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Genome-wide analysis of the *FKBP* gene family and the potential role of *GhFKBP 13* in chloroplast biogenesis in upland cotton

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Abstract

Background In plants, FK506-binding proteins (FKBPs) have been shown to participate in various biological processes such as photosynthetic system reaction, stress response, and growth and development. However, the roles of *FKBPs* in cotton are less well known.

Results In this study, we investigated *FKBP* family genes on a genome-wide scale in four *Gossypium* species. A total of 147 *FKBP* genes were identified from these four *Gossypium* species and placed into three classes based on phylogenetic analysis. Collinearity analysis indicated that whole-genome duplication events and segmental duplication events were the main sources of gene amplification during the evolution of *FKBP* genes. Conserved motif, expression profiles and cis-acting elements prediction of the *GhFKBPs* analysis revealed that *GhFKBP*s were differentially expressed in different tissues and under abiotic stress. qRT-PCR analysis showed that some *GhFKBPs* were predominantly expressed in leaves. The analysis of cis-acting elements prediction revealed that MYB, MYC and ERE related binding sites in the promoters of *GhFKBP* genes were the most abundant. Furthermore, the composition and distribution of these cis-acting elements exhibited differences between homologous *GhFKBP* gene pairs. Silencing of *GhFKBP13* in cotton resulted in disruption of chloroplast structure and starch metabolism disorders.

Conclusions Taken together, 147 *FKBP* family genes in four *Gossypium* species are comprehensively characterized, and *GhFKBP13* play a critical role in chloroplast biogenesis in upland cotton.

Keywords *Gossypium hirsutum*, *GhFKBP*s, Chloroplast biogenesis, Starch and sucrose metabolism

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Background

FK506-binding proteins (FKBPs) are part of the peptidyl-proline isomerase (PPIase, EC5.2.1.8) superfamily, catalyzing protein folding and maturation by converting cis-trans configurations of amino acid residues [1]. FKBPs are highly homologous receptor-binding proteins that are widely distributed in all organisms, including bacteria, fungi, animals and plants [2–5]. FKBPs can be divided into single-domain and multidomain FKBPs based on their sequence structure. Single-domain FKBPs have a single FK506-binding domain (FKBD) which contains approximately 110 amino acids that provide the active site and the receptor site, whereas multidomain FKBPs have an additional FKBD containing a tetrapeptide repeat (TPR) or other functional domains, including a C-terminal calmodulin or helix-binding domain [6, 7].

With the continuous improvement of plant genome sequencing and the advancement of bioinformatics, identification of gene families in plant genome databases has become viable, and gene family analysis facilitates the characterization of gene members, their chromosomal positions, and probable functions. *FKBP* family genes have been reported in different species [8–16]. These proteins are localized mostly in the chloroplast, cytoplasm, nucleus, mitochondria, peroxisome and endoplasmic reticulum, implying that FKBPs have a variety of biological functions. *AtFKBP20-2* is located in the chloroplast thylakoid lumen and is required for the accumulation of the PSII supercomplex in *Arabidopsis thaliana* (*A. thaliana*); genetic disruption of *AtFKBP20-2* resulted in slower plant development [17]. The *AtFKBP15-1*/15 – 2, which are localized to the endoplasmic reticulum, are expressed prominently in the vascular bundles of the root basal meristem region, and an *AtFKBP15-1*/*15 – 2* double mutant had more lateral roots than did Col-0 or single mutants [18]. Protein interaction studies indicated that *AtFKBP15-1*/*15 – 2* participate in the control of lateral root number by inhibiting the catalytic activity of vacuolar invertase 2 (*VIN2*) [18]. In addition, *FKBP*s were also reported to play an impotant role in plant development [19–21], hormone signaling [22], ribosomal RNA gene expression [23], heat stress responsiveness [24–26] and drought and salt stress [27–28] etc. Consequently, there remains intense interest in understanding their function in plant-related research.

As an oil crop and an important source of natural textile fibers, cotton plays an important role in agriculture and industry around the world. However, its production is mainly limited by various environmental stress conditions. Analysis of gene families could contribute to understanding the diversity of genes and environmental adaptability in plants. Over the past decade, an increasing number of cotton genomes have been assembled and many cotton gene families have been extensively characterized recently [29–37]. *FKBPs* have been shown to play important functions in plant growth and development. Whereas, the *FKBP* genes family in cotton has not been fully characterized. In this study, we identified *FKBP* family genes in four *Gossypium* species and investigated their phylogeny, chromosomal location, synteny relationships and gene structure. The *GhFKBPs* expression profiles were analysis by bioinformatics and qRT-PCR methods. According to the *GhFKBP* genes expression results and previous studies, we performed a preliminary exploration of the function of *GhFKBP13* in chloroplast development and its possible regulatory mechanism. The results provide a basis for understanding the biological role of *FKBPs* in chloroplast development in upland cotton and provide a better opportunity for the utilization of the *FKBP* genes in enhancing photosynthetic light utilization in elite cotton cultivars.

Materials and methods

Identification of *FKBP* gene members

The cotton genome sequence data of *Gossypium arboreum* (*G. arboreum*, ICR), *Gossypium hirsutum* (*G. hirsutum*, WHU) were downloaded from Gossypium Resource and Network Database(GRAND, https://grand.cricaas.com.cn/). *Gossypium raimondii* (*G. raimondii*, HAU), *Gossypium barbadense* (*G. barbadense*, ZJU) were downloaded from Cotton FGD (Cotton Functional Genomics Database) (http://www.cottonfgd.org/). FKBP protein data of *A. thaliana* were downloaded from TAIR (http://www.arabidopsis.org/). The Hidden Markov Model (HMM) of FKBP (Pfam ID: PF00254) was downloaded from the Pfam database (http://pfam.xfam.org/). Then, Gene IDs containing conserved FKBP domains with an e-value threshold of 0.01 were extracted from the four *Gossypium* species using HMMER 3.3.2 software (http://hmmer.org/). Finally, the Batch SMART program of TBtools 1.09 (https://github.com/CJ-Chen/TBtools) was used to confirm whether the candidate protein sequences contained the FKBP core domain.

Phylogenetic and collinearity analyses of four cotton species

To investigate how the *FKBP* genes differ among the four cotton species, the protein sequences of *G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. raimondii* and *A. thaliana* were compared via the ClustalW function of MEGA-X software (https://www.megasoftware.net/docs). A phylo genetic evolutionary tree was subsequently constructed via maximum likelihood estimation (MLE) with 1000 bootstrap replicates. To study the collinearity between different species in cotton, Tbtools software(One step MCScanX-super Fast) was used to conduct collinearity analysis between different cotton FKBPs, and Advanced circos plot in Tbtools was used to visualize the results.

Conserved protein domains, chromosomal location

For *FKBP* family genes of *G. hirsutum*, the MEME website (http://meme-suite.org/) was used to identify conserved protein sequences. Ten motif parameters were selected, and the other parameters were unchanged. The generated MAST file was subsequently used to construct protein domain maps via TBtools. The gff files and gene IDs of *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. raimondii* were also generated via TBtools software to construct a map of the chromosomal locations of the family members.

Cis-acting elements analyses of *GhFKBP*s

The 2000 bp upstream sequences of the GhFKBP genes' start codon (ATG) were analyzed to identify the cis-acting elements in the putative promoter regions, and the cis-acting elements were identiffed with the online program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression analyses of *GhFKBP*s

The expression of *GhFKBP* family members in various tissues/organs and sodium chloride and PEG-induced drought stress was determined through previously published RNA sequencing data from Cotton Omics Database (http://cotton.zju.edu.cn/10.rnasearch.html) and is summarized as heatmaps. The regulation transcription factors of *GhFKBP*s were predicted using PlantRegMap(http://plantregmap.gao-lab.org/go.php/) with *G. raimondii* as the target species.

