BMC Genomics

RESEARCH Open Access

Genome-wide analysis and functional validation of the cotton FAH gene family for salt stress

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Abstract

Background Fatty acid hydroxylases (FAHs) are a family of enzymes that includes fatty acid hydroxylases, carotenoid hydroxylases, and sterol desaturases. Fatty acids are highly important for plants. They are the main source of energy storage and the main component of the cell membrane. Saturated fatty acids can be divided into two categories: saturated fatty acids and unsaturated fatty acids. FAHs play a pivotal role in enhancing plant salt tolerance by modulating fatty acid metabolic pathways, thereby improving cell membrane stability and antioxidant capacity.

Results In this study, we identified a total of 129 FAH gene family members in four cotton species, namely, *Gossypium hirsutum*, *Gossypium darwinii*, *Gossypium arboreum*, and *Gossypium raimondii*. The FAH genes were divided into five subgroups via evolutionary analysis. FAH genes located in the same subgroup presented similar gene structures and a consistent distribution of conserved motifs through the analysis of evolutionary trees, gene structures, and conserved motifs. Chromosomal localization analysis of the FAH gene family revealed that it has undergone chromosomal segment duplication events. Analysis of *cis*-acting elements suggested that the FAH gene may be involved in regulating biotic and abiotic stresses, plant growth and development, signaling pathways, and other physiological processes. The RT–qPCR results revealed significant differences in the expression levels of FAH gene family members under salt stress conditions compared with those in the control group. Additionally, we successfully silenced *Gohir.A03G045300* through VIGS experiments, and the results indicated that the silenced plants were more sensitive to salt stress than the control plants were. This suggests that *Gohir.A03G045300* may be involved in the response of cotton to salt stress.

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Conclusions A total of 129 FAH genes were identified in four Gossypium species through bioinformatics analysis. Gene silencing of FAH members in *G. hirsutum* revealed that the FAH gene family plays a crucial role in the response of cotton to salt stress.

Keywords Cotton, Fatty acid hydroxylases, Salt stress, Gene knockdown, RT–qPCR

Background

Fatty acid hydroxylases (FAHs) are a family of enzymes that includes fatty acid hydroxylases, carotenoid hydroxylases, and sterol desaturases. Plant carotenoids are lipid-soluble pigments commonly found in the membranes of chloroplasts and chromoplasts, and they are also essential substances for plant survival. It protects chlorophyll from photooxidative damage caused by strong light and serves as an essential structural component of the photosynthetic antenna and reaction center complexes. Additionally, some carotenoids are key components of certain pigment–protein complexes [1], and they play crucial roles in both photosynthesis and photoprotection [2]. β-Carotene hydroxylase is involved in the synthesis of lutein through the hydroxylation of β-carotene, making it a key enzyme in the carotenoid biosynthesis pathway. Lutein and zeaxanthin play important roles in scavenging free radicals [3, 4]. The gene sequence of β-carotene hydroxylase is relatively conserved and has been identified in *Arabidopsis thaliana* [5], *Capsicum annuum*, and sweet potato [6].

Studies have shown that the β-carotene hydroxylase gene plays an important role in the response to abiotic stress. In rice, the β-carotene hydroxylase gene DSM2 enhances resistance to drought and oxidative stress by increasing the biosynthesis of lutein and abscisic acid [7]. In addition, the FAH family also includes C-5 sterol desaturase and C-4 sterol methyl oxidase [8, 9], which play important roles in cholesterol biosynthesis and plant cuticular wax biosynthesis [10]. The catalytic center of these two enzymes contains a conserved domain sequence of HXHH (X is any amino acid), which can coordinate two iron ions and integrate into the endoplasmic reticulum to regulate the transmembrane transport of substances [11]. Phytosterols are important components of cell membranes and lipid rafts and play important roles in various physiological and biochemical processes and stress resistance during plant development [12] in response to various biotic and abiotic stresses. After salt stress, *A. thaliana* enhances its salt tolerance by reducing the contents of brassicasterol and stigmasterol, increasing the ratio of sitosterol to stigmasterol, and increasing the permeability of the cell membrane [13]. Moreover, the total sterol content in the roots of soybean decreased by 50%, and the saturated fatty acid content increased significantly after salt stress [14].

In addition, phytosterols are involved in the synthesis of brassinolides (BRs) as signaling molecules, regulating the expression of phytosterol synthase genes and thereby regulating plant tolerance to stress [15]. It can also serve as a component of the cell membrane or a key element of lipid rafts, participating in the regulation of plant responses to stress. When plants are subjected to salt stress, their membrane structure and function first change. Phytosterols exist primarily in the form of free sterols within the plant, where they interact with phospholipids and membrane proteins in the cell membrane under the influence of free sterols. This interaction enhances the adaptability of the plant membrane system to environmental stress. Fatty acids are highly important for plants. They are the main source of energy storage and the main component of the cell membrane. Saturated fatty acids can be divided into two categories: saturated fatty acids and unsaturated fatty acids. Unsaturated fatty acids can be further divided into monounsaturated fatty acids with only one double bond and polyunsaturated fatty acids containing two or more. At present, an increasing number of studies have shown that fatty acids and their derivatives participate in various signal transduction pathways by forming double bonds at specific positions to produce unsaturated fatty acids and then participate in the regulation of biotic and abiotic stresses in plants [16]. Many related functional genes have been identified and verified. Wei et al. reported that the *PtFAD3/7/8* gene, which is induced by abiotic stress, plays an important role in regulating the transformation of linoleic acid and linolenic acid and is crucial for resisting osmotic stress [17]. Shi et al. isolated rice fatty acid desaturase gene 2 (*OsFAD2*) from rice, and the overexpression of *OsFAD2* in the rice vegetative stage revealed that the cold tolerance of transgenic plants significantly increased and that the grain yield greatly improved [18]. In this study, candidate genes of the FAH family in four different cotton species were identified, a phylogenetic tree of FAH genes was constructed according to their genome files and protein sequences, and the evolutionary selection relationships of FAH gene members were analyzed. In addition, the physical and chemical properties, subcellular localization prediction, gene structures, conserved motifs, collinearity and *cis*-acting element distributions of the cotton FAH family members were analyzed. Moreover, the expression of FAH members in upland cotton after salt stress was further detected. The function of cotton FAH family members was further verified via VIGS experiments. This study helps elucidate the evolution and function of the FAH superfamily in cotton and provides useful information for

further exploration of the molecular mechanism of how the FAH superfamily is involved in fatty acid synthesis, sterol biosynthesis and the carotenoid pathway.

Results

Identification of FAH gene family members in cotton

A total of 129 FAH gene family members were identified from four cotton species, including 43 members in *Gossypium hirsutum*, 42 members in *Gossypium darwinii*, and 22 members in *Gossypium raimondii* and *Gossypium arboreum*. In addition, the physicochemical properties, including the starting position of the genes on the chromosome, gene length, molecular weight, and isoelectric point of the proteins encoded by the 129 genes identified, were analyzed (Supplementary Table S1). We found that the number of amino acids encoded by FAH family members ranged from 147 to 857, and the average number of amino acids was 410. The molecular weights of the FAH family proteins ranged from 17410.1 Da to 97252.8 Da, with an average molecular weight of 7432 Da. The theoretical isoelectric point of these proteins ranged from 6.96 to 9.66, and the average isoelectric point was 8.36, which was weakly alkaline. To understand the location of this family in cells, we predicted the subcellular localization of these 129 FAH members (Supplementary Fig. S1). The results revealed that all FAH family members were highly expressed on the plasma membrane of plant cells, indicating that the FAH family may play an important role in the plasma membrane.

