker for selection in yeast. AH109 contains ADE2 and HIS3 screening markers. The transformed yeast cells were cultured on SD/-Trp medium and SD/-Trp-His-Ade medium for 30 h at 30 °C, and the growth of yeast cells was observed.

2.8. Yeast One-Hybrid Assay

For yeast one-hybrid assays, the ORF of \textit{GbTCP4} was inserted into pGADT7-Rec2 (Clontech, Palo Alto, CA, USA) as the effector (GbTCP4-pGADT7). Three tandem repeats of TCP I (TGGGTCCCACAT), TCP II (TTGTGGGCCCCT), the mutated TCP I (TAGATTCTAAAG), and the mutated TCP II (TGGGAGACTCG) were synthesized with reference to previous studies [15]; different fragments containing different TCP motifs of the promoter sequences of \textit{AtCPC}, \textit{AtCAD5}, and \textit{GbTCP4} were amplified from genomic DNA. These sequences were cloned into pHIS2 (Clontech, Palo Alto, CA, USA) upstream of the reporter gene HIS3. Co-transformation of pHIS2 with or without the TCP \textit{cis}-element sequences and GbTCP4-pGADT7 into Y187 cells was performed as described by Yu et al. (2017) [19]. The pGADT7 vector carries the LEU2 nutritional marker for selection in yeast. The pHIS2 vector carries the TRP1 nutritional marker for selection in yeast. The transformed yeast cells were cultured on SD/-Trp-Leu medium and SD/-Trp-His-Leu medium supplemented with 10 mM or 30 mM 3-amino-1,2,4-triazole (3-AT) for 30 h at 30 °C, and the growth of yeast cells was observed. Primer sequences are shown in Supplementary Table S1.

2.9. Generation of Transgenic Arabidopsis Plants

To construct a constitutively overexpressing plant expression vector driven by the cauliflower mosaic virus 35S promoter (35S CaMV), the ORF of \textit{GbTCP4} was cloned into the pCAMBIA3301 vector (Cambia, Canberra, Australia). Agrobacterium-mediated \textit{Arabidopsis} transformation was performed as described by Ni et al. (2013) [20]. Transgenic \textit{Arabidopsis} seeds were screened using glufosinate. Three homozygous T3 generation transgenic \textit{A. thaliana} plants were selected for further experiments.

2.10. Root Hair Observation and Measurement

For root hair analyses, Col-0 and 35S:GbTCP4 seeds were planted on Murashige and Skoog (MS) medium plates. When two true leaves emerged, 30 seedlings from Col-0 and 35S:GbTCP4 were placed in a new MS medium plate. After 1 week of upright growth, the root hair density and root hair length were measured at 1 cm above the main root tip. Photographs were taken under a Classica SK200 digital microscope (Motic, Xiamen, China). All experiments were repeated three times.

2.11. Trichome Observation and Measurement

For trichome analyses, Col-0 and 35S:GbTCP4 seeds were planted on MS medium plates. When two cotyledons grew, 30 seedlings from Col-0 and 35S:GbTCP4 were placed in nutrient soil (nutritional soil:vermiculite:perlite, 1:1:1). When the first two rosette leaves appeared, the number of leaf trichomes was counted. The stem trichomes on the main stem internodes were counted from the bottom of 1-month-old soil-grown seedlings. Images were collected under a SteREO Discovery.V20 microscope (Zeiss, Oberkochen, Germany). All experiments were repeated three times.
2.12. Phloroglucinol Staining

Thirty seedlings from three 35S:GbTCP4 transgenic Arabidopsis lines and WT Arabidopsis were planted in nutrient soil and used for phloroglucinol staining after 6 weeks of growth. Fresh stems of Arabidopsis were taken, and a 1 cm thick sample of the same part of the transgenic and WT stems was cut with a blade. A 1% phloroglucinol-alcohol solution was dropped on the cross-section of the sample and left for 2 min. The sample was dried with 32% concentrated hydrochloric acid solution until the sample cross-section appeared red. The sample staining results were observed with a Nikon Eclipse TS2R microscope (Nikon, Tokyo, Japan) and were imaged.

