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The cytochrome P450 gene *GhCYP94C1* is involved in drought stress in upland cotton (*Gossypium hirsutum* L.)

Lijiao Gu[1,2], Pengyun Chen[3], Shuxun Yu[4]**

[1]*Institute of Forest Biotechnology, Forestry College, Hebei Agricultural University, Baoding, P.R. China*
[2]*Hebei Key Laboratory for Tree Genetic Resources and Forest Protection, Baoding, P.R. China*
[3]*Beijing Innovation Center for Crop Seed Technology, Ministry of Agriculture and Rural Affairs, Key Laboratory of Crop Heterosis Utilization, Ministry of Education, College of Agronomy and Biotechnology, China Agricultural University, Beijing, P.R. China*
[4]*State Key Laboratory of Cotton Biology, Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, P.R. China*

**Corresponding authors: gulijiao1990@126.com; ysx195311@163.com*

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Abstract: Cytochrome P450 proteins belong to one of the largest families of enzyme proteins in plants and play important roles in plant growth and development and the stress response. In our previous studies, a cytochrome P450 gene, *GhCYP94C1* (cytochrome P450 94C1), was functionally characterized as a positive regulator of seed germination, main root elongation and early flowering. However, whether the gene has other potential functions remains to be further explored. In our study, expression analysis showed that *GhCYP94C1* was highly expressed in roots and was suppressed by drought treatment. Endogenous silencing of *GhCYP94C1* via virus-induced gene silencing (VIGS) increased drought resistance in cotton plants, which was accompanied by the upregulated expression of the abscisic acid (ABA) biosynthesis gene nine-cis-epoxycarotenoid dioxygenase 9 (*GhNCED9*) during drought stress. Our findings suggested that *GhCYP94C1* may play an important role in drought resistance. Combined with previous research results, the present results provide a theoretical basis for future breeding of new cotton varieties with early maturation and drought resistance.

Keywords: ABA signaling network; drought tolerance; expression analysis; qRT–PCR; VIGS assay

Cotton is an economically important crop and a source of textile raw material (Liu et al. 2000) and is cultivated under various climates around the globe (Noreen et al. 2020). Water is a major component of plants and is necessary for plant nutrient transport, chemical and enzymatic reactions, cell expansion and transpiration (Meshram et al. 2022). Water deficit or drought is the main abiotic factor limiting plant growth and crop productivity. Under drought stress, the boll weight per plant, boll number per plant or lint percentage of cotton decreased, which resulted in a significant decrease in seed cotton yield and lint yield (Meshram et al. 2022). Several studies have shown that drought stress leads to the loss of 6 kg/ha of cotton fibre in the vegetative period, 15 kg/ha in the flowering period and 4–8 kg/ha in the mature period (Paytas 2018). Previous studies showed that the actin depolymerizing factor *GhADF1* could be induced by PEG6000 and improves drought resistance and fibre yield in RNAi cotton plants (Qin

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et al. 2022). Thus, drought stress can reduce the fibre yield and quality of cotton, making it necessary to identify drought resistance genes to improve cotton drought tolerance.

Cytochrome P450 is the largest family of enzyme proteins and occurs widely in plants, animals, fungi and bacteria (Nelson et al. 1996). In plants, cytochrome P450 plays important roles in phytochemical defence mechanisms, hormone synthesis and secondary biomass metabolism (Donaldson & Luster 1991; Werck-Reichhart 1995). Furthermore, cytochrome P450 genes are involved in the metabolic synthesis of endogenous substances such as phenylpropanoids, phytohormones, terpenes, flavonoids, lignins and alkaloids, which are involved in resistance to insects and pathogens. Cytochrome P450 catalyses the detoxification and degradation of exogenous substances such as insecticides, herbicides and environmental pollutants (Deng & Hatzios 2002; Bhatnagar et al. 2003; Yu et al. 2004; Moore & Kroger 2010; Cresnar & Petric 2011). Previous reports have shown that cytochrome P450 plays important role in abiotic stresses. For example, a spinach cytochrome P450 gene, *SoCYP85A1*, improved drought tolerance in transgenic tobacco plants by regulating the transcripts of stress-related genes and balancing intracellular reactive oxygen species (ROS) levels (Duan et al. 2017). Rice cytochrome P450 gene *dss1* mutants showed improved drought resistance, which might be due to the accumulation of abscisic acid (ABA) and metabolites (Tamiru et al. 2015). The sunflower *HaCYP93A1* gene was induced by high salinity, and the high expression of *HaCYP93A1* under salt stress was significantly correlated with jasmonic acid (JA) biosynthesis genes, suggesting that *HaCYP93A1* may participate in the salt tolerance pathway by regulating the JA signalling pathway (Wang et al. 2017). Research on P450 family genes in cotton has mainly focused on growth and disease resistance; for example, *GhCYP703A2* functions in sporopollenin formation and fertility (Ma et al. 2022), *GbCYP86A1-1* improves the resistance of cotton to *Verticillium dahliae* (Wang et al. 2020), and silencing *GhCYP749A16* resulted in sensitivity in the tolerant cotton cultivar Stoneville 474 via a virus-induced gene silencing (VIGS) assay (Thyssen et al. 2018). However, there are few reports on the role of P450 genes in the drought resistance of cotton.

