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ORIGINAL RESEARCH

Silencing *GhFAR3.1* reduces wax accumulation in cotton leaves and leads to increased susceptibility to drought stress

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Abstract

Fatty acyl-CoA reductases (FAR) are involved in plant wax synthesis and play important roles in plant growth and development. However, little information is available for cotton *FAR* genes. In this study, we carried out a genome-wide identification of *FAR* genes in Upland cotton (*Gossypium hirsutum* L.) and found 10 *GhFARs* that form five pairs of homoeologs (*GhFAR2A* to *GhFAR3.4D*) distributed on six chromosomes. The 10 *GhFARs* were separated into two subfamilies. Most *GhFARs* showed tissue-specific expression patterns, and at least one *GhFAR* of each pair of homoeologs was relatively highly expressed in at least one of the tissues investigated. *GhFAR3.1* was highly expressed in leaves. The function of *GhFAR3.1* in wax accumulation and drought tolerance was analyzed using virus-induced gene silencing (VIGS). Silencing *GhFAR3.1* reduced the total wax content and relative water content of leaves by over 60% and 13%, respectively, suggesting a role of *GhFAR3.1* in wax synthesis and protection against water loss. Compared to the well-watered conditions, drought stress induced significant accumulation of wax in leaves of wild-type plants but not in leaves of *GhFAR3.1* silenced plants, leading to less water holding capacity in *GhFAR3.1* silenced plants and plant wilting. Silencing *GhFAR3.1* had no effect on the expression levels of the wax biosynthesis pathway genes *KAS*, *KCS*, and *LACS* (upstream *GhFAR3.1*), but reduced the transcript level of its downstream gene *WSD*. Together, these results suggest that leaf wax content is important for water retention and drought tolerance and that *GhFAR3.1* is essential for wax synthesis in cotton leaves. These results also provide the basis for further study on the molecular regulation mechanism of *GhFARs* in cotton development and surface lipid synthesis.

KEYWORDS
drought response, *GhFAR*, *Gossypium hirsutum*, VIGS, wax content

1 | INTRODUCTION

Upland cotton (*Gossypium hirsutum* L.) is an important industrial crop in the world. With the frequent occurrence of extreme environmental issues such as global warming, the impact of drought on cotton production is of major concern. The effect of drought stress on plant growth is mainly manifested in the loss of water in plant cells through stomatal transpiration on the surface of leaves,

which eventually leads to the decrease of cell osmotic potential. Plant cuticular wax is a critical barrier of plant self-protection and plays an important role in alleviating the damage of drought stress to plants (Bondada et al., 1996; Cameron et al., 2006; Kim et al., 2007; Kosma et al., 2009; Kim et al., 2006). As an important strategy of plants to adapt to environmental stresses, the content of plant epidermal wax is significantly induced by drought stress (Oliveira et al., 2003).

Cuticular wax is the mixture of saturated hydrocarbon backbones, for example, alkanes, fatty alcohols, aldehydes, and ketones (Greer et al., 2007; Rowland et al., 2006; Schneider et al., 2016). Fatty acyl reductase (FAR), first found in Jojoba (*Simmondsia chinensis*), is a key enzyme involved in the synthesis of long-chain fatty alcohols (Müller & Riederer, 2005). In *Arabidopsis thaliana*, eight genes encoding FAR have been identified, and five of them have been emphatically analyzed and investigated (Rowland & Domergue, 2012). *AtFAR1*, *AtFAR4*, and *AtFAR5* are responsible for the production of suberin-associated C22:0, C20:0, and C18:0 primary alcohols, respectively (Domergue et al., 2010). *AtFAR2*, also named as MALE STERILITY2, encodes an enzyme that specifically recognizes C16:0-ACP substrate for the production of C16:0 primary fatty alcohols found in pollen cell walls (Chen et al., 2011). *AtFAR3/CER4* encodes FAR involved in the production of C24:0 and C26:0 long-chain primary fatty alcohols of stem and leaf cuticular waxes, *AtFAR3/CER4* produces 28:0 and 30:0 fatty alcohols found in *Arabidopsis* cuticle, in addition to 24:0 and 26:0 (Rowland et al., 2006). Rice (*Oryza sativa*) homolog of *AtFAR2*, *DPW* (*Defective Pollen Wall*), is specifically expressed in tapetum cells and microspores, and is required for the synthesis of primary fatty alcohols essential for the biosynthesis of anther cuticle and pollen sporopollenin. Loss-of-function mutations in *DPW* cause defective tapetum development and function (Shi et al., 2011). Several genes, including *TaFAR1*, *TaFAR2*, *TaFAR3*, *TaFAR4*, *TaFAR5*, *TaFAR6*, *TaFAR7*, and *TaFAR8*, encoding FARs catalyzing the synthesis of long-chain fatty alcohols have also been identified in common wheat (*Triticum aestivum*) (Chai et al., 2018; Wang et al., 2016; Wang, Wang, Sun, Hegebarth, et al., 2015; Wang, Wang, Sun, Wang, et al., 2015). *TaFAR1*, *TaFAR5*, *TaFAR6*, *TaFAR7*, and *TaFAR8* are localized in the endoplasmic reticulum and involved in the synthesis of primary alcohols in wheat cuticle wax. Overexpression of *TaFAR5* in tomato leads to the accumulation of C26:0, C28:0, and C30:0 primary alcohols (Wang, Wang, Sun, Wang, et al., 2015). In addition, *TaFAR6*, *TaFAR7*, and *TaFAR8* responded to drought, cold, and other stresses (Chai et al., 2018).

The effects of water stress on the epicuticular total wax content and wax compounds of cotton leaves, bracts, and boll have been reported (Bondada et al., 1996). The relationship between the content and compositions of cuticle wax in cotton fiber and fiber quality has also been assessed (Thompson et al., 2017). Despite the importance of FARs in plant growth, development, and stress response, up to now, few studies reported *FAR* genes, let alone their function, in cotton. In this study, on the basis of the genome-wide identification of *FAR* genes in the Upland cotton (*G. hirsutum*) genome, we investigated their expression patterns in various vegetative and reproductive tissues and found high expression of *GhFAR3.1* in leaves. We then characterized the role of *GhFAR3.1* in drought response by silencing its expression using the virus-induced gene silencing (VIGS) approach and found that the silencing of *GhFAR3.1* resulted in the reduction of wax content in leaves and consequently more water loss. These results indicate that *GhFAR3.1* is essential for the accumulation of wax that contributes to the development of cotton leaf cuticle and drought tolerance.

