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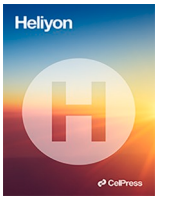

Genome-wide identification of glutamate receptor-like gene family in soybean

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ABSTRACT

Glutamate receptor-like genes (*GLRs*) are essential in the growth and development of plants and many physiological and biochemical processes; however, related information in soybean is lacking. In this study, 105 GLRs, including 67 *Glycine soja* and 38 *Glycine* max GLRs, were identified and divided into two clades (Clades II and III) according to their phylogenetic relationships. GLR members in the same branch had a relatively conservative motif composition and genetic structure. Furthermore, the soybean *GLR* family mainly experienced purification selection during evolution. *Cis*-acting element analysis, gene ontology, and Kyoto Encyclopedia of Genes and Genomic annotations indicated the complexity of the gene regulation and functional diversity of the soybean *GLR*. Moreover, transcriptome data analysis showed that these *GLRs* had different expression profiles in different tissues, and Clade III members had higher and more common expression patterns. Additionally, the expression profiles under jasmonic acid treatment and salt stress indicate that the GLR participated in the jasmonic acid signaling pathway and plays a role in salt treatment. This study provides information for a comprehensive understanding of the soybean GLR family and a reference for further functional research and genetic improvement.

1. Introduction

Glutamic acid is a widespread primary amino acid in organisms that plays an essential role in protein metabolism [1]. As an excitatory neurotransmitter, it is regulated by glutamate receptors (GluRs) [2]. GluRs were speculated to have regulatory effects in mammals as early as the 1950s. Later, they were found to regulate various neurological, mental, and emotional disorders and play critical roles in almost all aspects of brain function, thereby attracting increasing attention [3]. The glutamate receptor-like gene (GLR) sequence in plants is highly homologous with the animal GluR sequence. Moreover, phylogenetic and sequence comparisons have shown that they share a common ancestry [4]. With the identification of 20 *Arabidopsis thaliana* (*A. thaliana*) GLRs (AtGLRs) in 1999 [5], GLRs have been continuously identified in other plants, including 36 *Gossypium hirsutum* (*G. hirsutum*) GLRs (GhGLRs) [6], 13 *Oryza sativa* (*O. sativa*) GLRs (OsGLRs) [7], and 16 *Zea mays* (*Z. mays*) GLRs (ZmGLRs) [8]. Plant GLRs have critical physiological functions and play key roles in growth, development, and various physiological and biochemical pathways, including coping with biotic and abiotic stresses. For example, *OsGLR3.4* can regulate rice root growth and promote nitrate absorption by the roots [9]; *AtGLR3.4* mediates abiotic stimulation of *A. thaliana* to external touch and cold [10]; *AtGLR1.2* and *AtGLR1.3* initiate the downstream CBF/DREB1 cold response pathway through endogenous jasmonic acid (JA) accumulation under cold stress, enhancing cold tolerance

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[11]; and *AtGLR3.3* confers resistance to *Pseudomonas syringae* pv *tomato* DC3000 and *Hyaloperonospora arabidopsidis* in *Arabidopsis* [12,13]. These previous findings indicate that GLRs could be a strong candidate to significantly improve traits through genetically engineered breeding.

Soybean is a major crop worldwide and an essential source of edible oil and plant-based protein. Moreover, it has been used for nutrition in humans and animals and has industrial applications [14]. However, its yield and quality are hindered by various environmental factors, such as drought, high salt, alkali, and biotic stress [15]. Therefore, it is important to study the molecular mechanisms of soybeans' responses to various stresses to cultivate resistant varieties. Furthermore, given the versatility and importance of *GLRs*, targeting these factors may have practical applications.

GLRs have been intensely studied in different plants and cash crops; however, relevant information on soybean—a significant cash crop—is relatively scarce. In this study, we systematically identified and characterized the soybean *GLRs* and their expression patterns in different tissues under hormone induction and biological stress. These results can provide helpful information for further functional analysis, application of soybean *GLRs*, and breeding of improved varieties.

2. Materials and methods

2.1. Soybean GLRs identification and phylogenetic analysis

Genomic information of *Glycine* max (*G. max*) (Wm82. a2. v1) and *Glycine soja* (*G. soja*) (ASM419377v2) were downloaded from NCBI and Phytozome [16], respectively. To comprehensively retrieve GLRs in soybeans, we obtained the possible *G.* max (GmGLRs) and *G. soja* GLRs (GsGLRs) with 20 AtGLRs acquired from the Arabidopsis Information Resource (https://www.arabidopsis.org/) as the search sequence (Table S5). Next, the Hidden Markov Model (HMM) files (PF00060, PF00497, PF01094, PF01609, and PF10613) associated with GLR were obtained from the Pfam database (http://pfam.xfam.org/) and were searched against the soybean protein data, using an e-value of $\leq$ 1e-5 as the criterion. The resultant sequence was treated as a union and subsequently validated utilizing the CDD [17] and SMART tools [18].

To better understand the phylogenetic relationship between soybean GLRs, we used the GLR protein sequences from *G. max*, *G. soja*, *A. thaliana*, *O. sativa*, *Solanum lycopersicum* (*S. lycopersicum*), *Saccharum* hybrid cultivar R570 and *Saccharum spontaneum* (*S. spontaneum*) to construct phylogenetic trees. These sequences were aligned using Mafft7 [19], and a maximum likelihood (ML) tree was constructed using IQtree software [20] with 1000 guided replications. The results were visualized using MEGA X [21].

2.2. Molecular characteristics, chromosomal localization, and selection pressure analysis

The number of amino acids, molecular weight (MW), isoelectric points (pI), instability index, aliphatic index, and grand average hydropathicity (GRAVY) of all finalized acquisition sequences were calculated using the ExPASy tool (https://www.expasy.org/). Additionally, we predicted subcellular localization using the WoLF PSORT server (https://wolfpsort.hgc.jp/) and obtained the transmembrane domain and signal peptide using DTU Health Tech (https://services.healthtech.dtu.dk/). Furthermore, we used the gff3 annotation file to analyze the chromosomal localization of MG2C. Next, a Multiplex Collinear Scanning Toolkit (MCScanX) was used to detect segmental and tandem duplication genes in soybean GLRs [22] and visualized using Tbtools [23]. MEGA X was performed to evaluate the non-synthetic (Ka) and synonymous substitution rates (Ks) and calculate the Ka/Ks ratio between the homologous gene pairs. The selection mode was determined based on the Ka/Ks ratio. Lastly, the divergence time (million years ago, Mya) was calculated using the formula below.

$$T = Ks/2\lambda \times 10^{-6}$$

where λ was assumed to be 6.1×10^{-9} [24].

2.3. Conserved motif, gene structure, function annotation, and cis-regulatory elements analysis

Ten conserved motifs of the proteins were analyzed using the MEME online tool (https://meme-suite.org/meme/) with default parameters. Furthermore, we obtained the intron-exon distributions of soybean *GLRs* using the GFF annotation files from the soybean genome using Gene Structure Display Server 2.0 (GSDS2.0) [25]. Next, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomics (KEGG) annotation were performed by submitting the protein sequences to eggnog-MAPPER [26]. Lastly, the 2000 bp sequences upstream of the translation initiation site of *GsGLRs* and *GmGLRs* were used as the query sequence, and the *cis*-acting regulatory elements (CAREs) were predicted using the PlantCARE database [27]. The results were visualized using GSDS2.0.

