

Genome-wide expression analysis of LACS gene family implies GHLACS25 functional responding to salt stress in cotton

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Abstract

Background: Long-chain acyl-coenzyme A synthetase (LACS) is a type of acylating enzyme with AMP-binding, playing an important role in the growth, development, and stress response processes of plants.

Results: The research team identified different numbers of *LACS* genes in four cotton species (*Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium raimondii* and *Gossypium arboreum*), indicating a certain diversity of *LACS* genes among different cotton species. By analyzing the structure and evolutionary characteristics of *LACS* genes, the *GHLACS* genes were divided into six subgroups, and a chromosome distribution map of the family members was drawn, providing a basis for further research classification and positioning. Promoter cis-acting element analysis showed that most *GHLACS* genes contain plant hormones (GA, MeJA) or non-biological stress-related cis-elements, suggesting that *GHLACS* genes may be involved in the regulation of plant growth, development, and stress response processes. The expression patterns of *GHLACS* genes under salt stress treatment were analyzed, and the results showed that *GHLACS* genes may significantly participate in salt stress response through different mechanisms. The research team selected 12 *GHLACS* genes responsive to salt stress for tissue expression analysis and found that these genes are expressed in different tissues.

Conclusions: *GHLACS25* was chosen for in-depth study, and it was confirmed that the *GHLACS25* gene plays an important role in salt stress response through virus-induced gene silencing (VIGS) and induced expression in yeast cells. Overall, this study provides important clues and evidence for understanding the function of *LACS* genes in cotton and their role in growth, development, and stress response processes. It is hoped that future research will further explore the mechanism of action of *LACS* genes in cotton and explore their potential application value in improving stress resistance traits.

Background

Long-chain acyl-CoA synthetase superfamily, also referred to as acyl activating enzymes, long-chain acyl-CoA synthetases (LACSs) are a significant type of enzyme [1, 2]. Most enzymes in this superfamily catalyze reactions through a two-step mechanism. The initial procedure engages in the amalgamation of ATP with free fatty acids, leading to the creation of an adenylated intermediate; the ensuing step ties this intermediate with the thioester bond of CoA to produce acyl-CoA [2]. In general, *LACS*s partake in the esterification process of long-chain free fatty acids (LCFAs), giving rise to the formation of fatty acyl-CoA thioesters. These products can subsequently enter numerous metabolic pathways, including but not limited to lipid storage, membrane lipids, surface lipids, and fatty acid degradation [3]. Furthermore, some *LACS*s have been identified as transporters of fatty acids between different subcellular compartments, indicating their important role in intracellular lipid metabolism [3, 4].

The substrate of LACS is fatty acid, which is very important for plant metabolism and resistance to abiotic and biological stresses. Fatty acids serve both as an energy source and a protective surface layer against multiple stresses in plants [5, 6]. It is known that *Arabidopsis thaliana* is a widely studied model

plant, and its *LACS* gene family includes 9 members [7]. Numerous *LACS* genes, confirmed to play a significant role in the production of lipids in the stratum corneum, are integral for plants as they minimize non-stomatal water loss. This crucial function enhances the plant's resistance to stress. Several single or double mutants of *Arabidopsis LACS*, such as *AtLACS2*, *AtLACS1 AtLACS2*, *AtLACS4 AtLACS8*, and *AtLACS4 AtLACS9* have been identified so far. These mutants demonstrated enhanced stratum corneum permeability, elevated water loss, and heightened susceptibility to drought [1, 8–10]. Due to functional redundancy, high-level *AtLACS* mutants usually show higher sensitivity to drought. For example, the triple mutant *AtLACS1 AtLACS2 AtLACS4* is a good example. It was found that the transpiration rate of triple mutant *AtLACS1 AtLACS2 AtLACS4* exceeded that of single mutant or double mutant [4, 10]. In addition, in other species besides *Arabidopsis*, studies have described the role of *LACS* in stress resistance. Research discovered that ectopic expression of two *LACS* (*MdLACS2* and *MdLACS4*) in apple resulted in decreased epidermal permeability, reduced water loss, and increased resistance to drought and salt stress in transgenic plant [11]. Furthermore, another study demonstrated improved tolerance to PEG, NaCl, and ABA treatments in callus altered by *MdLACS1* [12]. The survival and thriving of plants in any given environment is attributed to the equilibrium between genetic adaptability and phenotypic plasticity, with abiotic environmental elements potentially triggering a stress response. These adaptive and responsive abilities can improve the reproductive adaptability of plants in the ecological environment and may be transformed into stable agricultural output. In a word, these results show that *LACS* activity is indispensable in plant growth, development and stress resistance.

Gossypium hirsutum is a kind of cultivated cotton, which is not only famous for its high fiber yield, but also has the characteristics of salt and alkali tolerance, and is the main pioneer crop in saline-alkali areas. However, the growth and yield of cotton are seriously restricted by external environment and internal factors. More and more studies show that *LACS* family proteins play an important role in seed germination, oil synthesis and stress response. Nonetheless, outside environmental influences and internal elements considerably limit the proliferation and productivity of cotton. Increasing research demonstrates that proteins from the *LACS* family have a significant function in the processes of seed germination, oil creation, and stress reaction. However, there is relatively little research on *LACS* protein during cotton growth and development. In this study, the *LACS* family of cotton was comprehensively reviewed, and the *LACS* genes of four major cotton varieties were preliminarily identified. In *Gossypium hirsutum*, we further studied one of *LACS* genes and preliminarily identified its tolerance to saline-alkali environment. By analyzing the tolerance of some *LACS* genes to environmental stress, this study provides a new perspective for the evolution and in-depth study of this gene family. Our results provide an important basis for further revealing the mechanism of action of this family gene and the biological function of *LACS* gene in growth and development.

Results

Identification of *LACS* family members

In order to identify the members of *LACS* gene family in cotton, we used the hidden Markov model of PF05001 as the query condition, and compared the proteome and genome of *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii* with local blast software. In this process, we screened according to the domain AMP-binding, and determined the family members by incomplete and gene knock-out without this domain. Finally, the number of genes identified in *G. hirsutum* (*GHLACSs*), *G. barbadense* (*GBLACSs*), *G. arboreum* (*GaLACSs*) and *G. raimondii* (*GrLACSs*) was 38, 39, 22 and 20 respectively. According to the number of genes in four cotton varieties, it is determined that the *LACS* gene in cotton belongs to conservative type, which is basically consistent with the evolution of allotetraploid cotton[13].they were named *GHLACS1-38*, *GBLACSC1-39*, *GaLACS1-22*, *GrLACS1-20* according to their position on the chromosome.

Then we analyzed the physical and chemical properties of *LACS* family members, such as protein length, protein molecular weight, isoelectric point, protein hydrophilicity and hydrophobicity (Table.S1). We found that in *G. hirsutum*, the length of proteins encoded by *Lacs* family members ranged from 535aa (*GHLACS13*) to 762aa (*GHLACS36*). The molecular weight ranges from 59.29kDa (*GHLACS27*) to 85.11kDa (*GHLACS36*), and the isoelectric point ranges from 5.3 (*GHLACS17*) to 8.17 (*GHLACS2*). The encoded protein's length and molecular weight also fluctuate within a specific range in other cotton species. Furthermore, we have also made predictions about the subcellular location of these family members. The results showed that 25 genes in the *GHLACS* family were predicted to be located in plastids, 6 in cytoplasmic matrix, 4 in endosperm, 2 in nucleus and 1 in cell membrane.

Analysis on the Evolutionary Relationship of Family Members

In order to study the evolutionary relationship of *LACS* protein family, you have compared the full-length protein sequences, using 128 protein sequences from *Arabidopsis thaliana* and four cotton species (Fig.S1). The evolutionary relationship between these sequences was constructed by MEGA7 software, and the rootless phylogenetic tree was generated (Fig. 1A). In the results, you divided *LACS* protein into six subgroups, and observed that subgroup B and subgroup F contained a large number of genes, with 8 and 9 *GHLACS* genes respectively. In addition, you also found that in *Arabidopsis*, except for subgroups D and E, the other four subgroups all contain the *LACS* gene of *Arabidopsis*. It is worth noting that according to the number of genes contained in four cotton varieties in each subgroup, it is found that the number of tetraploid cotton variety *G. hirsutum* is almost twice that of diploid cotton variety, This offers evidence indicating that *G. hirsutum* is the product of hybridization between two diploid cotton varieties (*G. arboreum* and *G. raimondii*). Additionally, we conducted an analysis of the homologous genes of *G. hirsutum*, and it was found that these genes evolved slowly after repeated events, which was conservative at the protein level. In the phylogenetic tree, all species have gene pairs from the same node, indicating that all species' *LACS* genes have experienced gene duplication events, which eventually led to the expansion of *LACS* genes. The replication of genes varies across different species and populations. Specifically, upland and island cotton both display a significantly higher gene count compared to other

species, indicating a substantial evolutionary expansion of the *LACS* gene family in these two types of cotton. Furthermore, we constructed a phylogenetic tree that illustrates the evolutionary relationship between *Arabidopsis thaliana* and upland cotton (Fig. 1B), and found that there was no *ATLACS*s gene in subgroup E, while the *LACS* gene in subgroup E might be unique to *G. hirsutum*.