2 | MATERIALS AND METHODS

2.1 | Characterization of the *GhFAR* gene family

To identify the *GhFAR* family genes in the *G. hirsutum* genome, we used the amino acid sequence of AtFAR2 (or MS2) (GenBank accession no. NM_112032) of *Arabidopsis thaliana* to search for its homologs in the annotated proteins of the TM-1 genome (Zhang et al., 2015) that was downloaded from CottonGen (https://www.cottongen.org) using BLASTP (E value ≤ 10^{-10}). The conserved domains of the candidate GhFARs were further confirmed by the Pfam database (http://pfam.xfam.org).

The theoretical molecular mass and isoelectric point of GhFARs were calculated by ExPASy (http://web.expasy.org/compute_pi/). Multiple sequence alignment of GhFARs was performed with MEGA 7.0 and the phylogenetic tree was constructed by the neighbor-joining algorithm of MEGA 7.0 with 1,000 bootstrap replicates. The TBtools v0.66443 Software was used to locate and display the distribution of the *GhFAR* genes on the cotton chromosomes based on their genomic coordinates downloaded from CottonGen (https://www.cottongen.org). The exon-intron structures of the *GhFAR* genes were diagrammatically represented by the GSDS program (http://gsds.cbi.pku.edu.cn/).

The expression profiles of individual *GhFAR* in various tissues were investigated based on the transcriptome data generated previously in our laboratory (NCBI accession number PRJNA672231, PRJNA565616, PRJNA640994). The generated clean reads were aligned to the *G. hirsutum* TM-1 reference genome (Zhang et al., 2015), and Htseq v0.6.1 was used to measure the number of reads aligned with each gene. The expression levels of individual genes were quantified by FPKM. The heatmap showing the expression levels of *GhFARs* was generated using the pheatmap package in RStudio.

2.2 | Virus-induced gene silencing

Total RNA was isolated from cotton leaf tissue (*G. hirsutum* cv. Xinluzao 33), and then 1μg RNA was reverse transcribed to obtain first-strand cDNA using an EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TRANS). A 304-bp fragment was amplified using the primers of 5′-CGGGGTACCTATGGCTTCCGCTACTTCAC-3′ and 5′-CCGGAATTCCTGTCATTCGCAACTTCTCG-3′ (the underlined nucleotides represent the restriction sites of *Eco*R I and *Kpn* I) that targets both homoeologs of *GhFAR3.1*, that is, *GhFAR3.1A* and *GhFAR3.1D*.

The PCR cycles were performed as follows: 94°C for 3 min, followed by 32 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final cycle of 72°C for 10 min. The amplified product was ligated into the pGEM-T easy vector to get pGEM-T-*GhFAR3.1*, and the nucleotide sequence of the cloned fragment was subsequently confirmed by sequencing.

Tobacco rattle virus (TRV) derived vectors, pTRV1 and pTRV2, were used for VIGS (Liu et al., 2002). pTRV2::*GhCHLI* (encoding magnesium protoporphyrin chelatase subunit I) was used as a positive control (Gu et al., 2014). pGEM-T-*GhFAR3.1* plasmid was digested by restriction endonucleases *EcoR* I and *Kpn* I to obtain the cDNA fragment of *GhFAR3.1*, which was then cloned into the pTRV2 plasmid using the standard DNA recombinant technique to construct the VIGS vector pTRV2::*GhFAR3.1*. Plasmids pTRV2::*GhFAR3.1*, pTRV2::*GhCHLI*, pTRV2 (as a negative control), and pTRV1 were, respectively, transferred into the *Agrobacterium tumefaciens* strain GV3101 by electroporation. *A. tumefaciens* carrying pTRV1 or pTRV2::*GhFAR3.1* construct was mixed in a 1:1 ratio and infiltrated into cotyledons of 10-days-old seedlings of Xinluzao 33. Control plants were infiltrated with pTRV2 or pTRV2::*GhCHLI* (mixed with pTRV1 in a 1:1 ratio). The VIGS experimental procedure was carried out according to the protocol previously described (Xiong et al., 2019).

2.3 | Measurement of the wax content and relative water content in leaves

The total wax content was measured using chloroform extract from leaves of cotton plants 4 weeks after infiltration. Briefly, the total wax was extracted from 4 cm^2 fresh leaves in a glass bottle containing 10 ml chloroform for 1 min. Then, the leaves were taken out and the chloroform was evaporated under nitrogen gas. The total wax content was obtained using the following formula: Wax content of leaf (g/cm^2) = $(W_2 - W_1)/S$, where W_2 is the bottle weight after nitrogen drying (g); W_1 is the empty bottle weight (g); S is leaf area (cm^2). To measure the relative water content, the weight of fresh leaves (W_f) detached from cotton plants was measured and then the leaves were immersed in water for saturation. After 12 hr, leaves were taken from water and measured to get the water-saturated weight (W_t) after removing residue water on the leaf surface by gentle wiping with tissue paper. The leaves were then dried at 65°C until a constant weight (W_d) was achieved. Leaf relative water content was determined using the following formula: Relative water content (%) = $(W_f - W_d)/(W_t - W_d)$, where W_f is the fresh weight of leaf (g); W_d is the dry weight of leaf (g); W_t is the weight of leaf saturated with water (g). Both experiments were repeated three times.

2.4 | Drought stress

When the true leaves of the cotton seedlings infiltrated with pTRV2::*GhCHLI* appeared yellow, the maximum soil water-holding capacity of the matrix was determined for the pots with plants of wild-type, infiltrated with pTRV2 or pTRV2::*GhFAR3.1*. Two treatments of water content, 10% (representing drought) and 70% (representing well-watered) of soil water-holding capacity, were imposed on wild-type plants and those carrying pTRV2 or pTRV2::*GhFAR3.1*, respectively. Soil moisture was controlled by the weight measurement method according to Hanson (2009). Drought treatment lasted for two weeks, and each treatment was set up with three replications.