2.4. GLR expression analysis by RNA-seq data

We obtained the corresponding transcriptome data from the NCBI database to understand *GmGLR* expression patterns in different tissues, salt stress, and hormonal induction processes. The transcriptome data accession number for different tissues was PRJNA869516, including leaves, roots, seeds, stems, and pods at the mature stage (R6 stage) and flowers at the flowering stage (R2 stage). Regarding salt stress, the acquired transcriptome data corresponded to 100 M salt stress (SRR21151086, SRR21151087, and SRR21151088) and no stress (SRR21151089, SRR21151092, and SRR21151093). For hormonal induction, 2-week-old soybean roots

grown in the dark were treated with JA (PRJNA218821) and indole-3-acetic acid (IAA) (PRJNA218823) and without phytohormone (PRJNA218830). All selected sequence reads are listed in Table S1. Trimmomatic [28] was used for data quality control, and the cleaned reads were mapped against the genome database using the HISAT2 program [29]. Lastly, GLR expression levels were expressed as fragments per kilobase of exon per million fragments, normalized by Log2 transformation in each data group and were visualized by Tbtools. Genes with a fold-change value ≥ 2 were defined as having significant differences in expression.

3. Result

3.1. Identification of soybean GLRs and protein property analysis

We identified 105 GLRs in soybeans (67 GsGLRs and 38 GmGLRs). The physicochemical properties of all predicted soybean GLRs are presented in Table S2. Briefly, the predicted MW extended from 12.78 kDa (KD) (GsGLR2.11b) to 167.88 KD (GmGLR2.2b). Additionally, pI increased from 5.35 (GsGLR2.12) to 9.31 (GsGLR2.13a). The grand average of hydropathicity (GRAVY) ranged from −0.272 (GsGLR2.11b) to 0.239 (GmGLR3.10); among the 105 proteins, 36 were hydrophilic (GRAVY index <0). Furthermore, the number of transmembrane domains ranged from 0 to 7, and 51 proteins had an instability index of 40. Almost all of these proteins, excluding GmGLR2.12b, GmGLR3.10, GmGLR3.14, GsGLR2.1d, and GsGLR2.11b, were predicted to be located in the plasma membrane. These changes might be closely related to the amino acid composition and level in the soybean GLR members.

3.2. Phylogenetic analysis

We constructed an ML tree using 67 GsGLRs, 38 GmGLRs, 20 AtGLRs, 13 OsGLRs, 9 ShGLRs, 34 SsGLRs and 13 SlGLRs to systematically understand the evolutionary relationship of GLRs in soybean (Table S3 and Table S4). As shown in Fig. 1, the GLR members involved in constructing the evolutionary tree, including all soybean GLRs, were explicitly divided into two large subsets (Clades II and III). Clade I in *Arabidopsis*, rice, tomato and sugarcane was grouped into the Clade II population. Based on phylogenetic tree grouping and chromosome position, we named the soybean GLRs listed in Table S2, including 20 GmGLRs and 34 GsGLRs in Clade II and 18 GmGLRs and 33 GsGLRs in Clade III.

3.3. Conserved motifs and gene structure of soybean GLRs

The motif was closely related to the protein's function. We found 10 motifs in soybean GLRs (Fig. S1). Motifs 1, 3, and 6 belonged to the Lig_chan superfamily and contained four transmembrane regions (M1, M2, M3, and M4). Motifs 2, 5, and 9 contained ligand-binding domain residues belonging to the periplasmic binding protein type 2 superfamily. Motifs 4, 8, and 10 contained another

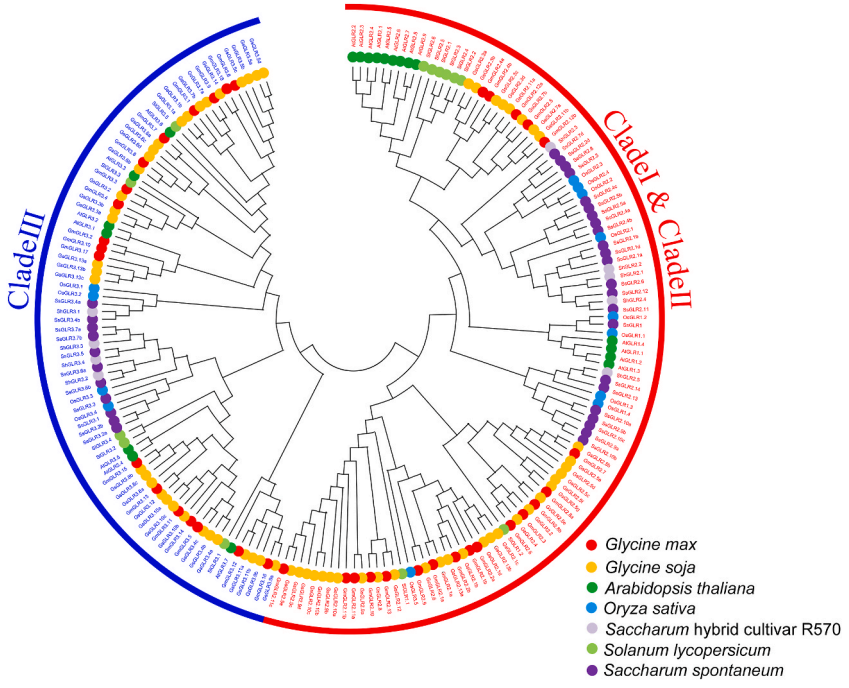

Fig. 1. Phylogenetic tree of GLRs from Arabidopsis, rice, and soybean. All protein sequences were aligned using MAFFT, and the ML tree was constructed using IQtree with 1000 bootstrap replicates. Different colors indicate different subgroups or species.

class of ligand-binding domain residues (periplasmic binding protein type 1 superfamily). Furthermore, among the 105 identified GLRs, 51 belonged to Clade III, among which 46 contained all 10 motifs, accounting for 90.20 %, and the other 54 belonged to Clade II, among which 42 contained motifs 1,2, 3, 4,6,8,9, and 10, accounting for 77.70 % (Fig. 2A and B). Moreover, regarding the gene structure, the number of CDS varied from 2 to 11 (Fig. 2C). *GmGLR3.2* had the highest CDS number among all genes, whereas *GmGLR2.12b* and *GsGLR2.11b* had the lowest number, containing two each.

3.4. Chromosomal distribution and gene duplication analysis of soybean GLRs

We clarified the evolutionary relationship of the GLR family in soybean by analyzing the chromosomal distribution, tandem duplication, and collinear relationships between *G.* max and *G. soja*. Fig. 3A shows that 65 *GsGLRs* were unevenly distributed in 11 *G. soja* chromosomes, 2 *GsGLRs* (*GsGLR2.13a* and *GsGLR2.13b*) were located on unmapped scaffolds, and 38 GmGLRs were randomly distributed on 12 *G.* max chromosomes (Fig. 3B). For *G. soja*, Chr11 possessed the most *GsGLRs* (16), and Chr04 carried only one signal. Regarding *G. max*, Chr13 contained the most *GmGLR* members (13), and Chr01, Chr02, Chr04, and Chr11 had only one member each.