Phylogenetic and evolutionary selection analysis of the cotton FAH gene family

To study the evolutionary relationships among the members of the cotton FAH family, a phylogenetic tree was constructed using the FAH protein sequences of the four *Gossypium* species mentioned above (Supplementary Fig. S2). According to the study of genetic distance, 129 members of the FAH family were divided into five subgroups. The largest subgroup was Group 1, with a total of 49 members. In this branch, *G. hirsutum*, *G. darwinii*, *G. arboreum* and *G. raimondii* had 16, 15, 9 and 9 members, respectively. The smallest subgroup was Group 2, with only 6 members. The other three subgroups had 36, 13 and 26 FAH family members, respectively. Members of the same branch tend to have more similar evolutionary relationships and similar functions. According to the phylogenetic tree, most of the homologous genes between allotetraploid cotton and diploid cotton clustered together in the same group, indicating that the cotton FAH gene family is expanding.

To further study the selection events of the FAH family in the evolutionary process and determine whether there is selective pressure on the protein-coding genes of the family, we used KaKs_Calculator2.0 software to calculate the Ka (nonsynonymous substitution) and Ks (synonymous substitution) values of FAH homologous gene pairs in cotton and the Ka/Ks ratio (Supplementary Table S2). The results revealed that in the development and evolution of all FAH gene pairs, the incidence of synonymous base substitutions was much greater than that of nonsynonymous base substitutions, and the Ka/Ks ratio was far less than 1, so it was not affected by natural selection. The results revealed that these genes experienced purifying selection during evolution.

Analysis of the gene structure and conserved motifs of the FAH gene family

To better understand the evolutionary relationships among different members of the FAH gene family, we constructed a phylogenetic tree with an intron–exon structure and conserved motifs for the four cottons on the basis of the FAH protein sequence. The FAH gene region features a rich distribution of introns, with each FAH gene containing at least one intron. Compared with the other four subgroups, the FAH gene in subgroup 1 contained more introns, with a maximum of 11 introns. The diversity of the FAH gene structure indicates that different selection events may have occurred during the process of gene evolution. Notably, in the four cotton species, the genes closely related and closely related in the phylogenetic tree tended to have more similar exon and intron arrangements, indicating that the exon–intron structure is highly correlated with the phylogenetic relationship between FAH genes.

Conserved motifs are often related to the function of proteins. To reveal the characteristic motifs of the FAH gene family, MEME software was used to identify the conserved motifs in the FAH protein. A total of 10 conserved motifs, named motifs 1 to 10, were identified in the FAH gene family (Supplementary Fig. S3). The motif sequences are presented in Supplementary (Figs. S4-S7). Through the analysis of conserved motifs, we found that different motifs were missing in all 129 FAH gene family members. However, the FAH genes in *G. hirsutum* contained motif 3 and motif 5, the FAH genes in *G. darwinii* contained motif 4 and motif 6, the FAH genes in *G. arboreum* contained motif 7 and motif 3, and the FAH genes in *G. raimondii* contained motif 5 and motif 4. The FAH gene was highly conserved in five different subgroups of the family, and the number and arrangement of motifs in the group were similar.

Chromosome localization and collinearity analysis of the cotton FAH gene family

The position distribution map of the FAH gene family on the chromosomes of the four species of cotton was drawn via TBtools software (Fig. 1). The results revealed that 21 of the 43 FAH gene family members in *G. hirsutum* were

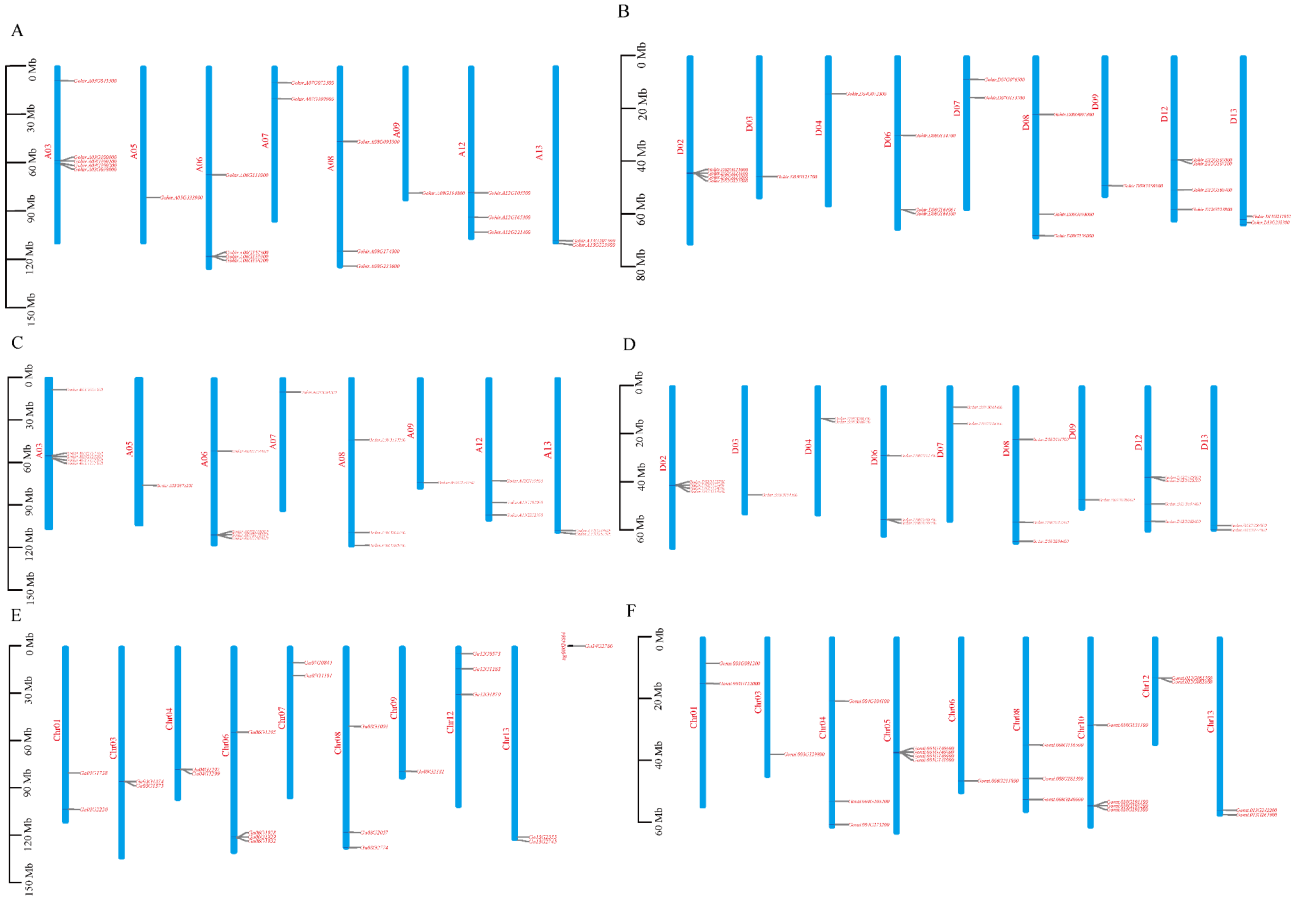

Fig. 1 Distribution of FAH genes in At genome of *Gossypium hirsutum* (**A**), Dt genome of *Gossypium hirsutum* (**B**), At genome of *Gossypium darwinii* (**C**), Dt genome of *Gossypium darwinii* (**D**), the genome of *Gossypium arboreum* (**E**) and the genome of *Gossypium raimondii***(F)**

