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The Cotton Cinnamyl Alcohol Dehydrogenase 6 Functions in Developing Cotton Fibers

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Abstract

Cotton fiber is the most important source used natural raw in the textile industry and its productions play a pivotal role in the global economy. Cinnamyl alcohol dehydrogenase (*CAD*) plays crucial roles in the development of cotton fibers, but its functional mechanisms remain largely unknown. Research on improving fiber quality of cotton crop is important. Owing to a fiber-specific *CAD* gene, designated *GhCAD*6 from upland cotton (*Gossypium hirsutum* L.) is expressed predominantly in fiber cells, especially at the secondary wall thickening stage of fiber development, we characterized the function of *GhCAD*6 in cotton fiber development. Through cloning *GhCAD*6 and analyze expression of *GhCAD*6 in cotton fiber development by real-time RT-PCR. *GhCAD*6 overexpression construct was introduced in upland cotton variety Xinluzao 36 using *Agrobacterium tumefaciens*-mediated transformation. A number of *GhCAD*6 transgenic plants were obtained and a single copy of *GhCAD*6 was integrated into the recipient genome of cotton fiber. The expression of *GhCAD*6 in transgenic plants was higher with compared to that in the wild type and the *GhCAD*6 transgenic cottons resulted in changing the content of the fiber cell structural composition phenylpropanoid. Although these transgenic lines in cell wall composition was not obvious difference, except only the phenylpropanoid content in the mature fiber of transgenic plants being slightly lower, the mature cotton fiber cells of transgenic lines had a quite smooth, fine, and dense surface, which led to be changed in the fiber length, strength, uniformity and micronaire value and improved the fiber quality in transgenic lines. These results demonstrate that *GhCAD*6 is involved in regulating cotton fiber development and is a promising candidate gene to improve fiber quality in cotton through genetic manipulation.

Keywords

Cotton Fiber, Phenylpropanoid Metabolism, Cinnamyl Alcohol

Dehydrogenase (*CAD*), Fiber Quality, Structural Composition

1. Introduction

Cotton (*Gossypium spp.*) is the most important source used natural raw in the textile industry and its productions play a pivotal role in the global economy. Cotton fiber is formed from partial single ovule epidermal cells of the outer integument, which undergo differentiation, protuberance, elongation, secondary wall thickening and dehydration maturation [1]. The main indices of cotton fiber quality include fiber length, strength, and fineness. Cotton fiber length is one of the most important indices of fiber quality, which is closely related to spinning quality. When other qualities are identical, spinning count positively correlates with fiber length. Cotton fiber strength refers to the strength of the cotton fiber to resist tensile fracture. The fiber strength positively correlates with the quality of yarn and cloth when other indices are identical. Fiber fineness is closely related to yarn strength. When spinning yarn using fine mature fiber, yarn strength is high because of the large number of fiber roots in the yarn, large indirect contact surface, and tight cohesion [2]. The cross-section of the mature cotton fiber is generally irregular and oval and the biochemical components of its cell wall are related to cotton fiber yield, quality, and utilization. It appears to be composed of a primary wall, secondary wall, and middle cavity from the outside to inside. Owing to the lack of parent materials, good germplasm, and innovative technologies, the improvement of cotton fiber quality index is not adequate. With the development of science and technology, there is an urgent requirement to cultivate high-quality cotton varieties by combining conventional breeding and biotechnological techniques. Cloning and functional analysis of development-related genes in cotton fiber are the basis to achieve this goal.

The phenylpropanoid pathway is one of the important secondary metabolic pathways in plants [3]. Previous studies have shown that the main structural components of cotton fibers include abundant structural polysaccharides and small quantities of phenylpropanoids and the phenylpropanoid pathway is the second-largest metabolic pathway after carbohydrate metabolism in cotton fiber [4] [5]. Some key enzymes of the phenylpropanoid metabolic pathway are predominantly expressed in the secondary wall during the elongation period of cotton fiber [6]-[11]. Cinnamyl alcohol dehydrogenase (CAD) plays a key role in phenylpropanoid metabolism. CAD affects the last step of the phenylpropanoid pathway and functions on the synthesis of guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units [12] [13]. Two kinds of phenylpropanoid structural monomers, G and S units, are present in cotton fiber [4]. It has been reported that several *CADs* participate in regulating the process of cotton fiber development [14] and found that *GhCAD*1 and *GhCAD*6 were predominantly expressed during the secondary