Additionally, we identified 15 and 8 segmental duplication events in *GsGLRs* (Fig. 4A, Table 1) and *GmGLRs* (Fig. 4B, Table 1),

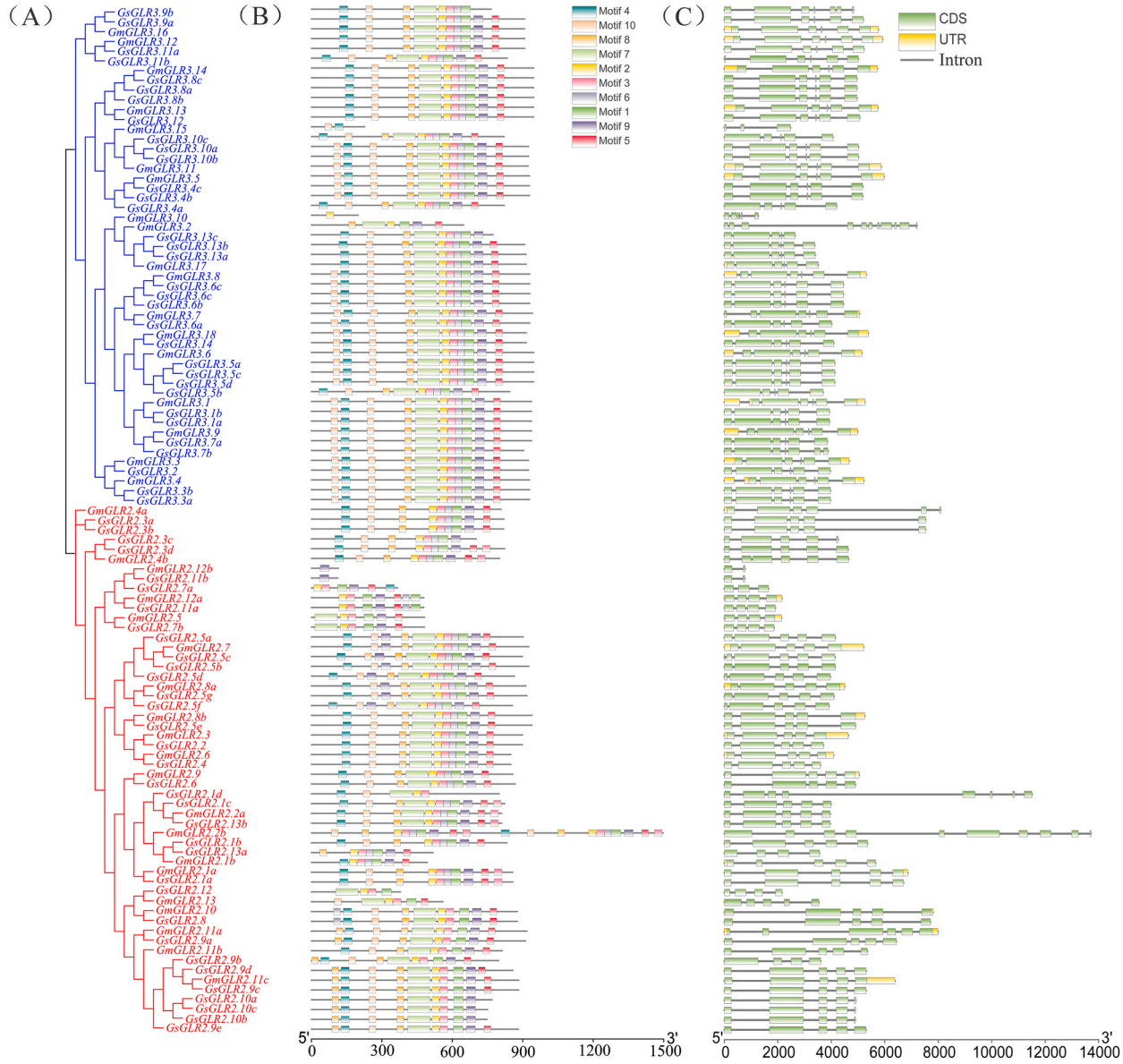

Fig. 2. Schematic diagram of the phylogenetic tree (A), conserved motif (B), and gene structure (C) of GsGLRs and GmGLRs. Genes from the same subtribe were indicated by the same color.