In our previous studies, *GhCYP94C1*, a downstream target of the leaf senescence-related gene *GhWRKY27*, was identified by ChIP-seq, yeast one-hybrid and electrophoretic mobility shift assays (Gu et al. 2019). The *GhCYP94C1* gene can promote seed germination, main root elongation and early flowering in overexpressed *Arabidopsis thaliana* (Tian et al. 2022). *GhCYP94C1* gene expression was higher in early-maturing cotton varieties than in non-early-maturing varieties (Gu et al. 2019), and *GhCYP94C1* can cause a delayed flowering phenotype in VIGS cotton plants (Tian et al. 2022). These results suggest that the *GhCYP94C1* gene may be an important factor affecting the growth and flowering time/early maturation of cotton, and whether this gene has other potential functions needs to be further explored. In this study, we analysed the expression pattern of the *GhCYP94C1* gene under drought treatment and found that *GhCYP94C1* expression was inhibited by drought stress. *GhCYP94C1* repression enhanced drought tolerance in VIGS cotton plants. In addition to the functions of the *GhCYP94C1* gene in the growth and early flowering of cotton, our results highlight its role in drought resistance, which provides a theoretical basis for breeding early-maturing and drought-resistant cotton varieties in the future.

MATERIAL AND METHODS

Plant materials and drought treatments. CCRI74 cotton plants were planted in the field of the Cotton Research Institute of the Chinese Academy of Agricultural Sciences (Anyang, Henan, China). For the expression analysis of the *GhCYP94C1* gene in different tissues, roots and stems were collected from 14-day-old seedlings, and the first true leaves newly grown from the seedlings under normal conditions were harvested. For drought treatment, CCRI74 seeds were germinated in pots (tillage soil : nutrient soil = 2 : 1) in a greenhouse at 28 °C and 16 h light/8 h dark photoperiod. The 10-day-old seedlings were irrigated with 15% polyethylene glycol 6000 (PEG6000, osmotic agent to simulate drought), and the cotyledons were collected at 0, 2, 4, 6, 8, and 12 h for the expression of *GhCYP94C1* mRNA under drought stress. Samples were collected and quickly frozen in liquid nitrogen, and there were three experimental replicates.

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT–PCR). Total RNA from each sample was extracted using the RNAprep PurePlant Kit (Polysaccharides & Polyphenolics-rich) (Tiangen, China). The PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) was used for cDNA synthesis. Relative expression levels were identified using an ABI 7500 Real-time

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PCR system and TB Green™ Premix Ex *Taq*™ (Tli RNaseH Plus) (TaKaRa, Japan). The 20-μL reaction system was comprised of: 10 μL TB Green Premix Ex *Taq* (Tli RNaseH Plus) (2×), 0.4 μL (10 μM) PCR forward primer, 0.4 μL (10 μM) PCR reverse primer, 0.4 μL ROX Reference Dye II (50×), 2 μL cDNA template and 6.8 μL ddH_2O. The reaction procedure was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. *GhActin* was used as an internal reference gene. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ algorithm (Schmittgen & Livak 2008). All reactions were conducted with three replicates. The primers used for qRT–PCR analysis were as follows: *GhActin*-F: ATCCTCCGTCTTGACCTTG, *GhActin*-R: TGTCCGTCAGGCAACTCAT; *GhCYP94C1*-F: CTCCTTCATCTTCTTCACTTTCACG, *GhCYP94C1*-R: TGCCGAGGATATGAATATGGATTGT; *GhNCED9*-F: CCCAGTAGACGGTCCGATAA, *GhNCED9*-R: ATCCCAAACCTTGACATCTTG (a marker gene related to the ABA signalling pathway).