wall thickening of cotton fiber [4]. Although *GhCAD*1 and *GhCAD*6 homology is very high, owing to the different catalytic activities of the two enzymes their functional substrates are different. *GhCAD*6 plays a major role in the process of phenylpropanoid synthesis and *GhCAD*6 expression increases during the secondary wall formation in cotton fiber, while *GhCAD*1 has only a compensatory function during the fiber development. Li *et al.* [15] showed that *GhCAD*6 is located on cotton chromosome 26 and involved in the metabolism of phenylpropane units and cell wall phenolic acids in cotton fibers. Therefore, we want to improve cotton fiber quality by introducing *GhCAD*6 into cotton plants.

In this study, we obtained the stable *GhCAD*6-transformed plants of T_6 generation after six generations of kanamycin selection via containing neomycin phosphotransferase II gene (*NPT II*) driven by 35S promoter and *GhCAD*6 driven by E6 promoter and transferred to Xinluzao36 using *Agrobacterium tumefaciens*-mediated transformation and further characterized that the cotton *GhCAD*6 (GenBank: EU281305.1) can significantly change the formation of cell wall cross-linking structure of cotton fiber and over-expression of *GhCAD*6 can improve fiber quality in transgenic cotton plants. These findings suggest that *GhCAD*6 may involve in regulating cotton fiber development and could be used as a promising gene resource to improve fiber quality in cotton through genetic manipulation.

2. Materials and Methods

2.1. Experimental Materials

Xinluzao36, an upland cotton variety, was used in the present study.

2.2. *GhCAD*6 Gene Expression Pattern

Total RNA was extracted from the leaves of the transgenic and control plants at the seedling stage and fibers of the transgenic plants and WT at 5, 10, 15, 20, and 25 days post-anthesis (DPA) using the hot boric acid and proteinase K method [16]. SuperScript® II Reverse Transcriptase (*Invitrogen*) was used to synthesize cDNAs from the mRNAs. The specific primer QGhCAD6A/QGhCAD6AS was designed for fluorescent quantitative PCR and cotton *GhUBQ7* was used as the internal reference gene. Advanced Universal SYBR Green Supermix (*Bio-Rad*) was used for Real time RT-PCR. Each sample was analyzed three times. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Construction of *GhCAD*6 Expression Vector and Cotton Transformation

To construct the sense *GhCAD*6 expression vector, the coding region of *GhCAD*6 was cloned into the cloning vector (pGEM-T Easy; *Promega*, USA) and further inserted into the plant expression vector pCAMBIA2300 under the control of the cotton fiber cell-specific E6 promoter between the *Bam*HI and *Sac*I restriction

sites. The primers of *GhCAD*6 used for plant expression vector construction are listed in Table 1. The recombinant vector (*GhCAD*6: pCAMBIA 2300) was transformed into *Agrobacterium tumefaciens* (strain LB4404) and was cultured on LB solid medium with suitable resistance at 28˚C for 2 days. Single colonies were selected and inoculated in 50 mL LB liquid medium containing 50 mg/L kanamycin, 100 mg/L Rifampicin, and 20 mg/L Streptomycin with shaking at 250 rpm and 28˚C for 12 - 18 h. When the OD_{600} of the culture reached 0.5 - 0.8, it was centrifuged at room temperature, 3000× *g*, and 5 min. The precipitate was diluted with 10% sucrose solution to prepare a solution with 0.2 - 0.4 OD_{600}, which was gently sprayed on the stigma of cotton flowers during anthesis and pollination. The entire flower was covered with paper to retain moisture. The seeds of the transgenic plants were harvested after maturation.

Table 1. The primers used in this study.