Distribution of *LACS* gene family members on chromosomes

In order to further study the genetic differences of *LACS* gene family members, we drew the chromosome distribution map (Fig. S2-A) and statistical map (Fig. S2-B) of *LACS* gene family members by using the gff3 file and gene ID information of the genome. Through our research, we discovered that the *LACS* genes in *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium arboreum*, and *Gossypium raimondii* are located on the chromosomes specific to each respective cotton variant. Furthermore, 118 family members were found on varying specific chromosomes, with only the *GaACS22* gene located on a scaffold. This result shows that the genetic evolution process of *LACS*s gene is mature and stable. We discovered a gene in *Gossypium hirsutum*, located in a similar position on chromosome 1 of both subgroup A and subgroup D, by examining the distribution position of genes. This discovery is the same as *Gossypium barbadense*, and *GHLACS16* and *GHLACS34* are homologous genes in evolutionary relationship, and the two genes have similar promoter action elements. Notably, subgroup A of this cotton seed contains two additional genes compared to subgroup D. Furthermore, chromosomes A05 and D05 exhibit a higher gene count compared to other chromosomes in the genome.

The number of genes in *Gossypium barbadense* differs between the two subgroups. More genes are found on chromosomes 1 and 5 of both subgroup A and D compared to the other chromosomes. Interestingly, there is no gene distribution on chromosomes 4,6,12 and 13 of subgroup A and chromosomes 2,8,12 and 13 of subgroup D of two tetraploid cotton species, which may be related to the chromosome deletion of two cotton species during evolution. In *Gossypium arboreum*, one gene was mapped to scaffold, and the number of genes on Chr01 and Chr05 was 4, while the gene on Chr08 was deleted. However, in *Gossypium raimondii*, there are more genes on Chr01 and Chr05, with 3 and 4 genes on the corresponding chromosomes, while Chr02, Chr08, Chr012 and Chr13 of this cotton species have no *LACS* family gene distribution. Notably, we found that the deletion of *LACS* gene family was consistent with that of two tetraploid cotton varieties, both on chromosomes 2, 8, 12 and 13, which confirmed to some extent that the donor species of D subgenome of two tetraploid cotton varieties mentioned in previous studies were *Gossypium raimondii* [14]. The allocation of *LACS* genes across the 13 chromosomes among different cotton species exhibits an uneven distribution. Interestingly, there is no apparent correlation between the number of genes allocated to each chromosome and the respective chromosome length.

Motif and gene structure analysis of conserved protein of *GHLACS*

The MEME online website was utilized to examine the amino acid sequence encoded by the *GHLACS* gene, which revealed a total of 10 motifs (Fig. 2) were identified, which perform different functions and

are distributed in the sequences of various subgroups. In subgroup A, the length of gene sequence is quite different, including 7–10 motif, and their gene structures are also quite different. However, subgroup B and subgroup C have similar motifs, and both subgroups contain 10 motifs and frequent intron intervals. Most members of subgroup D include motif 6, 2, 4, 7, 5, 3, 1 and 8, and the intron length of this subgroup is generally long. As for the members of subgroup E, except *GHLACS13*, they all contain motif 6, 2, 4, 9, 5, 1 and 8. Interestingly, *GHLACS13* contains two motifs 9. Except for *GHLACS7* and *GHLACS27*, all the members of subgroup F contain 10 motifs, excluding motif 8. There are similar motifs in the same subgroup, which indicates that the protein structure in a specific subgroup is conservative, but the functions of most conservative motifs remain to be clarified. However, there is little difference in exon length of gene coding sequence, which indicates that the gene is conservative to some extent. Each subgroup showed high consistency in gene structure and phase distribution, which further proved the reliability of homologous grouping relationship.

Cis-acting elements and expression analysis of promoters

To investigate the importance of various promoter elements in abiotic stress response, we examined the evolutionary relationship sequence of the *GHLACS* gene family along with the sequence of the 2kb region preceding the initiation codon of its members (Table S3). Our analysis identified the response elements of the *GHLACS* gene family to abiotic stresses such as plant hormones, drought, light, and low temperature (Fig. 3). Among them, the number of light response elements is the largest, followed by MYB response elements. Most members contain MeJA response elements and salicylic acid response elements, while about half members contain gibberellin response elements. Notably, jasmonic acid pathway has been proved to be related to stress response [15, 16]. Furthermore, research has demonstrated the involvement of methyl jasmonate (MeJA), a plant growth and development regulator, in the regulation of gene expression in plants [17–19]. Exogenous MeJA can promote the accumulation of osmotic substances such as proline and soluble sugar, which is beneficial to the osmotic adjustment of plants to adapt to waterlogging stress [20]. In addition, under the condition of salt stress, the use of MeJA can enhance the salt tolerance of crops. Enhancing crop salt tolerance not only mitigates the impact of salt stress on crop growth and development but also indirectly modifies the soil environment [21]. A large number of cis-acting elements are related to growth and development and plant hormone response, which indicates that *GHLACS* gene may involve very complex regulatory patterns in the transcription process. Within the same gene subgroup, the homeopathic elements contained in gene promoters are different, even the homologous genes with high similarity are different. Through the analysis of promoters, we can acquire a more profound comprehension of how the gene family responds to diverse plant hormones. which in turn aids in comprehensively understanding the regulatory network of the *GHLACS* gene family.

We used real-time quantitative research method to focus on the expression pattern of *LACS* gene during seed development and germination. The results showed that the expression of most *LACS* genes was relatively low, and only a few *LACS* family members responded significantly to salt stress. The expression difference between *LACS* members in subgroup A and subgroup B is the most significant, which indicates that these two subgroups are very important for plants to adapt to adverse environment. In particular,

GHLACS20 was significantly up-regulated and *GHLACS38* was significantly down-regulated under stress. Interestingly, members of subgroup D hardly responded to salt stress. Intriguingly, subgroup D members displayed minimal response to salt stress. The expression of the reaction varies among different members, much like how the expression of a single gene can differ across various tissues. We hypothesize that this could be tied to the diverse functional elements each member possesses, as well as each tissue's unique adaptability to their surroundings

Collinearity analysis

We carried out a collinearity analysis on genes from four cotton species (*G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii*) to delineate their positional relationship, homology, amplification process, and arrangement sequence on the same chromosome. Firstly, we Blast the genome protein sequences of various cotton species, Then the homologous gene pairs were found by MCScanX [22], Combined with the chromosome length files between genomes, it was finally presented by CirCos [23]. The results are shown by a circle diagram (Fig. S3). A total of 489 repetitive gene pairs were identified in four cotton species (Table.S4). There are 35 pairs of segmental duplications and 454 pairs of whole-genome duplications. We found that out of the 38 genes in upland cotton, there are 40 gene pairs (Fig.S3-c), with 11 pairs being segmental duplications and 29 pairs being whole-genome duplications. In the 39 *LACS* genes of island cotton, there are 42 gene pairs (Fig.S3-h), with 11 pairs being segmental duplications and 31 pairs being whole-genome duplications. Interestingly, there are no tandem duplications in the *LACS* genes of the tetraploid cotton species. In *G. arboreum*, 22 genes formed 8 pairs of *LACS* homologous genes (Fig.S3-d), all of which were fragment repeats. At the same time, four pairs of *LACS* homologous genes (Fig.S3-a) were formed from 20 genes in *G. raimondii*, all of which were fragment replication. These results indicate that fragment replication /WGD is the main reason for gene amplification, and tandem replication does not seem to play a role in the evolution of *LACS* gene family. In our analysis of diploids (Fig. S3-f), it was found that *G. raimondii* had a total of 31 gene pairs. As for tetraploids, there were 108 gene pairs between *G. barbadense* (Fig. S3-e) and 70 gene pairs between *G. arboreum* and *G. hirsutum*. Moreover. the relationship between *G. barbadense* (Fig. S3-g) and *G. hirsutum* (Fig. S3-j) involved 68 gene pairs, while there were 58 gene pairs (Fig. S3-b) and 60 gene pairs (Fig. S3-i) connecting *G. raimondii* to *G. hirsutum* and *G. barbadense*, respectively.

Determination and analysis of selective pressure (Ka/Ks)

During the evolution of protein, replicated genes may undergo three functional changes: non-functionalization, where they lose their original function; sub-functionalization, where they partially lose their former function; and new functionalization, where they acquire a new function [24]. We leverage the computation of the synonym substitution rate (Ks) for synonym (Ka) to deduce the level of selective constraint, and additionally examine the gene pairs' selection pressure during the evolutionary process. We calculated the Ka, Ks, and Ka/Ks values for 357 pairs of homologous genes (Table.S5) of ten combinations of four cotton varieties (Ga-Ga, Ga-Gr, GB-Ga, GB-GB, GB-Gr, GH-Ga, GH-GB, GH-GH, GH-Gr, Gr-Gr). $Ka/Ks < 1$ is considered as purification selection, which indicates that natural selection eliminates harmful mutation and keeps protein unchanged. A Ka/Ks ratio greater than 1 indicates positive selection,

suggesting that natural selection plays a role in protein evolution. This causes the mutation location to become rapidly fixed in the population, subsequently accelerating gene evolution. On the other hand, a Ka/Ks ratio of 1 indicates neutral selection, implying that natural selection does not impact the mutation process [25]. By utilizing this Ka/Ks ratio, we assessed the selection pressure on recurring gene pairs. The findings revealed that Ka/Ks was greater than 1 in 10 gene pairs and less than 1 in 347 gene pairs. Furthermore, among the 303 gene pairs, the Ka/Ks values ranged from 0 to 0.49. Our research results show that the Ka/Ks ratio of about 97% *LACS* gene pairs in four cotton varieties is less than 1 (Fig. 4). This suggests that the genes within this group are incredibly preserved, thoroughly filtered and chosen, asserting their roles as crucial and challenging to alter.