distributed in the At subgenome and were distributed in A03, A05–A09, A12 and A13; 22 genes were distributed in the Dt subgenome and were distributed in D02–D04, D06–D09, D12 and D13. Most of these genes are distributed at both ends of the chromosome. In *G. darwinii*, which is also tetraploid, we also found that the FAH gene has a similar distribution on the chromosomes. Among the 42 FAH gene members, 20 were distributed on chromosomes A03, A05-A09, A12 and A13 of the At subgenome, and 22 were distributed on chromosomes D02-D04, D06-D09, D12 and D13 of the Dt subgenome. Notably, four genes on chromosome A03 of *G. hirsutum* and *G. darwinii*, two allotetraploid cottons, presented homologous genes on chromosome D02. This phenomenon may be due to the large fragment replication of chromosomes in upland cotton during evolution. The above results suggest that the At and Dt genomes of *G. hirsutum* and *G. darwinii* may have originated from the same two ancestors and that the FAH gene family is relatively conserved in evolution, but some gene deletions still occur. In the At genome-donor diploid cotton *G. arboreum*, 22 FAH genes were distributed on chromosomes A01, A03-A04, A06-A09, A12 and A13. In *Gossypium raimondii*, which is a diploid donor of the Dt genome, 22 FAH members are distributed on chromosomes D01, D03-D08, D12 and D13.

Collinearity analysis can explain the homology between genes well, and collinear homologous sequences may have similar functions. Therefore, MCScanX and Circos software were used to analyze and map the collinearity of the FAH gene families of the four different cotton varieties. In the *G. arboreum* genome, the FAH gene family has collinear genes located between chromosomes A08, A09 and A12 and between chromosomes A04 and A06 (Fig. 2C). The collinearity of the FAH gene in *Gossypium raimondii* occurred mainly among the D01, D05, D08, D09, D10 and D12 chromosomes (Fig. 2D). In the tetraploid cotton species *Gossypium hirsutum* and *Gossypium darwinii*, most of the collinear relationships between genes occur on the same chromosome or between homologous chromosomes (Fig. 2A-B). Moreover, we also found that the FAH gene has a collinear relationship with the A03 and D02 chromosomes of the two tetraploid cotton species, which is similar to the previous distribution of FAH homologous genes on chromosomes.

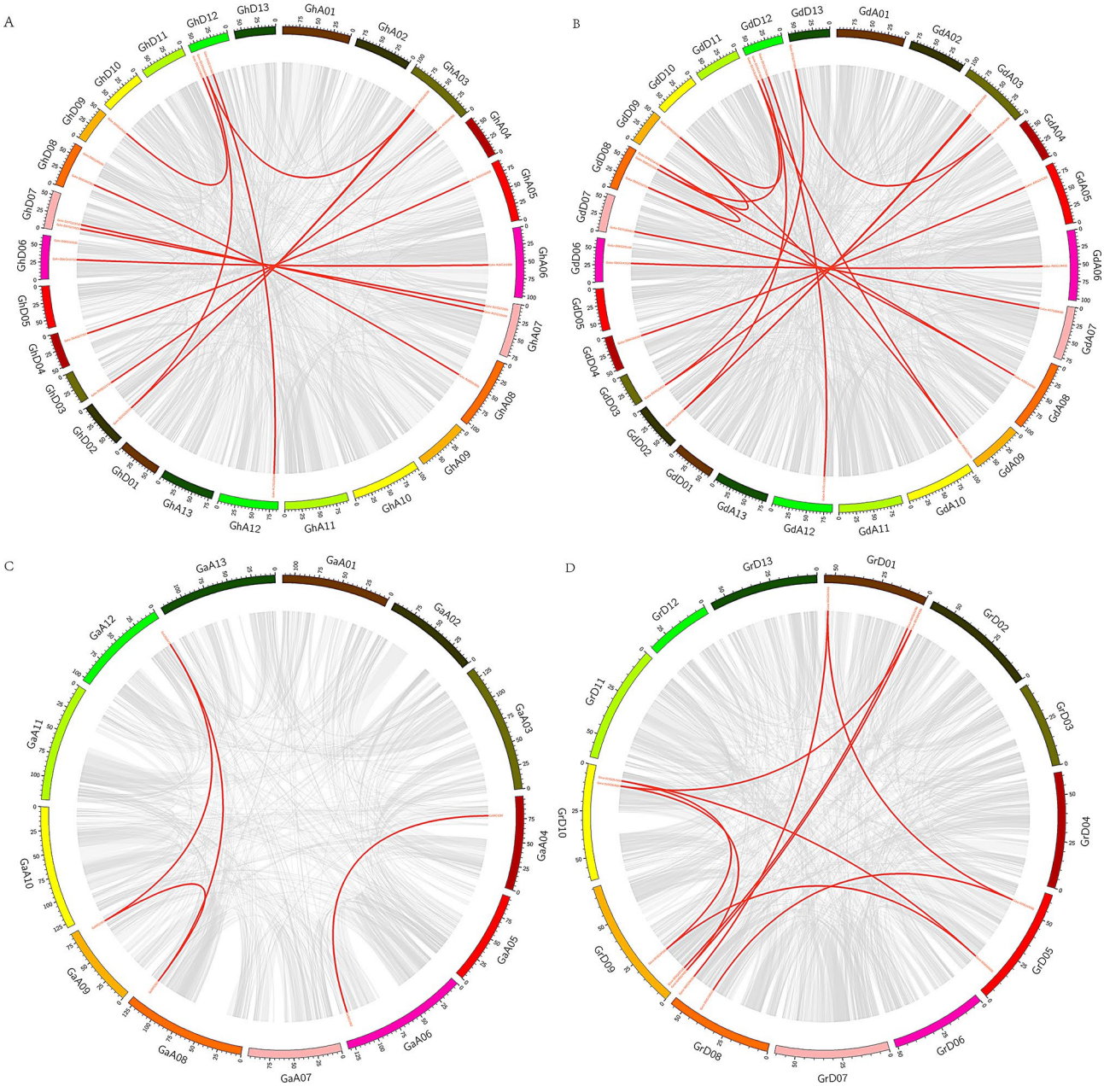

Fig. 2 Collinearity analysis of cotton FAH genes in *Gossypium hirsutum* (**A**), *Gossypium darwinii* (**B**), *Gossypium arboreum* (**C**), and *Gossypium raimondii* (**D**). Note: The gray lines represent the collinearity relationships of all genes within the genomes of *Gossypium hirsutum* (**A**), *Gossypium darwinii* (**B**), *Gossypium arboreum* (**C**), and *Gossypium raimondii* (**D**). The red lines represent the collinearity relationships of the FAH gene family across the genomes

Analysis of *cis*-acting elements of the cotton FAH gene family

To understand the potential function of the FAH gene family, this study analyzed the 1500 bp promoter sequence upstream of the FAH gene to detect *cis*-acting elements (Fig. 3). In the FAH gene family, various *cis*-acting elements, including the basic TATA box and CAAT box, are involved in the response to both biotic stress and abiotic stress. Additionally, elements involved in abiotic stress and signal transduction, such as MYB, ABRE, CAT-box, and HD-zip1, have been identified. The binding sites of ABREs and MYBs play a role in the abscisic acid response, whereas the CAT box is a *cis*-regulatory element associated with meristem expression. HD-zip is involved in the regulation of adverse stress in plants. These results clearly indicate that FAH genes may be involved in the regulation of biotic or abiotic stresses, plant growth and development, signal transduction pathways, and other physiological processes.

