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Based on the whole genome clarified the evolution and expression process of fatty acid desaturase genes in three soybeans

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ABSTRACT

Soybean is an important oil crop cultivated worldwide. With the increasing global population crossed with growing challenging cultivation conditions, improving soybean breeding by selecting important traits is urgent needed. Genes coding for plant fatty acid desaturases (FADs) genes are major candidates for that, because they are involving in controlling fatty acid composition and holding membrane fluidity under abiotic stress. Here, 75 *FADs* were found in three soybean genomes, which were further classified into four sub-groups. Phylogenetic tree, gene structure, motif and promoter analysis showed that the *FAD* gene family was conserved in the three soybeans. In addition, the numbers of omega desaturase from Chinese cultivated varieties were significantly higher than those in Chinese wild soybean and ancient polyploid soybean, respectively. However, it was the opposite for the sphingolipid subfamily. These results indicated that each subfamily was subjected to different selection pressures during cultivation and domestication. As the extra genes of the subfamily were very close to other family members' positions on chromosomes, they should be produced by duplication. The *cis*-element analysis of *FAD* promoter sequences revealed that upstream sequences of *FAD* contained abundant light, hormone and abiotic stress responsive *cis*-elements, suggesting that the quality of soybean could be improved by regulating these stresses. Expression analysis of Chinese wild soybean under salt stress showed that *GsDES1.1*, *GsDES1.2*, *GsFAD2.1* and *GsSLD1* in leaves and *GsSLD2*, *GsSLD5* and *GsSLD6* in roots were not closely related to salt stress response. Therefore, we explored the significant role of conserved, duplicated and neofunctionalized *FAD* in the domestication of soybean, which contributes to the importance of soybean as a global oil crop.

1. Introduction

Plant fatty acid desaturases (FADs) can be classified as: soluble FADs and membrane-bound FADs, with most of them being identified as the latter type [1–2]. Soluble desaturases with two conserved D/EXXH histidine boxes, and membrane-bound desaturases generally contain three histidine boxes, H(X)3-4H, H(X)2–3HH and H/Q(X)2–3HH [3]. These histidine boxes and iron ions possibly constitute the catalytic center of the desaturase [4]. The soluble FADs are mainly found in plant plastid, such as SAD/FAB2 [3]. The membrane-bound FADs exist in plant, animal and many other organism and located in plastids or in endoplasmic reticulum, such as FAD subfamilies including Omega-6-Fatty acid desaturases (FAD2 and FAD6), Omega-3-Fatty acid desaturases (FAD3, FAD7 and FAD8), Palmitate desaturase (FAD4), Palmitoyl-monogalactosyldiacyl-glycerol Delta-7-desaturase (FAD5), Delta-8-fatty acid desaturase (SLD1), Sphingolipid delta-4 desaturase (DES1), Zeta carotene desaturase (ZDS1) and Delta-7-sterol-C5(6)-desaturase (STE1) [5–6]. The membrane bound *FADs* can further be classified based on their roles into four different subfamilies by various roles, such as first, omega, front-end and sphingolipid desaturases [7–8,14]. To date, *FAD* genes have been identified and characterized in so many species, such as *Arachis hypogaea* [15], *Sesamum indicum* [9], *Gossypium hirsutum* [7], *Brassica napus* [2,8], and *Juglans regia* [30]. In soybean, members of the *FAD* gene family have been reported by Chi et al. [16] using the older version of *Glycine* max (http://www.phytozome.net/soybean) using whole-genome shotgun sequencing. Soybean is an important oil crop, and with the development of sequencing technology, the genome of *Glycine max* has been up to four versions (http://www.phytozome.net). The genomes of wild and cultured soybean species have been sequenced using new sequencing methods [17–18]. Transcriptome data for soybeans have been published [19–22]. However, there have been no reports on members of the *FAD* gene family identified by the updated genomic data till date.

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Plant FADs catalyze the desaturation of fatty acids in many ways and play significant roles in regulating the fatty acid composition and adjusting membrane fluidity under some abiotic stress [23–30], especially under salt stress [7–8,31]. Wang et al. [9] reported that overexpression of the *LeFAD3* in tomatoes dramatically increased resistance to salt stress. In *Arabidopsis*, *AtFAD2* and *AtFAD6* were induced under salinity stress [10–11]. In sunflower, expression levels of 8 FAD genes in roots could be obviously enhanced with the increase of salinity concentrations [8]. *FADS1* and *FADS2* encode rate-limiting enzymes in the metabolism of ω-3 and ω-6 fatty acids, respectively [12]. Reinprecht et al. [13] found that *FAD3* determines the linolenic acid level, which is related to the development of off-flavors and low stability in soybean oil.

Soybean was domesticated in China about 6000–9000 years ago [32]. Since bottlenecks and human selection, cultivated soybeans have much lower genetic diversity than their wild counterparts [33–34]. This reduced variation has potentially caused the loss of some genes involving in different environmental tolerance [35]. However, wild soybeans that have a high allelic diversity may therefore be a resource of genes for adapting to certain environmental conditions to be reintroduced into cultivated soybeans via breeding, and this can be done because there is no reproductive block between wild and domesticated soybeans [35]. Williams 82 is a cultivated soybean developed in the 1980s, it is landraces that used as progenitors for cultivars development in Korea [17,36]. "Zhonghuang 13" is a soybean cultivar bred by Chinese scientists in 2001, it is derived from cultivar accessions that exhibits high yield capacity and high stress tolerance [17]. The *Glycine soja* W05, a Chinese wild soybean accession, has a high salt tolerance in wild soybean [18]. So, comparative analysis of *FAD* gene families in these three soybean varieties can reveal the evolution process of *FAD* gene family in the domestication process of soybean will contribute to cultivated soybean breeding. Moreover, excessive dissolved salts in soils affect plant growth and crop yield [37]. There are 8% salt-affected soils of the world's total land area [38], and it is estimated that the area of agricultural land affected by salinization will double by 2050 [39]. Therefore, identification of the salt tolerant genes in wild soybean W05 will contribute to cultivated soybean salt tolerant breeding and maintaining sustainable production.

In this study, a total of 75 *FADs* were identified from the three soybean genomes, which were further divided into four subgroups by phylogenetic analysis. There was no evolutionary relationship among the four subgroups, but they all had a very conserved FAD desaturase domain, and the classes and distributions of conserved motifs were similar in the four subgroups. We also performed an analysis of chromosome localization, conserved domain, gene structure and promoter features. Twenty-three *FAD* genes from Chinese wild soybean used to elucidate their expression patterns in response to salt stress using published transcriptome data. We found some omega desaturase subfamilies of Chinese cultivated soybean to be much larger than expected and assumed that increasing neofunctionalization of the subfamily was due to different selection pressures during cultivation and domestication. This study aimed to lay the foundation to explore the functions of soybean *FAD* genes and new schemes to improve soybean quality.

2. Materials and methods

2.1. Identification of FAD proteins in three soybeans

The genome data of *Glycine soja* W05 (Chinese wild soybean), Gmax_ZH13 (Chinese cultivated soybean), and *Glycine max* var. Williams 82 (palaeopolyploid soybean) were downloaded from Genome Warehouse (GWH) database in BIG Data Center (http://www.wildsoydb.org/Gsoja_W05/download/, https://bigd.big.ac.cn/search?dbId=gwh&q=GWHAAEV00000000&page=1 (v2)) and Phytozome database (http://www.phytozome.net), respectively. The FAD protein sequences of rice from RGAP release 7 (http://rice.plantbiology.msu.edu/) and *Arabidopsis thaliana* from TAIR release 10 (http://www.arabidopsis.org) were used as query to conduct BLASTp and tBLASTn against the three soybean genomes with an E-value ≤ 1e −20 as the criterion. After removing the redundant sequences, the putative FAD protein sequences were submitted to NCBI Conserved Domains Database (https://www.ncbi.nlm.nih.gov/cdd/), Pfam database (http://pfam.xfam.org), SMART database (http://smart.embl.de/) and Protein Subcellular Localization Prediction Tool (PSORT, http://psort.hgc.jp/) for further verification.

2.2. Evolution analysis of FAD gene family in three soybeans

Full-length protein sequences of all FADs were used for multiple sequence alignment using Muscle software with default parameters. To detect the statistical reliability, we constructed a Maximum likelihood (ML) tree using MEGA7 with the 1000 bootstrap replicates [40].

2.3. Gene structural and motif analyses of FAD genes in three soybeans

To further clarify the structural variety of *FAD* genes, we predicted their coding sequences, the intron-exon structures using the online tool Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). The motifs among the *FAD* gene members were identified using the online program MEME (Multiple Expectation Maximization for Motif Elicitation) version 4.11.1 (http://meme-suite.org/index.html). Visualization of the conserved motifs in FADs was performed using the TBTools software [41].

2.4. Analysis of cis-acting element and location of FAD genes

Upstream sequences (1.5 kb) of the *FAD*-coding sequences were obtained from the genome sequences and then submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify regulatory elements [42]. The gene promoters of *FADs* were explored using the online program Gene Structure Display Server (GSDS) 2.0 (http://gsds.cbi.pku.edu.cn/) [43]. The chromosomal positions of the *FAD* genes were obtained from the soybean genome browser. Visualization was carried out using the TBTools software [41].