Specific expression of *LACS* genes

In order to gain a deeper understanding of the role of the *GHLACS* gene in plant growth and evolution, we initially selected 12 genes from different subgroups based on the phylogenetic tree. Subsequently, we employed qRT-PCR technology to analyze their expression patterns in various tissues, including young roots, hypocotyls, and cotyledons. The results showed that (Fig. 5), most *GHLACS* members showed different degrees of response to salt stress, while *GHLACS24* and *GHLACS38* had almost no response to salt stress in all parts. Specifically, *GHLACS23* only responded to salt stress in cotyledons, *GHLACS3* responded in young roots and cotyledons, while *GHLACS5*, *GHLACS16* and *GHLACS20* responded in hypocotyls and cotyledons. In addition, *GHLACS25*, *GHLACS31* and *GHLACS32* all showed a certain degree of response. In particular, the *GHLACS25* gene demonstrated a robust response to salt stress across all time intervals and in various types of tissues. This response was notably more distinct in both the hypocotyls and cotyledons. Therefore, we decided to focus on this gene.

Interaction network of *GHLACS* proteins

To gain insights into the function of the *GHLACS* protein, we employed the STRING database to explore its interaction network and analyze the protein sequences of homologous genes in *Arabidopsis* (Fig. 6). By studying the *ATLACS* protein in *Arabidopsis thaliana*, we were able to infer the potential function of the *GHLACS* protein. Research findings show that the protein equivalent to *GHLACS25* is *ATLACS4* as per the study results. The fact that *AtLACS4* interacts with *AtLACS*, a closely related member of the same family in *Arabidopsis thaliana*, suggests that *GHLACS* may also be involved in cellular lipid synthesis and subsequent breakdown through β -oxidation [26]. *AT5G60335* is a hydroxyl thioester dehydratase protein, which interacts with *ATLACS4*, suggesting that *GHLACS25* may be involved in the dehydration process of 3-hydroxyl acp intermediate [27]. Additionally, the relationship between *LACS4* and *FATB* could be crucial in providing saturated fatty acids necessary for plant growth and seed development [28]. The interaction between *ECH2* and *LACS4*, which encode monofunctional alkenyl coenzyme A hydratase, indicates that *GHLACS25* may be involved in the degradation of cis-unsaturated fatty acids [29]. In *Arabidopsis thaliana*, *ECH2* mutant leads to growth defects in seedlings [30], and *ECH2* plays a role in ethylene signal transduction [31]. These findings underscore the significance of ethylene not only in the regulation of plant growth and development, but also in how plants respond to various stresses [32, 33]. Interestingly,

in cotton, the interaction between a GHECH2 protein and a GHECH2-like protein and GHLACS25 was verified by double-luciferase detection and yeast two-hybrid technique (Fig. 7), which further explained the important role of LACS protein in growth and development and resistance to stress.

VIGS silencing of GHLACS25

Cotton cotyledons were infected with no-load (EM) and plant virus-induced gene silencing (VIGS) for 10–15 days when cotton cotyledons were completely unfolded. Subsequently, we detected the expression of *GHLACS25* gene in wild-type (WT), empty-load (EM) and VIGS plants by qRT-PCR (Fig. 8A). The results showed that the expression level of *GHLACS25* gene in the leaves of wild-type and no-load plants was similar, and it was significantly higher than that of VIGS plants that hardly expressed *GHLACS25* gene, which indicated that *GHLACS25* gene had been successfully silenced. Then, we treated wild-type, empty-load and VIGS plants with 200mM NaCl. After 2 days of treatment, it was found that all three plants wilted to varying degrees, and the stress degree of infected wild-type and empty-load plants was obviously more serious than that of uninfected wild-type plants, and the stress degree of VIGS plants was the most serious, followed by empty-load plants and then wild-type plants (Fig. 8B and C). To some extent, this result shows that the tolerance of *G. hirsutum* plants to salt stress is affected after the *GHLACS25* gene is silenced. **Fig. 8** VIGS inquiry experiment. **A** Expression of *GHLACS25* gene in leaves infected by VIGS; **B and C** Phenotype of cotton infected by VIGS under salt stress for 2 days

Yeast induced expression

Because the response to salt stress at the whole plant level is considered to depend largely on the tolerance mechanism of cells [34]. Therefore, the conservatism of the basic transport mechanism makes *Saccharomyces cerevisiae* a model system with considerable value for understanding the ion balance in plants [35]. In our research, it was found that PYES2-GHLACS25 and PYES2 were grown in SG-Ura with agar with different salt concentration gradients for 5 days, and we can clearly see that the growth of PYES2-GHLACS25 strain was weaker than that of PYES2 (Fig. 9A). Then, we cultured PYES2-GHLACS25 and PYES2 on SG-Ura broth medium with different salt concentration gradients for 24 hours, and then measured the OD value with spectrophotometer. The relative growth rate of cells was calculated by OD value (Fig. 9B). The growth conditions of PYES2 and PYES-GHLACS25 were different under different salt concentrations. With the increase of salt concentration, the growth inhibition of PYES2 yeast is more serious than that of PYES-GHLACS25 yeast, and this difference is more and more obvious. Combining the two experimental results, we concluded that the salt tolerance of yeast cells was increased after the expression of *GHLACS25* was induced in yeast.

Materials and methods

Plant materials and growth conditions

Cotton seeds were cultured in a greenhouse at 28 °C (16 h light and 8 h dark) from the Han M263 material preserved by the Cotton Research Institute of the Chinese Academy of Agricultural Science. The tobacco variety was Benji tobacco, which was cultured in a greenhouse at 28 °C (16 h light for 8 h dark).

Identification of members of upland cotton gene family

First, cotton genome data were downloaded from Cotton FGD website (<http://www.cottonfgd.org/>), including genome data file, CDS sequence file, protein data file and GFF annotation file. Next, the hidden Markov model (HMM file) of AMP-binding model (Pfam:PF00501) was obtained from PFAM website (<https://pfam.xfam.org/>) to identify LACS members in cotton. Under the environment of Windows system, you can run the HMMER 3.0 program, and use AMP-binding model to carry out the *hmmsearch* program to compare and screen the genome data of four cotton varieties. Combined with 9 *LACS*s sequences of *Arabidopsis thaliana*, local blast was carried out to obtain the initial members of *LACS*, and the sequences containing C, N and CN residues were removed by CD search on NCBI (<https://www.ncbi.nlm.nih.gov/structure/CDD/wrpsb.cgi>), and finally the members of *LACS* gene family of four cotton species were obtained. We used <http://bioinfo.usu.edu/Plant-mSubP/> to predict the subcellular localization information of *GHLACS*s. In addition, we also used Cotton Functional Genome Database (Cotton FGD) to search for other characteristics of *LACS* gene. In addition, we also use the online tool EXPASY-PROT PARAM (<https://web.expasy.org/protparam/>) to analyze information such as isoelectric point (pI), molecular weight (MW), exon/intron length, average hydrophilicity and charge. These methods provide various data and information for research, which is helpful to fully understand the characteristics and functions of *GHLACS*s gene and its encoded protein. This information will play an important supporting role in our research. SubPlant (<http://bioinfo.edu/Plant-mSubP/>) was used to predict the subcellular location of *GHLACS*s. Additionally, the Cotton Functional Genome Database (Cotton FGD) was utilized to search for other features of the *LACS* gene. To analyze Isoelectric point (pIs), Molecular weight (MWs), exon/intron length, mean hydrophilicity, and charge, we employed the online website ExPasy-ProtParam (<https://web.expasy.org/protparam/>)

Evolutionary analysis of gene system

The target sequence file is submitted to MEGAX in fasta format, and the relationship between sequences is analyzed by neighborhood connection method[36]. By analyzing 1000 random data sets, Bootstrap analysis is used to evaluate the tree topology of neighbor connection data [37]. The phylogenetic tree is constructed with MEGAX, and the generated Newick file is beautified with the online website EvolView (<https://www.evolgenius.info/evolView-v2/>).

Chromosome localization

The TBtools software was utilized to map the chromosome position of family members using the *G. hirsutum* reference genome gff3 file and gene ID file. Similarly, the position of the *LACS* gene in the chromosomes of *G. barbadense*, *G. arboreum*, and *G. raimondii* was successfully mapped using the same method. To obtain the genome sequences of *G. barbadense*, *G. arboreum*, and *G. raimondii*, we downloaded them from the Cotton FGD (<http://www.cottonfgd.org/>).

Protein conserved domain and gene structure analysis

By analyzing the *LACS* family gene of *Gossypium hirsutum*, the conserved sequence of the protein was successfully identified using the MEME website (<http://meme-suite.org/>). For this analysis, a maximum motif parameter of 10 was used while keeping the other parameters unchanged, resulting in the generation of the *LACS* family domain file. To visualize the protein domain, the Domain MAST file, gene evolution relationship file, and *Gossypium hirsutum* genome GFF3 file downloaded from the MEME website were employed in conjunction with TBtools [38].