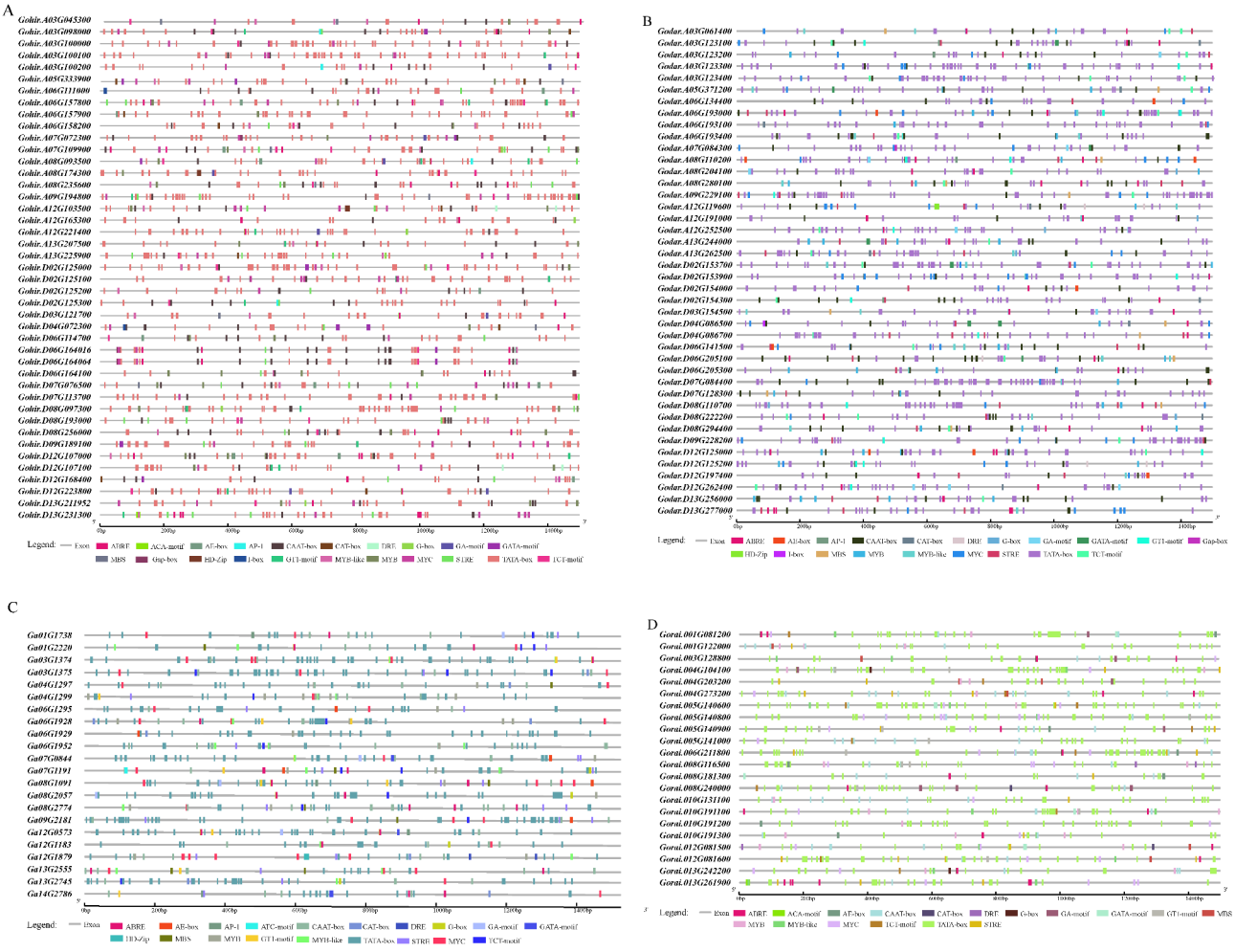

Fig. 3 Analysis of the *cis*-acting elements of FAH genes in *Gossypium hirsutum* (**A**), *Gossypium darwinii* (**B**), *Gossypium arboreum* (**C**) and *Gossypium raimondii* (**D**)

RT–qPCR analysis of the cotton FAH gene family

Through the published transcriptome data of upland cotton (Supplementary Table S3), the expression of 43 FAH genes at different time points after salt stress was analyzed (Fig. 4). The results revealed that the FAH gene of upland cotton was strongly affected after salt treatment. According to the transcriptome data, eight FAH genes were randomly selected for RT–qPCR verification (Fig. 5). We found that the expression levels of the eight selected *GhFAH* genes significantly changed after salt stress. Among them, *Gohir.A03G100200*, *Gohir.A05G333900*, *Gohir.A12G221400*, *Gohir.D07G113700* and *Gohir.D02G125200* were significantly upregulated under salt stress. *Gohir.A03G045300*, *Gohir.A12G165300*, and *Gohir.D13G211952* was significantly downregulated. The RT–qPCR results for these eight genes were consistent with the trend of transcriptome expression. In summary, the FAH gene can quickly regulate its expression via transcription within 24 h after salt stress to cope with tolerance to salt stress.

Virus-induced gene silencing (VIGS) of FAH genes leads to increased sensitivity of plants to salt stress

Virus-induced gene silencing (VIGS) is an effective method for studying gene function. To explore the role of the FAH gene family in cotton, we constructed a VIGS vector for silencing *Gohir.A03G045300* in upland cotton 'Xiang FZ031'. Approximately two weeks after Agrobacterium infection, the true leaves of TRV2:*CLA1* plants presented an albino phenotype (Fig. 6A), indicating that the VIGS vector was active and effective. In addition to the positive control plants, we used 200 mM salt solution to test the stress tolerance of the cotton plants and observed their phenotype after 14 days. The growth status of the TRV2:*Gohir.A03G045300* plants was much slower than that of TRV2:00. We subsequently measured the shoot fresh weight and plant height of TRV2:*Gohir.A03G045300* and TRV2:00 (Fig. 6B, D, E). Compared with those of the control plants, the plant height and fresh weight of the silenced plants were significantly lower. We obtained true leaves from TRV2:*Gohir.*