2.5 | qRT-PCR analysis

Total RNA was extracted from cotton leaves using RNAiso Plus (Takara), and then 1 μg RNA was reverse transcribed to obtain cDNA. The transcript levels of genes were analyzed by qRT-PCR using the LightCycler® 480 II (Roche). Each reaction was performed in 10 μl volume using SYBR Green Master Mix (Takara) under the following PCR conditions: 94°C for 1 min followed by 40 cycles of 94°C for 15 s, 59°C for 15 s, and 72°C for 20 s. The cotton poly-ubiquitin gene *GhUBQ7* was used as an internal control. All gene-specific primers for qRT-PCR were designed using the Primer 6.0 program (Table S1). All qRT-PCR reactions were performed in triplicate. The relative expression levels of target genes were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

3 | RESULTS

3.1 | Genome-wide identification of *GhFAR* in cotton

Using the protein sequence of AtFAR2 from *Arabidopsis thaliana* as a query and BLASTP, we identified 10 FAR candidates among the annotated proteins of *G. hirsutum*. Their FAR identity was confirmed by searching the Pfam database. The length of the GhFAR protein sequences ranged from 370 to 608 amino acids. The predicted molecular weight (MW) of the GhFAR proteins ranged from 42.17 to 67.37 kDa, and the theoretical isoelectric point (PI) ranged from 7.66 to 8.90 (Table 1).

Multiple sequence alignment showed that the entire amino acid sequences' identity of GhFAR families was 55.94% (Figure 1). All GhFARs contain the NAD-binding-4 and FAR_C domain. The two conserved motifs, GXXGXX(G/A) and YXXXK reported to be present in the NAD-binding-4 domain of published FARs (Kavanagh et al.,2008; Marchler-Bauer et al.,2011), were found in all GhFARs except GhFAR3.4D that lacks the YXXXK motif and GhFAR3.1A that lacks the GXXGXX(G/A) motif (Figure 1). These results suggest the conservation of FARs during the history of plant evolution. A phylogenetic tree was constructed for FAR proteins from *Gossypium hirsutum*, *Arabidopsis thaliana*, *Glycine max*, *Brassica rapa*, and *Oryza sativa* to investigate the relationship among the FARs from these five plant species (Figure 2). In line with the reported results in *Arabidopsis* (Schwacke et al., 2003), these FARs could be divided into A, B, and C three subfamilies; however, none of the 10 GhFARs belongs to subfamily A, in which five of the eight AtFARs reside. Eight (GhFAR3.3A/GhFAR3.3D, GhFAR3.1A/

TABLE 1 The *GhFAR* genes in *Gossypium hirsutum*

Gene name	Gene ID	GenBank accession no.	Chromosome and coordinates	CDS length (bp)	No. of amino acid	MW (KDa)	PI
GhFAR2A	*Gh_A09G1215*	XM_016813925	A09:64020476...64023173	1,827	608	67.35	8.40
GhFAR2D	*Gh_D09G1221*	XM_016815965	D09:39188736...39191421	1,827	608	67.37	8.40
GhFAR3.1A	*Gh_A07G0991*	XM_016888527	A07:18,799,634...18805068	1,113	370	42.17	8.56
GhFAR3.1D	*Gh_D07G1069*	XM_016897778	D07:15,147,674...15152535	1,476	491	55.81	7.66
GhFAR3.2A	*Gh_A08G0044*	XM_016883727	A08:400,528...406052	1,476	491	55.56	8.90
GhFAR3.2D	*Gh_D08G0086*	XM_016864405	D08:709,291...714605	1,476	491	55.47	8.76
GhFAR3.3A	*Gh_A09G0895*	XM_016813517	A09:57,428,273...57434513	1,458	485	55.79	8.83
GhFAR3.3D	*Gh_D09G0921*	XM_016816428	D09:34,589,431...34592847	1,470	489	55.93	8.83
GhFAR3.4A	*Gh_A09G0880*	XM_016838929	A09:56,860,820...56865476	1,530	509	58.77	8.90
GhFAR3.4D	*Gh_D09G0903*	XM_016817642	D09:34,288,150...34291989	1,452	483	55.42	8.86

GhFAR3.1D, GhFAR3.2A/GhFAR3.2D, GhFAR3.4A/GhFAR3.4D) of the 10 GhFARs belong to subfamily C that contains a single *Arabidopsis* FAR (AtFAR3). These results suggest that most cotton FARs are more closely related to AtFAR3 than to other *Arabidopsis* FARs.

The 10 *GhFAR* genes are located on three pairs of homoeologous chromosomes, that is, A07/D07, A08/D08, and A09/D09, with six *GhFARs* found on chromosomes A09 and D09 (Table 1; Figure 3). Chromosome A07, A08, D07, and D08 each contains a single *GhFAR* (*GhFAR3.1A*, *GhFAR3.2A*, *GhFAR3.1D*, and *GhFAR3.2D*, respectively). According to the number of introns and exons, the 10 *GhFARs* could be divided into three groups (Figure 4), largely consistent with their phylogenetic relationship (Figure 1). *GhFAR3.1D*, *GhFAR3.2A*, and *GhFAR3.2D* contain 10 exons and 9 introns. *GhFAR3.1A* contains eight exons and seven introns. There are 11 exons and 10 introns in *GhFAR3.4A* and *GhFAR3.4D*, and 10 exons and 9 introns in *GhFAR3.3A* and *GhFAR3.3D*. However, there are only nine exons and eight introns in *GhFAR2A* and *GhFAR2D* (Figure 4).