Collinearity Analysis of LACSs Genes in Four Cotton Species

The gff3 file of the target genome was prepared and integrated with TBtools software to generate the protein sequence alignment of the genome. Subsequently, gene pair files for each combination of two species (Ga-Ga, Ga-GH, Ga-Gr, Ga-GB, GB-GH, GB-Gr, GB-GB, Gr-GH, Gr-Gr, GH-GH) were obtained. The TBtools software was employed to analyze the collinearity relationship of repetitive gene pairs among the four cotton species (*G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. raimondii*). Finally, the collinearity results were visualized using the chromosome length file and genome comparison file.

Analysis of GHLACSs promoter region and differentially expressed genes

Firstly, we utilized the TBtools software to extract the upstream 2000bp DNA sequence of the *GHLACSs* gene from the genome sequence fa file. Next, we identified and predicted the cis-regulatory elements present in the promoter region of the *GHLACSs* gene using the Plant CARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). During this process, we focused on screening and analyzing cis-acting elements that were associated with abiotic stress, plant hormone metabolism, and plant growth and development. Finally, the relevant charts were redrawn using the TBtools software[38].

The salt-tolerant cotton material (Han M263) seeds were generously provided by the State Key Laboratory of Cotton Biology. To extract total RNA, we followed the instructions of the RNA EASY spin Plus plant RNA rapid extraction kit from Beijing Tiangen Biotechnology. The three tissues, namely young roots, hypocotyls, and cotyledons, were used for RNA extraction. Next, cDNA synthesis was performed using the Transscript all-in-one first-strand cDNA synthesis supermix for qPCR, following the provided guidelines for one-step gDNA removal. For the qRT-PCR experiment, we utilized the Roche 480 equipment and TransStart Top Green qPCR SuperMix fluorescence quantitative kit. The processing results were calculated using the $\Delta\Delta C_t$ method[39] to calculate the processing results, and choose TBtools software for data analysis.

Luciferase complementation assays (LCA)

Using the primer (Tab.S2) provided in the attached table, we cloned the coding region of *GHLACS25* into pCAMBIA1300-cLUC, and constructed the GHLACS25-cLUC vector. At the same time, the coding regions of *GHECH2* and *GHECH2-like* were cloned into pCAMBIA1300-nLUC to construct GHECH2-nLUC and

GHECH2-like-nLUC vectors. Following a 3–4 weeks cultivation period, tobacco (*Nicotiana tabacum*) plants were prepared. An appropriate amount of *Agrobacterium* containing the target gene and the interacting gene vector was introduced into resistant LB medium (containing Kanamycin at 50 mg/L and Rifampicin at 25 mg/L) for culture until the OD 600 reached approximately 0.8-1.0. Subsequently, the two types of bacterial liquid were mixed in equal proportions. After collecting and resuspending the bacterial liquid through centrifugation, it was injected into tobacco leaves. Prior to applying the substrate near the injection site, the infected plants were cultured in darkness for 24 hours, followed by normal cultivation for 2 days. Finally, fluorescence was observed using a luminescence imaging system.

Yeast twohybrid assays (Y2H)

The coding region of *GHLACS25* was successfully cloned into the pGADT7 vector (AD-GHLACS25), while the coding regions of *GHECH2* and *GHECH2-like* were separately inserted into the pGBKT7 vector (BD-GHECH2 and BD-GHECH2-like, respectively). Subsequently, the constructs pGADT7 (AD-GHLACS25), pGBKT7 (BD-GHECH2), and pGBKT7 (BD-GHECH2-like) were introduced into Y2HGold chemically active cells (Weidi, YC1002). To identify positive bacteria, SD-Trp-Leu (DDO) medium was used for screening, followed by transferring the colonies to SD-Trp-Leu-His-Ade (QDO) medium. After inoculation, the cultures were allowed to grow for 3 days, and photographs were taken to observe the results.

Virusinduced gene silencing (VIGS) in upland cotton

The linear pYL156 vector was obtained by using BamHI and SacI restriction sites, and the cloned vector T-GHLACS25 was used as a template to amplify with primer GHLACS25-V to obtain the VIGS fragment. in-FuSion technology was used to insert the VIGS silent fragment of *GHLACS25* into the linear pYL156 vector. When planting Han M263 until two cotyledons spread flat, VIGS was injected on the back of the leaves with a disposable medical syringe (the injection area was over 80%), and then the seedlings were cultured in the dark at 28°C for 24 h. After the treatment, the normal growth of seedlings was ensured at a suitable temperature (28 14 h/25 10 h).

Construction of GHLACS25 Expression Plasmid for Yeast

The coding region of *GHLACS25* was cloned into vector PYES2(PYES2-GHLACS25) and then PYES2(PYES2-GHLACS25) was transferred into INVSC1 chemically active cells (Weidi, YC1050). Next, screening by SD -Ura solid medium showed that the positive bacteria were backward. We let PYES2-GHLACS25 and PYES2 grow on SG-Ura with agar medium with different salt concentration gradients, and took photos five days later. Secondly, we put PYES2-GHLACS25 and PYES2 on SG-Ura broth medium with different salt concentration gradients. After 24 hours of culture, the absorbance was measured at the wavelength of 600nm to calculate the relative growth rate of yeast.

Discussion

The ever-evolving high-throughput sequencing technology has facilitated the sequencing of a growing number of plant genomes. These scientific advancements greatly assist researchers in conducting

genome-wide analysis of crucial gene families, as well as in the identification of essential genes related to distinct characteristics or biological processes. Simultaneously, the results of these studies have accelerated the progress and realization of the comprehensive plant genome sequencing project. The number of LACS (long-chain fatty acid synthase) genes identified in *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii* is 38,39,22 and 20 respectively. The gene count in tetraploid cotton species such as *G. hirsutum* and *G. barbadense* is roughly equivalent to the combined gene count in two diploid cotton species like *G. arboreum* and *G. raimondii*. This observation partially elucidates the origin of tetraploid cotton species. Through the analysis of motif and gene structure, we infer that *G. hirsutum*'s LACS gene family is relatively conservative, that is, the gene sequence and structure are relatively stable. In addition, the researchers also found that gene replication events may be caused by polyploidy, tandem and segmented replication[22]. In our chromosome mapping analysis, we found that LACS genes were scattered on each chromosome in six subgroups. It is worth noting that the localization of LACS gene was not found on chromosomes 2, 8, 12 and 13 in D subgroup of diploid, which is consistent with the distribution of LACS gene on chromosome A subgroup of tetraploid. Interestingly, the distribution of LACS gene was not found on chromosome 8 in subgroup A of diploid. However, in subgroup A of the two tetraploids, LACS gene was not distributed on chromosomes 4, 12 and 13 except chromosome 8.

Replication events (tandem duplication, segmental duplication, and whole-genome duplication) play an important role in gene amplification [40, 41]. The subfunctionalization model of repeated genes holds that after repeated events, each repeated gene only retains a part of the functions of its ancestors. The subfunctionalization model can be further divided into complete subfunctionalization and partial subfunctionalization. Complete subfunctionalization means that two repetitive genes retain part of the functions of their ancestors without overlapping functions. In other words, each repetitive gene is responsible for different functions in cells or tissues. In this case, the loss of each gene will lead to the loss of function, so the existence of two genes is complementary, and partial subfunctionalization means that there is partial functional overlap between two repeated genes. Although they may have some common functions, they may also have specific functional differences in some aspects. In this case, the existence of two genes increases the functional diversity and provides a wider range of adaptation.[42]. The subfunctionalization model of repetitive genes provides a mechanism to explain the functional changes and retention of repetitive genes during evolution. Through functional differentiation and subfunctionalization, repetitive genes can undertake more functions and provide greater adaptability to environmental changes and natural selection [43, 44]. This model provides an important theoretical basis for us to understand the evolution of gene families and the formation of functional diversity [45]. The high proportion of repetitive genes in plant genome just reflects the retention rate of repetitive modules in plants [46]. It is still a subject of great interest to explore the retention mechanism of repeated genes in plant genome. Whenever a replication event occurs, the whole gene sequence of cotton will be doubled, and these redundant genes will be selectively recombined or lost over time [13]. Some repetitive genes may be preserved in future generations, thus providing the most basic genetic information for the evolution of plants [47]. Through the calculation of the Ks value of replicated genes, it is found that with the increase of the age of replicated genes, genes with fewer cis-acting elements are more common. The

calculation of the Ka/Ks ratio revealed that approximately 97% pairs of *LACS* genes in four cotton varieties have this Ka/Ks ratio was less than 1, which indicated that the gene family had been strongly purified. Together with the outcomes from the analysis of collinearity (Fig.S3), We identified 454 genome-wide replications in four cotton species. Therefore, genome-wide replication is the main reason for the amplification of *GHLACS* gene.