Plant materials and qRT-PCR analysis of gene expression

The *G. hirsutum* cultivar "Jimian176" was selected for *GhFKBP* family gene expression analysis. Jimian176 seeds were grown in a greenhouse at 25 °C under a 16-h light/8-h dark cycle. The tissues of the roots, stems and leaves of four-leaf-stage cotton plants were immediately frozen in liquid nitrogen and stored at -70 °C. Total RNA was extracted from the samples by using the EASYspin Plus Plant RNA Kit (Aidlab, Beijing, China) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, China) according to the manufacturer's protocol. The primer pairs used were designed with Primer 5 (Table S1). qRT-PCR was performed by a C1000 Touch™ PCR (Bio-Rad, USA), and the reaction conditions were set as follows: 95 °C for 10 min, followed by 95 °C for 15 s and 58 °C for 1 min for thirty-five cycles, after which dissociation curve analysis was performed. Transcription quantitative analysis of gene expression used Histone 3 as reference gene for normalization(Table S1). All reactions for each sample were performed in triplicate with biological replicates. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Virus-induced gene silencing assays

Sequence alignment analysis showed that *GhFKBP13* has a high degree of homology in the A and D subgenomes, with a sequence similarity of 97.7%. However, *GhFKBP13* has limited homology with other *GhFKBP* genes, with sequence similarities between 42.5% and 51.9% with its closely related *GhFKBP* genes. To avoid the potential silencing of other homologues, a specific 300 bp fragment amplified from the *GhFKBP13* gene ORF was integrated into the TRV vector from General Biol Co., Ltd. (An Hui, China) to construct TRV-*GhFKBP13*. TRV-*GhFKBP13*, TRV-*GhCLA1* (indicator vector) and the TRV negative control vector were subsequently transformed into *Agrobacterium tumefaciens* GV3101. These constructed strains were mixed with the strain harboring pYL192 (a helper vector) (1:1 ratio, OD600 = 0.8) and coinjected into two fully expanded cotyledons of cotton seedlings. When injected TRV-*GhCLA* plants showed albino symptoms about two weeks later, the expression level of *GhFKBP13* in the TRV-*GhFKBP13* plants and TRV negative control plants was determined via qRT-PCR, and three biological replicates were used.

Transmission electron microscopy (TEM) observation

For TEM analysis, the leaves of VIGS-silenced *GhFKBP13* plants and control cotton plants were cut into 1 mm×1 mm pieces on each side of the main vein of the leaf. Leaf sections were washed with 4% glutaraldehyde in phosphate buffer for 5 min and then fixed with 4% glutaraldehyde in phosphate buffer at 4 °C for 4 h. Then, the leaf sections were rinsed with 0.1 M PBS 3 times and postfixed with 1% osmium acid prepared in PBS buffer at 4 °C for 1 h. The leaf sections were rinsed with 0.1 M PBS 3 times and dehydrated with an acetone series (30-50%-70-80%-90-95%-100%-100%) for 20 min each and then embedded in Spurr's medium before thin sectioning. The leaf sections were then further stained with uranyl acetate and examined with an H-7650 electron microscope (HITACHI Ltd., Tokyo, Japan).

Chlorophyll content assay

The total chlorophyll content in plants was determined using a spectrophotometric method. After silencing the target gene, we took 0.1 g cotton leaf and cut into slices, immersed it in 15 mL of 95% ethanol for 48 h at 4 °C in the dark. The concentrations of chlorophyll a(Ca) and chlorophyll b(Cb) and carotenoids(Cx + c) were measured at 665, 649 and 470 nm using a UV-1800PC spectrophotometer (Mapada Co., Ltd., China) and calculated according to the formula

Ca = 13.95A665-6.88A649, Cb = 24.96A649-7.32A665, and Cx + c=(1000A470-3.27Ca-104Cb)/229.

RNA sequencing(RNA-seq) analysis

Total RNA was extracted from VIGS-silenced *GhFKBP13* leaves and control plant leaves and subsequently sent to Personalbio Technology Co., Ltd. (Shanghai, China). Paired-end sequencing was performed on an Illumina NovaSeq 6000 sequencer. Differential expression was calculated using DESeq2 software. Genes with an absolute log2-fold change (VIGS silenced/TRV control) > 1 and a p value < 0.05 were considered to be significantly differentially expressed genes (DEGs). Gene Ontology (GO) enrichment analysis and KEGG enrichment analysis were performed on the differentially expressed genes (DEGs) using ClusterProfiler (adjusted p value < 0.05).

Results

Identification of the *FKBP* gene family and characteristics of the *FKBP* genes in *G. Hirsutum*

A total of 48, 47, 28 and 24 *FKBP* genes were ultimately identified from *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. raimondii*, respectively (Table S2). All these genes were named according to their orthology with reported isoforms in *A. thaliana*(He et al., 2004) and labeled A/D based on their location on the A_t subgenome or the D_t subgenome(Table S3). For more than two proteins with a sequence orthologous to the same *A. thaliana* protein, lower-case extension letters were added to the name according to the order on the chromosome.

The primary characteristics of the *G. hirsutum* FKBPs were analyzed, including the number of FKBP_C domains, protein length (aa), molecular weight (MW), isoelectric point (pI) and the subcellular location of the predicted proteins. The number of FKBP_C domains in the *G. hirsutum* FKBP family ranged from 1 to 3, the protein length ranged from 112 aa to 629 aa, the MWs ranged from 12.06 kDa to 70.95 kDa, and the pI values ranged from 4.84 to 9.57. The subcellular localization of the proteins was predicted by WoLF and TargetP online, and the results showed that GhFKBP proteins were mainly located in nucleus(24.4%), cytoplasm(28.5%) and chloroplast(30.6%)(Table S4).

Phylogenetic analysis of the *FKBP* genes in four *Gossypium* species and *A. Thaliana*

To better elucidate the evolutionary relationships of cotton FKBPs, all FKBP amino acid sequences from the four *Gossypium* species and *A. thaliana* were used to construct a phylogenetic tree. As expected, the cotton FKBPs and *Arabidopsis* homologs were classified into three groups (Fig. 1). Among these branches, group I had the fewest members (23), including FKBP16-3, FKBP13, FKBP16-2 and HEN. In addition to *G. arboreum* having only one HEN gene, there were clearly no other *Gossypium* species with an identified HEN gene. In group II, there were 51 members, including FKBP16-1, FKBP19, FKBP20-2, FKBP17-1, FKBP17-2, FKBP16-4, FKBP18, and AtTIG. Group III had 97 members, more than half of the total number of FKBP family members. This group included FKBP15-1, FKBP15-2, FKBP43, FKBP53, FKBP12, FKBP20-1, FKBP42, FKBP62, FKBP72 and FKBP65. Among these genes, *Arabidopsis* FKBP65 and FKBP62 were less closely related to other homologous FKBP proteins in *Gossypium* species. Overall, the FKBP proteins are very conserved in *Gossypium species* and *A. thaliana*.

Chromosomal distribution and collinearity analysis of *FKBP* genes in four *Gossypium* species

To understand the chromosomal distribution of the *FKBP* genes, we created a physical map of the chromosomal distribution of the *FKBP* genes in the four *Gossypium* species (Fig. 2). The *FKBP* gene family members were found to be distributed on specific chromosomes, except for *GaFKBP13-1*, which was mapped to the scaffold (contig00019673), and the number of *FKBP* genes on each chromosome ranged from 1 to 4. In *G. arboreum*, the *FKBP* genes were located on all chromosomes except chr 12, whereas in *G. raimondii*, they were located on all chromosomes except chr02 and chr13. In the tetraploid cotton species *G. hirsutum* and *G. barbadense*, the gene distributions were not uniform. *FKBPs* genes were not present on chrA04, chrA10, chrA13, chrD03 and chrD13 in *G. hirsutum*; on the other hand, *FKBPs* were not present on chrA03, chrA13, chrD03 or chrD13 in *G. barbadense*.