3.2 | Expression pattern of *GhFARs*

To get insight into the potential function of *GhFARs*, we analyzed the expression level of each *GhFAR* in different cotton tissues (Figure 5). *GhFAR3.3A* was mainly expressed in ovary and pistil. *GhFAR2A* was relatively highly expressed in anthers and was lowly or not expressed in other tissues. *GhFAR3.1A* and *GhFAR3.1D* had a similar expression pattern in all tissues, that is, mainly expressed in leaves and rapid elongating fibers. *GhFAR3.2A* and *GhFAR3.2D* seemed to be expressed in most tissues investigated, but also showed specific expression in certain tissues, such as anther, flower, and mature seed. *GhFAR3.4D* was mainly expressed in pistils and leaves, while the expression level of *GhFAR3.4A* was very low in all tissues investigated. The results indicate that most *GhFARs* seem to be preferentially expressed in different tissues and that the two homoeologs of some *GhFAR* genes were differentially expressed in some tissues, suggesting that different *GhFAR* genes, even homoeologous *GhFARs*, may function differently in the development of cotton. In leaves, three *GhFARs*, that is, a pair of homoeologs *GhFAR3.1A* and *GhFAR3.1D* as well as *GhFAR3.4D*, had a much higher expression level than others, suggesting that these three genes are the major ones involved in converting fatty acids to fatty alcohols in leaves.

3.3 | Silencing of *GhFAR3.1* decreased total wax content and relative water content in cotton leaves

To know whether *GhFAR3.1* plays a role in the wax synthesis and protection of water loss in cotton leaves, and therefore potentially drought tolerance, we knocked down the expression level of *GhFAR3.1* using VIGS with a 304-bp fragment targeting both homoeologs of *GhFAR3.1*. We used plants infiltrated with *A. tumefaciens* carrying pTRV1/pTRV2::*GhCHLI* that targeted a gene encoding a ChlI subunit of magnesium chelatase (the key enzyme for chlorophyll synthesis) as a positive control for the VIGS experiment as the newly emerging leaves of those plants would turn yellow (due to the lack of chlorophyll) if the VIGS approach worked properly (Figure S1). To make sure the success of VIGS, we further analyzed the expression level of *GhFAR3.1* in the plants infiltrated with pTRV1/pTRV2::*GhFAR3.1* using qRT-PCR. Compared to the wild-type (WT) plants and the plants infiltrated with *A. tumefaciens* carrying pTRV1/pTRV2 (plants-pTRV2::*00*), the plants infiltrated with *A. tumefaciens* carrying pTRV1/pTRV2::*GhFAR3.1* (plants-pTRV2::*GhFAR3.1*) had a significantly low ($p < .01$) expression level of *GhFAR3.1* (Figure 6), suggesting successful inhibition of *GhFAR3.1*.

We then performed a drought treatment for the VIGS plants. Compared to their respective control plants under the well-watered conditions, drought treated WT and plants-pTRV2::*00* had a significantly increased expression level of *GhFAR3.1* ($p < .01$) (Figure 7) and total wax content ($p < .05$) (Figure 8a), suggesting that *GhFAR3.1* was induced by water stress and that *GhFAR3.1* could play a positive role in response to drought by enhanced biosynthesis of wax. Consistent with this observation, the expression level of *GhFAR3.1* in plants-pTRV2::*GhFAR3.1* was also increased after drought treatment, but the induction was not significant compared with that in well-watered plants. Consequently, leaves

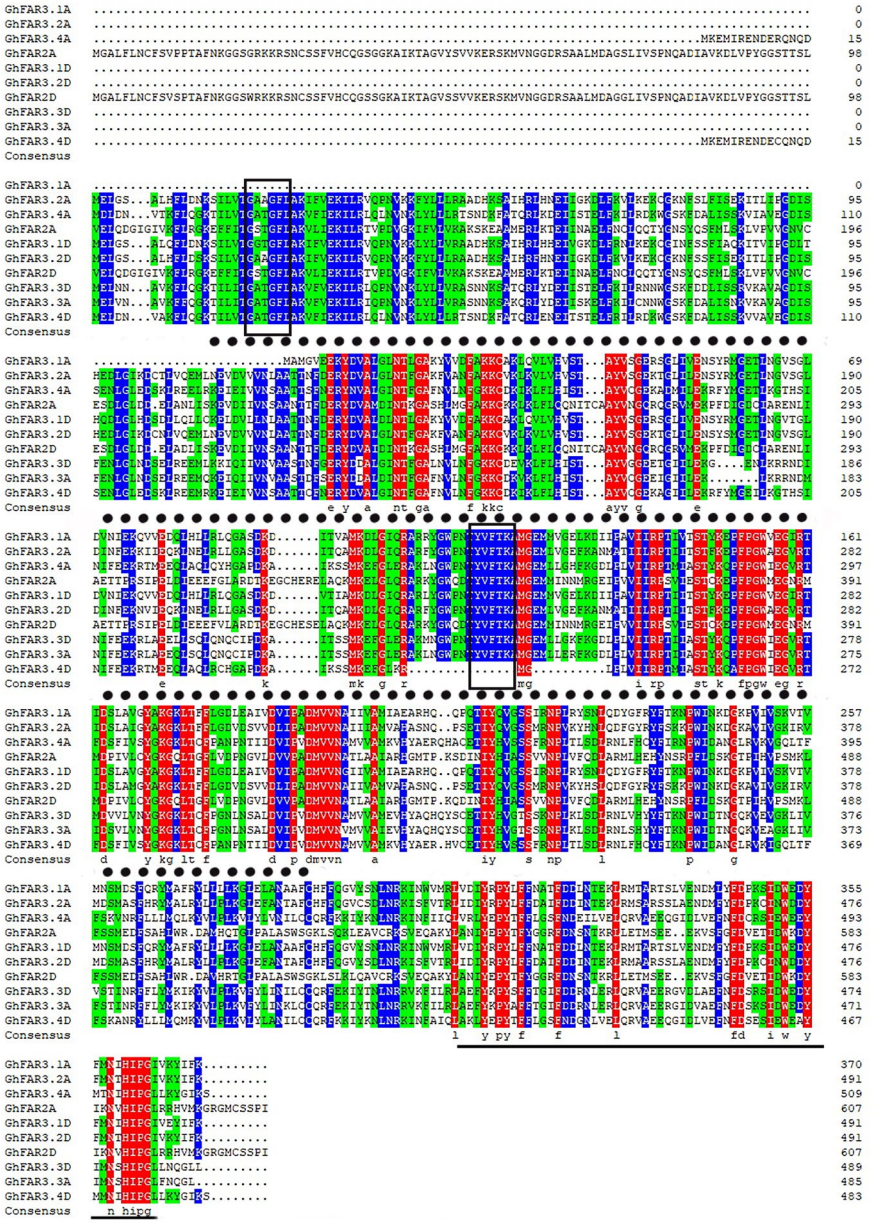

FIGURE 1 Alignment of *GhFAR* sequences. The NAD-binding-4 and sterile domains are indicated by black dot and solid lines, respectively. The two conserved motifs [GXXGXX(G/A) and YXXXK] within the NAD-binding-4 domain are indicated by black boxes

of plants-pTRV2::*GhFAR3.1* showed symptoms of wilting and yellowing after 14 d due to very low expression level of *GhFAR3.1* (Figure 7) and significantly reduced total wax content (Figure 8a).