2.13. Determination of Lignin Content

The lignin content of the 35S:GbTCP4 transgenic Arabidopsis line and WT Arabidopsis stems was determined according to the procedure of the Plant Lignin ELISA Kit (Mlbio, Shanghai, China). All experiments were repeated three times.

2.14. Statistical Analyses

Statistical analysis of the numerical data was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA). For multiple pairwise comparisons, the data were compared using a one-way ANOVA test. *p < 0.05 was considered statistically significant, and **p < 0.01 was considered extremely significant.

3. Results

3.1. Cloning and Sequence Analysis of GbTCP4

The GbTCP4 gene was cloned from Xinhai 21 according to the cotton fiber transcriptome data of the sea-island cotton cultivar Xinhai 21. The ORF of GbTCP4 is 1335 bp in length and encodes 444 amino acids with a predicted MW of 48.05 kDa and a pI of 7.16. The amino acid sequence of GbTCP4 contains a conserved TCP domain between 36 and 99 amino acids. Phylogenetic tree analysis found that GbTCP4 clustered with AtTCP3 and AtTCP4 in one branch, belonging to the CIN subclass of the class II TCP protein (Figure 1A). Multiple sequence alignment revealed that GbTCP4 shares 47.07% and 50.43% overall amino acid identity with AtTCP3 and AtTCP4, respectively. The amino acid sequence alignment of the TCP domain of GbTCP4 and AtTCP4 indicates that their amino acid identity is 95.31%, and there are only three different amino acids (Figure 1B). Moreover, it is predicted that there is a nuclear localization signal (PRDRRVR) in the basic structure of the TCP domain of GbTCP4.
Figure 1. Phylogenetic tree analysis and sequence alignment of GbTCP4. (A) The phylogenetic tree of GbTCP4 and Arabidopsis TCP family proteins. The accession numbers of the proteins are as follows: AtTCP1, AT1G67260.1; AtTCP2, AT4G18390.1; AtTCP3, AT1G53230.1; AtTCP4, AT3G15030.1; AtTCP5, AT5G60970.1; AtTCP6, AT5G41030.1; AtTCP7, AT5G23280.1; AtTCP8, AT1G58100.1; AtTCP9, AT2G31070.1; AtTCP10, AT2G37000.1; AtTCP11, AT1G68800.1; AtTCP12, AT3G02150.2; AtTCP13, AT1G58600.1; AtTCP14, AT3G27010.1; AtTCP15, AT5G19070.1; AtTCP16, AT3G27010.1; AtTCP17, AT5G08070.1; AtTCP18, AT5G81910.1; AtTCP19, AT3G35560.1; AtTCP20, AT5G51910.1; AtTCP21, AT5G8330.1; AtTCP22, AT1G207010.1; AtTCP23, AT1G35560.1; AtTCP24, AT1G30210.1. (B) Comparison of amino acid sequences of GbTCP4 and AtTCP4. Black indicates identical amino acids. The horizontal line indicates the conserved teosinte branched1/cycloidea/proliferating cell factor1 (TCP) domain.
3.2. GbTCP4 Protein Localizes to the Nucleus

To analyze whether the GbTCP4 transcription factor is localized in the nucleus, the *Agrobacterium*-mediated method was used to transiently express 35S:GbTCP4-GFP in onion epidermal cells, and cells transformed with 35S:GFP served as a control. As shown in Figure 2A, compared with that transformed with 35S:GFP, the onion epidermis transformed with 35S:GbTCP4-GFP showed green fluorescence only in the nucleus. The subcellular localization results indicated that the GbTCP4 protein was localized to the nucleus.