The long-chain acyl-CoA synthase (LACS) is a significant subset of the acyl-CoA synthase family. Known for its ability to convert long-chain or ultra-long-chain fatty acids into their corresponding thioesters within plants, this enzyme plays a crucial role in numerous lipid-related metabolic processes. Changes in LACS activity will result in an array of phenotypic modifications such as male sterility, fusion of organs, unusual epidermal structures, postponed seed germination, alterations in seed oil composition, and resilience to diverse environmental pressures like drought, salt stress, hypoxia and biological stress [48]. Several *AtLACS*s gene mutants have been found in *Arabidopsis thaliana*, which show the characteristics of increased cuticle permeability, increased water loss and drought sensitivity, suggesting that these *LACS* genes are involved in stress regulation and have overlapping function[8–10]. Our study found that the promoter region of cotton *GHLACS* gene contains rich response elements to light, low temperature, drought and various hormones (such as MeJA, SA, ABA, IAA and GA). Furthermore, numerous elements correlating with hormone response have been identified within the promoter region of the *GHLACS* gene. Hormones like ABA, MeJA, and GA are pivotal in managing plant responses to a variety of stress situations [49]. The presence of these elements aligns with the role of the *GHLACS* gene in augmenting cotton's resistance to saline stress conditions, as demonstrated in *GHLACS25*. We speculate that *LACS* family genes may be involved in abiotic stress response and hormone regulation pathway in plants. Revealing that in different plants like *AtLACS1* and *AtLACS2* of *Arabidopsis thaliana*, *LACS* could potentially have a function in the plant's response to abiotic stress. They seem to have concurrent roles in the keratin and wax synthesis and provide protection to plants from different environmental pressure[1].

In plants, LACS protein mainly exists in a few subcellular structures such as endoplasmic reticulum, plastid, peroxisome and cytoplasm. Plastid is the main place for fatty acid synthesis. *LACS* is widely distributed in eukaryotes, and its activity can be detected in subcellular structures such as plastids, microsomes, mitochondria and peroxides [50, 51]. Several *LACS* genes show varying forms of expression in diverse plant tissues and perform various functions. For a study, twelve *GHLACS* genes were examined. *GHLACS24* and *GHLACS31* showed high levels of expression in the roots. In contrast, *GHLACS11*, *GHLACS24*, and *GHLACS26* were predominantly expressed in the leaves, while *GHLACS25* was mainly expressed in both stems and leaves. We know that *PoLACS4* in peony can quickly respond to drought and salt stress, and may activate a series of subsequent adaptation and resistance reactions [52]. The study of *MdLACS2* in apple shows that it can reduce epidermis permeability and water loss, thus promoting the drought resistance of transgenic plants [53]. Ectopic expression of *MdLACS4*, a long-chain acyl-CoA synthase gene can promote the accumulation of cuticle wax and improve the resistance of plants to abiotic stress [11]. *GmLACS2-3* also plays an important role in preventing water loss [54]. Combined with the analysis of expression profile, we selected *GHLACS25* gene which responded positively to salt stress and expressed at high level in roots and leaves. The gene in cotton was silenced using VIGS technology,

which also triggered its expression in yeast We also verified the interaction between GHLACS protein and GHECH2 protein related to plant growth and development [30]. The conclusive findings indicated a potential involvement of the gene in the regulatory process of cotton growth, development, and salt stress.

Conclusion

In the above research, we identified the LACS family genes of four cotton varieties (*G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. raimondii*) and analyzed their evolutionary relationships. By comparing the gene structure, chromosome distribution, phylogenetic relationship, cis-acting elements and collinearity of *GHLACS* gene in *G. hirsutum*, we have a deeper understanding of the cotton *LACS* gene family. At the same time, by analyzing the cis-acting elements of *GHLACS* gene promoter, we speculate that *GHLACS* gene may be involved in stress defense response, abiotic stress and plant hormone signal transduction mechanism. On this basis, we selected 12 *GHLACS* genes for simple tissue specificity analysis based on the real-time quantitative data in the previous period, and conducted VIGS silencing experiment and yeast-induced expression experiment on *GHLACS* genes that responded significantly under salt stress. Finally, we determined that *GHLACS25* gene may be involved in the response of upland cotton to salt stress, which provided a strong basis for studying the molecular mechanism of *LACS* gene in plants under abiotic stress.

Abbreviations

LACS

Long-chain acyl-coenzyme A synthetase

ECH2

Enoyl coenzyme hydratase 2

IAA

Auxin

VIGS

Virus induced gene silencing

EM

Empty vector

WT

Wild type

At

Arabidopsis thaliana

GH

Gossypium hirsutum

GB

Gossypium barbadense

Ga

Gossypium arboreum

Gr

Gossypium raimondii

MEME

Multiple Em for Motif Elicitation

MeJA

Methyl Jasmonate.

Declarations

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Author contributions

M.K designed the project, He was the first corresponding author. Y.X conceived and designed the experiments, writing original draft. S.F provided Methodology, Software. Y.H were responsible for writing-review and editing. D.Z and Y.W provided the Methodology. J.P and M.K were mainly responsible for overseeing and revising the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The genomic data of cotton, *Arabidopsis thaliana* in the article can be downloaded

from Cotton FGD ([https:// cotton fgd.org/](https://cottonfgd.org/)) and TAIR ([https:// www.arabidopsis.org/](https://www.arabidopsis.org/)) respectively. The analysis software, analysis methods and datasets generated are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

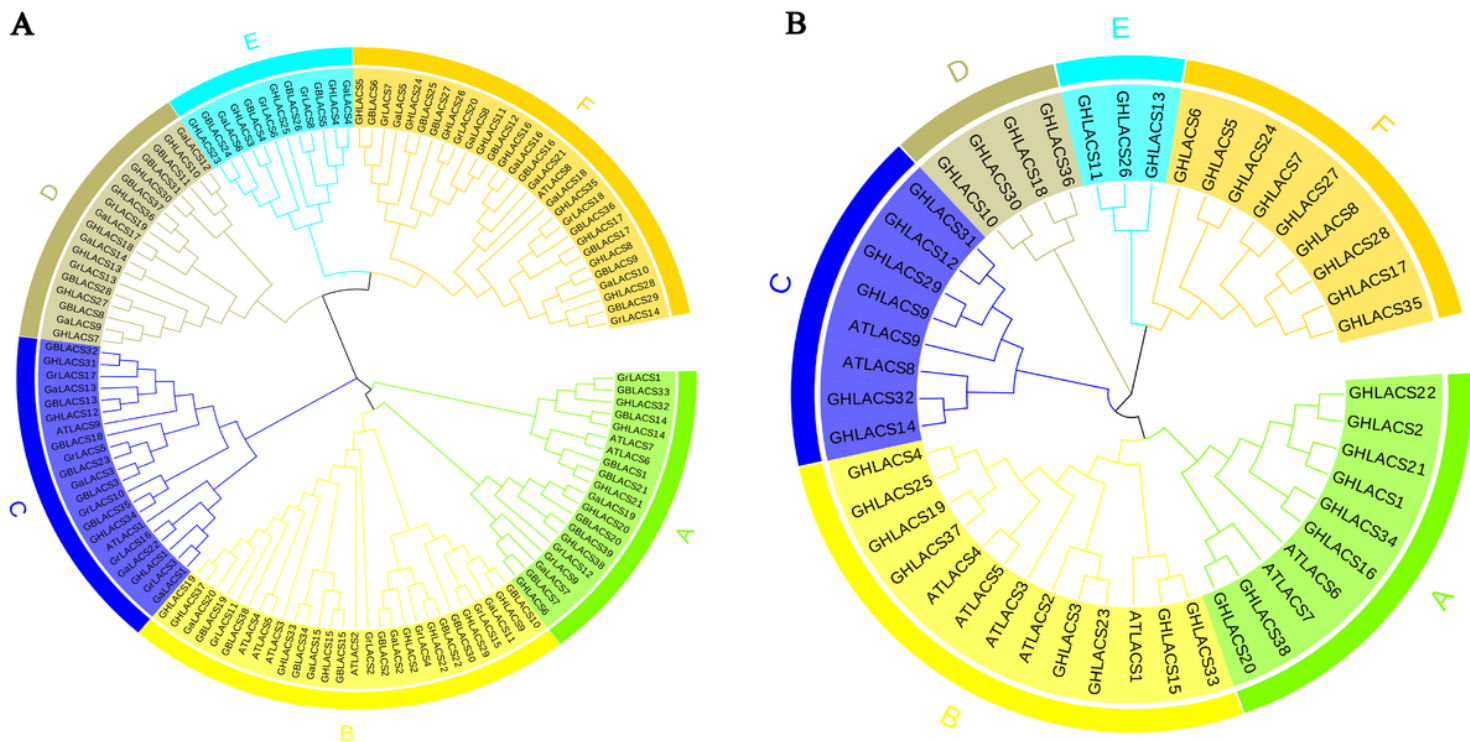


Figure 1

Phylogenetic tree of *LACS* family members. **A** Four cotton species (*G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. raimondii*) and *Arabidopsis* *LACS* gene phylogenetic tree; **B** The phylogenetic tree of *G. hirsutum* and *Arabidopsis* *LACS* genes.