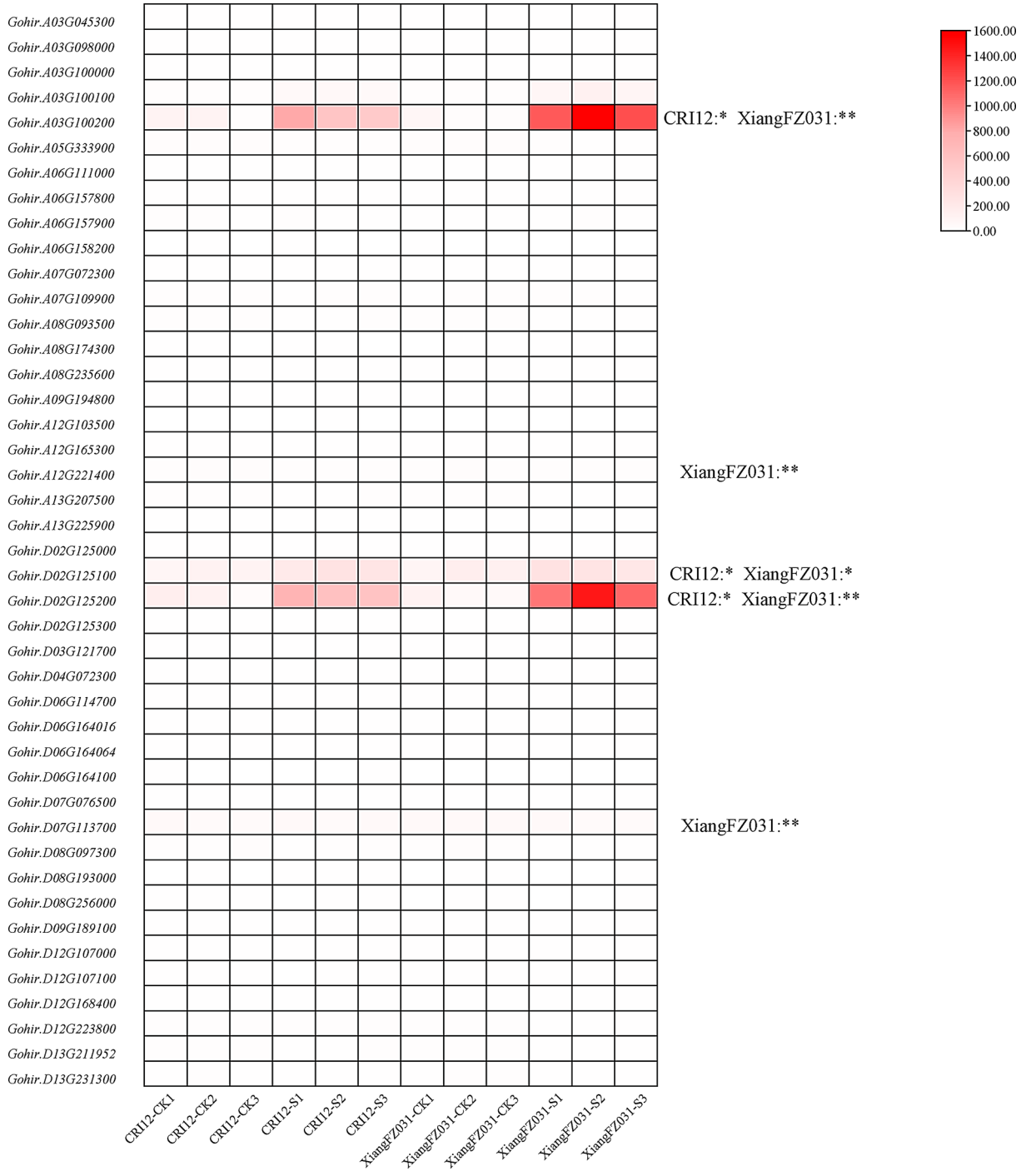

Fig. 4 The expression levels of FAH genes in different periods after salt stress in *Gossypium hirsutum*. Heatmap showing gene expression in the leaf tissues of the CRI12 and XiangFZ031 varities. The colors range from red to white, indicating the differential gene expression levels from high to low. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$. Not labeled indicates no significant difference

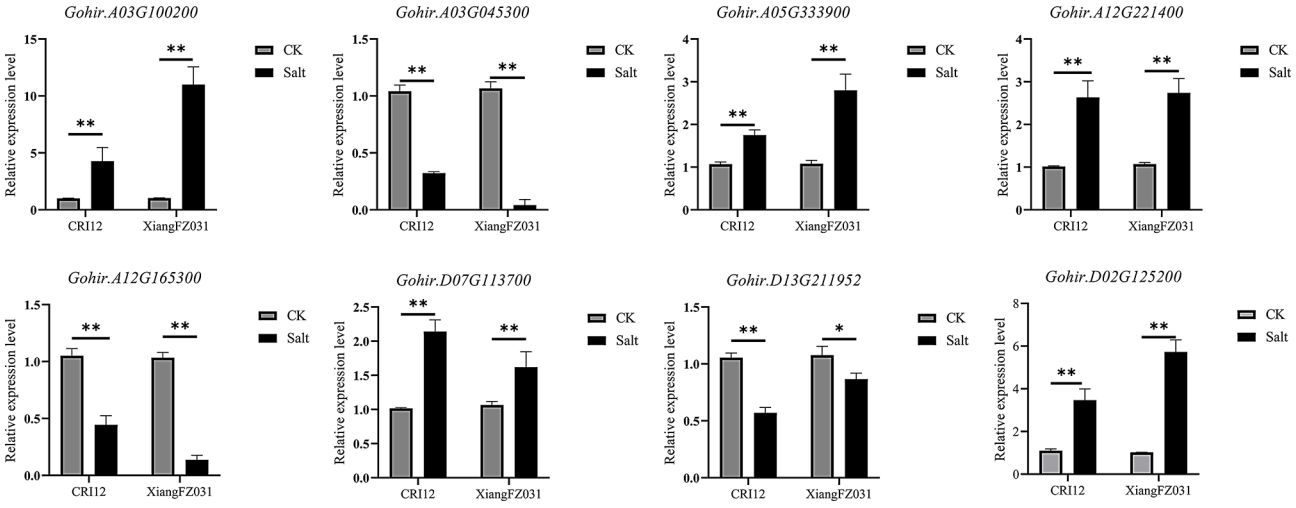

Fig. 5 Quantitative analysis of FAH gene expression in *Gossypium hirsutum* after salt stress. The calculation results were analyzed via a t test. The asterisk indicates that there is a significant difference at the 0.05 probability level according to the t test. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$