VIGS assay of *GhCYP94C1* in cotton. The 202-bp VIGS fragment of *GhCYP94C1* was amplified using the following primer sequences: *GhCYP94C1-Xba*I-F: CTAGTCTAGACTCCTTCATCTTCTTCACTTTCA; *GhCYP94C1-Bam*HI-R: CGCGGATCCCCGAGGATATGAATATGGATTGT. The fragments were integrated into the pYL156 vector and transformed into GV3101 competent cells. GV3101 cells harbouring pYL156 (empty control), pYL156-*GhCYP94C1*, or pYL156-*CLA1* (positive control) were mixed with cells containing pYL192 (helper vector) with OD600 = 1.5 at a 1 : 1 ratio. Each mixture was manually injected into the underside of approximately 10-day-old CCRI74 cotyledons. After continued culture for approximately three weeks, the empty control and VIGS plants were subjected to 20% PEG6000 for the detection of drought stress. The inoculated plants were kept at 23 °C with a 16 h light/8 h dark cycle in a greenhouse. The experiment was performed according to Gao et al. (2011).

RESULTS

Expression pattern analysis of *GhCYP94C1* in different tissues and under drought stress. To identify the potential functions of *GhCYP94C1*, qRT–PCR was used to detect the expression levels of *GhCYP94C1* in roots, stems and leaves. As shown in Figure 1A, *GhCYP94C1* was highly expressed in roots, followed by stems and leaves. The expression of the *GhCYP94C1* gene in roots was significantly higher than that in stems and leaves under control condition. In addition, the cotton seedlings were subjected to drought treatment, and the results showed that the expression levels of *GhCYP94C1* were inhibited by drought stress at 2–24 h (Figure 1B). Compared with the control (0 h), the expression of *GhCYP94C1* was significantly decreased, suggesting that *GhCYP94C1* mRNA was inhibited by drought stress.

VIGS vector construction. The approximately 200-bp VIGS fragment of *GhCYP94C1* was amplified from the CCRI74 cotton variety via PCR. The pYL156 vector and gel extraction VIGS amplification fragment were double digested with *Xba*I and *Bam*HI, respectively. The double restriction products of the VIGS fragment and pYL156 vector were ligated by T4 ligase, and the VIGS fragment of *GhCYP94C1*

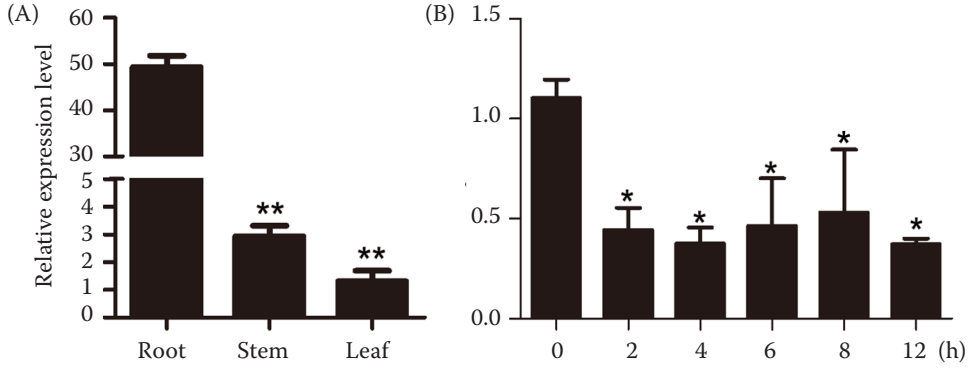

Figure 1. Expression of *GhCYP94C1* mRNA in different tissues and under drought stress: expression pattern of *GhCYP94C1* in the roots, stems and leaves under normal conditions (A), expression pattern of *GhCYP94C1* under drought conditions (B) The values are the means ± standard errors; **($P < 0.01$) indicates extremely significantly different; *($P < 0.05$) indicates significantly different

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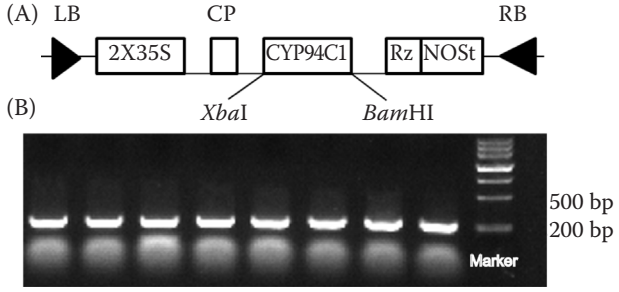