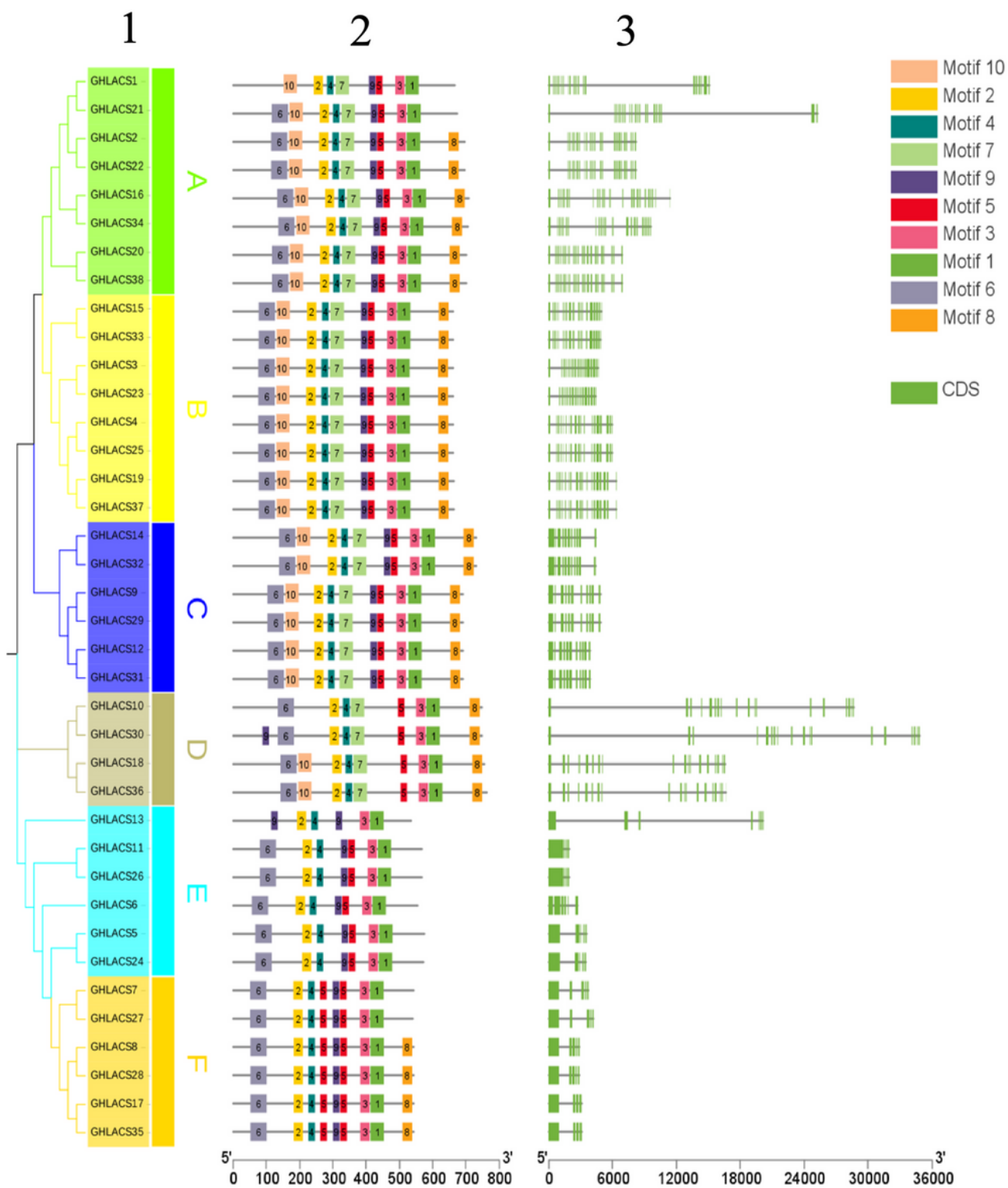


Figure 2

Phylogenetic tree, conserved domain and gene structure analysis of *GHLACS* gene.

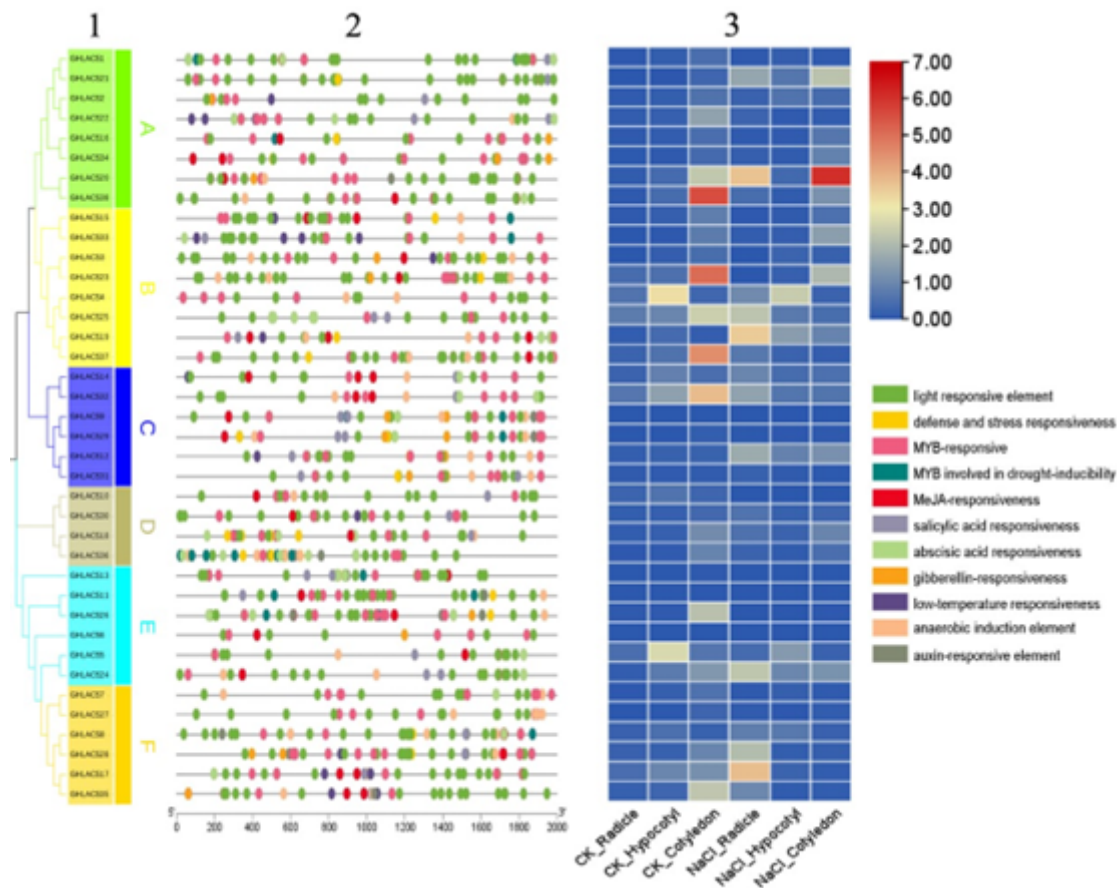


Figure 3

Analysis of *GHLACS*s family promoters and differentially expressed genes. **1** Phylogenetic tree of *GHLACS* gene; **2** cis-element in the promoter of *GHLACS* gene; **3** Differentiated genes of *GHLACS* expressed under water and salt stress.

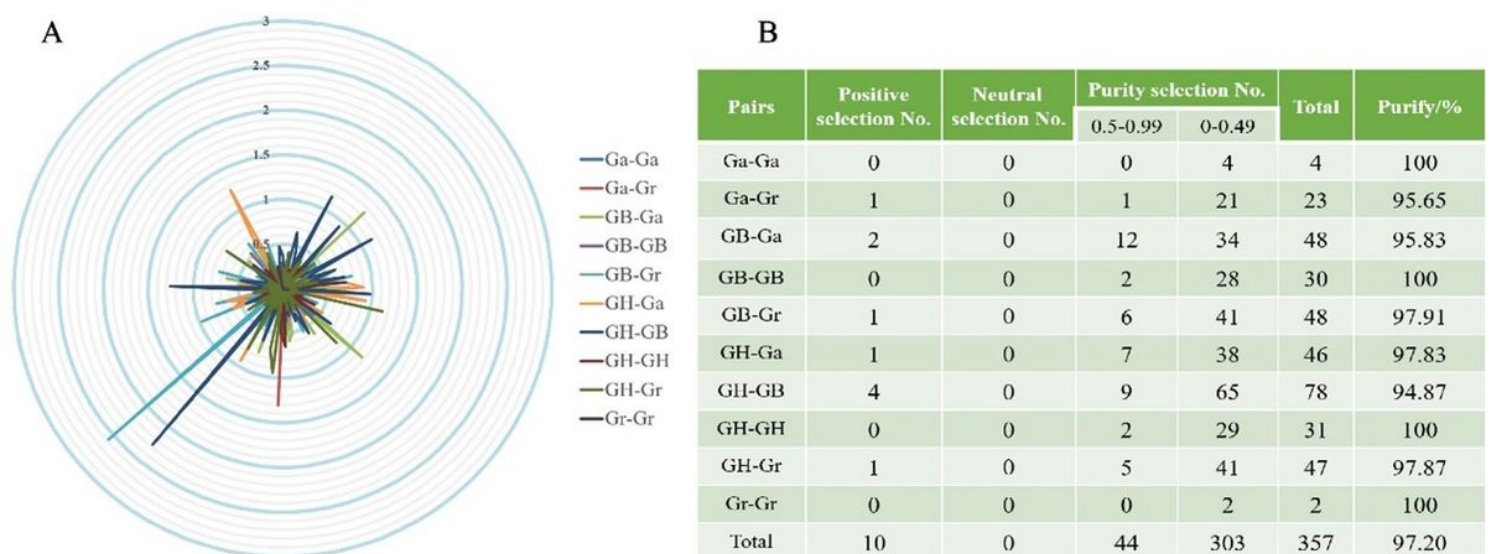


Figure 4

Selection pressure (Ka/Ks) analysis. **A** Select pressure (Ka/Ks) to analyze radar chart; **B** Prediction of Logarithmic Number of Repetitive Genes in Four Different Cotton Combinations