Name	Primer sequence (5' → 3')	Annealing temperature/˚C	Target fragment length (bp)
NPTIIS	GCACAACAGACAATCGGCTGCTC	52	496
NPTIIAS	GCCATGGGTCACGACGAGATCC	52	
GhCAD6S	TGTGCAGGGGTGACAGTTTAC	52	501
GhCAD6AS	CCCAATAAAACTCCCTGTAATCG	52	
QGhCAD6S	GTTCCTGGGCATGAAGTGGT	60	
QGhCAD6AS	TGCAACATCCAACAAGACAACC	60	
GhUBQ7S	AGAGGTCGAGTCTTCGGACA	60	
GhUBQ7AS	GCTTGATCTTCTTGGGCTTG	60	

2.4. Selections of *GhCAD*6 T_6 Transgenic Lines

When transgenic seeds were planted, the top leaves of the T_1 progenies of the transgenic plants were smeared with 0.5, 0.75, and 1% kanamycin sulfate solution every 5 days at the seedling stage. The response of the leaves to kanamycin was observed 3 days after being smeared. The leaves of the kanamycin-resistant plants were still green, while those of the kanamycin-sensitive plants were yellow in color. The progenies of the kanamycin-resistant plants were continuously selected and planted for six generations. The transgenic lines (T_6) and wild type (WT, Xinluzao36) were planted in Xinjiang Manas Molecular Breeding Experimental Station in 2016. Genomic DNA from T_6 transgenic plants leaves was extracted from the kanamycin-resistant or from WT plants using the Plant Genomic DNA Kit (TIANGEN, China) according to the manufacturer's instructions. The genomic DNA from T_6 transgenic plants was used to perform PCR to detect the presence of *GhCAD*6 gene. The PCR included a mixture from the Easy Taq PCR SuperMix (*TRANS*, China), 50 ng of genomic DNA from putative T_6 transgenic

or WT plants and the primers listed in Table 1. The PCR conditions were as follows: to detect *GhCAD*6 gene, 95˚C for 5 min, 40 cycles (94˚C for 45 s, 57˚C for 45 s and 72˚C for 1 min), and 72˚C for 10 min for final extension. Genomic DNA from WT plants was used as a negative control, while the original *GhCAD*6 gene expression vector used for cotton transformation was used as a positive control. The PCR products were analyzed by agarose gel electrophoresis.

For southern blot assay, total genomic DNA from the leaves of the *GhCAD*6 transgenic plants and CK was used as the template and negative control, respectively, while the plasmid E6-pCAMBIA-2300-GhCAD6 DNA was used as a positive control. The genomic and plasmid DNA were digested using the restriction endonucleases, *Bam*HI and *Xba*I, and the solutions were prepared according to the instructions of the digoxigenin-labeled hybridization kit I (Roche, China). Southern blot was carried out to detect the integration of the target gene in the genome of the progenies of transgenic plants.

RNA was extracted from the fibers of cotton bolls 20 DPA, and the expression of *GhCAD*6 in transgenic lines and WT was determined after reverse transcription into cDNA.

The primers used in these experiments are presented in Table 1.

2.5. The Content of Phenylpropanoid-Related Structural Components of Cotton Fiber

At the full-bloom stage, the cotton bolls from both strains were labeled and sampled at 5, 10, 15, 20, 25, 30, 40, and 50 DPA and naturally matured. After harvesting, cotton shells were removed quickly with tweezers and 1 - 2 bolls were wrapped in pre-prepared tinfoil paper to form a package, which was quickly frozen in liquid nitrogen and stored at −80˚C for future use.

The cotton fibers at different developmental stages were obtained and soluble substances were cleaned according to previously published methods [4]. The structural polysaccharide and phenylpropanoid content of the cotton fibers at different developmental stages was determined using the ethanol-nitrate and Klason methods. Sixteen lines of cotton fiber samples were collected and measured.

2.6. Visualization of Fiber

The quality of naturally mature cotton fibers was tested using a high volume instrument (HVI). Each treatment was performed three times. The surface morphology of natural mature cotton fibers was observed using scanning electron microscopy (SEM). The structural components related to phenylpropanoids in both the transgenic plants and WT fiber were analyzed using Fourier transform infrared spectroscopy (FTIR) according to previous methods [4].