2.5. Analysis of expression pattern of FAD gene family from wild soybean under salt stresses

RNA-seq data of wild soybean W05 under salt stress were obtained from the NCBI database under the accession NO. SRP184868. To obtain a comprehensive understanding of the gene expression patterns of *FAD* genes under 0.9% NaCl stress, whole-genome RNA sequencing of the roots and leaves of the W05 seedlings was performed. Effect of time course was analyzed after treatment for six durations (0, 1, 2, 4, 24 and 48 h). Total RNA was extracted using RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions: 1. 600 mg pestled tissue added 5 ml Lysis buffer (added β-ME) and homogenized; 2. Transfer 1 ml of the homogenate to a microcentrifuge tube and add 100 μl of 20% sarkosyl, then incubate at 70 °C water bath for 10 min; 3. Pipet 650 μl of the lysate directly onto a QIAshredderTM Spin Column placed in a 2 ml collection tube, centrifuge for 2 min at maximum speed (14,000–18,000 ×*g*); 4. Pipet 250 μl of the flow-through to a new 1.5 ml microcentrifuge tube, add 225 μl ethanol (96–100%) and mix well by pipetting, then discard remaining flow-through; 5. Pipet sample directly onto an RNeasy Mini Spin Column placed in a 2 ml collection tube and centrifuge for 45 s at ≥8000 ×*g* (≥10,000 rpm); 6. Add 500 μl Buffer RPE and centrifuge for 15 s at ≥8000 ×*g* (≥10,000 rpm) to wash the spin column membrane, discard the flow-through; 7.Repeate the step 6; 8. Add 30–50 μl RNAse-free water directly and centrifuge for 1 min at ≥8000 ×*g* (≥10,000 rpm) to elute the RNA. Followed by fragmentation and size selection, RNA was sequenced using the Illumina Hiseq 2000 platform to generate 180 bp paired-end reads. Hisat2 software was used to map

the reads against the *Glycine soja*: W05 genome. The FPKM value was log10 transformed. The average value was used to normalize the expression level of *FAD* genes between the roots and leaves of Chinese wild soybeans under different duration of salt stress. The heatmap packages (https://cran.r-project.org/web/packages/pheatmap) of R were used to draw the expression heatmap and determine the correlation between samples and genes.

2.6. Plant materials, growth conditions and abiotic stress treatments

Chinese cultivated soybean were used to analyze *FAD* gene expression under different abiotic stresses. It was selected based on their responses to abiotic stresses in our previous results of *cis*-acting elements prediction. Seeds were soaked overnight in water at room temperature and then sown in pots (16.5 cm diameter x 11 cm tall) containing a potting soil composed of a mixture of peat, perlite, and vermiculite (PRO-MIX BX, Premier Tech Horticulture) at a depth of approximately 2 cm. Seedlings were grown and watered regularly in a plant growth chamber with 18 h light/6 h dark at 22 °C. Lighting was 175 to 225 μmole m^{-2} s^{-1}, the temperature was maintained at 28 °C, and the relative humidity was approximately 55–60%. The nutrient solution was renewed every 7 days. At the 15th day irrigation was suspended to initiate the water deficit treatment: control plants (CK) were maintained at 70% and the stressed-plants (drought) were monitored periodically until soil field capacity reach 30–40%; salt treatment: the solutions were supplemented with NaCL (100 mM), according to the methods of Xu et al. [4] with some modification. And the heat and cold treatments were maintained temperature at 38 °C and 4 °C, respectively. After 24 h of treatment, the leaves of seedlings were harvested, immediately frozen with liquid nitrogen, and stored at −80 °C prior to RNA isolation. Each treatment was replicated three times.

2.7. FAD gene expression analyses under different abiotic stresses

The *FAD* genes were designed for expression profile analysis using qRT-PCR. For all samples, total RNA was isolated using the Takara Mini BEST Universal RNA Extraction Kit (Takara Co. Ltd) according to the manufacturer's protocol. The RNA concentration and quality were determined by a Nanodrop 2000 Spectrophotometer. For cDNA synthesis, 1 μg RNA was used along with PrimeScript RT Master Mix (Perfect Real Time, Takara Co. Ltd). Gene expression levels were quantified by the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher) and SYBR® Fast qPCR Mix (Takara Co. Ltd). The primer sequences used for qRT-PCR are listed in Table 2. The qRT-PCR reaction conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 30 s. The default setting was used for the melting curve stage. Three biological replicates were conducted for each selected sample. The expression profile bar charts were generated using Prism 8 software with average linkage clustering using the ΔΔCt values. The $2-\Delta\Delta Ct$ values were calculated as the relative expression levels according to the method described by Livak et al. [66] and the *Actin* was selected as the reference gene [67].

Table 2
Primers of soybean genes used for the qRT-PCR analysis.

Gene	PCR primer sequences
DES1.1	CACAACCTGGCCTTCTCAAC GACGGACTTCAGTGAGGCTA
DES1.2	CACAACCTGGCCTTCTCTAC CACAACCTGGCCTTCTCAAC
FAD2.2	ACTGATGTTCCTCCTGCCAA AGGCAGAAGGCTATGGTGAG
FAD2.3	TGCTAATGCACAAGACGCAA GTTGCAGCTCAAGTTCCCAA
FAD7.1	TTTAAGAGGTGGCCTCACCA CGCAGATCTTTCTGGCTCAC
FAD7.2	ACAACTGGCTTCTCTGGCTA TGATGGTGAGTTCTGTGGCT
FAD8.1	AAAGCATTGCTGGGTGAAGG AGCTCCCATGACCACAATCA
FAD8.2	CCAGCAGTGACTTGTTCGTT ATGGCCATGATGGTGCAAAT
FAD8.3	TTCTGGGCCCTCTTTGTTCT GCAATGGGTGCCAAGATTCA
FAD8.4	AAGTGGGAATCGGGAGTGAG GGACTTCCAAGGGTCCTTCA
SLD4	TGGGTTTGGTTCCCTCTGTT GTCCAATGTGCCACCAGTTT
SLD5	GGTGGAGCACCATCTGTTTC CGGCTTGGAACCATCTCTTG
SLD6	GGCCATTACAACGTGATGCT ACGACGAAGAAGGGCAAATG
SLD7	CGCAGCAGATGTGTATGAGG CTTGGGAGCCTAGGGAACAA
PAP26	ATGTGGTTGGCTTCCTTTCG AGCTAGTGATCCCTGCACTC
SAP13	TCGATGCGACCAGGTGTATT GCACTCGGTGTTGACATGAT
SZF2	ACCATTTCCGCATGTTCGAG GTCACCCTTCTTGCAACTCC
ADC	GCAGGAGGAGGAGGTTGATT AAGAATCTGAGCGGTGGTCA
bZIP17	GTGGTTCCGTGAAGGTCTTG GGGAGCTCATGAAGGGTTCT
CPK12	TCTGCAAGGAGGACTACGAC TAGTGTCCCTTCCGCACAAT
CYP86B1	GCAAGGTGGTGAGTCAAAGG CTTCAGCACCGTTCCATCTG
MYB70	AGCCAAGAAGCAAGTGCAAA TGGAGGCTGAGGTTCATTGT
GB3	GAGCAGCATTGACAGCAAGA CAACCAGCTGCTACAGCATT
NFYA5	AGCATCCACCAGAATTGCAT CCCTAACCCTGTTCAATGCG
AHP3	CAACTGCAGAGGCAACTTGT AATGCTTGGCTGGCCTAGTG
Hsp17.6A	SGCACGTGTTCAAGGCTGATA AGAACTTACCACTGCTACGC
BSD2	TTGTGAGGGAAATGGTGCAA AGAAAGCATGGTGTGGTAGC
Hsp23.5	GTCCTCTCTCATTGCGAAGC GATCGATGTCGGTGCTGTG
Hsf70B	ACTGTCCCTGCGTACTTCAA ACCACCACCAAGGTCAAAGA
Actin	GTGCACAATTGATGGACCAG GCACCACCGGAGAGAAAATA

3. Results

3.1. Identification of FAD genes in three soybeans

In total, 23, 29 and 23 *FAD* genes were identified in *Glycine soja* W05 (Chinese wild soybean), Gmax_ZH13 (Chinese cultivated soybean), and *Glycine max* var. Williams 82 (paleopolyploid soybean) using BLASTP method, respectively, using the BLASTP method. Here, *GmFADs*, *GsFADs* and *GwFADs* represent the *FAD* gene family members in paleopolyploid soybean, Chinese wild soybean and Chinese cultivated soybean, respectively. The members of the *FAD* gene family were named *ADSs*, *DESs*, *SLDs* and *FADs*, accordingly. However, there were 10 *FAD* genes rice and 17 *FAD* genes in *A. thaliana*, respectively. The length of the identified soybean *FAD* genes ranged from 1844 bp (*GmADS1*) to 6287 bp (*GmFAD2.2*), and the length of their proteins ranged from 319 (GsFAD2.2) to 455 amino acids (GsFAD8.1-GsFAD8.3 and GmFAD8.1-GmFAD8.3). Multiple alignment analysis was performed for FAD domain in the three soybeans to describe homologous domain sequences and the major amino acids within the FAD domain.

3.2. Phylogenetic analysis of the FAD gene family members in three soybeans, A. thaliana, and rice

We generated an unrooted phylogenetic tree based on full protein sequence alignment of FADs by the Maximum likelihood method to forecast the evolutionary relationships among *FAD* genes in the three varieties. However, 11 and 6 *FAD* genes were identified in *A. thaliana* and rice, respectively. As Fig. 1 shows, all desaturases analyzed in this study were divided into four subfamilies (first, omega, front-end, and sphingolipid), revealing that all came from a common ancestor before the divergence of the three soybeans.

The first desaturase subfamily contains nine ADS proteins, comprising five members from soybeans and four from *A. thaliana*. The omega desaturase subfamily had 40 proteins, including 30 GsFADs, 30 GmFADs, 19 GwFADs, 5 OsFADs, and 4 AtFADs. The front-end desaturase subfamily comprised 21 proteins, including 6 GsFADs, 6 GmFADs, 7 GwFADs, 2 OsFADs and no AtFAD. There were only eight DES proteins of soybean in the sphingolipid desaturase subfamily, one from *A. thaliana* and one from rice.