Figure 2. Molecular characterization of the GbTCP4 transcription factor. (A) Subcellular localization analysis of the GbTCP4 transcription factor. 35S:GFP is an empty vector control. The red arrow indicates the nucleus. (B) Analysis of the transcriptional activation activity of GbTCP4. pGBK7 is an empty vector control. (C) Analyses of the binding of GbTCP4 to TCP I (TGGGTCCCACAT), TCP II (TTGTGGGGGCCCCT), mTCP I (TAGATTCTAAAG), and mTCP II (TGCAGAGAGACTCG). (D) Analyses of the binding of GbTCP4 to the TCP-binding element in its promoter.
3.3. GbTCP4 Lacks Transcriptional Activation Activity

To detect whether GbTCP4 is capable of activating the HIS3 reporter gene in yeast, pGBK7 and GbTCP4-pGBK7 were transformed into AH109 yeast cells. Yeast cells transformed with pGBK7 and GbTCP4-pGBK7 grew normally on SD/-Trp medium (Figure 2B), indicating that both plasmids were transformed into yeast cells. However, yeast transformed with pGBK7 and GbTCP4-pGBK7 could not grow on SD/-Trp-His-Ade medium (Figure 2B). The above results indicate that GbTCP4 cannot activate the HIS3 reporter gene alone in yeast and may need to activate or inhibit the target gene together with other transcription components.

3.4. GbTCP4 Binds to Two Types of TCP-Binding Cis-Elements

To examine whether GbTCP4 binds to the normal and mutated TCPI/II binding cis-element sequences, yeast one-hybrid analyses were employed. In SD/-Leu-Trp medium, all transformed yeast cells grew normally, indicating that the transformation was successful. However, in SD/-Leu-Trp-His 10 mM 3-AT medium, only the yeast cells co-transformed with pHIS2-TCPI/II and GbTCP4-pGADT7 grew normally, indicating that GbTCP4 recognizes two types of TCP-binding cis-elements (Figure 2C).

3.5. GbTCP4 Binds to TCP-Binding Cis-Elements in Its Promoter

Using the PLACE network to analyze the promoter sequence of GbTCP4 [18], we identified five TCP-binding cis-acting elements in its promoter sequence (Supplementary Figure S1). Using the yeast one-hybrid method, as shown in Figure 2D, we determined that GbTCP4 binds directly to TCP-binding cis-elements in its promoter.

3.6. Expression Pattern of GbTCP4 in Cotton

qPCR was used to analyze the expression pattern of GbTCP4 in different tissues and organs of cotton. The qPCR results showed that the transcript level of GbTCP4 was the highest in flowers, while its transcript levels were lower in calyx, receptacle, leaf, hypocotyl, stem, and fibrous root tissues, and the lowest transcript level occurred in the main root (Figure 3A). qPCR showed that GbTCP4 was expressed in cotton fibers at different developmental stages, and it had the highest transcript level in cotton fibers at 5 days post anthesis (DPA), suggesting that GbTCP4 may be involved in regulating the elongation of cotton fibers (Figure 3B).
3.7. Overexpression of GbTCP4 Increases Root Hair Length and Density

To reveal the biological function of GbTCP4, it was overexpressed in Arabidopsis plants. We selected three representative homozygous transgenic plants for functional analysis. The GbTCP4 gene had a very high transcript level in the three homozygous 35S:GbTCP4 transgenic lines, whereas no transcript level expression of the GbTCP4 gene was detected in WT plants (Figure 4A). Because GbTCP4 has
a high transcript level during cotton fiber elongation, we examined its effect on Arabidopsis root hair development. Arabidopsis plants with two true leaves were placed in plates with MS medium for vertical culture. Approximately 7 d after vertical growth, compared with that of WT plants, the root hair length of the primary roots of three 35S:GbTCP4 transgenic lines significantly increased; the root hair lengths of the three 35S:GbTCP4 transgenic lines were 2.37, 2.44, and 3.03 times the WT root hair length, respectively (Figure 4B,C; Supplementary Table S2). Moreover, compared with that of WT plants, the root hair density of 35S:GbTCP4 transgenic plants significantly increased (Figure 4D; Supplementary Table S2). The above results suggest that the overexpression of GbTCP4 increases root hair length and density in the primary roots of Arabidopsis.

Figure 4. Constitutive overexpression of GbTCP4 in Arabidopsis increases root hair length. (A) Transcriptional levels of the GbTCP4 gene in three 35S:GbTCP4 transgenic Arabidopsis lines. The transcriptional levels were calibrated by the control (Col-0). AtUBQ3 was used as an internal reference gene. Values represent the means of three biological replicates, and error bars represent standard deviations. (B) Root hair phenotype from 7-d-old vertically grown seedlings of Col-0 and 35S:GbTCP4 transgenic lines. Scale bars: 100 μm. (C) Comparison of the root hair length on the roots from the Col-0 and 35S:GbTCP4 transgenic lines. (D) Comparison of the root hair density on the roots from the Col-0 and 35S:GbTCP4 transgenic lines. Values are means (±SE) of 30 seedlings for each of three independent experiments. Asterisks indicate significant differences (* p < 0.05; ** p < 0.01) relative to Col-0.