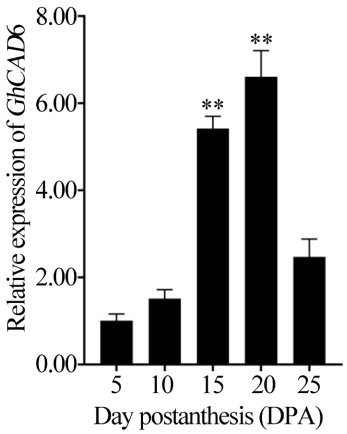
2.7. Data Processing and Mapping

All data were processed using Excel 2010. GraphPad Prism 5 was used to draw the diagrams.

3. Results

3.1. Relative *GhCAD6* Expression at Different Developmental Stages of Cotton Fiber

Owing to *GhCAD6* is expressed predominantly in fiber cells, especially at the secondary wall thickening stage of fiber development, we first checked the *GhCAD6* gene expression at different developmental stages of cotton fiber and the results are shown in Figure 1. The expression of *GhCAD6* gene in cotton fiber increased initially and then decreased with the development of the cotton fiber. The expression of *GhCAD6* gene was the highest at 20 DPA, but decreased rapidly at 25 DPA. Since 5 - 20 DPA is the key period for cotton fiber elongation, it indicates that *GhCAD6* gene plays an important role in fiber elongation.

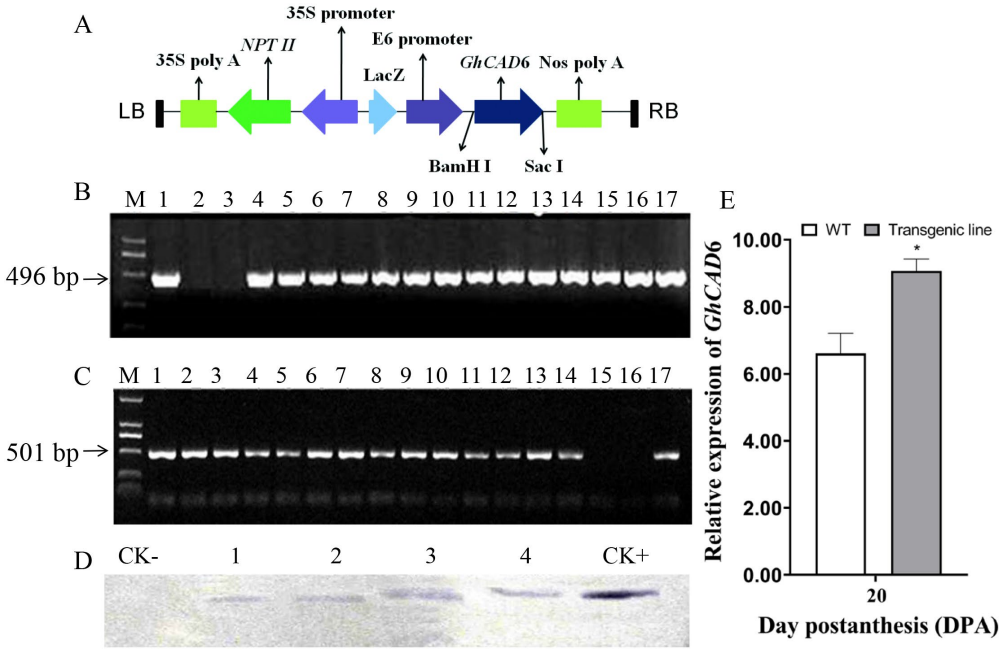


Note: The asterisk represents a very significant difference at the 0.01 level ($P < 0.01$).

Figure 1. Relative *GhCAD6* expression at different developmental stages of cotton fiber.

3.2. Generation and Identification of Transgenic *GhCAD6* Cotton Lines

Now that *GhCAD6* is preferentially expressed in cotton fiber, the full-length cDNA of *GhCAD6* was cloned and introduced into the expression vector driven by the control of the fiber-special E6 promoter (Figure 2A). We generated cotton transgenic *GhCAD6* plants by introducing the construct into cotton via *Agrobacterium tumefaciens*-mediated transformation. After the transformation of 1300 cotton flowers, a total of 4630 T_0 seeds were obtained and 31 kanamycin-resistant T1 generation lines were further selected. We confirmed the *GhCAD6* expression in 14 transgenic cotton lines by RT-PCR (Figure 2B and Figure 2C), which the transformation rate was up to 0.3%. Southern hybridization of the transgenic cotton plant genomic DNA further showed that a single copy of the *GhCAD6* gene was integrated into the recipient cotton plant genome (Figure 2D). We further tested the over-expression of *GhCAD6* gene in transgenic cotton lines by qRT-PCR and found that it was significantly higher than that in WT at 20 DPA (Figure 2E).