Concentrating on the analysis of soybean varieties, the phylogenetic tree (Fig. 2) illustrated that the *FAD* genes of *G. soja* W05, Gmax_ZH13, and *G. max* var. Williams 82 were clustered into four subfamilies. Most all three varieties of these subfamilies possessed *FAD* genes, indicating that they had a common ancestor before their divergence. However, there were differences in the number of members among the three varieties, with 23 members in *G. soja* W05, 23 in Williams 82, and 29 in *Gmax*_ZH13. There were 19 members of the front-end desaturase subfamily encoded by SLD genes, of which six were from *G. soja* W05, six from *G. max* var. Williams 82, and seven from Gmax_ZH13. There were

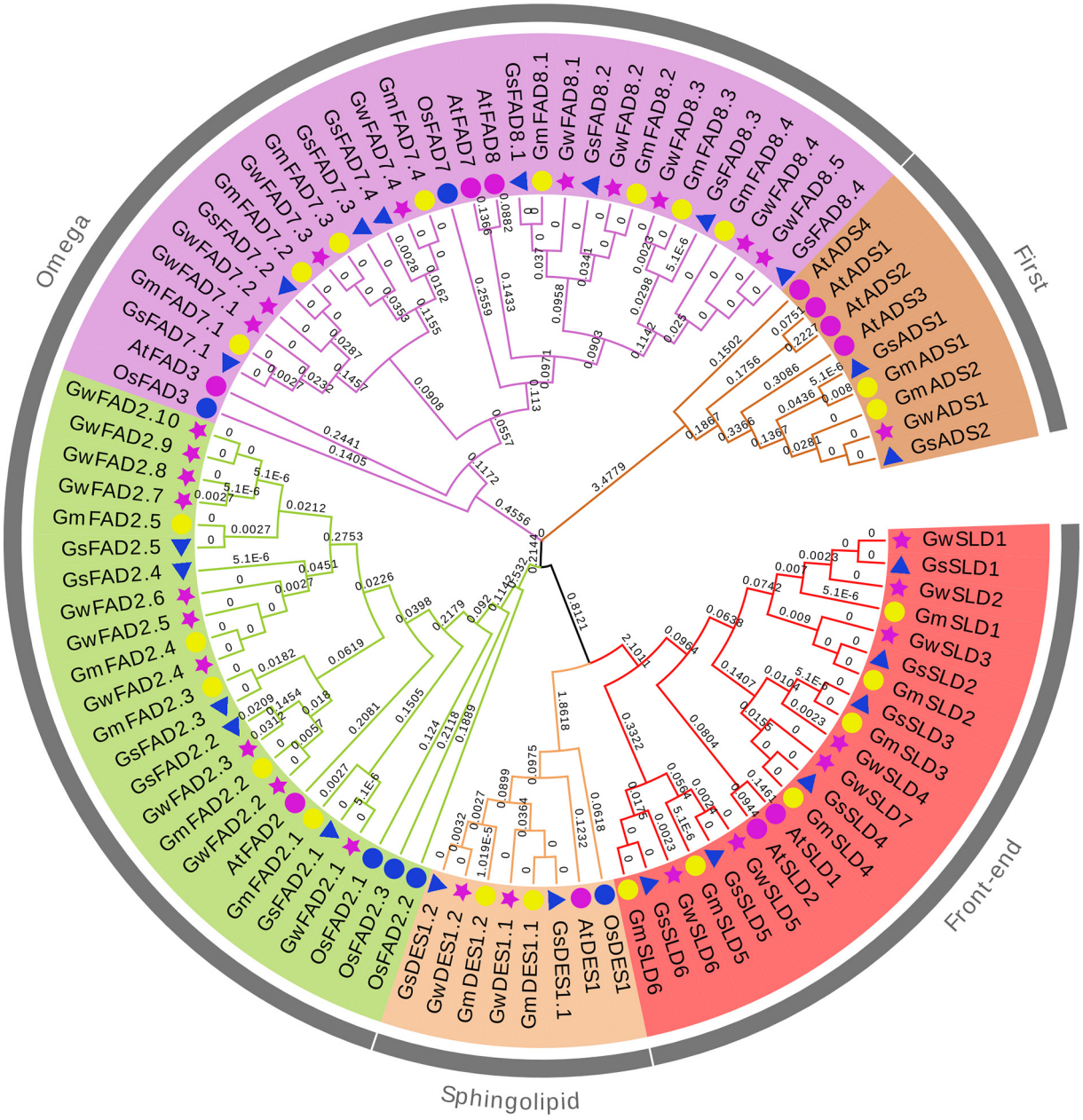


Fig. 1. Phylogenetic analysis of FAD proteins from *Arabidopsis*, rice and soybean. Maximum likelihood phylogeny of FAD proteins of three varieties was measured using MEGA7.0 program with bootstrap test (replicated 1000 times). The phylogenetic tree was constructed by the full-length protein sequences of FAD in *Arabidopsis*, rice and three soybean varieties (*Glycine soja* W05 (Chinese wild soybean), Gmax_ZH13 (Chinese cultivated soybean), and *Glycine* max var. (palaeopolyploid soybean)). And the *FAD* genes were marked purple circle, blue circle, blue triangle, purple star and yellow circle, respectively.

45 omega desaturase subfamily *FAD* genes, of which 13 homologs were from W05 and Williams 82 each, and 19 from Gmax_ZH13. In contrast, there were only two Sphingolipid subfamily proteins, named DES, from each of the three soybeans. There were five *ADS* genes in first desaturase subfamily: W05 and Williams 82 contained two members each, while *Gmax*_ZH13 only had one. These results indicated that there were abundant genetic variations in *FAD* gene family among the three soybean varieties.

3.3. Conserved motifs and gene structure analysis of FAD genes in three soybeans

To gain deeper understanding of the structural variation of *FADs*, the structures of the *FAD* genes were explored. As shown in Fig. 3C, 75 soybean *FAD* genes were grouped into three subfamilies, and similar exon-intron structural patterns were found. In the omega desaturase subfamily, many members of the *FAD* gene family contained relatively complex gene structures, with eight exons of *FAD7* and *FAD8* each. However, *GwFAD2.3* and *GsFAD2.2* contained three exons, while *GwFAD2.9* harbored two exons, and the other *FAD2s* genes contained only one exon. These results indicate that the gene structure of this gene subfamily is variable. In the *SLD* cluster, all of the genes had only one exon, while *GwSLD5* and *GsFAD5* possessed two exons. In the *ADS* cluster, each member had five exons and four introns (Fig. 3A). In the *DES* cluster, each member had two exons with only one intron. Interestingly, the exons and introns in genes of these two subfamilies were of the same length, which showed that the structure of these genes was relatively conserved in soybeans.

A structure map of all FAD proteins was visualized using MEME motif analysis results. As shown in Fig. 3B, FA desaturase of a fatty acid

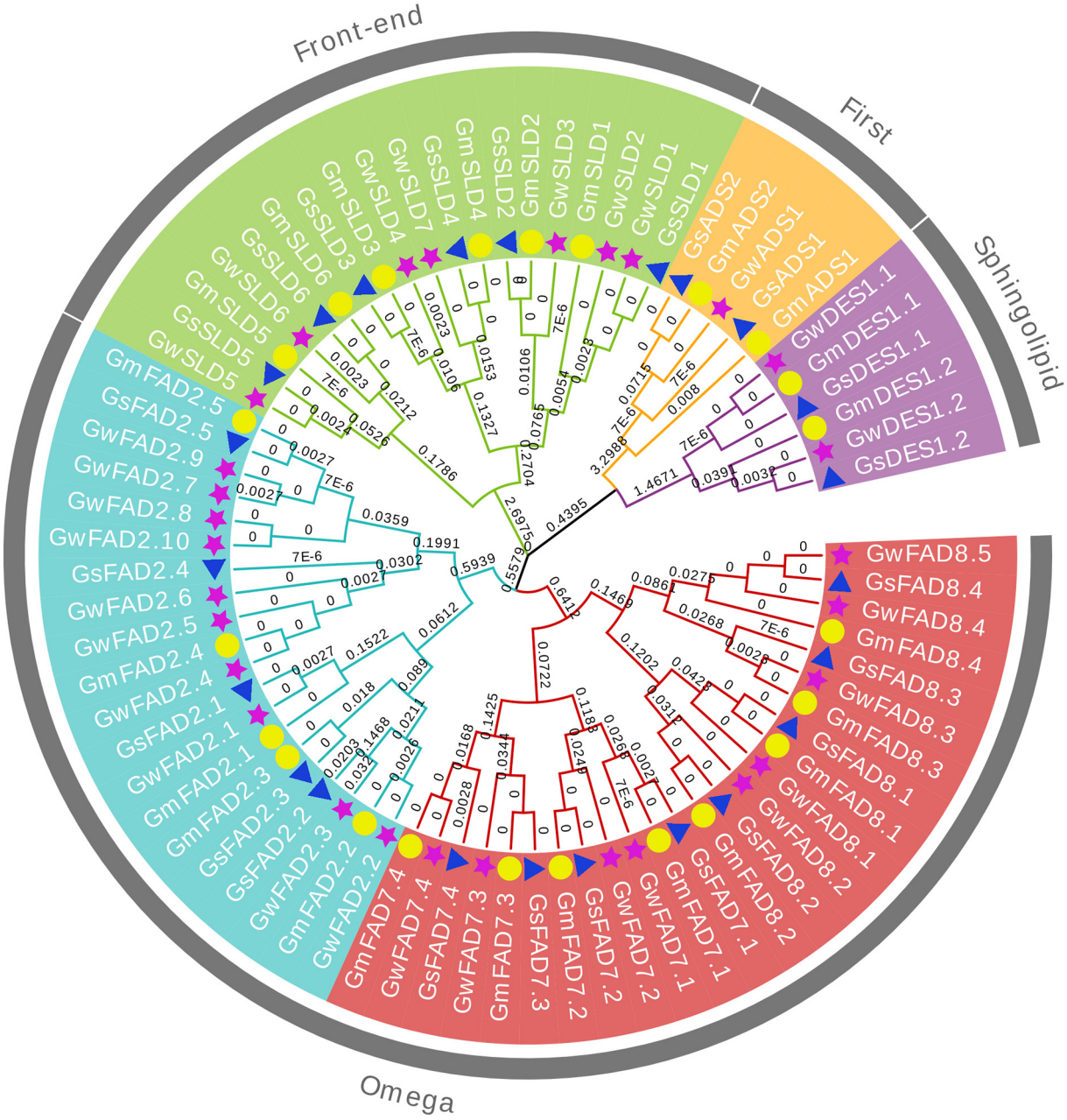


Fig. 2. Phylogenetic analysis of *FAD* genes from three soybeans. Maximum likelihood phylogeny of FAD proteins of three soybeans was carried out by MEGA7.0 program with bootstrap test (replicated 1000 times). The phylogenetic tree was drew based on the full-length protein sequences of FAD. The *Arabidopsis*, rice and three soybean varieties (*Glycine soja* W05 (Chinese wild soybean), Gmax_ZH13 (Chinese cultivated soybean), and *Glycine max* var. (palaeopolyploid soybean)). And the *FAD* gene families were marked blue triangle, purple star and yellow circle, respectively.