Collinearity analysis can reveal the origin and evolutionary history of gene families, including the duplication, transposition, and rearrangement processes of gene family members, as well as functional changes and adaptive evolution during the evolutionary history. Therefore, we performed a collinearity analysis of *FKBPs* gene in the four cotton species. The results showed that a total 253 gene pairs were identified by comparing the genomes and subgenomes of Ga-Ga(2 gene pairs), Gr-Gr(7 gene pairs), Ga-Gr(30 gene pairs), GhA_t-GhA_t(6 gene pairs), GhD_t-GhD_t(8 gene pairs), GhA_t-GhD_t(33 gene pairs), GbA_t-GbA_t(5 gene pairs), GbD_t-GbD_t(5 gene pairs), GbA_t-GbD_t(21 gene pairs), GhA_t-Ga(31 gene pairs), GbA_t-Ga(35 gene pairs), GhD_t-Gr(35 gene pairs) and GbD_t-Gr(29 gene pairs), and all the gene pairs belonged to whole-genome duplication (WGD) or segmentation (Fig. 3A and K, Table S5). It indicated that WGD/segmental duplication were the main sources of gene amplification during the evolution of the *FKBP* genes in four *Gossypium* species. The study revealed notable variations in the number of paralogous genes among different

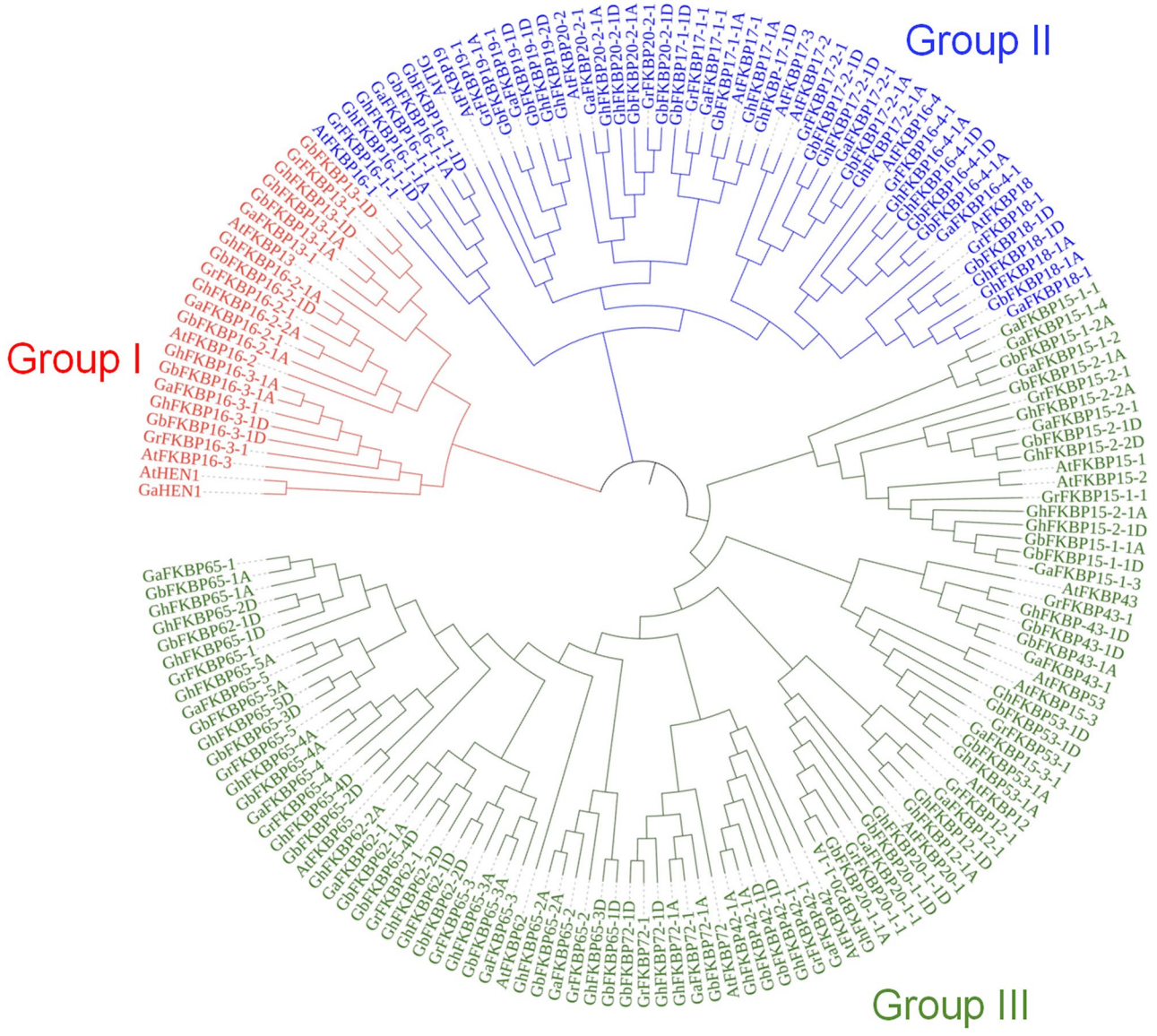

Fig. 1 Phylogenetic and evolutionary analysis of the *FKBP* gene family from *G.arboreum*, *G.raimondii*, *G.hirsutum*, *G.barbadense* and *A. thaliana*. Group I, Group II and Group III are distinguished by red, blue and green label respectively

genomes and subgenomes. For instance, *G. hirsutum* had a higher number of paralogous genes compared to *G. barbadense*, while *G. arboreum* had significantly fewer paralogous genes than *G. raimondii*. It indicated that *FKBP*s in four *Gossyium* species experienced different evolutionary pressure.

Conserved motif analysis, expression profiles and cis-acting elements prediction of the *GhFKBPs* in *G. Hirsutum*

To further explore the structure of GhFKBP family members, conserved motifs in the GhFKBPs were analyzed. Ten putative motifs were identified and conserved motif analysis revealed that motifs 1 and 7 were present in all the GhFKBP proteins in *G. hirsutum* except for GhFKBP65-1D, which lacks motif 7. In addition, despite the lack of some motifs in individual proteins, most members exhibited highly conserved motif structures and arrangements within the same group, whereas gene motifs varied greatly within different groups (Fig. 4A and B).

It is generally believed that gene expression characteristics are closely related to its function. The expression profiles of *GhFKBP*s was examined and the results showed that the *GhFKBPs* were differentially expressed in different tissues (Fig. 4C). We noted that most genes in group I were predominantly expressed in leaves. In group II and group III, most genes were predominantly expressed in root and stem tissues. On the whole, the expression pattern of *GhFKBP*s in group I showed opposite expression patterns with group II and group III. Analysis of induced