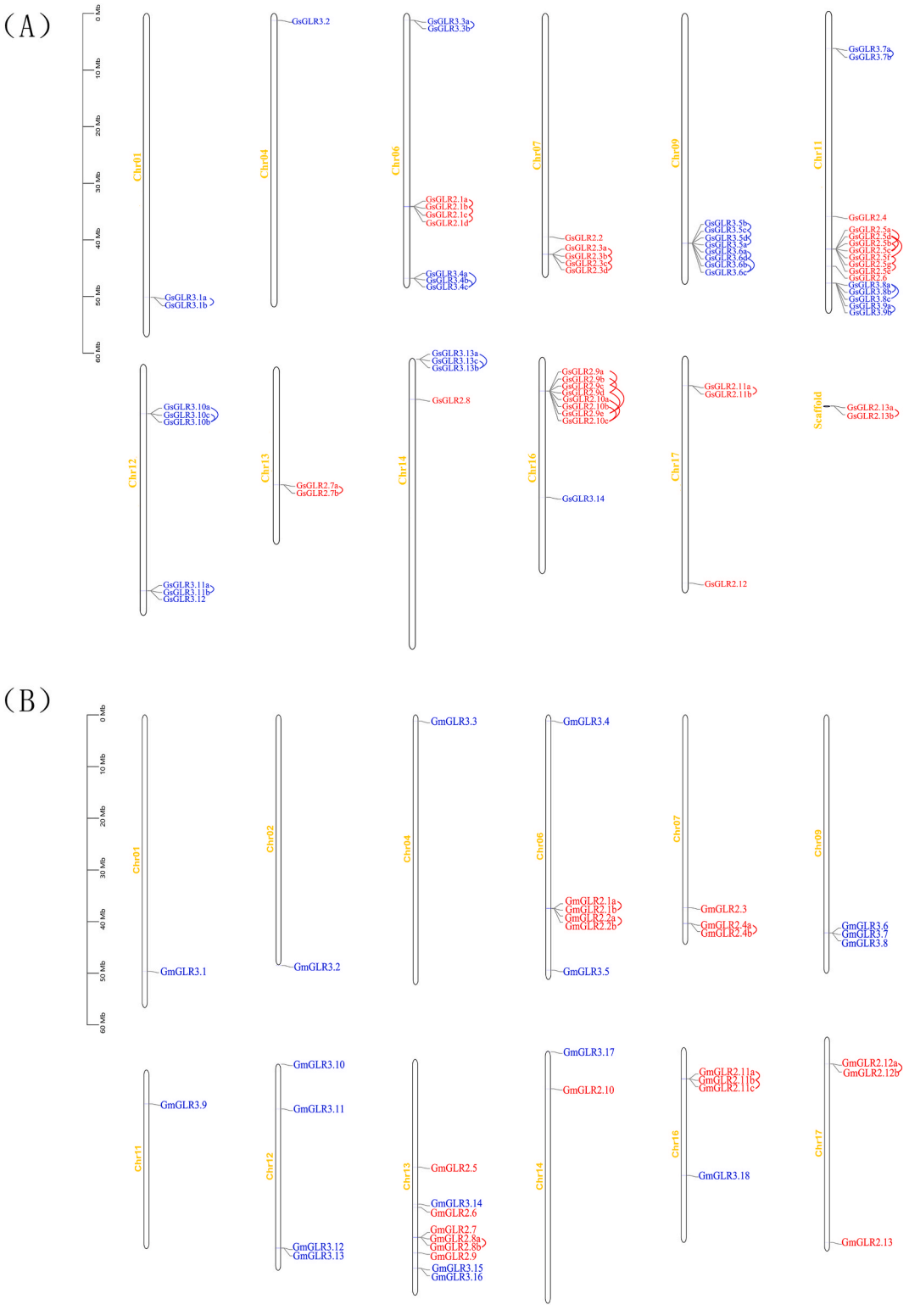

Fig. 3. Chromosomal distribution of GLRs in *Glycine max*. Chromosome size is indicated by its relative length. Chromosome numbers are shown at the top of each chromosome.

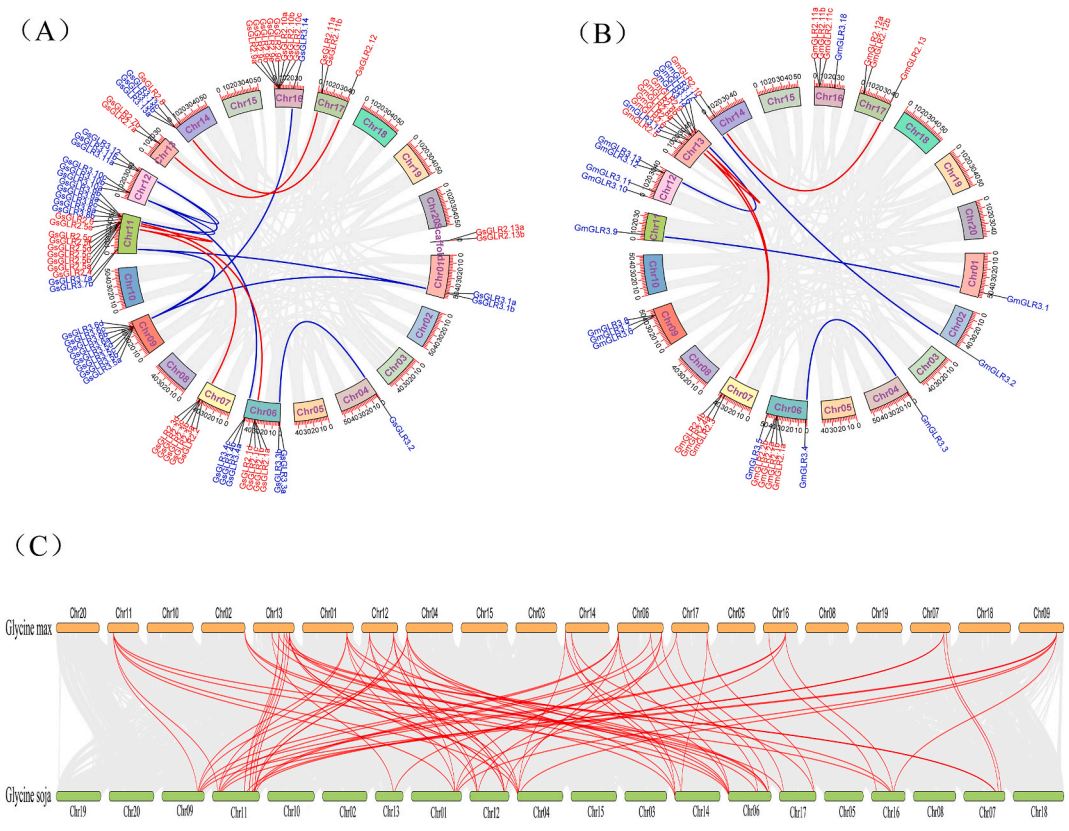

Fig. 4. Genome-wide synteny analysis of GLRs in *Glycine soja* and *Glycine max*. Genes from the same subtribe were indicated by the same color. (A) GsGLRs. (B) GmGLRs. (C) GsGLRs and GmGLRs.

Table 1
Ka/Ks and duplicated data analysis for segmental duplicated soybean *GLRs*.

Species	Gene ID1	Gene ID2	Ka	Ks	Ka/Ks	Mya
Glycine soja	*GsGLR2.1a*	*GsGLR2.6*	0.28	0.68	0.41	55.91
	GsGLR2.2	*GsGLR2.4*	0.08	0.14	0.61	11.43
	GsGLR2.4	*GsGLR2.5f*	0.42	1.63	0.26	133.31
	GsGLR2.7a	*GsGLR2.11a*	0.09	0.22	0.43	17.90
	GsGLR2.8	*GsGLR2.12*	0.03	0.15	0.22	12.02
	GsGLR3.10b	*GsGLR3.12*	0.15	0.50	0.31	41.11
	GsGLR3.1a	*GsGLR3.5c*	0.04	0.12	0.38	9.53
	GsGLR3.1a	*GsGLR3.7b*	0.02	0.08	0.25	6.30
	GsGLR3.2	*GsGLR3.3b*	0.03	0.15	0.17	12.50
	GsGLR3.4b	*GsGLR3.10a*	0.02	0.09	0.18	7.58
	GsGLR3.5a	*GsGLR3.14*	0.16	0.53	0.31	43.43
	GsGLR3.5c	*GsGLR3.7b*	0.02	0.12	0.15	9.85
	GsGLR3.8c	*GsGLR3.12*	0.16	0.50	0.32	40.84
	GsGLR3.8c	*GsGLR3.10b*	0.03	0.08	0.32	6.45
	GsGLR3.9a	*GsGLR3.11a*	0.45	1.55	0.29	127.32
Glycine max	*GmGLR3.1*	*GmGLR3.9*	0.02	0.09	0.28	7.28
	GmGLR3.2	*GmGLR3.17*	0.11	0.23	0.50	18.57
	GmGLR3.3	*GmGLR3.4*	0.02	0.08	0.25	6.25
	GmGLR3.13	*GmGLR3.16*	0.03	0.08	0.31	6.69
	GmGLR2.3	*GmGLR2.6*	0.09	0.14	0.64	11.29
	GmGLR2.3	*GmGLR2.7*	0.46	1.46	0.31	119.82
	GmGLR2.6	*GmGLR2.7*	0.45	1.48	0.30	121.62
	GmGLR2.10	*GmGLR2.13*	0.04	0.14	0.31	11.40

Note: Ka: non-synonymous substitution; Ks: synonymous substitution; Mya: million years ago.