desaturase were widely distributed other than motif 1, *FAD* members from the same groups preferred to share a similar motif composition model. For example, motifs 6 and 10 were unique to the front-end subfamily, whereas motifs 2, 3, 4, 5, and 7 were specific to the omega group. Moreover, motif 2 represented the FA desaturase of a fatty acid desaturase, while motifs 3 and 5 had no hits in the pfam database (http://pfam.xfam.org). Motif 4 was a domain of unknown function (DUF3474). Motif 7 belonged to the cellulose synt family of cellulose synthase (CesA) proteins, which are integral membrane proteins with several highly conserved residues containing some motifs that are essential to glycosyltransferase activity [44]. The proteins clustered into the subgroups, such as GmFAD8.1, GwFAD8.1, GsFAD8.1, GmFAD8.2, GwFAD8.2 and GsFAD8.2, showed highly similar motif distributions. Similar motif ranks among FAD proteins in subgroups show that the protein composition is conserved within a specific subfamily. Briefly, the conserved motifs and similar gene structures of the *FAD* members were more easily to be clustered into the same group. Together with the phylogenetic analysis results, these results strongly support the reliability of the group classifications.

3.4. cis-Elements in FAD promoters

To understand the potential function of *FAD* in abiotic stress responses, 1.5-kb upstream sequences of *FAD* genes were used to analyze *cis*-elements by PlantCARE. To understand the main function of members of the gene family and explore regulatory factors of the fatty acid

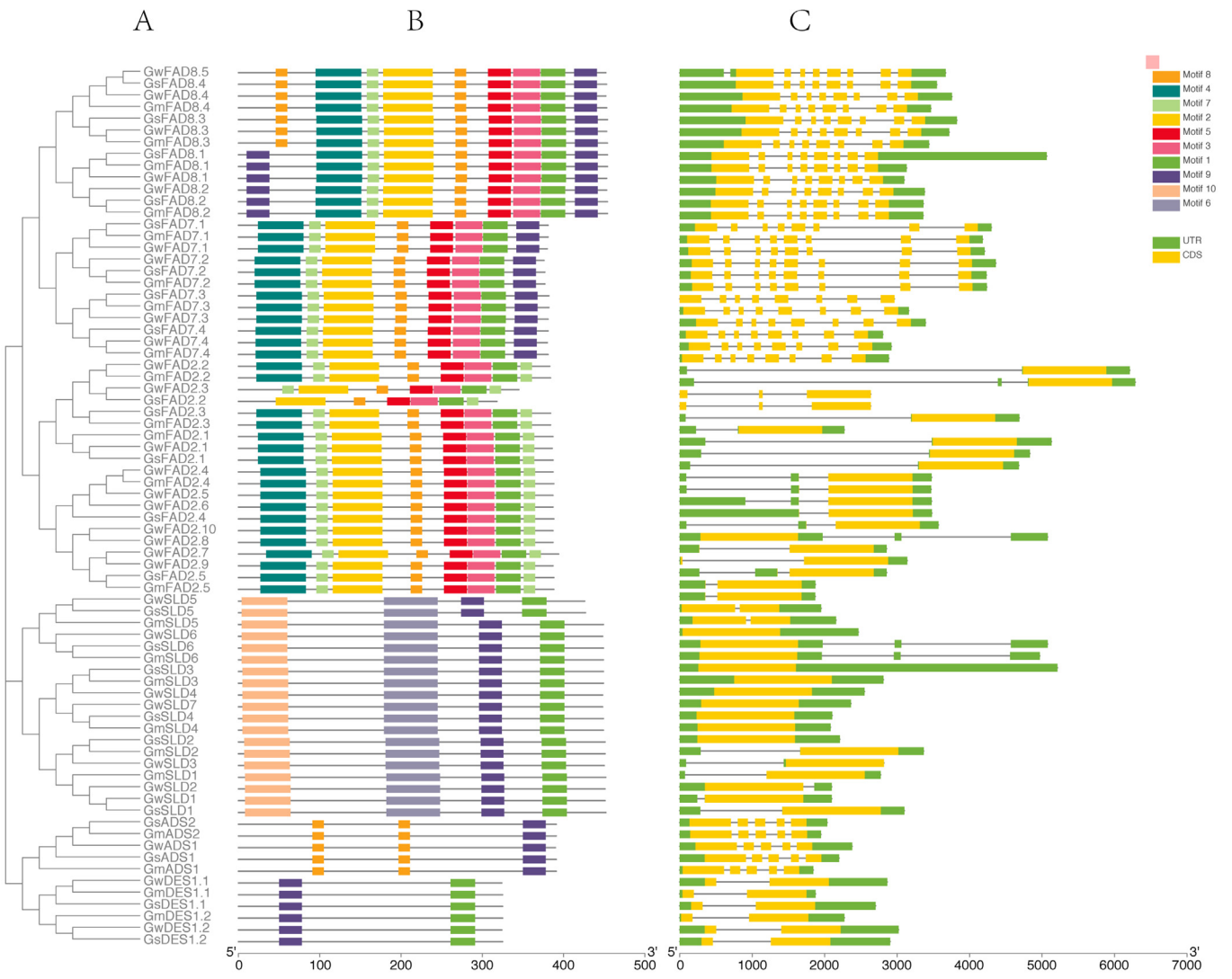


Fig. 3. Phylogenetic relationships, conserved motifs of FAD proteins and gene structures of soybean *FAD* genes. (A) Phylogenetic tree of FAD proteins in three soybeans. The unrooted Maximum likelihood phylogenetic tree was contructed with MEGA7 software using the all amino acid sequences of FAD proteins. (B) Exon and intron formation of *FAD* genes. Yellow boxes represented exons and black lined of the identical length mean introns. The up and down stream sequences of *FAD* genes were shown by green boxes. The sizes of exons could be estimated by the scale at bottom. (C) Arrangements of conserved motifs in FAD proteins of soybeans. Teen predicted motifs were shown by diverse colored boxes, and motif sizes were meant by the scale at bottom. For motif detailed information refered to Table 1.

synthesis pathway in soybeans, we selected nine *cis*-elements that were present in several gene members. Motifs of box4, G-box and GT1-motif were all the cis-acting regulatory elements involved in light responsiveness (Table 1). As Fig. 4 shows, there was at least one of the three light response *cis*-acting elements in the upstream of each soybean *FAD* gene. In addition to *GmDES1.1*, *GsDES1.1* and *GwDES1.1*, the box4 was distributed in the upstream of all *FAD* genes of three soybeans. The absence of the promoter sequence of the box4 in *DES1.1* gene of the three soybeans

Table 1
The main *cis*-elements in *FAD* promoters of three soybeans.

No.	Name	Sequence	Function
1	TGA-element	AACGAC	Auxin-responsive element
2	TC-rich repeats	ATTCTCTAAC	*cis*-Acting element related to defense and stress responsiveness
3	LTR	CCGAAA	*cis*-Acting element involved in low-temperature responsiveness
4	TCA-element	CCATCTTTTT	*cis*-Acting element related to salicylic acid responsiveness
5	ABRE	ACGTG	*cis*-Acting element was about the abscisic acid responsiveness
6	ARE	AAACCA	*cis*-Acting regulatory element essential for the anaerobic induction
7	circadian	CAAAGATATC	*cis*-Acting regulatory element related to circadian control
8	TGACG-motif	TGACG	*cis*-Acting regulatory element was about the MeJA-responsiveness
9	P-box	CCTTTTG	Gibberellin-responsive element
10	Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness
11	G-Box	CACGTT	*cis*-Acting regulatory element was about light responsiveness
12	GT1-motif	GGTTAAT	Light responsive element