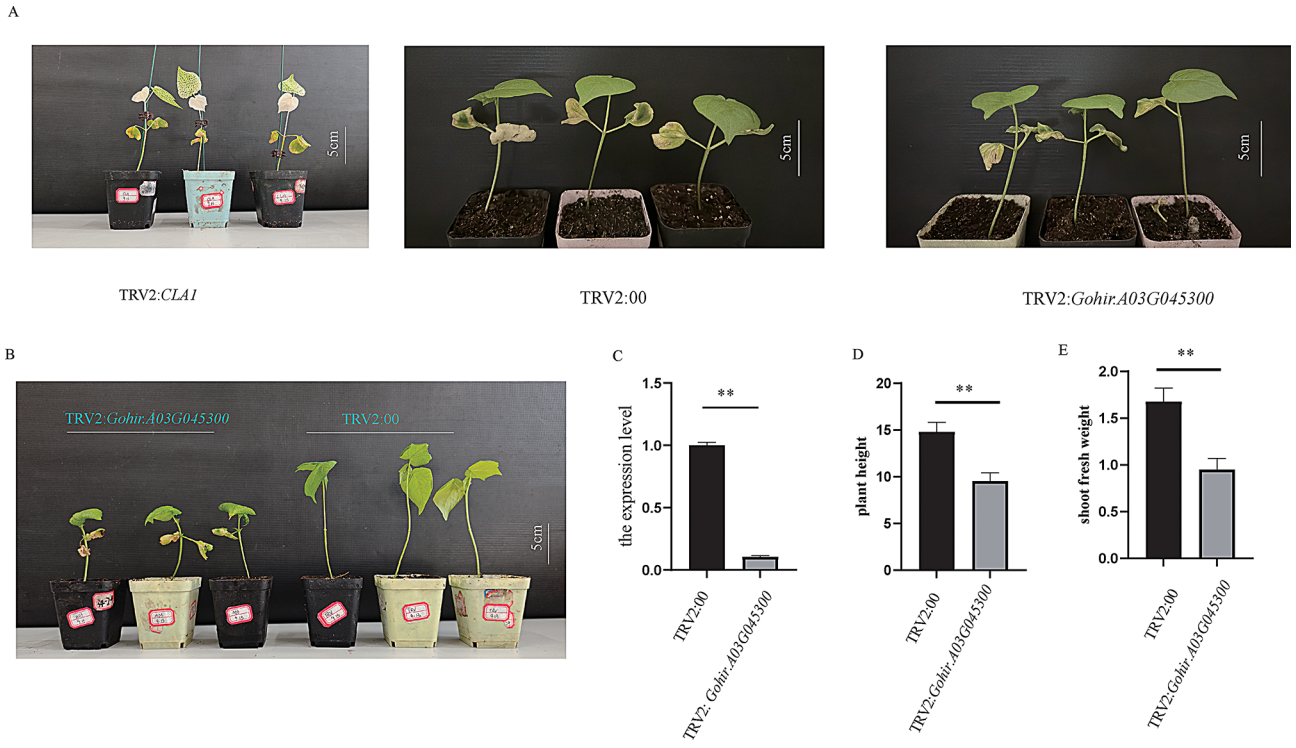

Fig. 6 VIGS experiment of the FAH gene family in *Gossypium hirsutum*. (**A**): The phenotype of the plant after 14 days of Agrobacterium infection. The left side is TRV2: *CLA1* (positive control), the middle is TRV2: 00 (negative control), and the right side represents TRV2: *Gohir.A03G045300* (the silenced plants). (**B**): Phenotypic comparison between negative control plants and the silenced plants. The right side is TRV2: 00 (negative control), and the left side is TRV2: *Gohir.A03G045300* (the silenced plants). (**C**) Comparison of expression levels of *Gohir.A03G045300* between the silenced plants and the negative control plants., (**D**) Comparison of plant height between the silenced plants and the negative control plants. (**E**) Comparison of shoot fresh weight between the silenced plants and the negative control plants The calculation results were analyzed via a t test. The asterisk indicates that there is a significant difference at the 0.05 probability level according to the t test. * indicates $P \leq 0.05$, * indicates $P \leq 0.01$

A03G045300 seedlings for RNA extraction to verify the effect of gene silencing. The expression levels of these genes in the TRV2:*Gohir.A03G045300* plants were significantly inhibited (Fig. 6C), which proved that gene silencing was successful. In summary, gene silencing experiments revealed that the TRV2:*Gohir.A03G045300* weakened its tolerance to salt stress after silencing, suggesting that *Gohir.A03G045300* may be involved in the response of cotton to salt stress.

Discussion

The fatty acid hydroxylase (FAH) family is a family of proteins containing fatty acids, carotenoid hydroxylases, and sterol desaturases, which are widely present in bacteria, plants, and animals. These proteins have been widely studied for their roles in plant growth and development [19] and in regulating biotic stress [20] and abiotic stress [21]. However, there are no reports on the regulation of the cotton FAH gene after salt stress. In this study, bioinformatics methods were used to systematically identify the members, evolutionary relationships, and structural characteristics of FAH genes in cotton, laying the foundation for further studies of the specific mechanism by which FAH genes regulate salt stress.

The formation of gene families is caused by genome-wide replication events or the polyploidization of genomes. This is a large-scale chromosome doubling event, which simultaneously increases the number of all genes in the species, resulting in many chromosome doubling fragments remaining in the genome [22]. A group of these genes are similar in structure and function and encode similar proteins [23]. Allotetraploid cotton (AADD) *G. hirsutum* and *G. darwinii* have undergone genome polyploidization events during evolution, and studies have shown that the At genomes of *G. hirsutum* and *G. darwinii* are derived from *G. arboreum* and that the Dt genome is derived from *G. raimondii* [24]. In this study, 43 and 42 FAH genes were identified in the allotetraploid cotton strains *Gossypium hirsutum* and *Gossypium darwinii*, respectively. Twenty-two FAH members were identified in *Gossypium arboreum*, and 22 FAH members were identified in *Gossypium raimondii*. The number of FAH genes in tetraploid upland cotton is roughly equal to the sum of the number of FAH genes in its two diploid ancestors. These findings indicate that there was no significant gene loss in the FAH gene family during the evolution of allotetraploid cotton. This phenomenon is similar to the results of genome-wide analysis of another PFK family, where 26 genes were identified in *Gossypium hirsutum* and *Gossypium barbadense*, and 14 PFK genes were identified in the diploid ancestors *Gossypium arboreum* and *Gossypium raimondii* [25].

According to the distribution of FAH family members on chromosomes, FAH members with similar structures and close locations on chromosomes A03, D02, A06 and D06 of tetraploid cotton exist. This may have been a tandem duplication event during the evolution of the FAH gene. Tandem duplication mainly occurs in the chromosome recombination region. These genes are closely arranged on the same chromosome, with similar sequences and similar functions [26]. Notably, we found four FAH members on the A03 chromosome of *Gossypium hirsutum*, namely *Gohir.A03G100100*, *Gohir.A03G100200*, *Gohir.A03G098000*, *Gohir.A03G100100*, and the homologous genes of these four members are located on chromosome D02, which is similar to the situation in the tetraploid *Gossypium darwinii*, probably because of fragment replication between chromosomes A03 and D02. Chromosome tandem duplication and fragment duplication may play important roles in the large-scale expansion of cotton species. A similar situation has been reported in the study of the cotton ABC gene family [27].

Ka/Ks evolutionary selection analysis and intragenomic collinearity analysis can be used to study evolutionary selection and tandem repeat events in gene families and then to study the evolutionary process of the family. Ka/Ks and collinearity analyses were performed on the FAH family members of four different cotton species. We found that in tetraploid cotton, the collinear region of FAH is concentrated mainly between homologous chromosomes. According to the Ka/Ks results, most genes underwent purifying selection during evolution. This finding indicates that base substitution in the coding sequence of these genes does not change the composition of the protein; thus, they are not affected by natural selection, and the function of these genes is well preserved [28]. Similarly, we also found that FAH family members have many gene pairs with a linear relationship between chromosomes A03 and D02. The results of collinearity, Ka/Ks, were consistent with the results of chromosome localization, indicating that many chromosome recombination events occurred on the homologous chromosomes of tetraploid cotton and that large chromosome duplications occurred on A03 and D02. Moreover, FAH genes are conserved in chromosome doubling and evolution. Fragment duplication has been important in evolution because many plants have multiple repetitive chromosomal blocks [29]. Furthermore, Arabidopsis has undergone two genome-wide duplications (WGDs) [30], and some studies have shown that the *GhAAI* gene family [31] and *GhARF* gene family [32] of cotton have also expanded. We concluded that large fragment replication and genome polyploidization are the main methods for the formation of the cotton FAH gene family. These findings improve our understanding of chromosome interactions, information exchange, and genetic evolution in cotton.