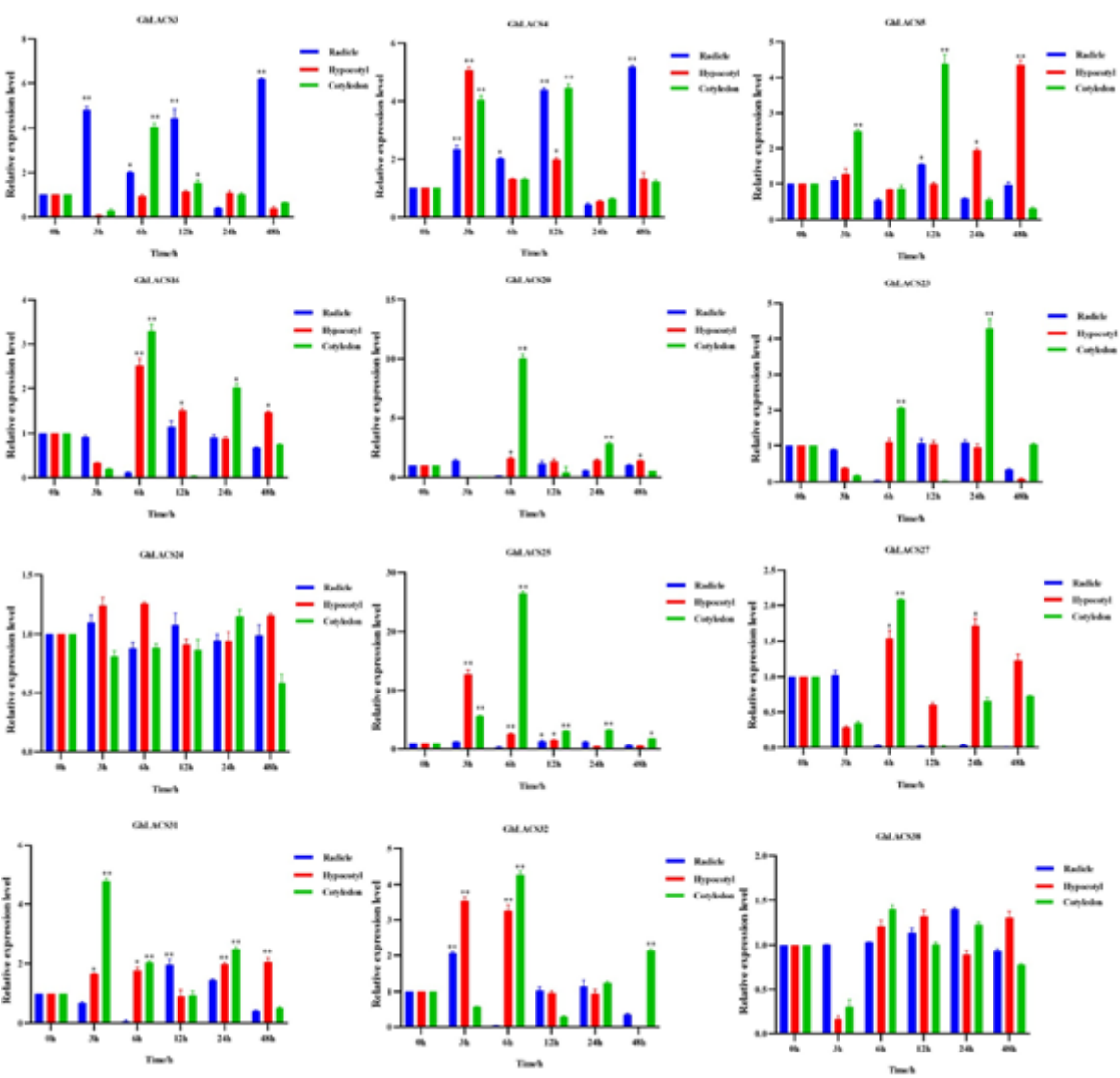


Figure 5

Expression level of *GHACS* gene in different tissues. Asterisks indicate significant differences (* P, 0.05; ** P, 0.01; Microsoft Excel Students' t test.)

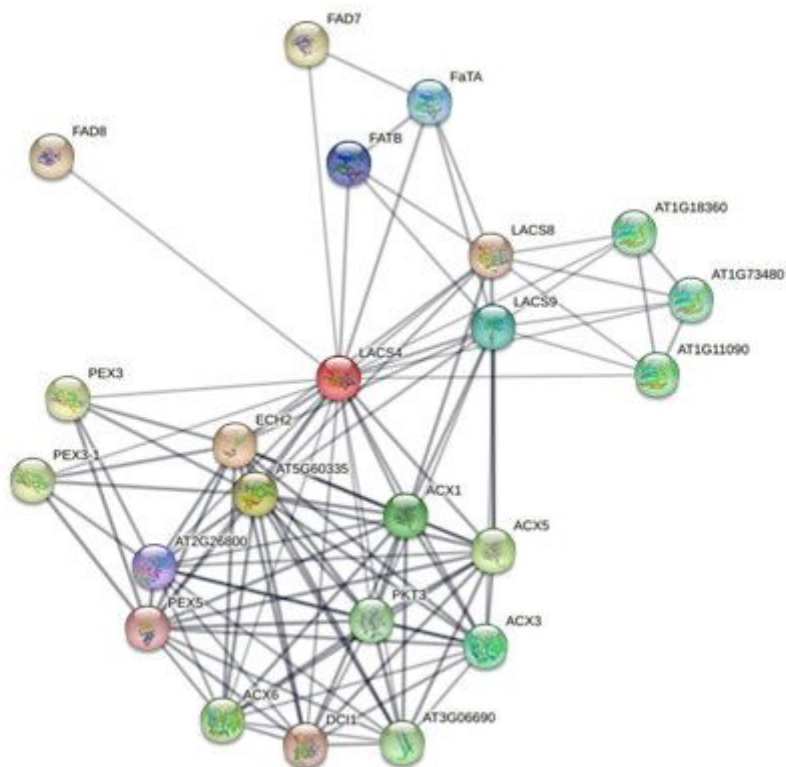


Figure 6

Interaction network of GHLACS proteins.

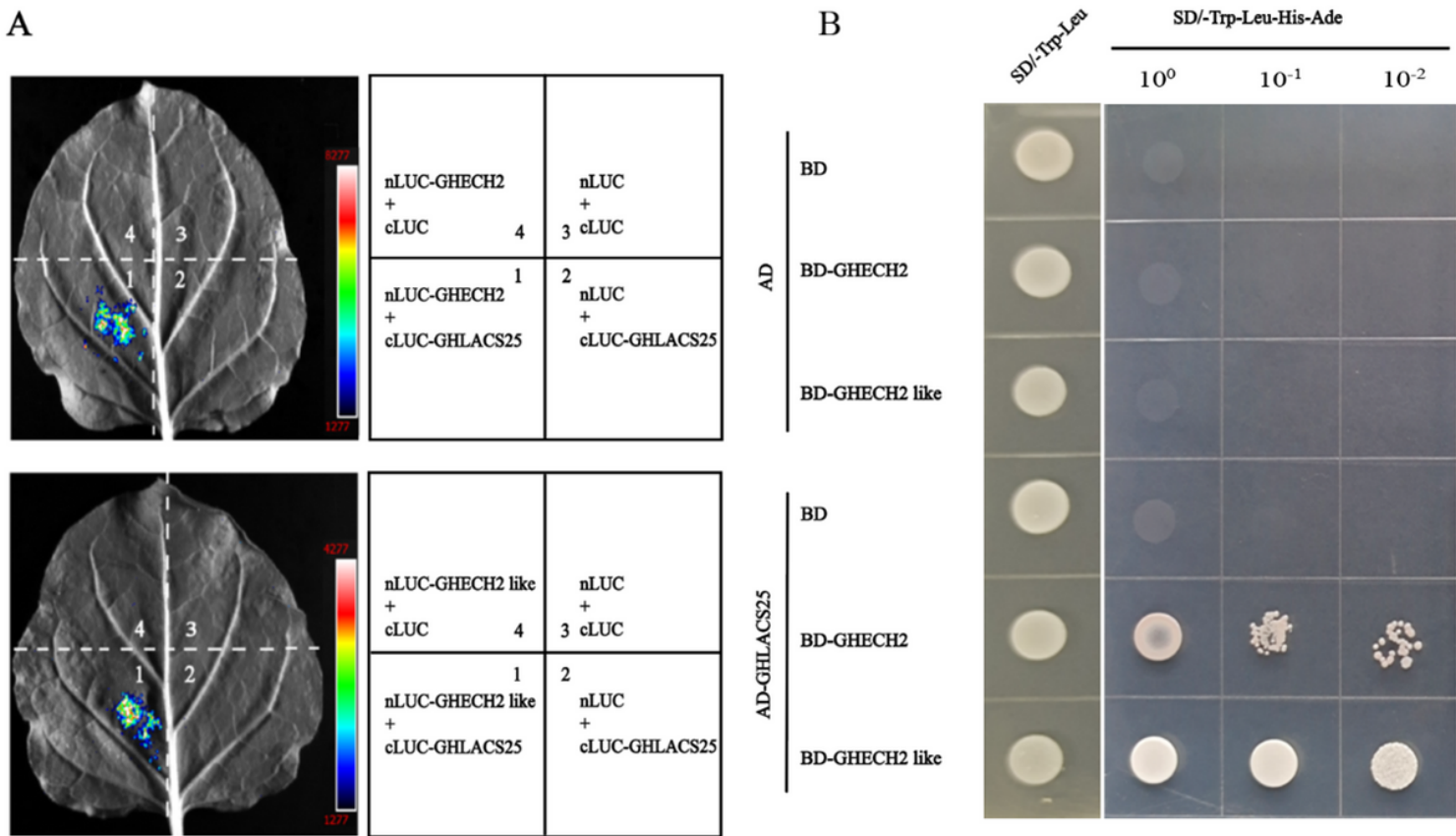


Figure 7

GHLACS25 interacts with GHECH2 and GHECH2 like. **A** Double fluorescein report detection experiment; **B** Yeast two-hybrid experiment