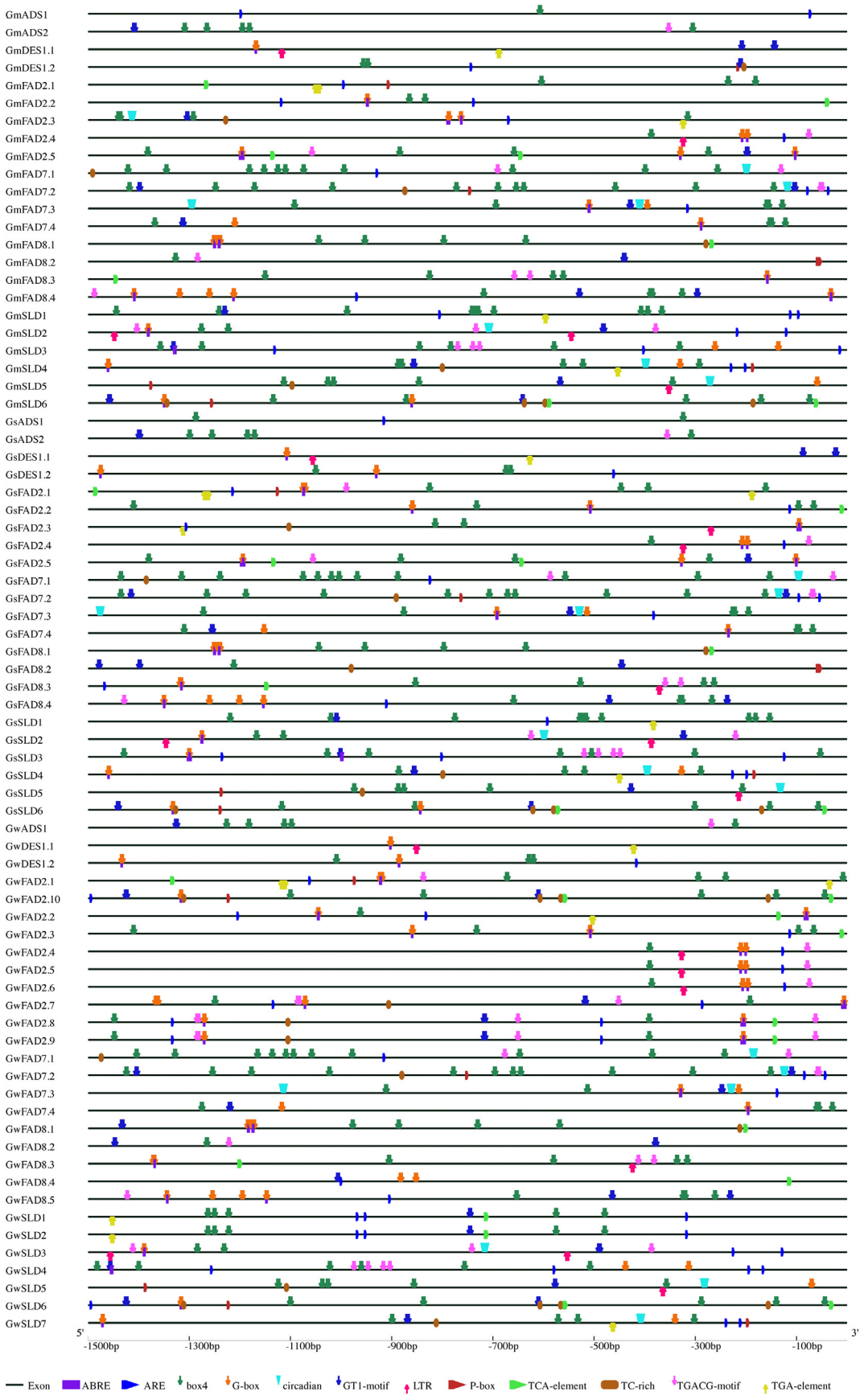


Fig. 4. *Cis*-elements of *FAD* promoters. Promoter sequences (−1500 bp) of *FAD* genes (promoter regions of *GwFAD2.9* were absent) are speculated on PlantCARE. The upstream length to the translation started site can be deduced on the basis of the scale at the bottom.

indicates that the *DES1.1* gene was not as sensitive as the other *FAD* genes of the three soybeans in the light-responsive process. Interestingly, in promoter sequences of the 75 *FAD* genes, the number and distribution sites of *cis*-acting elements related to light response were the most common *cis*-elements related to environmental response. In addition to the motifs of the box4, G-box and GT1-motif, other light-response elements were detected, including ACE, AE-box, ATC-motif, ATCT-motif, Box II, chs-CMA1a, GA-motif, Gap-box, GATA-motif, I-box, LAMP-element, LS7, MRE, P-box, TCCC-motif, and TCT-motif. This study indicates that light has a very important effect on soybean fatty acid desaturase accumulation. As described in Table 1, TGA-element, TCA-element, ABRE, TGACG-motif and P-box were the *cis*-acting regulatory elements involved in plant hormone responsiveness, responding to auxin, salicylic acid, abscisic acid (ABA), methyl jasmonate (MeJA), and gibberellin, respectively. They exist in different subfamilies and soybean varieties. In addition to *GmFAD2.1*, *GmFAD7.1*, *GmFAD7.2*, *GmFAD8.2*, *GsFAD7.1*, *GsFAD7.2*, *GsFAD8.2*, *GwFAD7.1*, *GwFAD7.2* and *GwFAD8.4*, all members of the omega subfamily had ABRE functional elements (Fig. 4). However, there was no ABRE motif found in any members of the first desaturase subfamily in the three soybeans. In the sphingolipid desaturase subfamily, except for *GmDES1.2,* other members presented at least one ABRE motif on the upstream sequences. In the front-end desaturase subfamily, except for the *GmSLD1*, *GmSLD5*, *GsSLD1*, *GwSLD1*, *GwSLD2*, and *GwSLD5*, the other members had at least one ABRE motif. This result revealed that ABA plays an important role in the expression of the members of the *FAD* gene family in soybean. However, it could not affect the expression of the genes in sphingolipid desaturase subfamily. Their distribution sites in each subfamily of the three soybean varieties were very similar, indicating that it was a relatively conservative *cis*-regulatory element. TC-rich is a *cis*-acting element involved in defense and stress responsiveness; there were promoter sequences of *GmDES1.2*, *GmFAD2.3*, *GmFAD7.1*, *GmFAD7.2*, *GmFAD8.1*, *GmSLD4*, *GmSLD5*, *GsFAD2.3*, *GsFAD7.1*, *GsFAD7.2*, *GsFAD8.1*, *GsFAD8.2*, *GsSLD4*, *GsSLD5*, *GwFAD2.7*, *GwFAD2.8*, *GwFAD2.9*, *GwFAD7.2*, *GwFAD8.1*, *GwSLD5* and *GwSLD7*, and four promoter sequences of *GmSLD6*, *GsSLD6*, *GwFAD2.10*, and *GwSLD6*. The results showed that in sphingolipid desaturase subfamily, the TC-rich element was lost in the two Chinese soybeans. This result might be due to geographical isolation, causing differences in the existence of TC-rich element. However, in the omega desaturase subfamily, only *GwFAD2.10* had more than one site of TC-rich elements, which revealed that the TC-rich element in the omega desaturase subfamily was subjected to large selection pressure in Chinese cultured soybean during breeding selection. In addition, the other three *cis*-acting elements involved in abiotic stress responses were also different in each gene subfamily of the three soybeans. This would be the result of long-term natural selection, which made the plants more adaptive to their environment.

3.5. Chromosomal location and duplication events of soybean FAD genes

Chromosome mapping of 75 soybean *FAD* genes revealed their locations on 20 chromosomes, except for Chr04, Chr05, Chr06, Chr12, and Chr16, which had no *FAD* genes (Fig. 5). *FAD* genes were distributed unevenly among the 15 chromosomes. Interestingly, the distribution of *FAD* genes among the three soybean varieties was similar. As shown in Fig. 5, in three soybean species, chromosomes Chr08, Chr09, Chr14, and Chr18 had the same number of *FAD* genes. Moreover, the four chromosomes of each harbored two, one, two and three *FAD* genes in the three soybean species, respectively. Chromosomes 10 and 20 each had three *GwFAD* genes, but each contained only one *GmFAD* and *GsFAD* genes. All these genes were belonged to the same cluster of the omega subfamily. These genes were new genes produced during soybean cultivation and domestication. The new genes are generated by replication during domestication because of their close distribution on chromosomes. *GmSLD2*, *GsSLD3*, *GwSLD3*, *GsFAD8.4*, *GmFAD8.4*, *GwFAD8.4* and *GwFAD8.5* belonged to chromosome 01. The *FAD8s* had an expansion in Chinese cultivated soybean, which was added to one. The positions of the two *GwFAD8*s were very close, indicating that the two genes should be produced through duplication during soybean cultivation and domestication. On the contrary, on chromosome 03, the number of *ADS* genes in Chinese cultivated soybean was lower than that in wild soybean and ancient polyploid soybean, and the number of genes in this subfamily was also deleted in Chinese cultivated soybean, which indicated that *SLD* genes on this chromosome were lost due to negative selection during cultivation and domestication.

3.6. Expression profiles of FAD genes in wild soybean under salinity stresses

The expression patterns of all 23 *GsFAD* genes in the transcriptome data, which was came from different salinity treatment durations of wild soybean leaves and roots, are shown in Fig. 6A and Fig. 7. Among 23 *GsFAD* genes. *GsFAD2.4* was not detected in any of the samples, which might be indicate a pseudogene or had special temporal and spatial expression patterns that were not examined in our libraries. Some genes exhibited preferential expression in the detected tissues. One gene (*GsFAD2.5*) showed highly transcript abundances in leaves, but not in roots. The expression of some genes showed significant trends during different treatments. The expression of most *FAD* genes shown increased in the roots and leaves of soybean after salt treatment, while less *FAD* transcript levels were downregulated in the roots and leaves. In leaves, *GsFAD8.2* showed induced transcript levels as salt treatment duration increased, while *GsADS1*, *GsADS2*, *GsFAD2.3*, *GsFAD7.2* and *GsFAD8.4* gene transcript levels in roots were dramatically decreased with an increase in treatment duration. These results indicated that *FAD* gene transcripts tend to be induced in the roots and leaves of soybean after salt treatment, suggesting that *FAD* genes play a key role in salt stress tolerance in soybean. Interestingly, *GsSLD1* and *GsSLD2* gene transcript levels were markedly upregulated after 1 and 2 h salt treatment in roots. These results illustrated that appropriate time and concentration of salt stress can induce the expression of *GsFAD* genes and promote fatty acid biosynthesis in soybeans. However, the expression of certain genes, such as *GsDES1.1*, *GsDES1.2*, *GsFAD2.1*, and *GsSLD1* in leaves after different treatment times and that of certain genes, such as *GsSLD2*, *GsSLD5*, and *GsSLD6* in roots after different treatment periods hardly changed. This indicated that they were not closely related to the salt stress response in soybeans.