Under the well-watered conditions, compared with WT and plants-pTRV2::00, the total wax content of the leaves of plants-pTRV2::*GhFAR3.1* was reduced by 66.67% and 62.50%, and the relative water content of the leaves was reduced by 14.78% and 13.83%, respectively (Figure 8). The higher the total wax content, the higher the relative water content. The reduction of the total wax content in plants-pTRV2::*GhFAR3.1* compared to that in both WT and plants-pTRV2::*00* was higher under the drought conditions than under the well-watered conditions due to the silencing of *GhFAR3.1*, consequently, the lowest relative water content was observed in leaves of plants-pTRV2::*GhFAR3.1* under the drought conditions (Figure 8). Under both conditions, the total wax content in leaves was positively correlated with the relative water content in leaves, but in both WT and plants-pTRV2::*00*, the relative water content of leaves was always higher under the well-watered conditions than under the drought conditions even though a lower wax content was observed under the former than the latter. In addition, in pTRV2::*GhFAR3.1*, despite no significant difference in the wax content under the well-watered and drought conditions, a significant difference in the relative water content was evident between these two conditions (Figure 8). These results suggest that water availability is more important than the total wax content in the determination of the relative water content.

3.4 | Effect of silencing *GhFAR3.1* and drought on the expression of wax biosynthesis-related genes

Waxes are mixtures of esters of long-chain carboxylic acids and long-chain alcohols. In de novo synthesis of fatty acids, β-ketoacyl-acyl

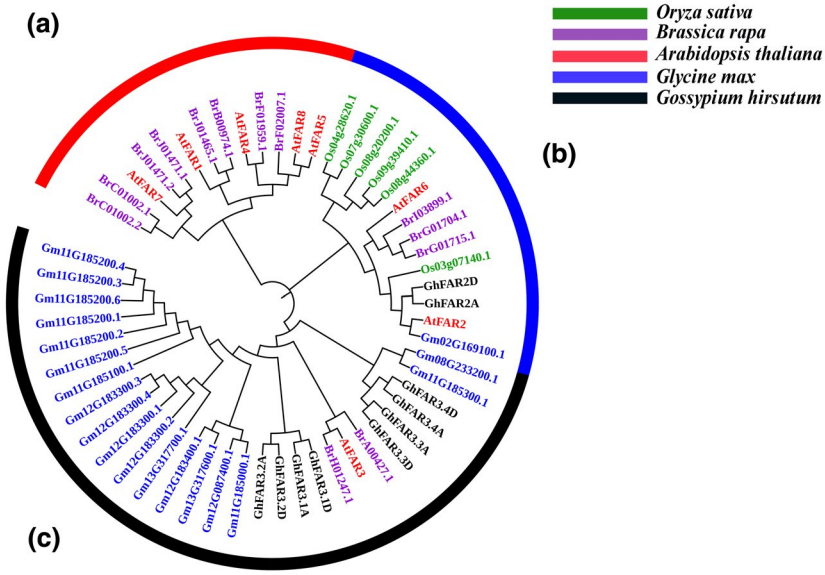

FIGURE 2 Phylogenetic relationship of the *FAR* genes from *Gossypium hirsutum*, *Arabidopsis thaliana*, *Oryza sativa*, *Brassica rapa*, and *Glycine max*. The NJ method of MEGA7.0 was used to construct the phylogenetic tree of the *FAR* genes. A, B, and C represent the three subfamilies

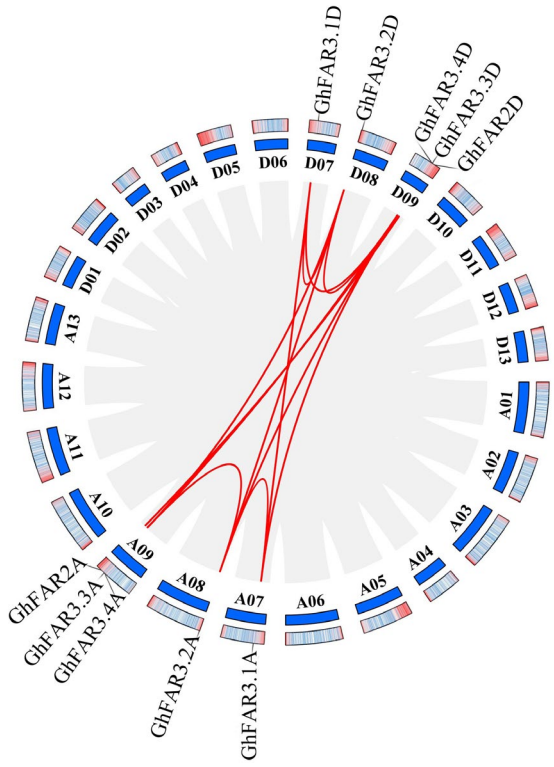

FIGURE 3 Genomic localization of the *GhFAR* genes

carrier protein synthase (KAS) is responsible for the polymerization of acyl-chain. Long-chain acyl-CoA synthetase (LACS) catalyzes free fatty acids to form fatty acyl CoA. In the synthesis of ultra long-chain fatty acids, β-ketoacyl CoA synthase (KCS) is the limiting-rate enzyme in the fatty acid chain elongation process and is responsible for catalyzing the reaction of C16 and C18 fatty acids to form C18 or C20-ketoacyl CoA. In synthesis of waxy components, FAR catalyzes the formation of primary alcohols from long-chain fatty acids in the acyl reduction pathway, and finally, wax esters are formed by wax ester synthase (WSD). We analyzed the effect of knocking-down of *GhFAR3.1* and drought treatment on the expression of *GhKAS*, *GhKCS*, *GhLACS*, and *GhWSD*.