Through the analysis of conserved motifs and gene structure members of the FAH gene family, it was found that FAH had different degrees of deletion in motifs but still had similar motif distribution patterns, especially among FAH members of the same subfamily. The number of introns contained in different subfamilies of the FAH gene was also quite different. It has been reported that introns may play a key role in the evolution of different species [33]. In the early stages of gene expansion, the introns of some genes are lost over time [34]; when

introns are subjected to weak selection pressure, genes without introns may evolve rapidly, and when a gene has more introns, it will promote species evolution and survival advantages [35]. Therefore, we speculate that some FAH genes in cotton have gradually lost introns over time and changed their functions during evolution.

In this study, the cotton FAH family members were predicted by subcellular localization, and all the members were found to be located on the plasma membrane, which means that FAH may be responsible for material transport and signal transduction between cells. Studies have shown that phytosterols, which are mainly components of the cell membrane, affect the structure and function of the cell membrane by mediating the biosynthesis of brassinolides (BRs), thereby increasing the adaptability of plants to abiotic stresses [36]. The prediction of *cis*-acting elements revealed that many regulatory elements, such as MYB, MBS, HD-zip1 and ABRE, are related to stress upstream of the cotton FAH gene promoter. MYB transcription factors have been widely reported to be associated with abiotic stress regulation in plants. It is known for its highly conserved MYB domain at the N-terminus, and its conserved sequence is CAACCA. In *A. thaliana*, after overexpression of the MYB12 gene, flavonoids significantly accumulate in transgenic plants, resulting in increased antioxidant capacity and drought tolerance in *A. thaliana* [37]. In addition, MYB transcription factors can regulate the expression levels of ABA signaling and SOS2 to increase plant salt tolerance [38]. During the germination stage of *A. thaliana* seeds, MYB7 significantly increases the germination rate under salt stress through negative regulation, and MYB42 enhances the salt tolerance of *A. thaliana* seedlings by positively regulating the expression of SOS2 [39].

Furthermore, the cotton FAH gene also has several elements related to light reactions, such as ACA motifs, G boxes, and GA motifs. In the process of plant evolution, complex mechanisms can be formed to perceive light intensity and other factors and affect a series of developmental events, such as seed germination and seedling formation [40]. The existence of these stress-related regulatory elements may be one of the important reasons for the rapid and significant differential expression of the cotton FAH gene after salt stress, but the specific regulatory mechanism is still unclear. Additionally, we further explored the function of the cotton FAH family through VIGS experiments. We silenced *Gohir.A03G045300* in XiangFZ031 cotton, and the results revealed that the growth of the silenced plants was significantly affected. Moreover, we identified the homologous gene *AT1G07420* in *A. thaliana*, which contains a fatty acid hydroxylase domain, and studies have shown that *AT1G07420* can regulate the growth and development of *A. thaliana* [41]. In summary, this FAH genome-wide analysis facilitates further exploration of the regulatory mechanisms of salt stress within this gene family and establishes a foundation for identifying high-yield, salt-tolerant germplasm resources in cotton.

Conclusions

Cotton is one of the most salt-tolerant crops, but excessive salt can cause ionic toxicity, osmotic stress and oxidative harm to cotton. In this study, a series of bioinformatics analyses were used to analyze the cotton FAH family, and the 129 FAH genes of four Gossypium species were divided into five subgroups. FAH genes located in the same subgroup presented similar gene structures and a consistent distribution of conserved motifs. Moreover, we used RT-qPCR to detect changes in the expression of FAH genes in cotton leaves after salt stress. Gene silencing experiments of *Gohir.A03G045300* revealed that this gene may be involved in the response of cotton to salt stress.

Materials and methods

Plant materials

The salt-tolerant line XiangFZ031 and the salt-sensitive upland cotton parent CRI12 were used as experimental materials. XiangFZ031, also known as Nationally Registered Cotton Variety No. 20,210,031, was developed by crossing *Gossypium darwinii*, a tetraploid wild cotton species with excellent salt tolerance, with an upland cotton variety CRI12. This process was followed by three backcrosses to CRI12 and marker-assisted selection from the BC3F8 generation. Healthy seeds of this species were selected and planted in pots at a ratio of 3:1 of nutrient soil and vermiculite. It was cultivated at 25 °C in a 16 h:8 h light: dark environment. When the second cotyledon fully expanded, the cotton plants were divided into control and experimental groups. The experimental group was exposed to 200 mM NaCl solution once. After 24 h, the first true leaves of the two groups of cotton plants were collected for subsequent RNA extraction and RT−qPCR experiments. For the VIGS experiment, the salt-tolerant strain XiangFZ031 was used as the material. Agrobacterium infection was performed when the two cotyledons of the plant were fully expanded. After the albino phenotype appeared, salt treatment was performed. Saline (200 mM) was added every two days, and the phenotype was measured after 14 days.

Identification analysis of FAH gene family members in cotton

The genomic data of the tetraploid cotton species *Gossypium hirsutum* (V2.1) and *Gossypium darwinii* (V1.0) and their common ancestor *Gossypium raimondii* (V2.1) were downloaded from the Phytozome V13 database (https://phytozome-next.jgi.doe.gov/), including CDSs,

gene annotation files and protein sequences [42–44]. The *G. arboreum* CRI genome and protein sequence were obtained from the CottonGen genome database (https://www.cottongen.org/) [45]. The Pfam number PF04116 of the FAH gene family was searched through the Pfam database (http://pfam-legacy.xfam.org/) [46], and the hidden Markov model (HMM) of the FAH gene family was downloaded. The sequences containing FAH protein domains in the protein files of the four cotton species were searched via HMMER 3.0 software and BLASTP. The E value was set to 1e-10 to screen candidate protein sequences. The candidate protein sequences were uploaded to the SMART database (http://smart.embl.de/) [47], Pfam database (http://pfam-legacy.xfam.org/) and CDD search in the NCBI database (https://www.ncbi.nlm.nih.gov/cdd/) [48] for reidentification. The amino acid sequences of all members of the FAH gene family of the four cotton species were analyzed via ExPASy proteomics server software (http://www.expasy.org) [49], and the amino acid length and isoelectric point (PI) were calculated. In addition, the subcellular localization of FAH family members was predicted via the WoLF PSORT website (https://wolfpsort.hgc.jp/) [50].

Construction and evolution analysis of the FAH gene family

To further analyze the relationships between the evolution of FAH family members, the obtained protein sequences were subjected to multiple sequence alignment via MEGA7 [51] software, and multiple sequence alignment was performed via MEGA7 with the Alignment - Align by ClustalW option, with default parameters. A phylogenetic tree was constructed via the neighbor–joining method with default parameters [52]. The online site Evolview (https://evolgenius.info//evolview-v2/#login) [53] was used to further modify and beautify the phylogenetic tree. This study also constructed a local index of the cotton FAH genome gene sequence, and compared the whole-CDS data with Blastp via the Blastall program with an E value of 1e-20, and the results of the genome comparison were obtained. The synonymous substitution rate (Ks), nonsynonymous substitution rate (Ka), and Ka/Ks of cotton FAH genes were calculated via Calculator 3.0 [54] to analyze the gene selection events of the FAH gene family during evolution and development.

Gene structure and conserved motif analysis

The MEME website (http://memesuite.org/) [55] was used to analyze the conserved motifs of FAH gene family members. The total number of parameters used to establish the search motifs was 10, the shortest motif length was 6 base pairs, and the longest motif length was 50. To analyze the structural information of FAH genes, the exons of FAH genes on chromosomes and the location information of the CDS, 3'-UTR and 5'-UTR were extracted. The online website GSDS (http://gsds.gao-lab.org/) [56] was subsequently used to analyze the structural information of the FAH gene family, and a gene structure map was drawn. Finally, TBtools software was used to merge and visualize the phylogenetic tree, gene structure and conserved motif images of the FAH genes.