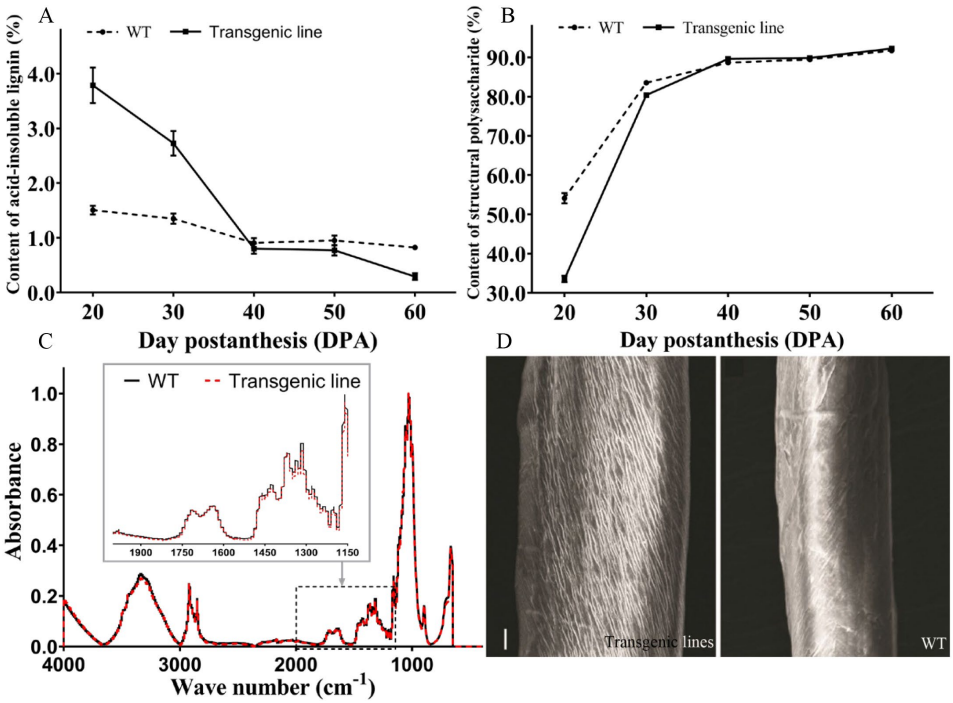
Note: **A**) Schematic diagram of the *GhCAD6* expression vector. LB and RB: left and right border of T-DNA; 35S ploy A: cauliflower mosaic virus polyadenylation signal; Nos poly A: nopaline synthase polyadenylation signal; NPT II: neomycin phosphotransferase gene; LacZ: b-galactosidase gene Z; E6: cotton fiber specific E6 promoter; *GhCAD6*: target gene; and *Bam*HI, *Sal*I, *Xba*I and *Sac*I: restriction endonuclease cutting site; **B**) Amplification of T6 generation transgenic cotton fiber DNA using NPT II primers; M: DL2000 Marker; 1: Positive control; 2: Non-transgenic control; 3: Negative control; 4 - 17: transgenic cotton lines; **C**) Amplification of transgenic cotton lines using *GhCAD6* primers; M: DL2000 Marker; 1 - 14: transgenic cotton lines; 15: Non-transgenic control; 16: Negative control; 17: Positive control. **D**) Southern blot hybridization of some *GhCAD6* transgenic cotton lines; CK−: negative control; 1 - 4: transgenic plant; CK+: positive control; **E**) qRT-PCR of *GhCAD6* in the transgenic lines and WT fiber. Fibers were collected at 20 DPA. Data are means ± SD, n = 3. The asterisk represents a significant difference at the 0.05 level ($P < 0.05$).

Figure 2. Molecular cloning and confirmation of transgenic *GhCAD6* cotton lines.