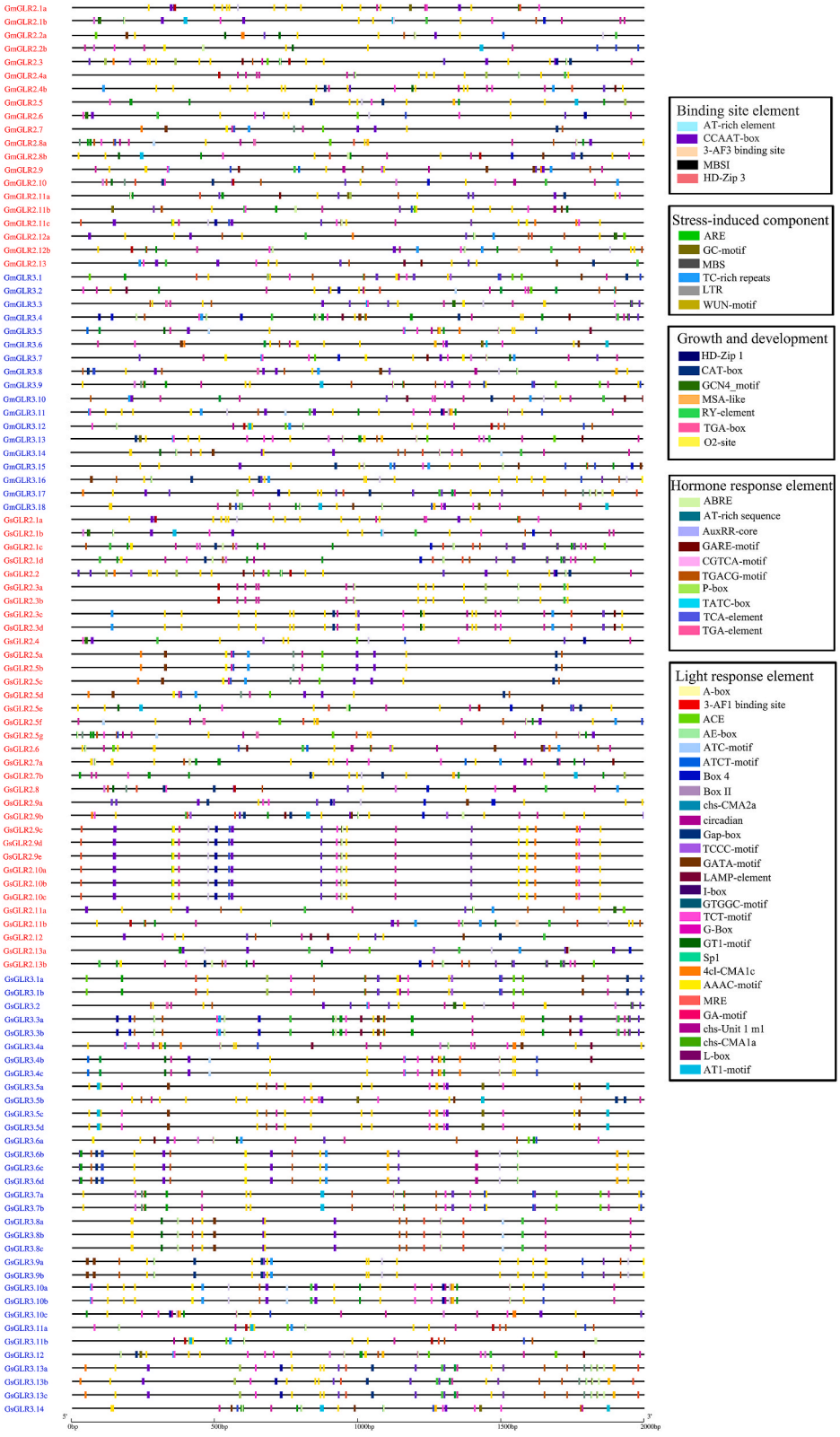

Fig. 5. The distribution of *cis*-acting elements in promoters of soybean GLR family members.

Fig. 6. GO and KEGG enrichment analyses of soybean GLRs. (A) The highly enriched GO terms in GsGLRs. (B) The highly enriched GO terms in GmGLRs. (C) The highly enriched KEGG pathways in GsGLRs. (D) The highly enriched KEGG pathways in GmGLRs. Pink represented extra functions in GsGLRs but not in GmGLRs, and green represented extra functions in GmGLRs but not in GsGLRs.

respectively. Regarding GsGLRs, Clades III and II subgroups had 10 and 5 duplicated gene pairs, respectively. Furthermore, in *G. max*, there were four duplicated gene pairs each in Clades II and III. Moreover, we found three segmental duplication events involving three members (*GsGLR3.1a/GsGLR3.5c/GsGLR3.7b*, *GsGLR3.8c/GsGLR3.10b/GsGLR3.12*, and *GmGLR2.3/GmGLR2.6/GmGLR2.7*).

Collinearity analysis between *GsGLRs* and *GmGLRs* revealed that 54 collinear lines existed (Table S5), which showed a strong ortholog of GLRs between *G. soja* and *G.* max (Fig. 4C). Additionally, exit 40 and 7 tandem duplications were found in *G. soja* and *G. max*, respectively; the details are listed in Table S2. Furthermore, we calculated Ka and Ks parameters between these duplicated gene pairs to explore the evolutionary dates and selection pressure acting on *GsLRs* and *GmGLRs* (Table 1 and Table S6). The Ka/Ks ratios of the gene pairs were all <1, indicating that soybean *GLRs* might be subjected to purification selection for retention. Lastly, the segmental duplication events of *GLRs* occurred approximately between 6.25 Mya (*GmGLR3.3/GmGLR3.4*) to 133.31 Mya (*GsGLR2.4/GsGLR2.5f*) with an average of 36.45 Mya.