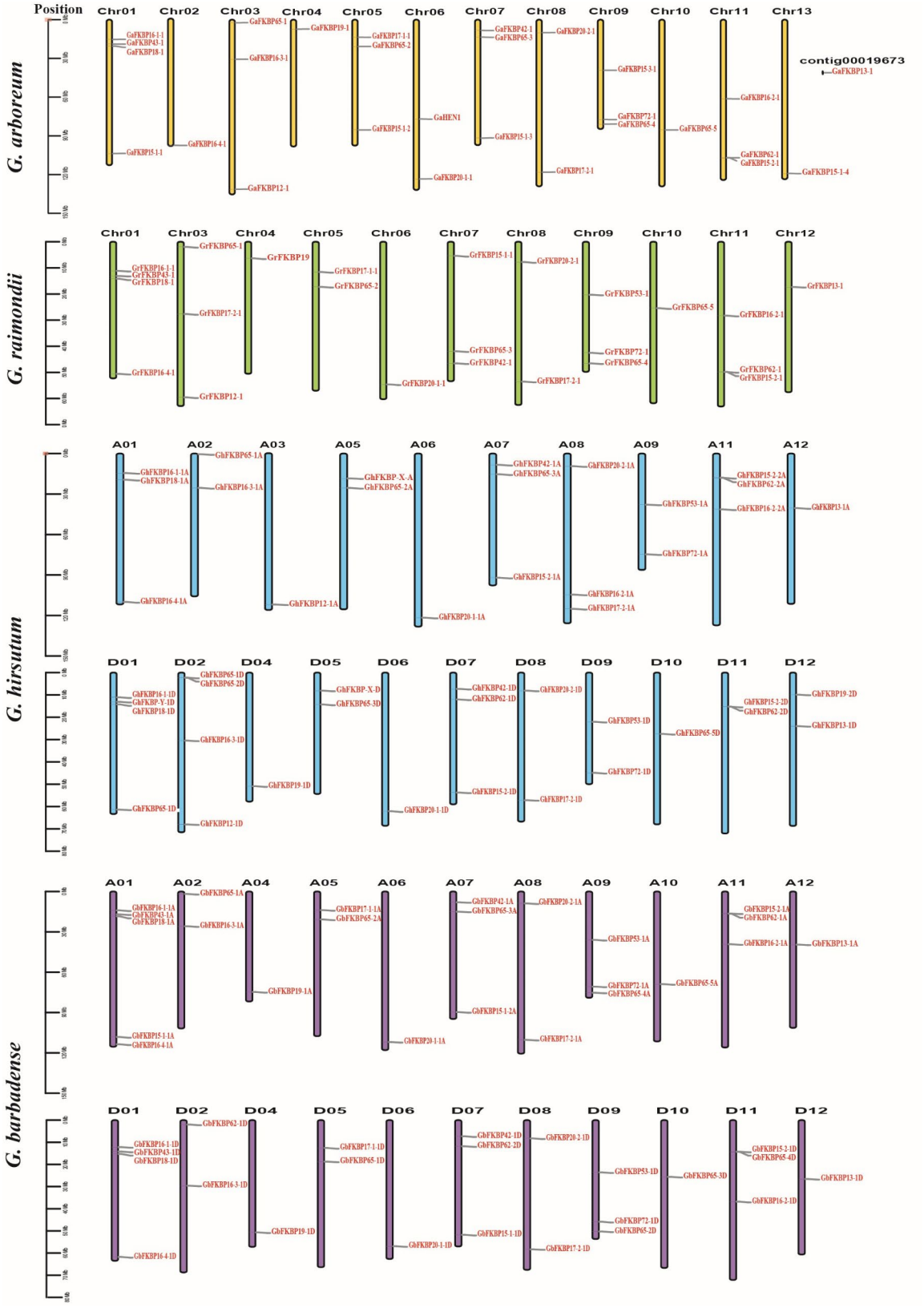

Fig. 2 Chromosomal locations of *FKBP* gene family of *G.arboreum*, *G.raimondii*, *G.hirsutum* and *G.barbadense*. The scale on the left is in mega-bases. The gene name on the left side of each chromosome corresponds to the approximate locations of each *FKBP* gene

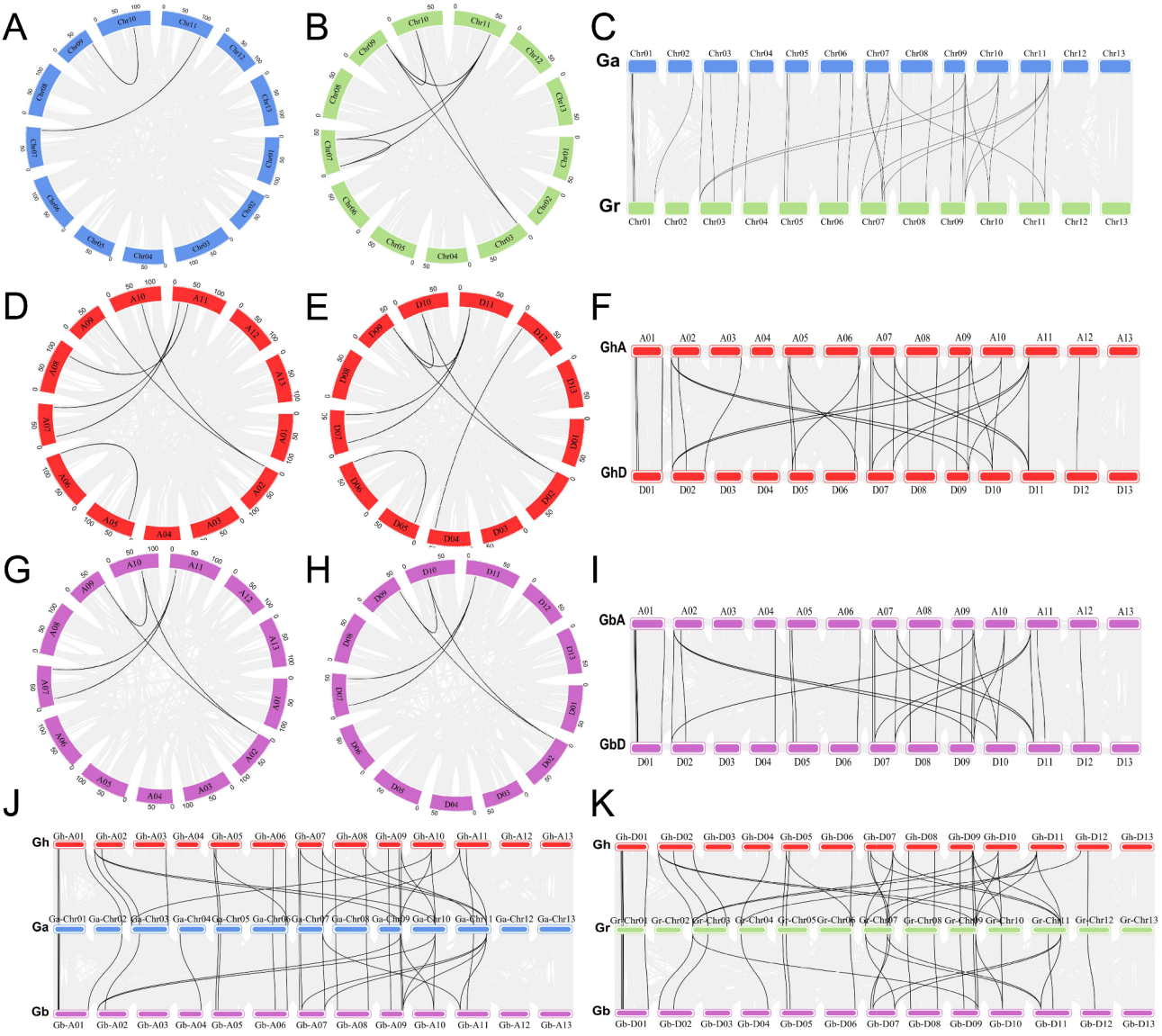

Fig. 3 The collinearity of *FKBP* genes within and among the four *Gossypium* species genomes or subgenomes. (**A**) Ga-Ga, (**B**) Gr-Gr, (**C**) Ga-Gr, (**D**) GhAt-GhAt, (**E**) GhDt-GhDt, (**F**) GhAt-GhDt, (**G**) GbAt-GbAt, (**H**) GbDt-GbDt, (**I**) GbAt-GbDt, (**J**) GhAt-Ga-GbAt, (**K**) GhDt-Gr-GbDt. (GhAt: A subgenome of *G. hirsutum*; GhDt: D subgenome of *G. hirsutum*; GbAt: A subgenome of *G. arbadense*; GbDt: D subgenome of *G. arbadense*; Gr: *G. raimondii*; Ga: *G. arboretum*)

expression of *GhFKBP*s by sodium chloride and PEG-induced drought stress showed that there was no obvious patten in group I, but there were more down-regulated genes than up-regulated genes. In group II and group III, most genes were predominantly up-regulated (Fig. 4D). In order to further verify the accuracy of gene expression, we selected 6 genes from group I for qRT-PCR analysis, and the results showed that the expression trend was consistent with the RNA-seq data (Fig. 5).