3.3. *GhCAD6* Alters the Content of Phenylpropanoid-Related Structural Components of Transgenic Cotton Fiber

To examine the effect on the cotton fiber about the changed phenylpropanoid content in the transgenic fibers cells, then we tested the changes in the content of fiber-related structural substances in cotton. We found that the acid-insoluble lignin percentage is gradually decreased during the development of cotton fibers, but the polysaccharide percentage is increased (**Figure 3A** and **Figure 3B**). Amazingly, the phenylpropanoid content of the transgenic cotton fibers was higher than that of the WT fibers at 20 - 30 DPA with the development of the cotton fiber, but the phenylpropanoid percentage in the transgenic cotton fibers was lower than that in the WT fibers after maturation, which led to the structural polysaccharide percentage was changed during the development of cotton fibers. The structural polysaccharide percentage in WT fibers was higher than that in the transgenic fibers at 20 DPA and the structural polysaccharide content rapidly increased at 20

- 30 DPA. The accumulation rate of structural polysaccharide in transgenic fibers was higher than that in WT fibers. Thus, the structural polysaccharide content of the transgenic fibers was slightly higher than that of the WT fibers. Figure 3C shows the average measured absorbance of the 14 transgenic lines compared to the WT. Although the FTIR showed no significant changes in the structural components related to phenylpropanoids in the WT and transgenic fiber, the absorption intensity of the phenylpropanoid structure-related functional groups in the WT fiber was slightly higher than that in the transgenic fiber, indicating that the phenylpropanoid content of the transgenic fibers was changed. Via the observation of SEM, the surface of mature cotton fibers of transgenic lines was smoother, finer, and compact than that of the WT (Figure 3D). The length and strength of transgenic fibers were higher than those of the WT (Table 2).



Note: **A)** The phenylpropanoid content in cotton fibers at different fiber developmental stages; **B)** The structural polysaccharide content in cotton fibers at different fiber developmental stages; **C)** Comparison of fiber cell wall components using FITR between the transgenic cotton lines and WT; **D)** Observation on the surface of mature cotton fibers in transgenic lines and WT. Bar = 2 μm.

Figure 3. Structural substances content and morphological phenotypes of the transgenic lines and WT fibers.

3.4. Fibers Fineness and Strength Are Improved in *GhCAD6* Transgenic Cotton Plants

To unravel the effect on the overexpression of *GhCAD6* in fiber cells, we focused on the fiber quality of *GhCAD6* transgenic cotton lines. The partial results of fiber quality after *GhCAD6* gene transformation were shown in Table 2 and resulted in the average fiber length, specific strength, uniformity index, and micronaire

value of the transgenic cotton lines were higher than those of WT fibers, except the extension rate was lower. The WT fiber length was 27.72 mm, while that of the transgenic plants was 30.46 mm, which was 6.0% - 13.9% higher than that of WT. The fracture specific strength of the WT fiber was 26.1 cN/tex, while that of transgenic plants was 28.0 - 31.4 cN/tex, with the average being 29.65 cN/tex, which was 7.3% - 27.2% higher than that of WT fibers. The uniformity index of the WT variety was 84.40%, and that of the transgenic plants ranged from 84.90% - 86.70%. Compared to those of WT fibers, the uniformity index and micronaire value of the fibers from transgenic plants increased by 0.59% - 2.73% and 2.61%, respectively, whereas the extension rate decreased by 4.16%.

Table 2. Effect of *GhCAD*6 over-expressing in cotton on fiber quality.

Materials		Fibre Length (mm)	Fibre Strength (cN/tex)	Uniformity Index (%)	Micronaire	Elongation (%)
Xinluzao 36		27.72	26.10	84.40	4.62	6.70
Transgenic lines	1	31.28	31.00	85.60	4.68	6.00
	2	30.99	30.40	84.90	3.93	6.20
	3	31.56	31.40	86.40	4.17	5.90
	4	30.78	30.80	84.90	4.47	6.00
	5	30.86	30.40	85.60	4.73	6.30
	6	30.83	30.20	86.30	4.45	6.20
	7	30.06	28.20	85.40	4.91	6.30
	8	29.96	28.80	86.70	4.79	6.00
	9	30.03	29.30	86.00	4.86	6.00
	10	30.15	29.10	86.30	4.76	6.10
	11	30.61	28.00	86.70	5.04	6.20
	12	29.93	28.70	86.60	4.93	6.30
	13	29.89	29.80	85.30	5.09	6.90
	14	29.55	29.00	85.20	4.84	6.80
Average value		30.46	29.65	85.85	4.69	6.23