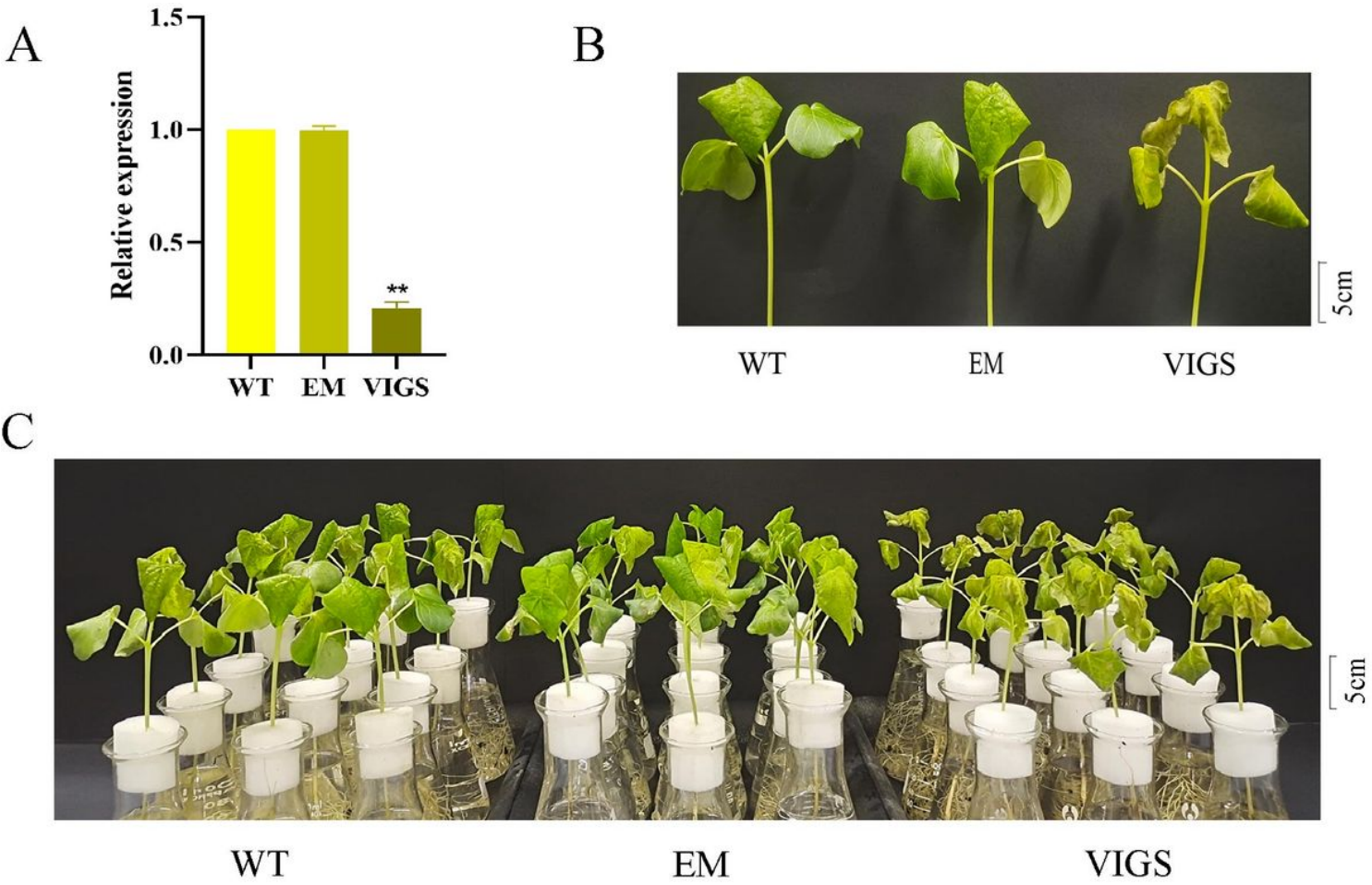


Figure 8

Interaction network of GHLACS proteins.

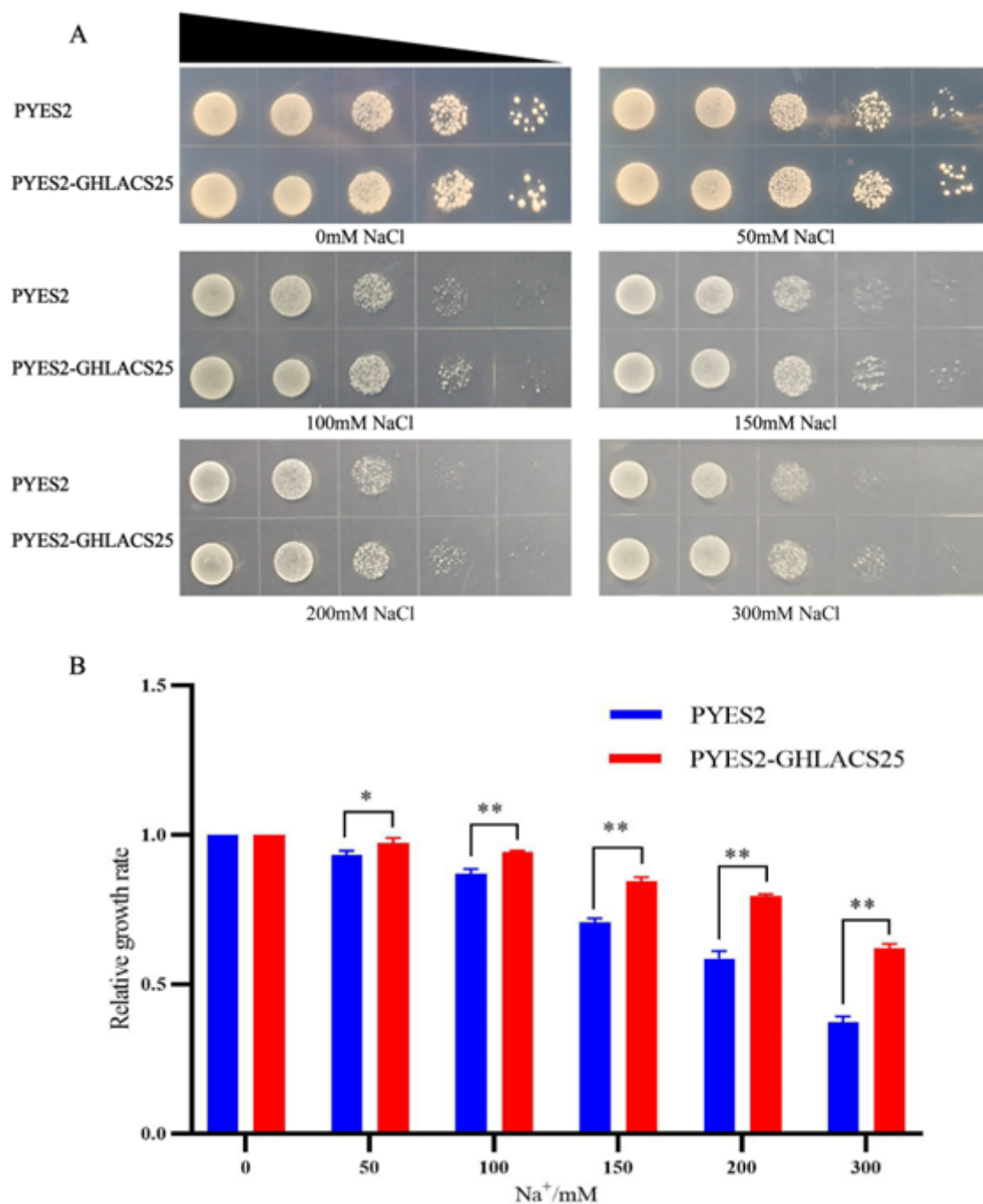


Figure 9

Functional analysis of PYES2-GHLACS25 in Na⁺ sensitive strain INVSC1. **A** growth status of expression vector PYES2-GHLACS25 and empty vector on SG-Ura with agar medium with different salt concentrations; **B** the relative growth rates of expression vector PYES2-GHLACS25 and empty vector on SG-Ura broth medium with different salt concentrations. The asterisk indicates the significant difference between PYES2 and PYES2-GHLACS25 (*, P, 0.05; **, P, 0.01; Microsoft Excel students' t-test)

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