Figure 2. Virus-induced gene silencing (VIGS) sequence amplification and vector construction: plasmid construction sketch of *GhCYP94C1* for VIGS assay (A), amplification of the *GhCYP94C1* VIGS fragment by PCR (B)
DNA marker III (200, 500, 800, 1 200, 2 000, 3 000, 5 000 bp) was used to mark the size of the band

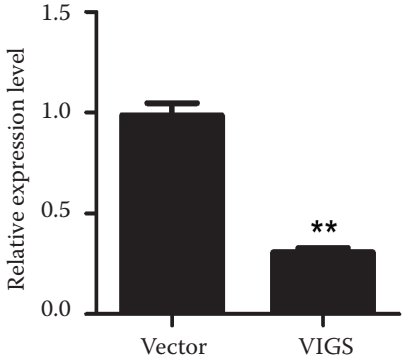

Figure 3. Expression of *GhCYP94C1* mRNA in empty vector and virus-induced gene silencing (VIGS) plants
The values are the means ± standard errors; **($P < 0.01$) indicates extremely significant differences

was inserted into the *Xba*I and *Bam*HI sites of the pYL156 vector (Figure 2A). The correctly sequenced recombinant plasmid was introduced into *Agrobacterium* GV3101 and further identified by bacterial liquid PCR. As shown in Figure 2B, the VIGS fragment band can be identified at the approximately 200 bp position, which is of the expected size.

Detection of *GhCYP94C1* gene silencing efficiency. Three weeks after injection, true leaves of empty-vector control and VIGS plants were collected, and total RNA was extracted to detect the expression level of *GhCYP94C1*. The results showed that the expression of *GhCYP94C1* in VIGS plants was extremely significantly lower than that in the empty-vector control plants. The expression levels of *GhCYP94C1* were reduced by 68.06% in the VIGS plants compared with the empty-vector control plants (Figure 3).

***GhCYP94C1* enhanced drought tolerance in VIGS cotton plants.** VIGS and empty-vector control plants with good growth and consistent growth status were selected for 20% PEG6000 treatment. After drought treatment, the leaves of both VIGS and empty vector control plants showed some degree of wilting. However, the empty-vector control plants wilted more severely than the VIGS plants (Figure 4A), suggesting that the *GhCYP94C1* VIGS plants had enhanced drought resistance.

Figure 4. Silencing of *GhCYP94C1* increased the drought tolerance of cotton plants via VIGS: Approximately 6-week-old VIGS plants after 20% PEG6000 treatment (A), relative expression levels of *GhNCED9* in control and VIGS plants before and after drought treatment (B)
VIGS – virus-induced gene silencing; CK – control; PEG – polyethylene glycol; the values are the means ± standard errors; **($P < 0.01$) indicates an extremely significant difference; ns – no significant difference

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In plants, ABA is an important hormone that mediates the drought response. When plants are subjected to drought stress, ABA will accumulate rapidly to regulate the drought resistance response. To further investigate the possibility of enhanced drought resistance in VIGS plants, we analysed the expression of the ABA synthesis gene *GhNCED9*. Our results showed that under normal growth conditions, the expression levels of *GhNCED9* showed no difference between the VIGS plants and the control plants (Figure 4B). However, after 20% PEG6000 treatment, the transcript accumulation of *GhNCED9* was elevated in both VIGS and control plants, and *GhNCED9* expression displayed a significant increase in VIGS plants compared with the control plants (Figure 4B).

DISCUSSION

The large cytochrome P450 (CYP450) superfamily was named based on the similarity of the amino acid sequences and the phylogenetic relationship. In plants, CYP450s can be divided into 11 clans, which are classified into two categories: single-family clans (CYP51, CYP74, CYP97, CYP710, CYP711, CYP727 and CYP746) and multifamily clans (CYP71, CYP72, CYP85 and CYP86) (Nelson & Werck-Reichhart 2011). CYP86 was the youngest clan, which contained three subfamilies, CYP86, CYP94 and CYP704 (Nelson & Werck-Reichhart 2011). Phylogenetic tree analysis showed that the *GhCYP94C1* gene belonged to the CYP94 subfamily of the P450 family (Tian et al. 2022). Tissue expression specificity analysis of the *GhCYP94C1* gene showed that the expression of this gene was higher in roots than in stems and leaves. The expression pattern of a gene is usually related to its function, and the site of expression indicates its potential function. Therefore, it is speculated that the *GhCYP94C1* gene may affect root growth and development. Tian et al. (2022) showed that the *GhCYP94C1* gene can cause overexpressed transgenic *Arabidopsis thaliana* to exhibit main root elongation phenotype. However, whether *GhCYP94C1* affects the root growth of cotton remains to be further explored.