4. Discussion

Due to phenylpropanoid biosynthesis that is involved in a variety of enzymes, phenylpropanoids are some of the important products of the phenylpropane metabolic pathway. CAD is one of the earliest studied enzymes that play a key role in phenylpropanoid synthesis [17]. Studies have shown that CAD is widely distributed in different tissues of various plants [13]. CAD plays an important regulatory role in lignin biosynthesis. Transgenic tobacco [18], popla [19], alfalfa [20], maize [21], pine [11], *Arabidopsis thanliana* [22], and other transgenic plants have been developed using genetic transformation to inhibit *CAD* gene expression and reduce *CAD* activity. Fornalé *et al.* [23] used RNAi to down-regulate *CAD* expres-

sion in maize and found that the total lignin content was not affected and that the lignin content in the midvein cell wall was decreased by 6.4%. In maize *bm*1 mutant plants, the total lignin content was only 80% of that in the wild-type plants [21]. The total lignin content in sorghum mutants was 15% - 25% lower than that in the wild-type plants [24].

Previous studies have shown that *GhCAD*6 gene participates in the metabolism of phenylpropanoid units and phenolic acids in the cell wall of cotton fiber, and further regulates the development of cotton [15]. In this study, we showed that over-expression of the *GhCAD*6 can significantly improve the length and specific strength of cotton fibers after the *GhCAD*6 gene was introduced into cotton receptors, which further proved the role of *GhCAD*6 gene in improving cotton fibers quality. *GhCAD*6 gene in transgenic lines was increased at 20 DPA, while the fiber quality of transgenic plants was significantly changed with compared to that of WT. This is in line with previous studies have reported that the genes introduced into the plants can interact with homologous genes in plants, which results in the change of the expression of both the targeted and host genes [25]-[27].

Phenylpropanoid metabolism is a very complex metabolic pathway involving many genes. After the *GhCAD*6 gene was transferred into the cotton receptor, its interaction with related genes may have led to higher expression level of *GhCAD*6 than that in WT. Thus, the phenylpropanoid and structural polysaccharide content of the fiber also changed after *GhCAD*6 transformation. However, the phenylpropanoid content of the transgenic fibers was higher and lower than that of WT fibers before and after 40 DPA, respectively. The structural polysaccharide content in the fiber from WT was higher than that in the fiber from transgenic plants at 20 DPA and it was almost similar in the two groups at 40 DPA. The reciprocal fluctuation of the phenylpropanoid and structural polysaccharide contents during fiber development may be the reason for the *GhCAD* transformation-induced change in cotton fiber quality. *CAD* exists in the form of a multi-gene family in plants, which can catalyze many different substrates. After *GhCAD*6 was transferred to cotton, it may have led to the interaction between other related genes and resulted in an increase in the total activity of *CAD*, owing to which the phenylpropanoid content in transgenic cotton lines was altered with that in the WT lines.

After *GhCAD*6 transfer into cotton, over-expression of *GhCAD*6 at the development fiber may have caused a change in phenylpropanoid content, which resulted in variation in cotton fiber quality. These results can provide a theoretical basis for further analysis of the mechanism by which *GhCAD*6 improves cotton fiber quality.

5. Conclusion

The main results were as follows: (1) *GhCAD*6 was integrated into the recipient genome of cotton fiber in the form of single copy; (2) The expression of *GhCAD*6 in the fiber of transgenic plants was higher than that in CK at the same develop-

mental stage. (3) In the cell wall of mature fibers, the content of phenylpropanoid compounds in transgenic plants was slightly lower than that in the CK, and the content of structural polysaccharides had no significant difference. (4) The surface of mature cotton fiber of transgenic progenies was smoother, finer and more compact than the CK; (5) The average length, specific strength and uniformity index of the fibers of transgenic *GhCAD6* plants were higher than those of the CK, the micronaire value increased and the elongation decreased.

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Author Contributions

Xuan Ding performed the experiments, analyzed the data; Wenran Hu designed the research, wrote and edited the manuscript. All the authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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