To further explore the relationship between samples and *FAD* gene expression patterns under different salt stress durations, we analyzed correlations among the samples (Fig. 6B) and *FAD* gene expression patterns (Fig. 6C). A high positive correlation between samples (Fig. 6B) of the 24 and 48 h treatment time in both roots and leaves was observed. In roots, there was a high positive correlation among the samples treated with salt for 1 and 2 h. The control and samples treated with salt for 24 and 48 h were clustered into the same group. However, in leaves, the correlation between the samples treated for 1 and 2 h was higher, while that between the control group and the samples treated with salt for 4 h was lower. Notably, the correlation between the control and salt-treated samples for 1, 2, 4, 12 and 24 h gradually decreases in leaves. In roots, the correlation between the control group and samples with longer salt treatments (1, 2 and 4 h) was lower, but the correlation between the control group and salt-treated samples for 24 and 48 h was higher. This indicated that the difference in of gene expression patterns in soybean leaves increased with increasing treatment time. In roots, the difference in *FAD* gene expression patterns was larger under short-term treatment, but it was not smaller under long-term treatment. This suggested that the roots of soybean were more sensitive to salt stress than leaves by changing the expression patterns of *FAD* genes in a short time. While it was relatively slow in the leaves, the

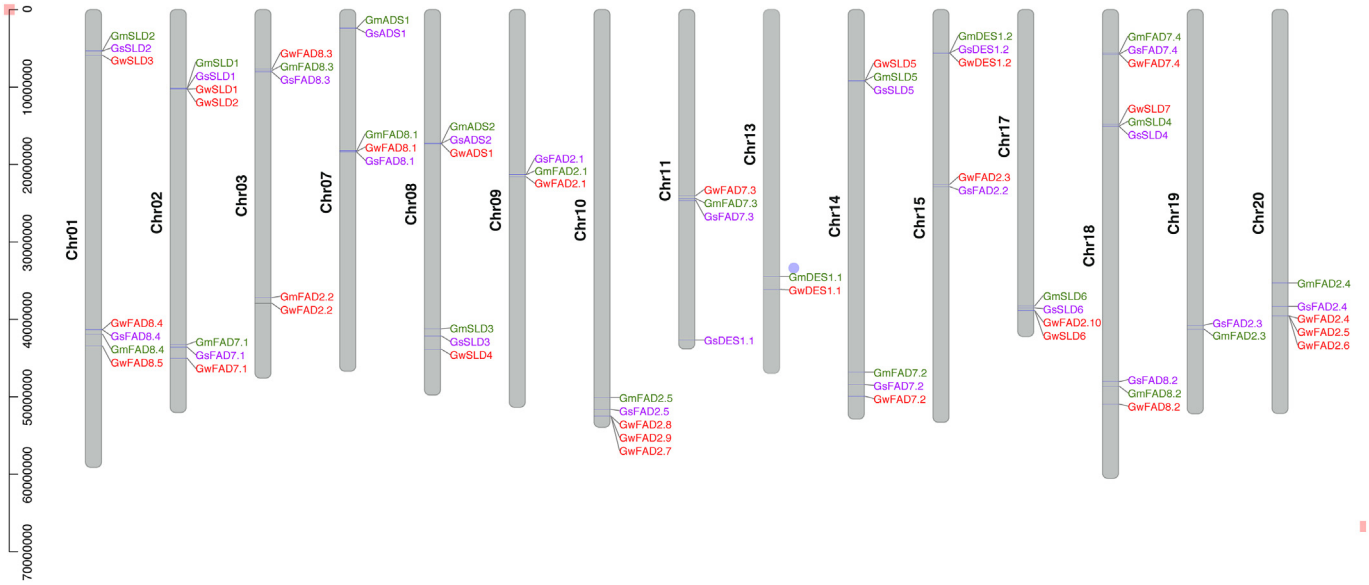


Fig. 5. Chromosomal distribution of *FAD* genes of soybean. The scale bar on the left meant the length (bp) of soybean chromosomes. The length of each chromosome was the longest of the three soybean genomes. The label of red, purple and green are represented the of the *FAD* genes in Gmax_ZH13, *Glycine soja* W05 and *Glycine max* var. Williams 82, respectively.

expression mode of *FAD* genes responding to salt stress changed more obviously over time. There were three groups based on the correlation of gene expression (Fig. 6C). The expression of *GsSLD2*, *GsSLD3*, *GsSLD5*, *GsSLD6*, *GsFAD2.2*, *GsFAD2.3*, *GsFAD2.5*, *GsFAD7.1*, *GsFAD7.4*, *GsFAD8.3*, *GsFAD8.4*, *GsADS1*, and *GsADS2* genes were significantly positively correlated. The genes among *GsDES1.2*, *GsDES1.1*, *GsSLD1*, and *GsFAD7.3* had the same expression relationship, indicating that they had similar functions in response to salt stress in soybean. However, they were negatively correlated with *FAD* genes in the other cluster. Interestingly, the expression was negatively correlated, which indicated that their expression in response to salt stress in soybean was mutually inhibited and may have opposite functions. Only two genes (*GsFAD7.2*, *GsSLD4*) had no significant relationship with the expression of other genes, indicating that they have a weak relationship with the expression patterns of other genes in response to salt stress.

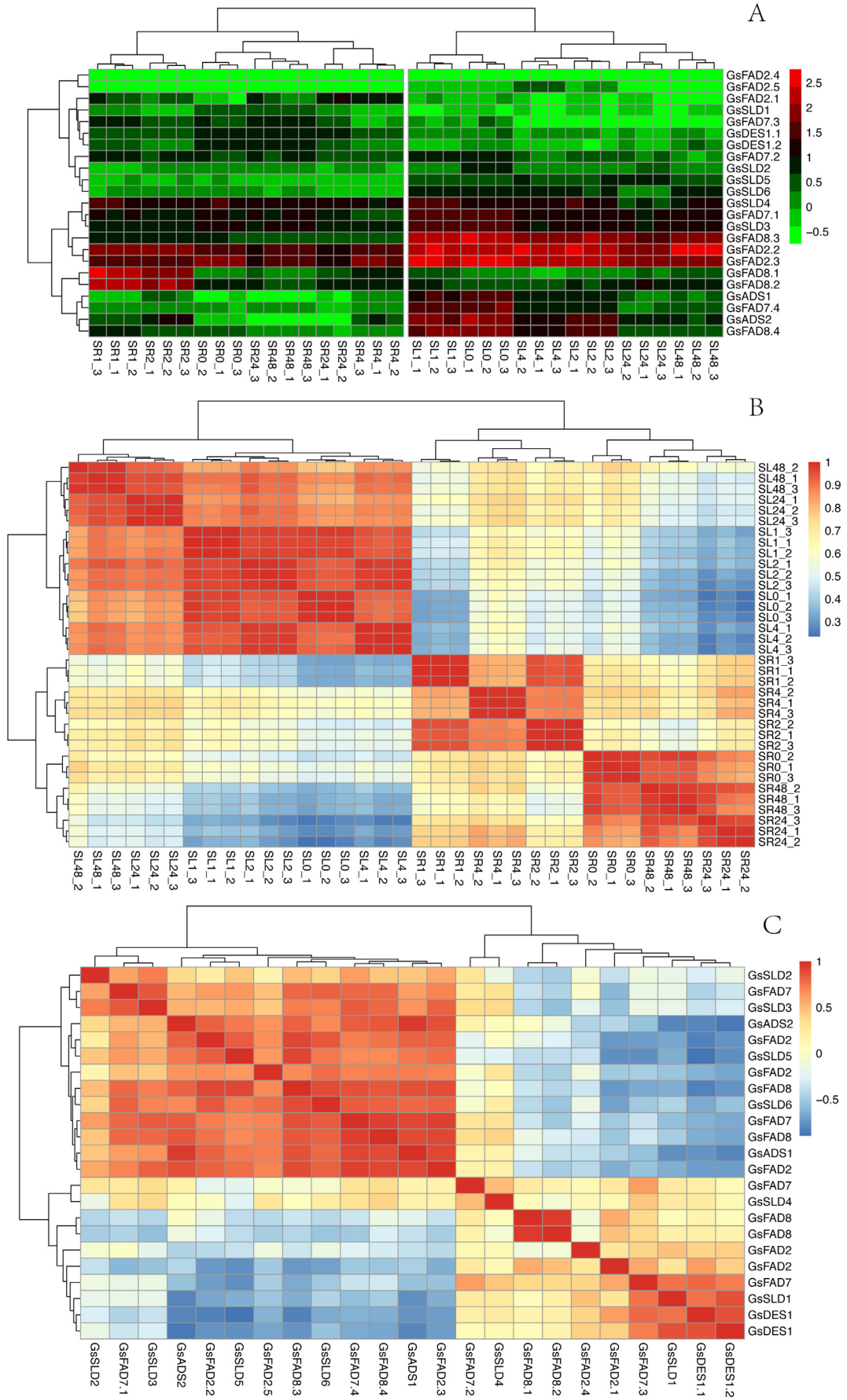
3.7. Expression of FAD genes altered under salinity, drought, cold and heat stresses

In this study, the expression of 12 *FAD* genes and 8 key salt responsive genes was significantly altered under the salinity stress (Fig. 8A). The greatest increase in expression was detected for *DES1.2*, *FAD8.1*, *SZF2* and *PAP26* under 100 mM NaCl treatment. *DES1.1*, *FAD2–3*, *FAD7–2*, *FAD8.2–4*, *SLD1–2*, *WRKY9*, *SAP13*, *ADC2*, *bZIP17*, *CPK12* and *CYP86B1* transcript levels were down-regulated with a spike of expression in the leaves. Drought application significantly decreased the expression of 4 *FAD* genes (*FAD2.2–3*, *FAD8.4* and *SLD6*) and 2 key drought responsive genes (*NFYA5* and *AHP5*) but upregulated the expression of *FAD7.1*, *FAD8.1–2*, *MYB70*, *CBF3* and *AHP3* in leaves as compared to the control treatment (Fig. 8B). The transcripts of 5 *FAD* genes (*FAD7.2*, *FAD8.1*, *FAD8.4* and *SLD4–5*) and one cold responsive genes (*COR15a*), exhibited an obvious increase with the cold treatment, while *DES1.2*, *SLD5*, *CBF1* and *ICE2* had a different expression pattern as compared to the control treatment (Fig. 8C). Further, heat stress increased the expression level of 4 *FAD* genes (*FAD2.2–3* and *FAD8.1–2*) and 5 genes associated with heat response (*Hsf17.6A*, *J3*, *Hsp23.5*, *Hsf70B* and *HsfA3*) (Fig. 8D). However, *SLD4*, *SLD7* and *BSD2* had the opposite expression pattern. These results indicated that *FAD* gene transcripts were mainly induced in the leaves of soybean after salt or drought treatment. The expression levels of most *FAD* genes were reduced under heat or cold stress. And their expression patterns were consistent with most of the key abiotic response genes we detected, suggesting that *FAD* genes play a crucial role in the salt, drought, cold and heat stress tolerance of soybean. The responses of these *FAD* genes to the abiotic stresses we detected were also consistent with our previous *cis*-element analysis results, this further proved the reliability of the promoter prediction results.