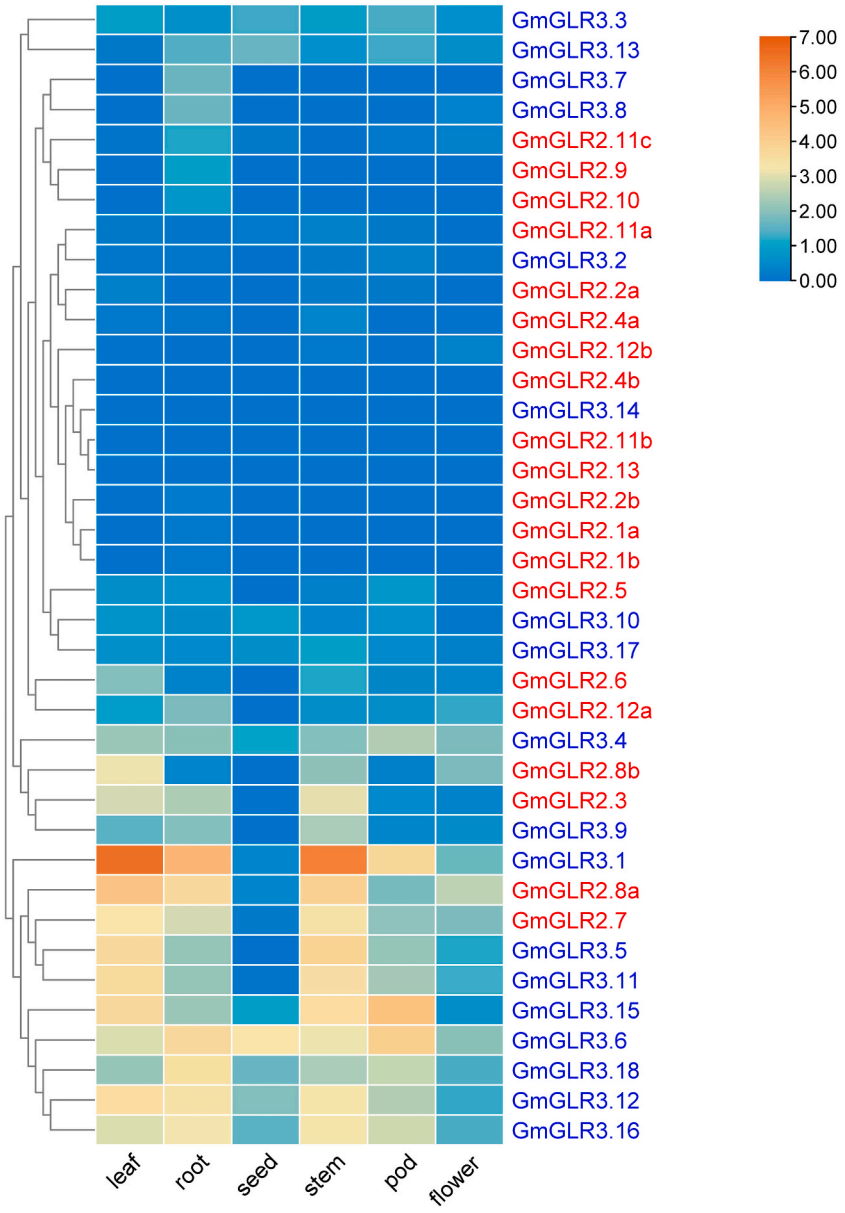

Fig. 7. GmGLR expression pattern in different tissues of *Glycine max*. Among these six tissues, the flower was in stage 2, and the other five were in stage 6.

3.5. Cis-acting regulatory elements

We predicted the CAREs to understand the potential transcriptional regulation of *GsGLRs* and *GmGLRs*. A total of 56 types of *cis*-acting elements were identified and divided into five categories (Fig. 5 and Table S7): binding site elements (5), stress-inducing elements (6), growth and development (7), hormone response (11), and light response (27). Furthermore, Box 4 element had the widest distribution in all *GmGLR* and 63 GsGLR promoters, whereas TGA-box was only found in the *GmGLR3.2* promoter. Notably, some elements were only found in *GsGLRs* or *GmGLRs*, for example, the 3-AF1 binding site and TGA-box in *GmGLR* promoters and 4 cl-CMA1c in *GsGLR* promoters. Additionally, *GsGLR3.3a* and *GsGLR3.3b* promoters contained the most regulatory elements (21), whereas only eight elements regulated the *GmGLR2.4a* promoter.

3.6. Gene ontology and Kyoto Encyclopedia of Genes and Genomics enrichment analyses

GO and KEGG enrichment analyses were performed to better understand the role of soybean GLRs at the molecular level. Furthermore, the GO annotations of soybean proteins were analyzed by molecular function (MF), cellular component (CC), and biological process (BP). In the MF ontology, GsGLRs and GmGLRs were enriched in 21 and 17 molecular functions, respectively. The four additional MFs in GsGLRs were ligand-gated ion channel (GO:0015276), ligand-gated channel (GO:0022834), ion-gated channel (GO:0022839), and gated channel activities (GO:0022836) (Fig. 6A and B). For the CC ontology, the highly enriched terms of GsGLRs and GmGLRs were consistent, focusing on the cell periphery, membrane, plasma membrane, and vacuole. Moreover, in BP ontology, many stresses and immunoregulation reactions were involved, and GmGLRs contained two other processes: positive regulation of response to stimulus (GO:0050896) and external stimulus (GO:0032103). Additionally, 13 different biological processes were found in the GsGLRs. The details are shown in Fig. 6A and B. These excess processes in GsGLRs may be conducive to adapting to unfavorable natural conditions and improving the viability of *G. soja*.

Furthermore, the KEGG pathway enrichment analysis revealed three highly enriched pathways participating in the diverse functions of soybean GLRs, including protein families (signaling and cellular processes), ion channels, and BRITE hierarchies (Fig. 6C and 6 D).

3.7. GmGLR expression patterns

To understand the GLR family expression pattern in *G. max*, we used transcriptome data to analyze their expression levels in different tissues. Fig. 7 shows that the expression patterns were similar between the seeds and flowers (R2 stage) and among the other four tissue types. Additionally, we observed low or moderate expressions of *GmGLR3.4*, *GmGLR3.6*, *GmGLR3.12*, *GmGLR3.16*, and *GmGLR3.18*, in all tissues. Moreover, *GmGLR3.6* and *GmGLR2.8a* exhibited the highest expression levels in seeds and flowers, respectively. Besides the above lowly- or moderately-expressed genes, *GmGLR2.7*, *GmGLR 2.8a*, *GmGLR3.1*, and *GmGLR3.5* showed low or moderate expression in four other tissues. A similar pattern was observed for *GmGLR2.3* and *GmGLR3.9* in leaves, stems, and roots, and *GmGLR2.8b* in leaves and stems. Notably, *GmGLR3.7* and *GmGLR3.8* expression levels were significantly higher in roots than in other tissues, suggesting that they may play a role in root development. Additionally, *GmGLR3.*1 had the highest expression in the leaf, stem, and root, and *GmGLR3.15* was found in the pod, indicating their essential role in the four tissues' development.

Furthermore, we plotted the average expression of Clades II and III genes across tissues (Fig. 8). The average expression of Clade III

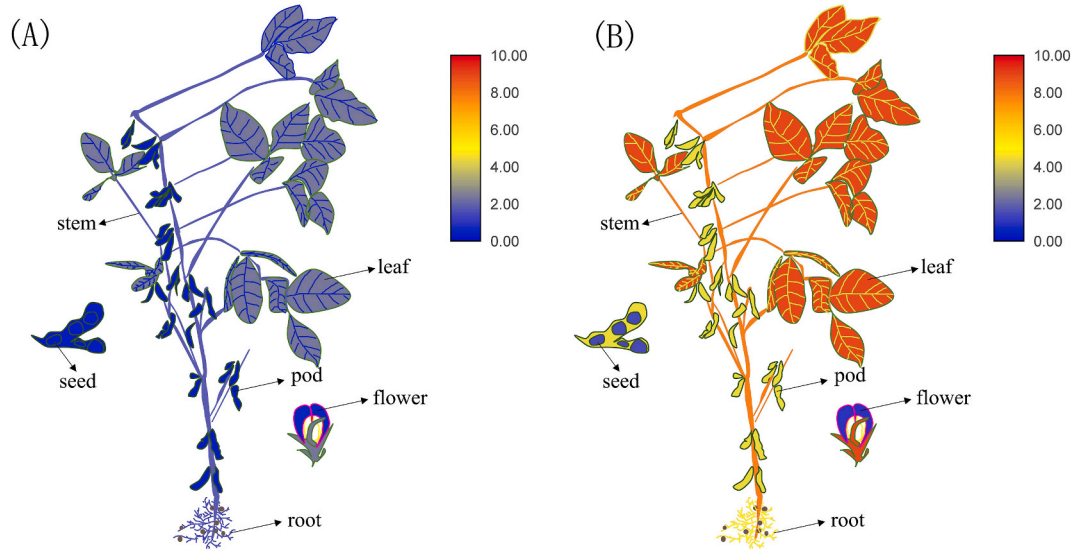

Fig. 8. Average expression of Clades II and III genes in different tissues of *Glycine max*. (A) for Clade II members and (B) for Clade III members.