The cis-acting elements analysis provide a powerful clue for defining stress receptive or tissue-specific expression behavior in different environments. In this paper, the cis-acting elements of 2000 bp upstream of the start codon in the promoter regions of *GhFKBP* genes were predicted. It can be seen from the results that the most abundant cis-acting elements were MYB, MYC and ERE-related binding sites in *GhFKBP* promoters (Fig. 6A). In addition, we found that the composition and distribution of cis-acting elements are significantly difference between *GhFKBP* homologous members. Such as there are 8 G-box and 7 ABRE binding sites in *GhFKBP20-2–1 A* promoter while only 1 G-box binding site and none ABRE binding site in *GhFKBP20-2-1D* promoter (Fig. 6A and B). Therefore, we speculate that these *GhFKBP* homologous genes may have different expression patterns in response to environmental changes.

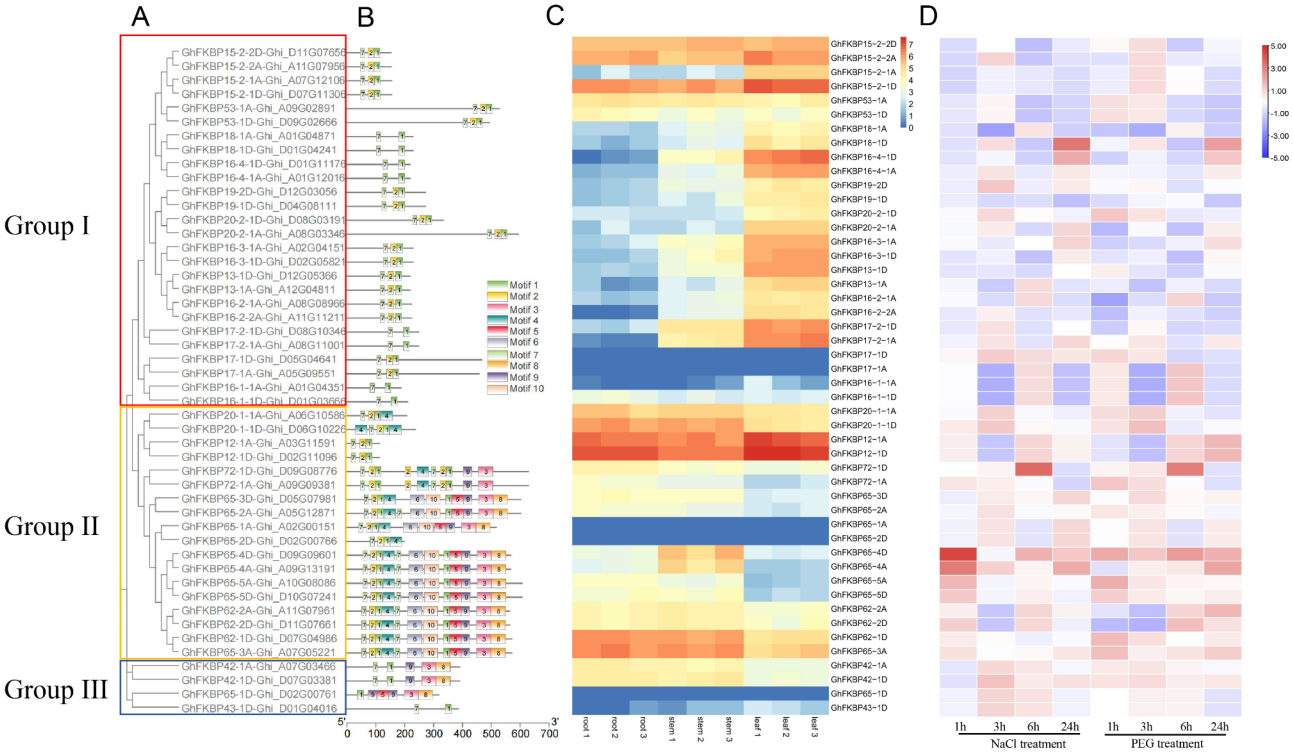

Fig. 4 Phylogenetic, conserved motifs and expression patterns of *GhFKBP* genes in *G.hirsutum*. (**A**) The phylogenetic analysis of GhFKBP members were based on the protein sequences and color boxes represent different groups. (**B**) Ten conserved motifs were analysis using MEME and TBtools software, while scale label represent the length of proteins. (**C**) The heat map was constructed based on transcriptome data (FPKM values) of root, stem and leaf tissue of G.hirsutum. Different colors represented the different expression levels of *GhFKBPs*. The scale bar on the right was marked according to the log2(FPKM value), and gene names were also shown on the right.Group I, Group II and Group III are distinguished by blue, yellow and red label respectively. (**D**) The heat map was constructed based on transcriptome data of sodium chloride and PEG-induced drought stress. Different colors represented the different expression levels of *GhFKBPs*. The scale bar on the right was marked according to the log2(foldchange)

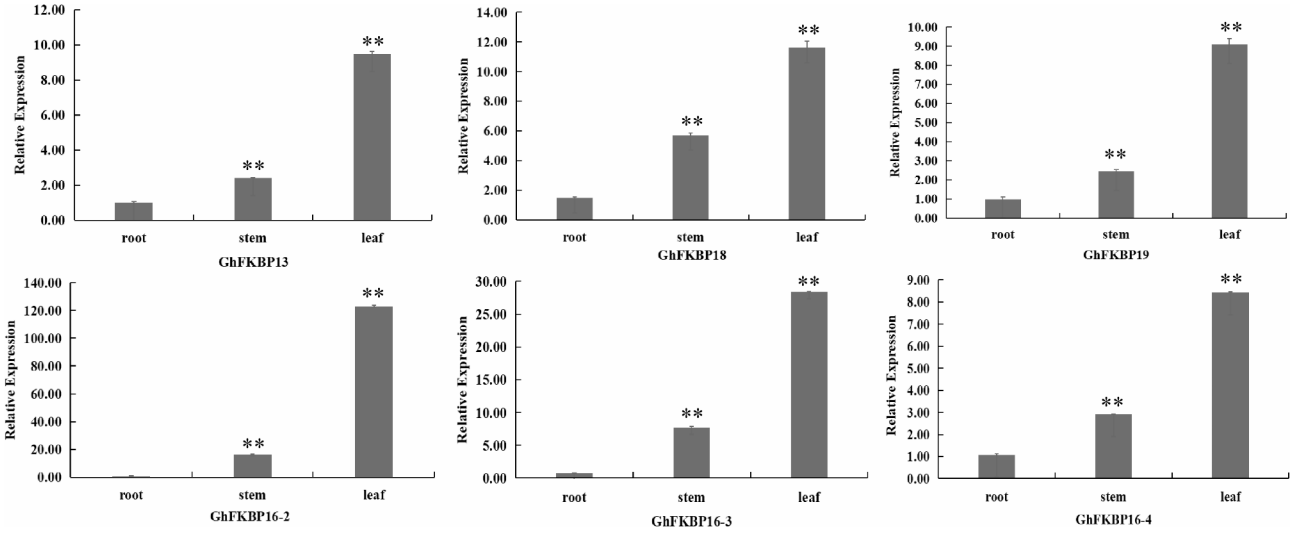

Fig. 5 qRT-PCR analysis of six selected genes in diferent tissues. Mean and SD values represent three independent replicates (Student's t test, $*P < 0.05$; $**P < 0.01$). (A)-(F) Relative expression levels of *GhFKBP13*, *GhFKBP18*, *GhFKBP19*, *GhFKBP16-2*, *GhFKBP16-3* and *GhFKBP16-4* in roots, stems, and leaves respectively.