4. Discussion

Research has shown that the cultivated soybean was domesticated from its annual wild relative (*Glycine soja* Sieb. and Zucc.) in China approximately 5000 years ago [45]. Gmax_ZH13 is a Chinese cultivated soybean generated by Chinese scientists in 2001. This germplasm is a hybrid progeny of cultivar accessions “Yudou 18” and “Zhongzuo 90052-76” by pedigree selection and has characters of high yield and stress tolerance[17] Williams 82, a Korea cultivated soybean, was first been developed in the 1980s [36]. Chi et al. [16] found 41 *FAD* genes from the Williams 82 genome (http://www.phytozome.net/soybean). In the current study, Chinese cultivated soybean had 29 *FAD* genes, which was more than 23 of the total number of both in the other two soybeans. The difference in total gene numbers of Williams 82 may result from different genomic versions and identification criteria and parameters of the BLAST software. There was no deletion of the four *FAD* subfamilies in the three soybeans. The number of the first desaturase gene subfamily in each soybean genome was almost the same as that in *A. thaliana* and rice, with opposite results in omega and front-end desaturases. This indicated that these two subfamilies came before the monocot-dicot divergence and had experienced a positive selection pressure during species evolution.

The enlarged subfamily of Chinese cultivated soybean provides an ideal option to research the evolution process of genes during the domestication. Genes from the omega subfamily were involved in abiotic stress response. Previous studies show that *FAD2, FAD6*, and *FAD8* in *A. thaliana* were implicated in salt tolerance [10–11,46]. Teixeira et al. [47] found that *PoleFAD7* and *PoleFAD8* expression was upregulated when peanuts were exposed to low temperatures. *FAD2* and *FAD6* were expressed in *A. thaliana* seedlings after salt stress [7]. Dar et al. [22] revealed the role and critical function of *FAD2* in response to cold and salt tolerance. Nguyen et al. [28] had found that membrane lipid


A
GsFAD2.4
GsFAD2.5
GsFAD2.1
GsSLD1
GsFAD7.3
GsDES1.1
GsDES1.2
GsFAD7.2
GsSLD2
GsSLD5
GsSLD6
GsSLD4
GsFAD7.1
GsSLD3
GsFAD8.3
GsFAD2.2
GsFAD2.3
GsFAD8.1
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2.5
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1.5
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0.5
0
−0.5
SR1_3 SR1_1 SR1_2 SR2_1 SR2_2 SR2_3 SR0_2 SR0_1 SR0_3 SR24_3 SR48_2 SR48_1 SR48_3 SR24_1 SR24_2 SR4_3 SR4_1 SR4_2
SL1_1 SL1_2 SL1_3 SL0_1 SL0_2 SL0_3 SL4_2 SL4_1 SL4_3 SL2_1 SL2_2 SL2_3 SL24_2 SL24_1 SL24_3 SL48_1 SL48_2 SL48_3
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SR48_2
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SR48_3
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SR24_1
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1
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C
GsSLD2
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GsADS2
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GsFAD2
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GsFAD2
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GsSLD2 GsFAD7.1 GsSLD3 GsADS2 GsFAD2.2 GsSLD5 GsFAD2.5 GsFAD8.3 GsSLD6 GsFAD7.4 GsFAD8.4 GsADS1 GsFAD2.3 GsFAD7.2 GsSLD4 GsFAD8.1 GsFAD8.2 GsFAD2.4 GsFAD2.1 GsFAD7.3 GsSLD1 GsDES1.1 GsDES1.2

polyunsaturation regulated by *FAD2* was related to endoplasmic reticulum (ER) stress tolerance in *A. thaliana*. Golizadeh et al. [29] reported that *FAD3* and lipid transfer protein 1 (*LTP1*) gene expression were upregulated in two wheat cultivars under cold stress. Moreover, *FAD7*-silenced tobacco plants have been reported to show stronger tolerance to high temperature than wild type plant, with higher dienoic to trienoic fatty acid ratios controlling membrane stability [48]. In *A. thaliana*, *FAD3* and *FAD7* were involved in drought and hypoxia stress signaling [49]. The numbers of the omega desaturase subfamily from Chinese cultivated varieties were significantly higher in four subclades as followers: omega, first, front-end, and sphingolipid (Fig. 2), with the main expansion occurring in *FAD2s* and *FAD8s*. The expansion of *FAD2s* and *FAD8s* genes could be explained by an adaptive advantage in a similar manner. However, in such cases, neofunctionalization may be required. The results of expression pattern and evolution results suggest that soybean *FAD2s* and *FAD8s* genes maintain a conserved function in environmental adaption, whereas they may have functionally diverged [16, 50]. Interestingly, the extent of genes may reveal transposable elements as a possible duplication mechanism, emphasizing the evolutionary significance of transposable elements [51]. However, the function of new genes from Chinese cultured soybeans needs to be further investigated.

For promoter analysis, 22 light-responsive *cis*-acting elements were predicted in 75 *FAD* genes, they were distributed in 855 sites, and were abundant in the upstream sequence of most genes. In *A. thaliana* chloroplasts, light-responsive control of the promoter region was in the ω-3 fatty acid desaturase gene (*FAD7*) [52]. In wheat, the increasing in *TaFAD7* gene's expression level was distinct in leaf development under light or dark conditions and the light-induced greening process of etiolated leaves [53]. Kargiotidou et al. [54] found that *FAD3* and *FAD8* of cotton were light-regulated at least at a transcriptional level, while the *FAD7* gene was adjusted by light via a post-transcriptional mechanism with specific changes in mRNA stability. Collados et al. [55] investigated the effect of light on the activity and regulation of plant fatty-acid desaturases responsible for *FAD3*, *FAD7* and *FAD8*, using soybean photoautotrophic cell suspension cultures. Physiological improvements in the efficiencies by which soybean canopies intercept light (ε_i), convert light energy into biomass (ε_c), partition biomass into seed (ε_p), and increase seed yield were observed [56]. Based on the results, we speculated that *FAD* gene expression is sensitive to light in soybean. Therefore, in agricultural production, light condition improvement may be increasing yield and fatty acid content of soybean seeds. It is well known that the environment has a significant influence on the growth and metabolism of plants. We had also predicted the *cis*-acting elements related to cold, drought, light, hypoxia, circadian, ABA, auxin, salt, defense, and stress response in the upstream of soybean *FAD* genes. The expression of *FAD8.1* and *FAD8.2* was induced under the four abiotic stresses (Fig. 8). The expression of *FAD2.2* and *FAD2.3* decreased under salt stress and drought stress, but increased under heat stress. The expression of *DES1.1* and *DES1.2* changed significantly under salt stress and cold stress, but the expression of *DES1.1* decreased and *DES1.2* increased under salt stress, while the expression pattern was opposite under cold stress. The consistency of these results with the prediction results of *cis*-acting elements indicates that the results of *cis*-acting elements analysis are reliable. Sivaramakrishnan et al. [57] reported that treatment of plants with appropriate concentrations of hormones (such as ABA and auxin) can effectively improve the biomass. Therefore, ABA and auxin hormones could improve the growth of soybean plants by increasing or decreasing the expression of the members of the *FAD* gene family. Moreover, environmental conditions were also an important factor restricting the yield and quality of soybean seeds. Korea is rich in hills and soil drainage is not smooth resulting in high salt content in the soil [65]. Although the other two soybean varieties are distributed in China, the environmental pressure of wild varieties will be greater due to human factors. The results also showed that *cis*-acting elements of *FAD* gene promoter sequences involved in abiotic stress response were also different in each gene subfamily of three soybeans, this may be due to the difference of their growth environment in nature.

One-fifth of irrigated agriculture was detrimentally influenced by soil salt. Salt stress is the first serious global abiotic stress, mainly due to NaCl, which hampers the growth and productivity of many crops by creating different cellular and biochemical changes [58–62]. Therefore, the development of salt-tolerant crops is to maintain continuous food production. Breeding improvement for salt-tolerant crops has not been elucidated due to insufficient understanding of the molecular basis of salt tolerance and deficiency of candidate genes that can regulate salt tolerance. Previous studies showed that *FAD2* and *FAD6* of *A. thaliana* were found to be salt tolerant [10–11]. On the other hand, *LeFAD3* upregulation could relieve salt tolerance in the early seedling stage [63]. Additionally, heterologous expression of sunflower *FAD2–1* or *FAD2–3* in yeast raised its tolerance to salt [64]. In this study, low expression levels of *GsSLD1* and *GsSLD2* were downregulated in all roots and leaves treated with salt. Interestingly, *GsSLD1* and *GsSLD2* gene transcript levels were markedly upregulated after 1 and 2 h of salt treatment in roots, which showed that the *FAD* genes may be tolerant to advisable levels to defend the cell membrane of soybean plants from salt stress. Our current studies showed that some *GsFAD* genes were differently expressed following salt treatment at different durations, explaining the involvement of *FAD* genes in environmental adaptation.