Chromosomal localization and collinearity analysis

The position information of the FAH genes on the chromosomes was obtained from four cotton gene annotation files, and then the position distribution map of the FAH genes on the cotton chromosomes was drawn via MapChart [57]. By comparing the sequences of all the FAH proteins, multiple Collinearity Scan toolkit (MCSCANX) [58] software was used to determine and analyze the duplication doubling events and collinearity between the FAH gene family in the cotton genome.

Analysis of *cis*-acting elements

To explore the related functions of gene expression regulation, the promoter sequences of 1500 base pairs upstream of the start codon of FAH genes were obtained from the genomic files of four cotton species. The *cis*-acting elements of genes were identified and analyzed via the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [59], and the results were visualized via the GSDS online website (http://gsds.gao-lab.org/).

Expression analysis of the FAH gene family under salt stress

Through the online *G. hirsutum* transcriptome public database, the expression of FAH gene family members after salt treatment at different time points was obtained, and the unit was FPKM. TBtools software was used to construct the expression heatmap. Ten genes were randomly selected from the FAH gene family members of upland cotton for real-time quantitative PCR (RT–qPCR) analysis. RT–qPCR primers for the FAH gene family were designed via Primer Premier 5 software (Supplementary Table S4). For the RT–qPCR experiments, a ChamQ SYBR qPCR Master Mix (LowROX Premixed) kit was used. The reaction volume was 20 μL. The amplification procedure was as follows: predenaturation at 95 °C for 30 s; denaturation at 95 °C for 10 s; and annealing at 60 °C for 30 s for 40 cycles. *GhUBQ* was used as an internal reference gene, and the relative expression level of the gene was quantified via the $2^{-\Delta\Delta Ct}$ method [60]. Three biological replicates and four technical replicates were used in the experiment. Finally, the GraphPad Prism software was used to visualize the RT–qPCR results.

Virus-induced gene silencing (VIGS) experiment

The upland cotton line XiangFZ031 was selected as the material for VIGS. Healthy seeds were soaked in carbendazim, sterilized, and then planted in pots mixed with nutrient soil and vermiculite (3:1). The climate room temperature was maintained at 25 °C, and the ratio of light to darkness was 16 h/8 h. When the cotyledons of the cotton plants had fully expanded and before the first true leaf tips had just appeared, the VIGS experiment was carried out. The primers designed by Primer Premier 5 were used for the VIGS reaction and ligated into the pTRV2 vector to obtain the recombinant expression vector. *Gohir.A03G045300* was amplified with an upstream primer (5'-AGTAGTCTGCCATTTCCATCCT-3') and a downstream primer (5'-CAATGTGCCTCAACTGTCTC TA-3'). The primer sequence was inserted into the pTRV vector and treated with the *Bam*HI-I and *Sac*I enzymes to construct pTRV2: *Gohir.A03G045300*. The resulting plasmid was subsequently transformed into *Agrobacterium tumefaciens* (GV3101). After positive clones were selected, the bacterial mixture was injected into the cotyledons of the cotton plants via a sterile syringe. After 24 h of dark treatment, the plants were transferred to the artificial climate chamber, and the positive control presented an albino phenotype after approximately two weeks. After that, the plants were treated with 200 mMof saline for 14 days, after which the plant height and shoot fresh weight were measured.

Abbreviations

FAH	Fatty acid hydroxylases
RT-qPCR	Quantitative real-time polymerase chain reaction
GSDS	Gene structure display server
HMM	Hidden markov model
MCScanX	Multiple collinearity scan toolkit
VIGS	Virus-induced gene silencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11450-y.

Supplementary Material 1: Fig. S1: Subcellular localization prediction distribution map of the FAH family in *Gossypium hirsutum* (A), *Gossypium darwinii* (B), *Gossypium arboreum* (C), and *Gossypium raimondii* (D). Note that the color and size of the circles indicate the reliability of the prediction results

Supplementary Material 2: Fig. S2: Phylogenetic relationships of FAH gene family members in *Gossypium hirsutum*, *Gossypium darwinii*, *Gossypium arboreum* and *Gossypium raimondii*. Each color represents a subgroup. Note: The phylogenetic tree was constructed via MEGA 7 neighbor-joining (NJ) with 1000 bootstrap replicates. The symbols in the figure represent different species of cotton. The star represents *Gossypium hirsutum*, the circle represents *Gossypium darwinii*, the triangle represents *Gossypium arboreum*, and the checkmark represents *Gossypium raimondii*. The numbers represent bootstrap values, ranging from 0 to 1.

Supplementary Material 3: Fig. S3: Phylogenetic tree, gene structure and conserved motif analysis of the FAH gene family in *Gossypium hirsutum* (A), *Gossypium darwinii* (B), *Gossypium arboreum* (C), and *Gossypium raimondii* (D). Note: The phylogenetic tree was constructed via MEGA 7 neighbor-joining (NJ) with 1000 bootstrap replicates. The conserved motifs in FAH proteins were identified via MEME software. The gray lines indicate nonconserved sequences, and each motif is indicated by a colored box. The lengths of the motifs in each protein are presented to scale. The exon–intron structure of the FAH gene is based on evolutionary relationships. Yellow rectangles indicate exons, and gray lines indicate introns

Supplementary Material 4: Fig. S4: Motif Sequence Logos in *Gossypium hirsutum*. The size of each letter at each position reflects the degree of conservation in the analyzed sequences: larger letters indicate greater conservation and smaller letters indicate greater variability at that position

Supplementary Material 5: Fig. S5: Motif Sequence Logos in *Gossypium darwinii*

Supplementary Material 6: Fig. S6: Motif Sequence Logos in *Gossypium arboretum*

Supplementary Material 7: Fig. S7: Motif Sequence Logos in *Gossypium raimondii*

Supplementary Material 8: Table S1: Physical and chemical properties of FAH gene family members

Supplementary Material 9: Table S2: Evolutionary selection analysis of FAH gene family members

Supplementary Material 10: Table S3: FAH gene accession numbers from NCBI in *Gossypium hirsutum*

Supplementary Material 11: Table S4: Primers used for RT–qPCR analysis in *Gossypium hirsutum*

Acknowledgements

The authors acknowledge School of Life Sciences, Nantong university for providing the laboratory and input facility for this experiment.

Author contributions

HG, WF and TGM performed most of the experiments and data analysis. YW, ZW, YX, YZ, JT, KZ, ZZ and WW helped in sample preparation and data analysis. RZ, JW and BW designed the experiments and edited the manuscript. All authors read and approved the final manuscript.

Funding

We appreciate financial help from Key Research and Development Project of Jiangsu Province, China (Modern Agriculture, BE2022364); Natural Science Foundation Project of Xinjiang Production and Construction Corps (2024DA027); State Key Laboratory of Cotton Bio-breeding and Integrated Utilization Open Fund (CB2024A06); The Practice Innovation Training Program Projects for College Students (202410304022Z, 2024131); High School Talent Student Training Program of Jiangsu Province (Su Qing Ke Fa [2024] No. 20).

Data availability

All the data generated in the current study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

All the seeds used for planting materials during the experiment were provided by our school and all the procedures were performed in accordance with international guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 2 December 2024 / Accepted: 5 March 2025
Published online: 18 March 2025

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