3.8. Overexpression of GbTCP4 Increases the Trichome Number of the Leaf and Stem

To study whether GbTCP4 regulates trichome formation, trichomes on the leaves and stems of WT and 35S:GbTCP4 transgenic plants were observed. Statistical analyses showed that, compared with WT plants, the three 35S:GbTCP4 transgenic lines had significantly increased trichome numbers on the first two rosette leaves, and the trichome numbers of the three 35S:GbTCP4 transgenic lines were 1.44, 1.48, and 1.77 times those of the WT plants, respectively (Figure 5A,B). Similarly, the trichome numbers on the main stem internodes from the bottom of three 35S:GbTCP4 transgenic lines were significantly higher than those of WT plants (Figure 5C,D). These results indicate that overexpression of the GbTCP4 gene in Arabidopsis increases the trichome number of leaves and stems.
Figure 5. Constitutive overexpression of GbTCP4 in Arabidopsis affects trichome formation. (A) Leaf trichome phenotype from the first two rosette leaves of 2-week-old soil-grown seedlings of the Col-0 and 35S:GbTCP4 transgenic lines. Scale bars: 500 µm. (B) Comparison of the trichome number on the first two rosette leaves from Col-0 and 35S:GbTCP4 transgenic lines. (C) Stem trichome phenotype from the main stem internodes from the bottom of 1-month-old soil-grown seedlings of Col-0 and 35S:GbTCP4 transgenic lines. Scale bars: 500 µm. (D) Comparison of the trichome density on the main stem internodes from the bottom of Col-0 and 35S:GbTCP4 transgenic lines. Values are means (±SE) of 30 seedlings for each of three independent experiments. Asterisks indicate significant differences (* p < 0.05) relative to Col-0.

3.9. Overexpression of the GbTCP4 Gene Increases Lignin Content in Transgenic Arabidopsis

Previous studies have found that overexpression of AtTCP4, a homologue of GbTCP4, increases the lignin content of transgenic Arabidopsis [21]. To analyze whether GbTCP4 is involved in the regulation of lignin content, lignin staining was performed on the 35S:GbTCP4 transgenic line and WT Arabidopsis using the phloroglucinol method. As shown in Figure 6A, the cross-sections of the three 35S:GbTCP4 transgenic line stems were darker, and the red color was denser than that observed for the WT plants, indicating that the three 35S:GbTCP4 transgenic lines had higher lignin content. The lignin content of the stems showed that the lignin content of the three 35S:GbTCP4 transgenic lines was significantly higher than that of the WT plants. Compared with that of WT plants, the lignin content of the three 35S:GbTCP4 transgenic lines increased by 17.13%, 18.77%, and 19.64%, respectively (Figure 6B). Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) catalyzes the final step in lignin precursor synthesis, reducing the cinnamyl aldehydes to their corresponding alcohols in the presence of NADPH [22]. Subsequently, qPCR was used to analyze the transcriptional level of the AtCAD5 gene in the three 35S:GbTCP4 transgenic Arabidopsis lines. The results showed that the transcript level of AtCAD5 was significantly elevated in the three 35S:GbTCP4 transgenic line relative to the WT plants (Figure 6C). In addition, analyses of cis-acting elements in the promoter revealed TCP-binding elements in the promoter of AtCAD5. Yeast one-hybrid results indicated that GbTCP4 can bind the TCP-binding elements in the AtCAD5 promoter (Figure 6D). These results indicate that overexpression of the GbTCP4 gene increases the lignin content of the stem of transgenic A. thaliana. Moreover, AtCAD5 increased transcript levels in the 35S:GbTCP4 transgenic Arabidopsis line.
Figure 6. Constitutive overexpression of the GbTCP4 gene increases lignin content in transgenic Arabidopsis stems. (A) Phloroglucinol staining of stem cross-sections of the Col-0 and 35S:GbTCP4 transgenic lines. xy: xylem; if: interfascicular fibers; co: cortex; ep: epidermis; bars: 100 µm. (B) Determination of lignin content in stems of the Col-0 and 35S:GbTCP4 transgenic lines. (C) Quantitative PCR (qPCR) analysis of transcriptional levels of the AtCAD5 gene in the Col-0 and 35S:GbTCP4 transgenic lines. The transcriptional levels were calibrated by the control (Col-0). AtUBQ3 was used as an internal reference gene. Values represent the means of three biological replicates, and error bars represent standard deviations. Asterisks indicate significant differences (* p < 0.05) relative to Col-0. The accession number of the gene is as follows: AtCAD5:At4g34230. (D) Analyses of the binding of GbTCP4 to the TCP-binding cis-element in the promoter of AtCAD5.