Overall, these above results provide insight into the potential functional roles of soybean *FAD* genes. The comprehensive analyses may help selected candidate *FAD* genes for further functional characterization, and for the genetic improvement in the agronomic characters and the environmental resistance of soybean.

5. Conclusions

A total of 75 *FAD* genes were identified in three soybean genomes. To predict the role of *FAD* genes in soybean, phylogenetic tree construction, and analysis of gene structure, motif, and promoter was conducted. The results showed that the *FAD* gene family was conserved in the three soybeans. The numbers of the omega desaturase family from Chinese cultivated varieties were significantly expanded, indicating that the family was subjected to serious selection pressures during cultivation and domestication. Promoter analysis showed that light, hormones, and abiotic stress played very important roles in fatty acid biosynthesis. Expression analysis of Chinese wild soybean under salt stress suggested that *FAD* genes had different sensitivities in response to salt stress. The functions of these genes in response to salt stress will be identified in a future study. We provide a basis for the development of markers for future breeding efforts and the identification of gene-editing targets to improve soybean performance. Furthermore, we continually observed putative neofunctionalization, a requirement to comprehend the emergence of new traits of soybean during evolution and domestication.

Fig. 6. Expression profiles of *FAD* genes in Chinese wild soybean under different treatment durations of salt (A) and relationship between the samples (B) and genes expression pattern (C). Fragments per kilobase of transcript per million mapped reads (FPKM) values of *GsFAD* genes were transformed by log10, and the heatmap was visualized by pheatmap in R. Note:SRX_Y is representing a sample representing biological repeat Y after salt stress treatment for X hours.

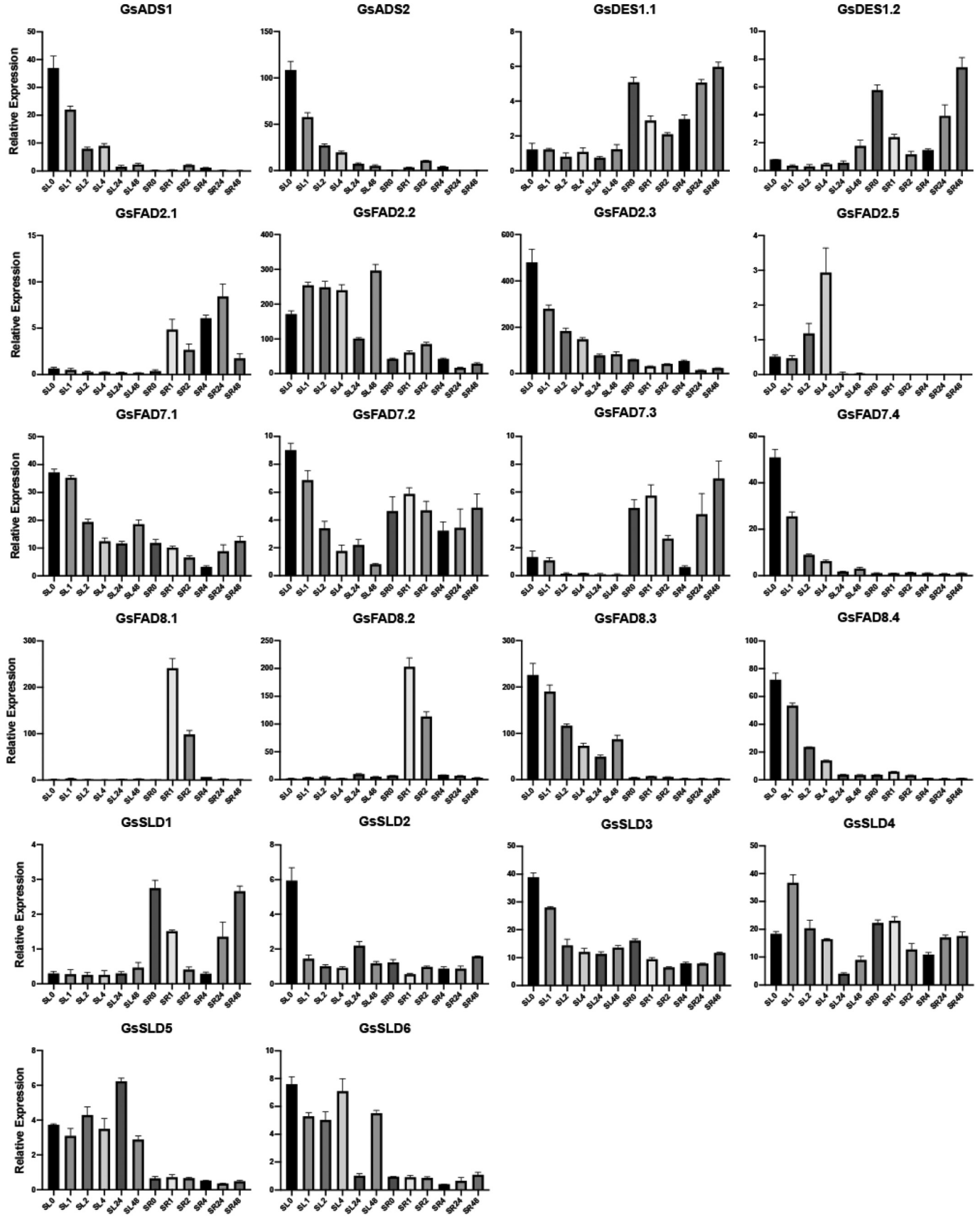

Fig. 7. Expression levels of *FAD* genes in Chinese wild soybean under different treatment duration of salt. Each column meant the mean ± SE of three independent experiments (each with three replicates).

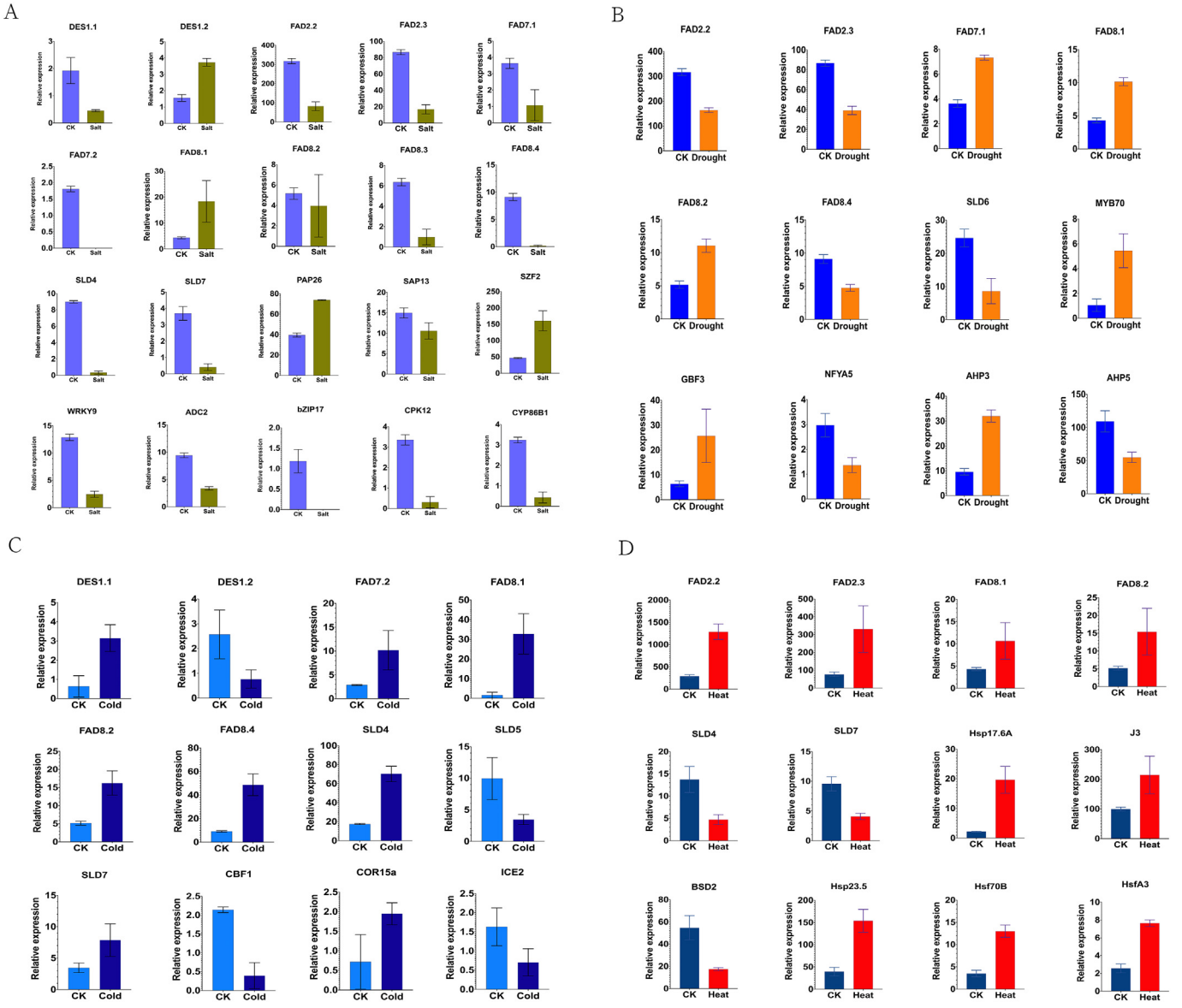

Fig. 8. The significantly expressed *FAD* genes and the stress responsive genes in soybean under salt (A), drought (B), cold (C) and heat (D) stresses. Each column represented the mean ± SE of three independent experiments (each with three replicates).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.05.161.

CRediT authorship contribution statement

B.Z., P.X. and Z.L. conceived and designed this study. H.Y., and W.L. conducted analysis. P.X. and W.C. contributed the analytical methods. B.Z. wrote the manuscript. P.X. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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