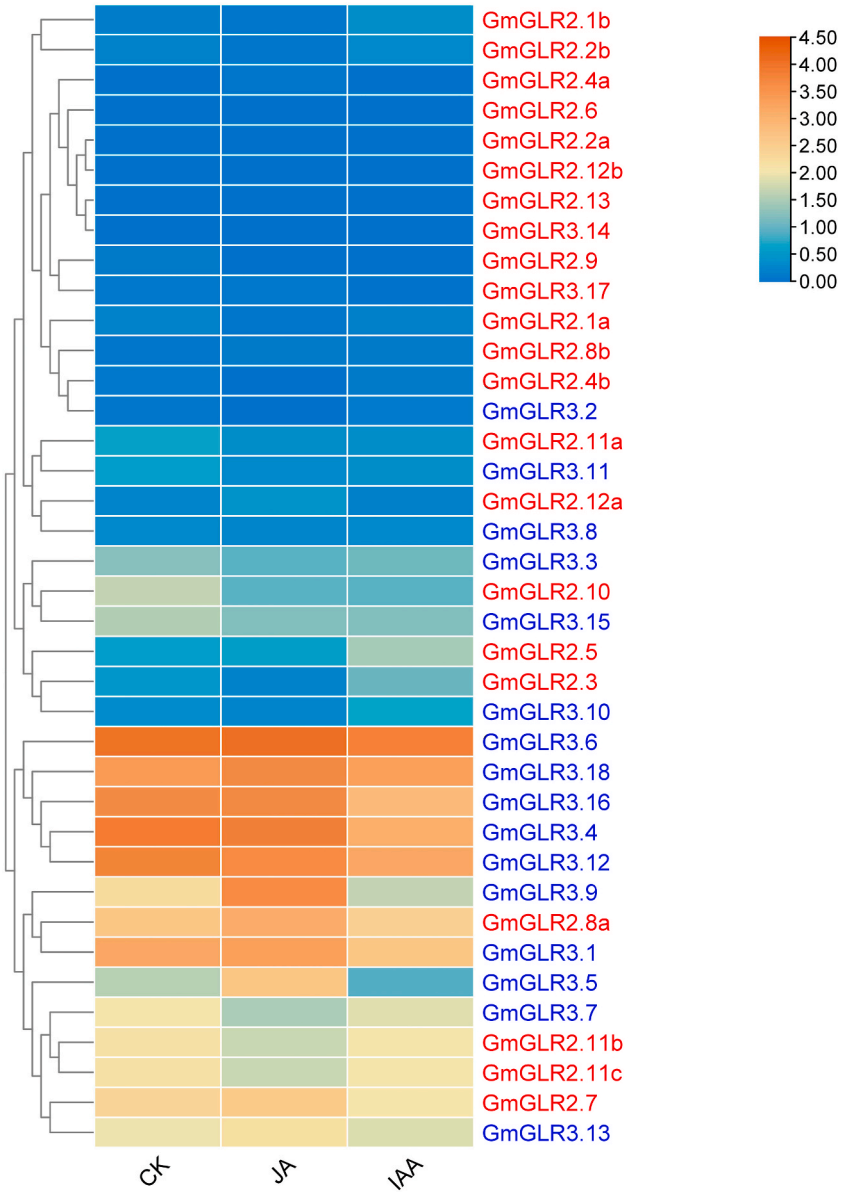

Fig. 9. GmGLR expression pattern in the face of phytohormone induction. CK: control group; JA: jasmonic acid; IAA: indole-3-acetic acid.

members (Fig. 8A) in each tissue was higher than that of Clade II (Fig. 8B), and the average expression of both clades in the seed and flower was lower than in the other four tissues. This result indicates that Clade III genes might participate in plant tissue development and have extensive functional divergence.

Additionally, we analyzed *GmGLRs* expression patterns under plant hormone induction and salt stress. Fig. 9 shows the *GmGLRs* expression patterns in 2-week-old soybean roots treated with JA, IAA, and without phytohormones. Moreover, *GmGLR3.5* and *GmGLR3.9* expression levels were significantly increased after JA treatment; however, no significant differences in gene expression levels were observed for IAA. Fig. 10 shows that *GmGLR2.5* was upregulated significantly under salt stress, suggesting its essential role in salt stress resistance.

4. Discussion

GLRs are essential for plant growth and development, signal transduction, and environmental stress adaptation. However, there is little information on GLRs in soybean. The GLR family has been characterized in some plants, including *A. thaliana* [5], *S. lycopersicum* [30], *S. Saccharum* [31], and *Malus. domestica* [32], most of which are divided into three branches by phylogenetic analysis. Moreover, there are four branches in *G. hirsutum* [6] and *O. sativa* [7]. A study pointed out that the relationship between subgroups I and II in