3.10. CPC is a Target Gene of GbTCP4

To identify the target gene of GbTCP4, the PLACE network was used to analyze whether promoter regions of some of the known transcription factor genes involved in root hair and trichome development contained TCP-binding elements [18]. Analyses of cis-acting elements in the promoter revealed TCP-binding elements in the promoter of CPC. Yeast one-hybrid results indicated that GbTCP4 can bind the TCP-binding elements in the CPC promoter (Figure 7A). Then, the transcript level of the CPC gene in the roots and leaves of the three 35S:GbTCP4 transgenic line were analyzed by qPCR. qPCR results revealed that, compared with that of WT plants, the transcript level of CPC in the roots of the three 35S:GbTCP4 transgenic line were elevated, and the transcript level of CPC in the leaves were reduced (Figure 7B). These results indicate that CPC is a target gene of GbTCP4.
Figure 7. CAPRICE (CPC) is a target gene of GbTCP4. (A) Analyses of the binding of GbTCP4 to the TCP-binding cis-element in the promoter of CPC. (B) Detection of the transcription levels of CPC gene in the roots and leaves of Col-0 and 35S:GbTCP4 seedlings by qPCR. The transcriptional levels were calibrated by the control (Col-0). AtUBQ3 expression was used as a control. Values represent the means of three biological replicates, and error bars represent standard deviations. Asterisks indicate significant differences (* \( p < 0.05 \)) relative to Col-0.

4. Discussion

4.1. GbTCP4 Regulates Root Hair Length and Trichome Number in Arabidopsis

Previous research has found that some TCP genes are highly expressed in cotton fibers [11,13]. For example, the transcriptome data and qPCR results showed that 15, 9, and 12 GhTCP genes from upland cotton were specifically expressed in the fiber initiation, elongation, and secondary cell wall deposition stages, respectively [13]. Similarly, 13 of the 36 G. arboreum TCP transcription factors are highly expressed at different fiber development stages [11]. Our present research also found that the GbTCP4 gene had a higher transcript level in rapidly elongating fibers, which suggests that it is involved in cotton fiber elongation. Because Arabidopsis leaf trichomes have been successfully used as an ideal model research system for the functional analyses of cotton fiber development-related genes [14,15], we constitutively overexpressed GbTCP4 in Arabidopsis to study its function in trichome development. We found that, compared with those of WT plants, the root hair length and trichome numbers of plants overexpressing GbTCP4 increased. These results strongly show that GbTCP4 regulates root hair length and trichome number in Arabidopsis.
4.2. Mechanism of the Action of GbTCP4 in the Regulation of Root Hair Length and Trichome Number in Arabidopsis