Under either the well-watered or the drought conditions, the expression levels of the three genes (*GhKAS*, *GhKCS*, and *GhLACS*) that encode enzymes catalyzing the reactions upstream of the reaction catalyzed by GhFAR in plants-pTRV2::*GhFAR3.1* were similar to that in WT and plants-pTRV2::*00*. In contrast, the expression level of *GhWSD* that encodes a wax ester synthase catalyzing the reaction downstream the reaction catalyzed by GhFAR was significantly lower in plants-pTRV2::*GhFAR3.1* than in WT and plants-pTRV2::*00* ($p < .01$) (Figure 9). These results indicate that the silencing of *GhFAR3.1* did not affect its upstream reactions in the wax biosynthesis pathway but led to reduced activity of gene encoding enzyme catalyzing reaction downstream the reaction catalyzed by GhFAR3.1, suggesting substrate-mediated induction of *GhWSD*. Under the drought conditions, the expression levels of *GhKAS*, *GhKCS*, and *GhLACS* were significantly induced in all three types of plants (i.e., WT, plants-pTRV2::*00*, and plants-pTRV2::*GhFAR3.1*, $p < .01$). For *GhWSD*, its expression level was also significantly induced by the drought treatment in WT and plants-pTRV2::*00* ($p < .01$) but not in plants-pTRV2::*GhFAR3.1* (increased slightly but not significant). These results indicate that the expression of *GhFAR3.1* and its upstream genes of the wax biosynthesis pathway is enhanced by drought treatment, and most likely, a result of the activation of the positive regulator(s) of the pathway rather than the structural genes themselves.

4 | DISCUSSION

Wax plays an important role in limiting water transpiration (Sánchez et al., 2001). Primary alcohols are major wax components in a wide range of plant species and are synthesized by acyl-reduction pathway, in which fatty acyl-ACP reductase encoded

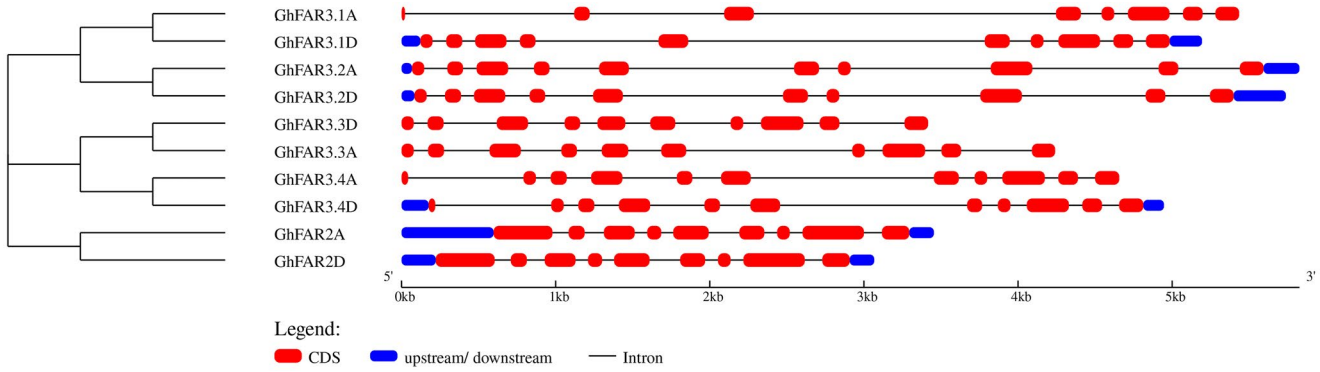

FIGURE 4 Gene structure of the *GhFAR* genes

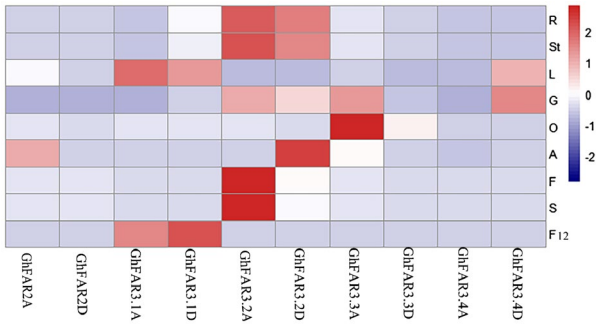

FIGURE 5 Expression levels of *GhFARs* in different tissues of *G. hirsutum*. Cotton variety "Xinluzao 33" was planted in the Cotton Research Institute of Shihezi University. R, roots at flowering and boll-forming stage. S, main stem near to the inverse fourth leaf. L, the inverse fourth leaf of cotton plants. G, gynoecium at 0 days post-anthesis; O, ovary at 0 days post-anthesis; A, anther at mature stage; F, flower at 0 days post-anthesis; S, mature seed; F_{12}, fibers at 12 days post-anthesis

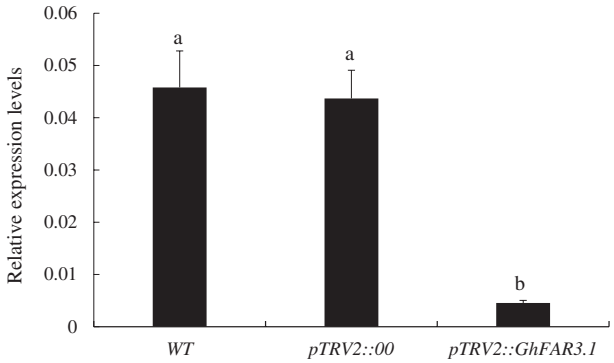

FIGURE 6 Comparison of the expression level of *GhFAR3.1* between VIGS plants and controls. WT, Wild type; pTRV2::*00*, plants infiltrated with pTRV2::*00*; pTRV2::*GhFAR3.1*, plants infiltrated with pTRV2::*GhFAR3.1*. Cotton leaves were used in the analysis. Relative expression levels refer to the expression value of *GhFAR3.1* to *GhUBQ7* (internal reference gene). For each quantification, three independent experimental repeats were used. Significant difference ($p < .01$) between different treatments is indicated by different letters (SPSS single factor ANOVA analysis)