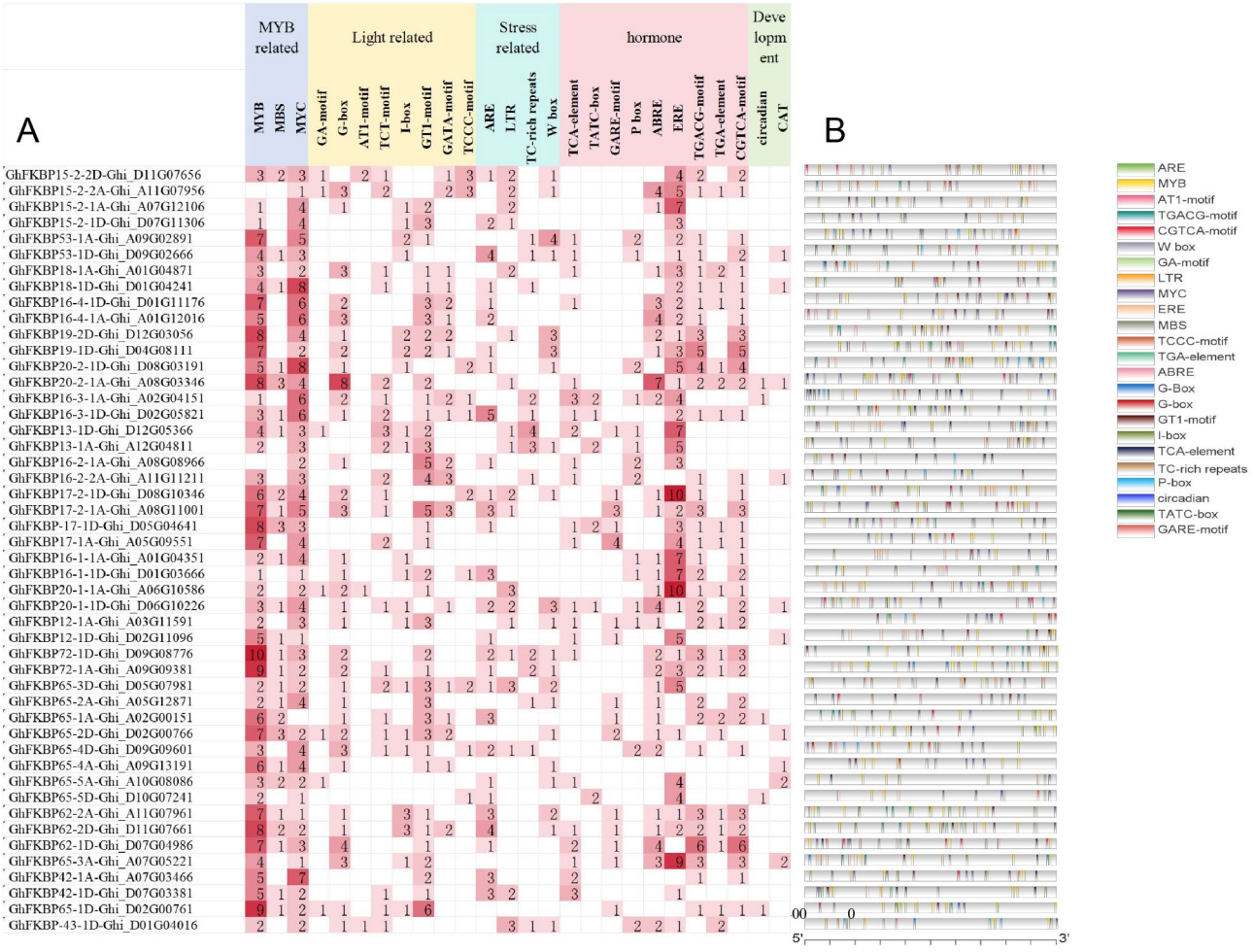

Fig. 6 Predicted cis-elements in the promoter regions of *GhFKBP* genes. (**A**) types and numbers of cis-acting elements in promoter regions 2000 bp from the transcription start site of GhFKBPs. (**B**) Postion of cis-acting elements in promoter regions. Motif names were shown nearby with different colors

Silencing of *GhFKBP13* disrupted the structure of chloroplasts and blocked starch metabolism

We noted that most of *GhFKBP* genes in group I were predominantly expressed in leaves, and some homologous genes of this group were reported to relate to the chloroplast development [17, 38–39]. Therefore, *GhFKBP13* were randomly selected in this group to further explore its function in chloroplast development. To obtain clues on the function of this gene, we performed VIGS analysis. TRV:*GhCLA* served as the positive control and TRV (empty vector) acted as the negative control. When the true leaves of the positive control plants were albino, the expression of *GhFKBP13* was determined via qRT-PCR in both the negative control plants and the *GhFKBP13* VIGS plants. The results showed that the leaves of the *GhFKBP13* VIGS plants were yellow–green, while those of the WT and negative control plants were green (Fig. 7A), and the expression level of *GhFKBP13* in the VIGS plants was significantly lower than that in the negative control plants (Fig. 7B). Additionally, to investigate whether the loss of leaf color is related to chloroplast changes, we used transmission electron microscopy (TEM) to observe the ultrastructure of chloroplasts in the first leaves of the VIGS and negative control plants (Fig. 7C). Compared with those in the negative control plants, the starch granules in the *GhFKBP13* VIGS plants exhibited expansion, and the chloroplasts were damaged. Moreover, in the *GhFKBP13* VIGS treatment, the chlorophyll a, chlorophyll b and carotenoid contents were significantly reduced (Fig. 7D). These results indicated that silencing *GhFKBP13* probablly interfered with starch metabolism, and led to destruction of the chloroplast structure.

Transcriptome analysis of the mechanism by which *GhFKBP13* regulates starch metabolism

To examine the impact of *GhFKBP13* inhibition on the global transcriptional profile, three biological replicates of leaves from which *GhFKBP13* was silenced and leaves from negative control plants were subjected to RNA-seq