Trichome initiation and patterning of Arabidopsis are activated by a MYB-bHLH-WD40 complex comprising three positive regulators (GL1, GL3, and TTG1) [23]. Conversely, in Arabidopsis, an R3-type MYB domain protein, AtCPC, negatively regulates trichome initiation and patterning. Arabidopsis overexpressing AtCPC has reduced or no trichomes on the leaves and more root hairs on the roots [24]. Moreover, TTG1 represses the activation of the CPC promoter by GL1 and GL3 [25]. Similarly, in cotton, GhTTG1 and GhTTG3 restore trichome formation in ttg1-mutant Arabidopsis plants [26]. GhCPC is homologous to AtCPC, and transgenic cotton lines overexpressing GhCPC showed delayed fiber initiation and reduced fiber length by a potential CPC-MYC1-TTG1/4 complex [27]. GhTCP22 and GhTCP14a can interact with GhMYB25/GhMYB23-GhGL3-GhTTG1 [13]. Our results showed that overexpression of GbTCP4 in Arabidopsis can activate the transcript levels of CPC in roots and inhibit the transcript levels of CPC in leaves. This may be because some of the TCP proteins, including the GbTCP4 protein, do not enable activation or inhibition by themselves and need to cooperate with other proteins to regulate the transcription of the target gene. Previous studies have found that two class I proteins, GhTCP22 and GhTCP14a, can interact with several transcription factors related to cotton fiber growth and development. Both can interact with GhSLR1, GhGL3, GhARF6, GhTTG1, GhMYB23, and GhMYB25 transcription factors. In addition, GhTCP14a can also interact with GhEIN3, GhBZR1, and GhMYB25L proteins [13]. Because cotton fiber development is similar to that of Arabidopsis trichome development, we speculate that the GbTCP4 gene may promote fiber elongation in cotton through a similar regulatory mechanism.

4.3. Overexpression of GbTCP4 Increases Lignin Content

Previous studies found that, when inhibition of AtTCP4 by miR319a was abolished, petals and stamens did not develop [28]. The AtTCP4 promoter drives the GUS reporter gene to be expressed in petals, unflowered pollen and carpels [29]. Similarly, we found that GbTCP4 has a high level of transcriptional expression in the flower, calyx, and receptacle of sea-island cotton. Recent studies have found that overexpression of miR319-resistant AtTCP4 increases the lignin and cellulose content of transgenic Arabidopsis [21]. Similar to this result, we found that, compared with WT plants, transgenic Arabidopsis plants that overexpress the GbTCP4 gene exhibit increased lignin content. Moreover, AtCAD5 increased transcript levels in the 35S:GbTCP4-3 transgenic Arabidopsis line. AtCAD5 was capable of efficiently catalyzing all five substrates, including p-coumaryl aldehyde, caffeyl aldehyde, coniferyl aldehyde, 5-hydroxyconiferyl aldehyde, and sinapyl aldehyde. AtCAD5 can be used to normally generate all three monolignols. Mutation of the AtCAD5 gene slightly delayed the syringyl lignin deposition rate [22]. We speculate that GbTCP4 may be involved in the regulation of AtCAD5 gene expression and thus may affect lignin content.

4.4. GbTCP4 Recognizes Two Types of TCP-Binding Cis-Elements

The TCP-binding cis-element sequences of the two classes are different but overlapping, but some TCP proteins show certain adaptability to recognition [8]. For instance, the class I transcription factor AtTCP16 can bind to TCP II binding sites [30]. In cotton, GhTCP14, a class I TCP transcription factor, can bind to TCP I and II binding sites [15]. Similarly, our results showed that GbTCP4, as a class II TCP transcription factor, could also bind to TCP I and II motifs. Previous research has shown that two classes of TCP proteins can bind to CG-rich sequences in target gene promoters, and speculations exist about a potential opposing relationship between class I and class II TCPs [8]. For example, GhTCP14 can recognize the promoters of PIN2, IAA3, and AUX1 [15]. Our results showed that GbTCP4 binds to the promoter of AtCPC and regulates its expression.
5. Conclusions

In conclusion, GbTCP4 acts as a transcription factor, localizes to the nucleus, and is capable of binding two types of TCP-binding cis-acting elements. GbTCP4 is involved in the regulation of *Arabidopsis* root hair and trichome development and lignin deposition and regulates the transcript levels of related genes. Our results not only increased understanding of the function of the sea-island cotton class II TCP protein but also provided candidate genes for improving cotton fiber length.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4425/10/9/726/s1, Figure S1: The promoter sequences of GbTCP4. The predicted TCP-binding site is represented by yellow, Table S1: Primer sequences used in this study, Table S2: Data for measuring the number and length of root hair.

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