by *FAR* genes converts fatty acyl-CoAs into primary alcohols (Rowland et al., 2006). *FAR* genes have been extensively investigated in many plant species due to their roles in wax biosynthesis. Heterologous expression of *BdFAR2* of *Brachypodium distachyon* in *Arabidopsis* mutant *cer4-3* resulted in a significant increase of the content of C26:0-OH in transgenic plants (Wang et al., 2018). Over-expression of *BdFAR2* and *BdFAR3* in *Arabidopsis* plants significantly increased the content of primary alcohols in transgenic lines but not the total amount of alkanes, fatty acids, and aldehydes (Wang et al., 2018). *TaFAR6*, *TaFAR7*, and *TaFAR8* of wheat had a sequence homology ranging from 45% to 50% compared to their *Arabidopsis* homolog *AtFAR3* (*CER4*). Over-expression of *TaFAR6*, *TaFAR7* or *TaFAR8* in rice and tomato resulted in an increase of wax crystals in rice leaf and tomato peel, and enhanced water holding capacity (Chai et al., 2018).

To identify *FAR* genes with a potential role in drought tolerance in cotton, we performed a genome-wide survey of *GhFARs* in the Upland cotton genome using the *Arabidopsis FAR* gene. A total of 10 *GhFAR* genes were identified. All of the 10 GhFAR protein sequences contain the two typical domains of FAR proteins, that is, the NAD-binding-4 and FAR_C domain (Doan et al., 2009; Teerawanichpan & Qiu, 2010), as well as the two conserved sequences, GXXGXX (G/A) and YXXXK (Kavanagh et al.,2008; Marchler-Bauer et al.,2011), except GhFAR3.4D that lacks the YXXXK motif and GhFAR3.1A that lacks the GXXGXX(G/A) motif, in the NAD-binding-4 domain, suggesting the conservation of FARs in cotton. Most *GhFARs*, even the two homoeologs of the same *FAR*, seem to be specifically expressed in different vegetative and reproductive tissues, suggesting a distinct function of *GhFARs*. Of the 10 *GhFARs*, *GhFAR3.1*, a homolog of *AtFAR3* (*CER4*), was mainly expressed in leaves and rapid elongating fibers. Previous studies had shown that cotton fiber cuticular waxes were mainly composed of very long-chain primary alcohols, which accounted for nearly 74% of the total waxes (Thompson et al., 2017). We reasoned that it might function in drought tolerance based on its expression patterns, and therefore it was selected for further functional characterization.

Most *GhFARs*, even the two homoeologs of the same *FAR*, seem to be specifically expressed in different vegetative and reproductive tissues, suggesting a distinct function of *GhFARs*. Of the 10 *GhFARs*,

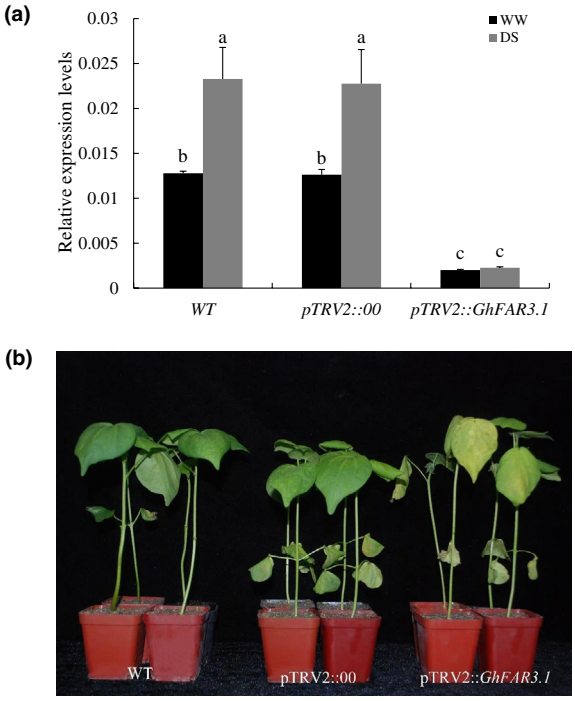

FIGURE 7 Comparison of the expression level of *GhFAR3.1* and plant phenotype between VIGS plants and controls after drought treatment. (a) The relative expression level of *GhFAR3.1* in WT, plants infiltrated with pTRV2::*00* and plants infiltrated with pTRV2::*GhFAR3.1*. (b) Phenotype of differently treated plants after drought treatment. WW, well-watered. DS, drought stress. Cotton leaves were used in the analysis. Relative expression levels refer to the expression value of *GhFAR3.1* to *GhUBQ7* (internal reference gene). For each quantification, three independent experimental repeats were used. Significant difference ($p < .01$) between different treatments is indicated by different letters (SPSS single factor ANOVA analysis)