Drought is one of the main abiotic stresses causing crop yield reduction. Previous studies showed that 83 *OsCYPs* were differentially expressed in response to drought stress using RNA-seq data in rice (Wei & Chen 2018). Under water deficit conditions, 82 and 39 significantly differentially expressed *CYP450* genes were detected in wheat and maize, respectively (Li & Wei 2020). In *Medicago truncatula*, 204 *MtP450* genes related to drought/salt treatment were analysed using weighted correlation network analysis (WGCNA), and 8 genes (*CYP72A59v1*, *CYP74B4*, *CYP71AU56*, *CYP81E9*, *CYP71A31*, *CYP704G6*, *CYP76Y14*, and *CYP78A126*) were identified as hub genes in drought stress (Xia et al. 2021). These results indicated that *P450* genes were closely related to the plant response to drought stress. The expression of *PacCYP707A1* was significantly downregulated during the dehydration of sweet cherry fruit, and *PacCYP707A1*-RNAi-treated fruits showed enhanced resistance to drought stress, along with an increase in ABA content (Li et al. 2015). In our study, the expression level of the *GhCYP94C1* gene was inhibited by drought, indicating that this gene may be able to enhance resistance to drought by reducing gene expression levels under drought conditions.

To test the functions of the *GhCYP94C1* gene in coping with drought stress in cotton, we constructed a VIGS vector to reduce the transcription levels of this gene in cotton. The VIGS recombinant vector was introduced into cotton, and three weeks later, we found that the expression level of the *GhCYP94C1* gene in cotton showed a very significant decrease. This demonstrated that the VIGS vector could work properly in cotton, and *GhCYP94C1* gene-silenced plants were successfully obtained. After 20% PEG6000 treatment, the VIGS plants with *GhCYP94C1* gene silencing showed a lower wilting phenotype than the control plants, indicating that the *GhCYP94C1* gene can increase the drought resistance of VIGS cotton plants.

To analyse the possible causes of improved drought resistance, we analysed the expression level of an ABA synthesis gene, *GhNCED9*. The results showed that *GhNCED9* was significantly higher in VIGS plants than in the control. ABA is a plant hormone that is involved in environmental stresses such as drought and high salinity and is essential for stress resistance (Fujita et al. 2011). The key step of ABA biosynthesis is catalysed by *NCED9* in plants (Tan et al. 2003). Overexpression of *AtNCED3* caused an increase in endogenous ABA content and an improvement in drought resistance (Iuchi et al. 2001). The expression levels of *GhNCED9* were elevated in VIGS plants during drought stress, indicating that the *GhCYP94C1* gene may be involved in the ABA signalling network. In addition, tissue expression analysis showed that the *GhCYP94C1* gene was

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highly expressed in roots compared with stems and leaves. In our previous study, ABA promoted the elongation of the main root and lateral root of cotton seedlings (Tian et al. 2022). Overexpression of the *GhCYP94C1* gene can promote main root elongation in transgenic *Arabidopsis thaliana* (Tian et al. 2022). In VIGS plants, the expression of the ABA biosynthesis gene *GhNCED9* during drought stress was elevated. Therefore, we hypothesized that *GhCYP94C1* might affect ABA synthesis by influencing the expression level of *GhNCED9*. The change in ABA content affected root development and ultimately improved the drought resistance of *GhCYP94C1* VIGS cotton plants. However, the effect of the *GhCYP94C1* gene on VIGS cotton roots, the pathways through which this gene participates in the regulation of the ABA signalling pathway, and the specific regulatory mechanisms remain to be further explored.

In conclusion, *GhCYP94C1* gene expression is downregulated under drought stress. Silencing *GhCYP94C1* by the VIGS technique can improve the drought resistance of VIGS cotton plants. These results indicated that the *GhCYP94C1* gene is a negative regulator of drought resistance. This study provides a new gene for gene editing knockout of *GhCYP94C1* to obtain drought-resistant cotton mutant materials. Our previous studies showed that *GhCYP94C1* positively regulates flowering. Combined with those results, this study also provides theoretical evidence for the cultivation of new early-maturing and drought-resistant materials in the future.

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