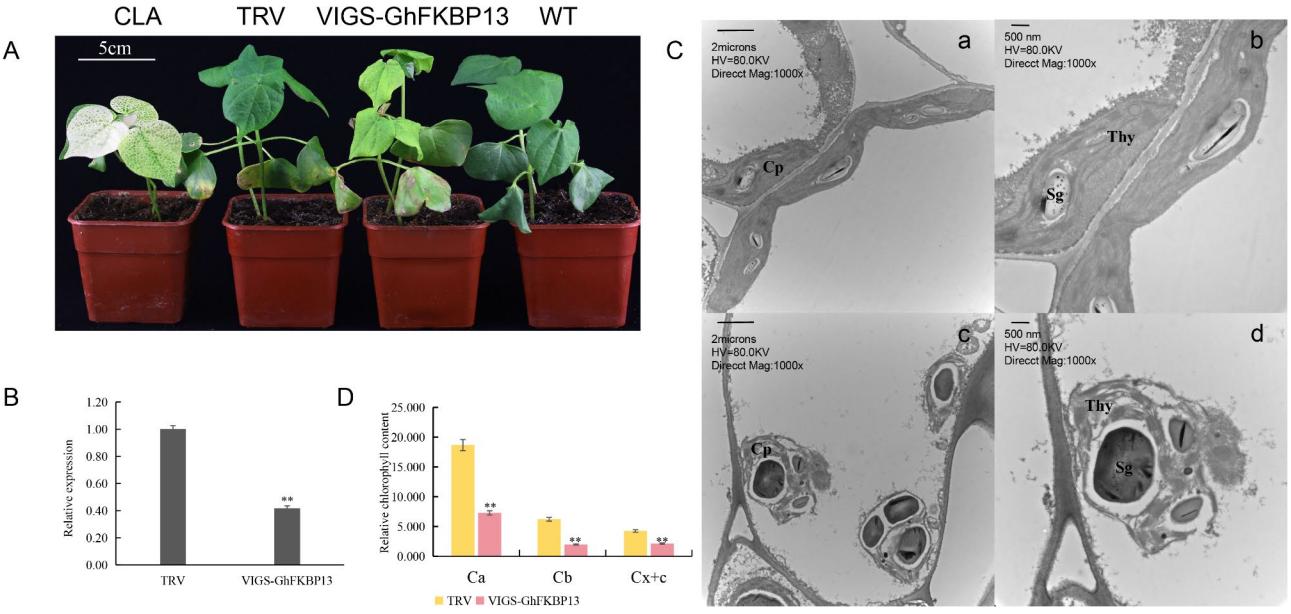

Fig. 7 Phenotypic, physiological and chloroplast structure changes in VIGS-*GhFKBP13* leaf of cotton. (**A**) Phenotype of positive control (TRV: GhCLA), empty control (TRV), VIGS-*GhFKBP13* plants (TRV: *GhFKBP13*), and WT (wild type). (**B**) The expression level of *GhFKBP13* in control and VIGS plants. (**C**) Transmission electron microscopic observation of the WT and VIGS plants. (**a**), (**b**) Ultrastructure of the chloroplast in wild-type leaves at different magnifications. (**c**), (**d**) Ultrastructure of the chloroplast in VIGS-*GhFKBP13* leaves at different magnifications. bar = 2 microns in (**a**) and (**c**), bar = 500 nm in (**b**) and (**d**). Cp chloroplast, Thy thylakoid lamellar, Sg starch granule. (**D**) Analysis of chlorophyll contents. Ca chlorophyll a, Cb chlorophyll b, Cx + c carotenoid. Mean and SD values in (**B**) and (**D**) represent three independent replicates (Student's t test, $*P < 0.05$; $**P < 0.01$)

analysis. A total of 1892 differential expression genes (DEGs) were obtained by comparing the *GhFKBP13*-silenced plants and the negative control plants; 871 DEGs were upregulated and 1021 DEGs were downregulated (Table S6, Table S7). The GO enrichment analysis showed that DEGs were most enriched in "response to stimulus", "response to stress", "extracellular region" and "response to chemical" (Fig. 8A Table S8). Moreover, DEGs related to chloroplast development and photosynthesis, as well as the starch and sucrose metabolism pathways, were also enriched, including "photosynthesis, light harvesting", "chloroplast thylakoid membrane protein complex", "sucrose-induced translational repression", and "starch catabolic process" related proteins (Table S9). KEGG enrichment analysis showed that 16 pathways were significantly enriched (Fig. 8B, Table S10). Among these pathways, DEGs are most significantly enriched in phenylpropanoid biosynthesis which is an important pathway for lignin biosynthesis and MAPK signaling pathway which plays an important role in the signal transduction processes of plant cells in response to environmental stress. It may be related to the adaptation to the changes in the environment in silencing *GhFKBP13* gene plant. In addition, we further analyzed the DEGs in starch and sucrose metabolism pathways, photosynthesis-antenna proteins, protein processing in endoplasmic reticulum pathways, which may related to the chloroplast biogenesis. In photosynthesis-antenna proteins pathway, all 7 DEGs of light-harvesting complex i/ii chlorophyll a/b binding proteins including *LHCB1*, *LHCB2*, *LHCB3* and *LHCB4* were down regulated. In the starch and sucrose metabolism pathway, 11 DEGs including *glycosyltransferase*, *β-amylase*, *β-fructofuranosidases*, *sucrose synthase*, *β-glucosidases* and *glycogen branching enzyme* were down regulated and 7 DEGs including *β amylases*, *glycosyltransferase*, *trehalose phosphate synthase* and *α amylase* were down regulated. In protein processing in endoplasmic reticulum pathways, 5 DEGs including *proteasome inhibitor*, *small heat shock protein 20*, *C3HC4 domain gene* and *disulfide-isomerase* were down regulated and 24 DEGs involving *small heat shock protein 20*, *heat shock protein 90*, *heat shock protein 70*, *heat shock protein 40*, *ATPase* and *ubiquitin fusion degradation protein* were up regulated. (Table S11).

Transcription factors are important regulators that play important roles in plant development. The identification of DEGs related to the transcripts of TF genes was subsequently conducted. A total of 182 TF-encoding genes from 32 different families were identified (Fig. 8C, Table S12). The top six DEGs in the TF-encoding gene family with the largest number of genes were MYB, ERF, NAC, WRKY, bHLH and bZIP TFs. Among the six TF families, the overwhelming majority of the ERF TFs were downregulated, while the majority of the NAC TFs were upregulated. Among the bHLH, MYB and bZIP TFs, more DEGs were downregulated than upregulated. However,

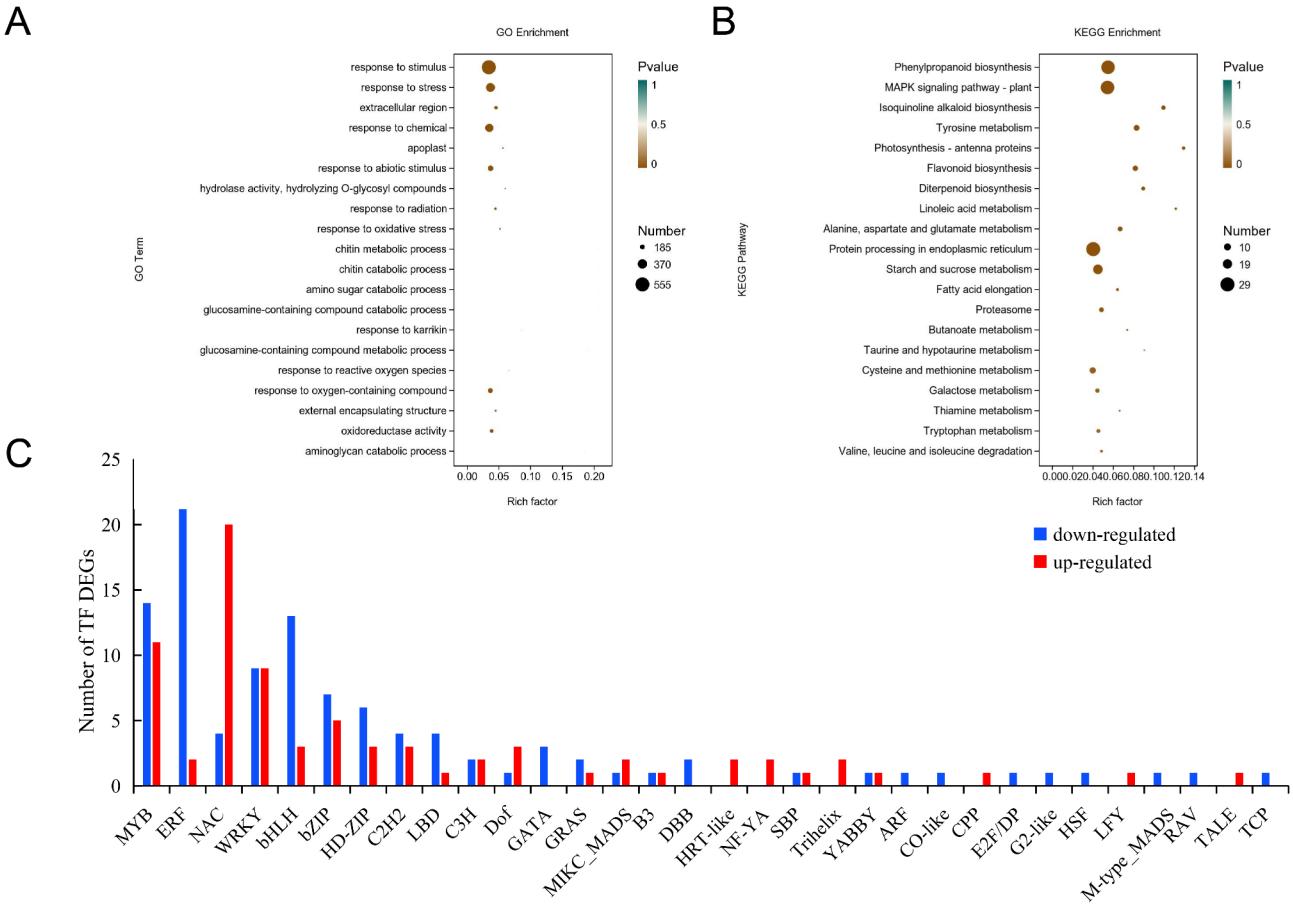

Fig. 8 Transcriptome analysis of differentially expressed genes (DEGs) in silencing *GhFKBP13* plants. (**A**) Gene ontology (GO) enrichment analysis of DEGs. (**B**) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs. (**C**) Number of transcription factors (TFs) analysis from the DEGs. The size of the circle represents gene numbers, and the color represents the Pvalue in (**A**) and (**B**)

for WRKY TFs, the number of upregulated DEGs was equal to the number of downregulated DEGs. In addition, to identify a possible *GhFKBP13* transcriptional regulator, we performed the prediction of TFs that regulate *GhFKBP13* expression, and 28 TFs were predicted, in which two TFs (zf-Dof and GATA transcription factor) were differentially expressed in *GhFKBP13*-silenced plants (Table S13). It speculated that these two TFs may play a role in the regulation of *GhFKBP13* expression. However, the mechanism underlying the interaction between *GhFKBP13* and two TFs (*zf-Dof* and *GATA* transcription factor) requires further verification.