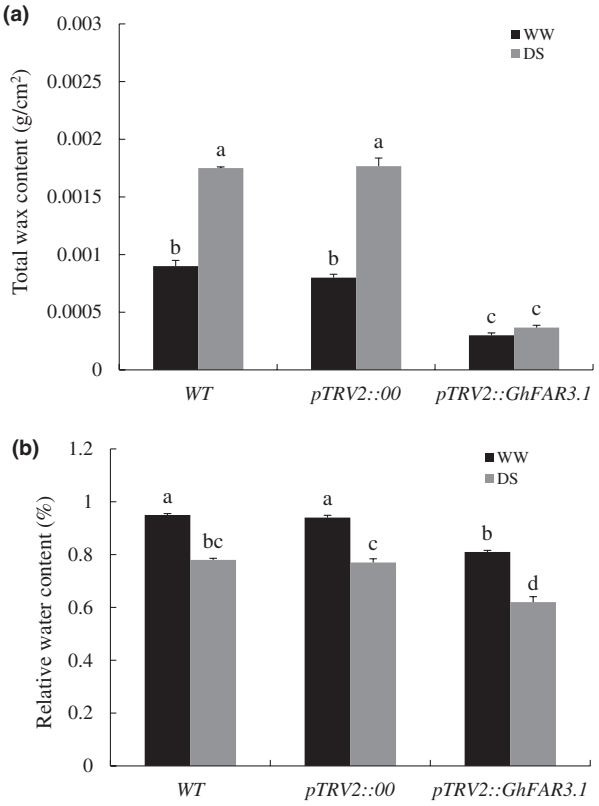

FIGURE 8 The relative water content and total waxy content in leaves from plants infiltrated with pTRV2::*GhFAR3.1* or pTRV2::*00* and un-infiltrated wild type. (a) The total waxy content in leaves from plants infiltrated with pTRV2::*GhFAR3.1* or pTRV2::*00* and un-infiltrated wild type. (b) The relative water content in leaves from plants infiltrated with pTRV2::*GhFAR3.1* or pTRV2::*00* and un-infiltrated wild type. WW, well-watered. DS, drought stress. For each quantification, three independent experimental repeats were used. Significant difference ($p < .05$) between different treatments is indicated by different letters (SPSS single factor ANOVA analysis)

GhFAR3.1, a homolog of *AtFAR3* (*CER4*), was mainly expressed in leaves and rapid elongating fibers. We reasoned that it might function in drought tolerance based on its expression patterns, and therefore it was selected for further functional characterization.

We used VIGS to silence the expression of *GhFAR3.1* in leaves, investigated the effect of silencing *GhFAR3.1* on the total wax content and the relative water content in leaves as well as drought response. We showed that the silencing of *GhFAR3.1* significantly decreased the accumulation of wax in leaves, consequently reduced the ability of water retention and drought tolerance (Figures 7 and 8). Our results also showed that drought stress was able to significantly enhance the total wax content in leaves and this ability was abolished in the *GhFAR3.1* silenced plants. In both well-watered and drought conditions, the total wax content was positively correlated with the relative water content in leaves, suggesting the protective role of wax in water retention. These results support the previous conclusion on the role of cuticular wax in plant drought resistance (Bondada et al., 1996; Cameron et al., 2006; Kim et al., 2007; Kwan et al., 2006).

It has been reported that *AtFAR3* of *Arabidopsis* could be activated by the *MYB94* transcription factor to increase the accumulation of cuticular wax (Lee & Suh, 2015). In this study, we found that the expression levels of the genes involved in the wax synthesis pathway, including *GhFAR3.1*, *GhKAS*, *GhKCS*, *GhLACS*, and *GhWSD*, were significantly increased under drought conditions. This result implies the activation of positive regulator(s), such as transcription factors, of the wax biosynthesis pathway by drought stress. Identification of such positive regulator(s) would greatly enhance our understanding of the mechanisms underlying drought tolerance involving wax and provide targets for improving drought tolerance of plants by genetic engineering. Proper wax accumulation in plant tissues is very important to ensure their protective function and to maintain the basic physiological activities of plants. Our results imply the potential function of *GhFAR3.1* in drought response. In addition, considering the differential expression patterns of most *GhFAR* genes, we speculate that they may function differently in cotton development, which should be investigated in the future.

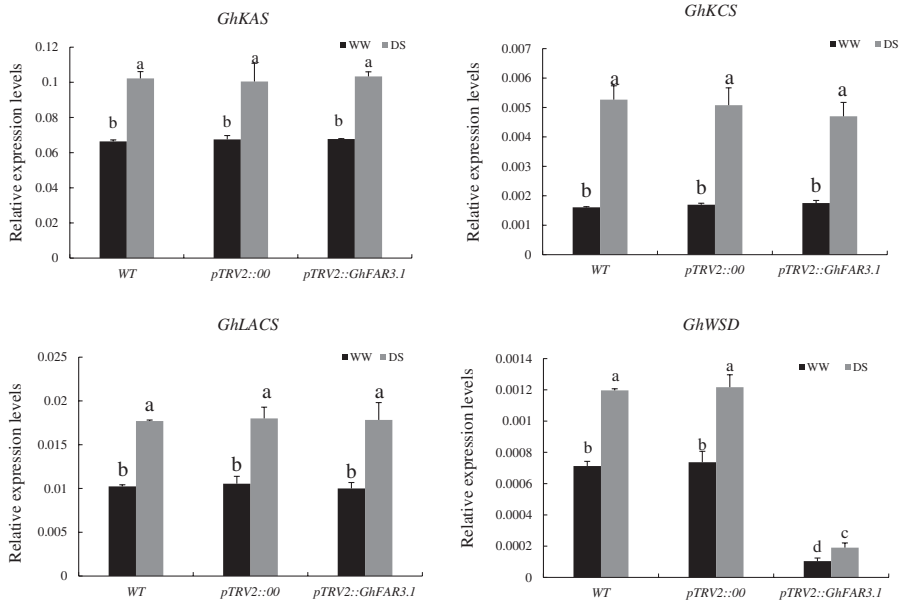

FIGURE 9 Changes of the expression level of wax biosynthesis-related genes in response to drought treatment. *GhKAS*, β-ketoacyl-acyl carrier protein synthase, *GhKCS*, β-ketoacyl CoA synthase, *GhLACS*, long-chain acyl-CoA synthetase, *GhWSD*, wax ester synthase. WW, well-watered. DS, drought stress. Cotton leaves were used in the analysis. Relative expression levels refer to the expression value of indicated genes (*GhKAS*, *GhKCS*, *GhLACS*, *GhWSD*) to *GhUBQ7* (internal reference gene). For each quantification, three independent experimental repeats were used. Significant difference ($p < .01$) between different treatments is indicated by different letters (SPSS single factor ANOVA analysis)

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Y-J. Lu., J.S., and F.L. designed the experiment. Y-J. Lu., X-Q.C and M-J.J. performed the experiment and took all measurements. Y-J. Lu. and F.L. analyzed the results. Y-J. Lu. and F.L. wrote the initial manuscript draft with revisions by X-Y.Z, F.X., and Y-J. Li. All authors approved the submitted manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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