Discussion

FKBPs are an important gene family that are widely present in prokaryotes and eukaryotes. In plant, *FKBPs* have been found to participate in various biological and physiological processes like photosynthetic system reaction, stress response, and growth and development etc [40–44]. However, the identification and analysis of the *FKBP* gene family in cotton genome have not been reported yet. In this study, *FKBP* genes were identified from *G. hirsutum(48 genes)*, *G. barbadense(47 genes)*, *G. arboreum(28)*, and *G. raimondii*(24). It was found that the number of *FKBP* genes in two tetraploid cotton species (*G. hirsutum* and *G. barbadense*) was observed to be almost double that of their progenitors (*G. arboretum* and *G. raimondii*) and there is good collinearity between species. These results further support the diploid origin of tetraploid cotton. We noted that *FKBP* genes in the A subgenome experienced gene loss during the evolution of tetraploid cotton, while the *FKBP* genes in the D subgenome is relatively conserved, which might be caused by asymmetric subgenome domestication. This speculated that *FKBP* genes between A and D subgenome might play different roles in the adaptive evolution of cotton. In addition, we found that all the family members arose from WGD or segmental duplication according to synteny block analysis (Table S5), which have shown similar results those some previous studies in cotton [32, 37]. This indicated that WGD or segmental duplication might be a main driver of gene expansion during cotton evolution.

The structure and pattern of protein expression are intricately linked to their function. In the analysis of phylogenetic tree, motif and tissue expression in *G. hirsutum*, it was found that most of *FKBP*s in group I had 2–3 conserved motifs and predominant of them were highly expressed in leaves (Fig. 5). Previous reports showed that some homologous *FKBP*s in group I play roles in chloroplast development. TaFKBP16-1 could interact with the subunit PsaL of PSI(photosystem I), and TaFKBP16-3 was shown to bind to Thf1(Thylakoid formation 1) and APO2(Accumulation of PSI-2), which were involved in the formation of photosynthetic membrane [38]. Peng et al. found that *Arabidopsis AtFKBP16-2* plays an important role in the formation of NDH-PS I subcomplexes [39]. Lima et al. showed that *AtFKBP20-2* participates specifically in the accumulation of the PSII upercomplex in the chloroplast thylakoid lumen [17]. In this study, we found that *GhFKBP13* can also affect chloroplast development. It is generally believed that genes with similar structures and expression patterns have similar biological functions. Therefore, we speculated that these GhFKBPs in group I may play important role in regulating chloroplast biogenesis. In additon, the *FKBP* genes have also been reported to play an important role in abiotic and biotic stress responses. We noted that most of *FKBP*s in group II showed ubiquitously expressed across all plant tissues, and many genes in this group were reported to be involved in abiotic stress responses. For example, overexpressing polar moss *PaFKBP12* in *Arabidopsis* showed enhanced resistances to salt, heat, and drought treatments [27]. In *Arabidopsis*, Overexpressing of maize *FKBP* gene *ZmFKBP20-1* significantly enhanced the tolerances to drought and salt [28]. *AtFKBP62* and *AtFKBP65* responded to high temperature stress and affect the accumulation of the heat shock transcription factor *HsfA2* [45]. In this study, based on gene expression analysis, it was found that these genes in group II showed an up-regulation trend under salt stress and PEG stress, suggesting that these genes may play an important role in responding to adversity stress.

Chloroplasts are important organelles for plant energy conversion and photosynthesis. Within chloroplasts, starch is the primary compound for storing energy in the form of grains and serves as the primary storage for excess carbohydrates produced during photosynthesis. Previous reports have indicated that various genes could interfere with starch metabolism and lead to chloroplasts destruction. For instance, silencing the *Nicotiana benthamiana* chloroplast thylakoid membrane protein TMP14 (*NbTMP14*) results in the destruction of most chloroplasts and an enlargement of starch granules [46]. In *Arabidopsis thaliana*, maltose-excess mutant mex1, which lacks the chloroplast envelope maltose transporter, could accumulate large amounts of starch in chloroplasts, leading to degradation of chloroplasts [47]. Rice mutant *lses1* (leaf starch excess and senescence 1) has an obvious excess starch phenotype in leaves, which may lead to chloroplast damage [48]. In this study, a similar phenomenon occured in the *GhFKBP13*-silenced cotton plants through transmission electron microscope observation. It implied that *GhFKBP13* is essential for maintaining chloroplast integrity and starch granule morphology in cotton. In addition, KEGG pathway enrichment analysis showed that starch and sucrose metabolism pathway was enriched in *GhFKBP13*-silenced plants, including *β-fructofuranosidases*, *sucrose synthase*, *amylase*, *β-glucosidases* and *glycogen branching enzyme*. Some of these genes have been previously reported to be associated with starch metabolism. For example, overexpression of *Arabidopsis sucrose synthase* in tobacco results in increased leaf starch [49]. β-Amylases (BAMs) are key enzymes of transitory starch degradation in chloroplasts, the loss of *BAM3* leads to starch-excess phenotype, which is further exacerbated by the loss of *BAM1* [50, 51], and *bam4* mutants have a starch-excess phenotype [52, 53]. Therefore, It is hypothesized that *GhFKBP13* may play an important role in chloroplast development and starch metabolism by regulating the genes involved in this pathway. However, the exact role of the *GhFKBP13* gene is still unclear, and further experiments will be conducted to investigate its function and regulatory network.

Conclusions

In this study, a total of 48, 47, 28 and 24 *FKBP* genes were identified from *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. raimondii*, respectively. Phylogenetic analysis showed that these FKBP family members can be divided into three groups and that FKBP family members were conserved in the evolution of cotton. The WGD/segmental duplication events played a vital role in the expansion of *GhFKBP*s. When the *GhFKBP13* gene was silenced using VIGS, the plant leaves turned yellow-green, and the contents of chlorophyll a, chlorophyll b and carotenoid were significantly reduced. Furthermore, electron microscopy observations revealed damage to the chloroplasts and packing with large starch granules in chloroplasts, indicating that *GhFKBP13* may play a critical role in chloroplast biogenesis. This study is the first to systematically analyze the cotton *GhFKBP* gene family and provides a new understanding the function of *GhFKBP*s in chloroplast biogenesis in upland cotton.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11293-7.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
HZ and YW conceived and designed the project. JL and ZG designed and performed the experiments and wrote the manuscript. GZ prepared materials. ML and ZA contributed bioinformatic analysis.

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Data availability
Data will be made available on request. The data presented in the study are deposited in the GSA, accession number CRA015118. Access it from the following link: https://bigd.big.ac.cn/gsa/browse/CRA015118.

Declarations

Ethics approval and consent to participate
Not applicable. The sampling of plant material was performed in compliance with institutional guidelines. The research conducted in this study required neither approval from an ethics committee, nor involved any human or animal subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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