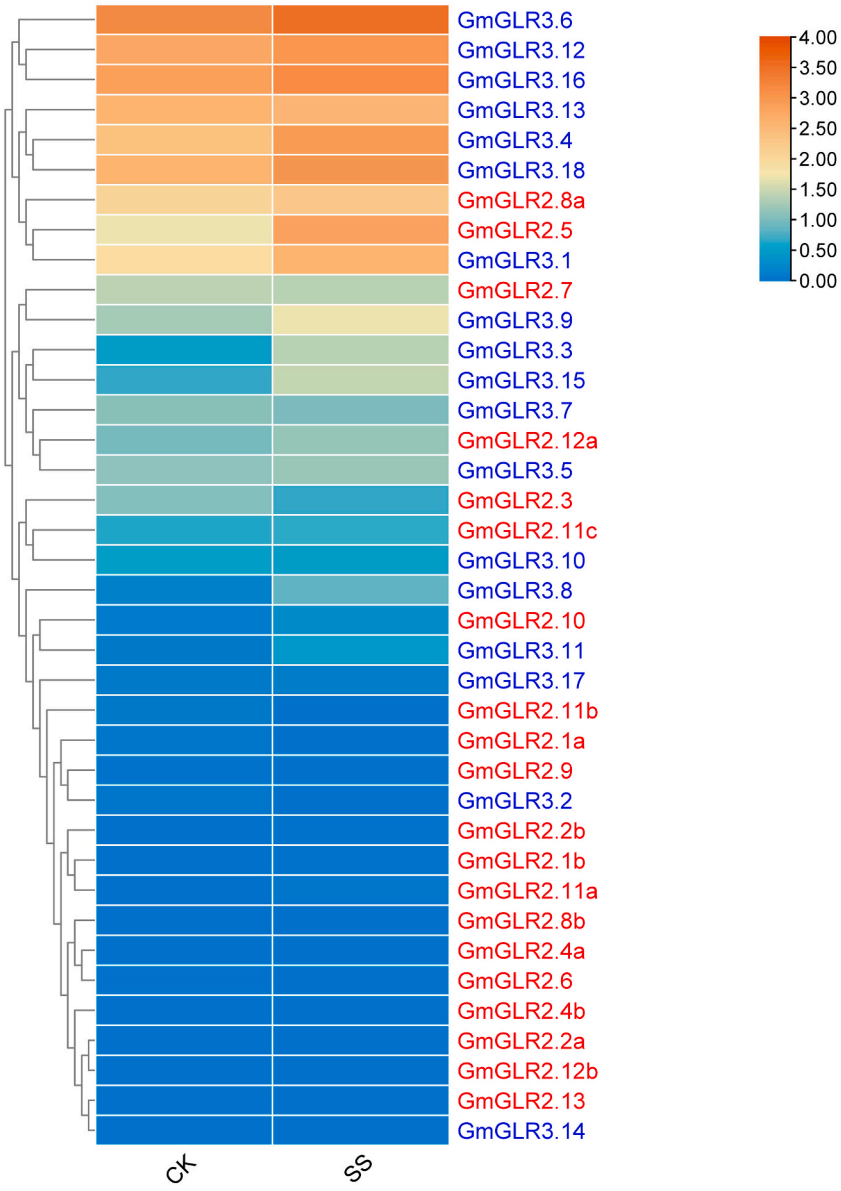

Fig. 10. GmGLR expression pattern under salt stress. CK: control group; SS: salt stress.

A. thaliana is a sister branch with a closer genetic relationship [33]. Furthermore, a recent study on GLRs in four Rosaceae plants classified subgroups I and II into a large class [34]. In our study, 38 GmGLRs and 67 GsGLRs were divided into two large subgroups (Clades II and III) according to their phylogenetic relationships, and the absence of Clade I was possibly due to the close sister relationship between Clades I and II in soybeans. We found that GLR members belonged to two large groups in soybean, consistent with recent reports of GLRs in *Z. mays* [8] and *Brassica rapa* (*B. rapa*) [35].

GLRs in *G. soja* and *G.* max display a relatively conservative distribution pattern on the chromosomes, with most members located on chromosomes of the same number and at equivalent positions, except for the loss or expansion of individual members in corresponding chromosomal locations. This distinct distribution reflects the different evolutionary history of GLR before and after the differentiation of *G. soja* and *G. max*. For instance, the similar distribution of GLRs on chromosome 11 of *G. soja* and chromosome 13 of *G. max*, considering the homologous relationships among these members and the length span of their distribution on the chromosomes, as well as the length differences of chromosomes 11 and 13 in the two species, suggests a chromosomal rearrangement event may have occurred after the divergence of *G. soja* and *G. max*. This inference has been confirmed in a previous study [36]. Gene structure variation promotes gene evolution [37]. In soybean GLRs, sister members with protein sequences clustering on the same branch exhibit highly similar numbers and lengths of introns, as well as similar protein motifs, indicating the conservation of gene evolution [38] and functional conservation [39] among these sister members, although their physicochemical properties do not show consistency.

Overall, even members clustered within the same sub-group have differences in gene structures, indicating functional differentiation and genetic diversity within the GLR gene family. Like previous studies on the GLR gene family [35,40], most soybean GLR members are localized to the cytoplasmic membrane. Some studies also suggest that GLRs can localize to organelles and perform certain functions. For instance, AtGLR3.4, localized in chloroplasts, is associated with photosynthesis [41]; mutations in the splice variant of AtGLR3.5, localized in the endoplasmic reticulum, can lead to endoplasmic reticulum deformation, ridge loss, and promote cell aging [42]. In this study, GsGLR2.1d/GmGLR3.10, GsGLR2.11b, and GmGLR2.12b/GmGLR3.14 are respectively localized to the cytoplasm, nucleus, and chloroplasts, and their functions remain to be further explored.

Plants may undergo significant changes in many aspects, such as physiological and biochemical functions, metabolic pathways, and even morphological characteristics, to adapt to the challenges of a constantly changing environment. Gene replication——which increases the abundance of genes, allows the generation of new genes, and expands new gene functions—plays a vital role in plants' evolution [43]. Additionally, tandem and segmental replication are important plant gene extension patterns [44]. In *G. soja*, we found that 15 pairs of *GsGLRs* were associated with segmental duplication and 40 with tandem duplications, indicating that tandem duplication was the main driving force for *GsGLR* evolution. Consistent with this result, the tandem repeat pattern of members of the *GLRs* family accounted for 44.12 %, 61.10 %, 61.76 %, and 77.50 % of repeat genes in pear, strawberry, plum, and peach, respectively, indicating that tandem repeats are likely the primary amplification mode of the GLRs family in plants [34]. In *G. max*, eight and seven members were associated with segmental and tandem repeats, respectively. Furthermore, we observed a significant reduction in the number of replicated genes compared with that in wild soybean, which may be associated with a significant loss of gene number during acclimation and breeding [45]. Compared with *G. max*, the widespread tandem repeat of the *GLR* in *G. soja* was conducive to the accumulation of rich genetic material and genetic diversity to adapt to the selection pressure of various complex environmental conditions in the outside world. The results of selection experiments indicated that the vast majority of soybean *GLRs* were subjected to purification selection to maintain their function [46]; however, two pairs of repetitive genes, *GsGLR2.5b/GsGLR2.5c* and *GsGLR2.10a/GsGLR2.10c* were strongly positively selected and underwent rapid evolutionary changes [47]. In soybean, two important genome-wide replication events occurred at 58–60 Mya and 12–13 Mya [48]. Our divergence time analyses showed that duplications of *GsGLR2.8/GsGLR2.12* and *GsGLR3.2/GsGLR3.3b* paralogous pairs occurred between 12 and 13 Mya, indicating that whole genome duplication (WGD) might participate in *GLR* gene amplification.

Furthermore, CAREs are a class of non-coding DNA sequences located in a gene's promoter region. The number and type of elements often indicate gene regulation and function differences [49]. Notably, little information is currently available regarding *GLR* CAREs. We found that the promoter region of the soybean *GLRs* contained five elements (a binding site element, stress-induced component, growth and development, hormone response element, and light response element) and had one more binding site element than the *GLR* CAREs in *B. rapa* [35] but was consistent with that in sugarcane [31]. Additionally, we compared the specific CAREs involved in *GLRs* among the three species (soybean, *B. rapa* and *S. lycopersicum*). Furthermore, Box III, SARE, motif I, and four light response elements (ACA-Motif, CH-Cmax 2b, PC-Cmax 2a, and SBP-Cmax 1c) were missing from soybeans. Box III is a protein binding site element found in a sequence related to salt stress in the TsVP1 promoter [50]. Moreover, motif I is related to cell development and root-specific regulation [51]. SARE is a salicylic acid-responsive element [49], and its absence in soybean *GLR* CAREs indicates that *GLR* may not directly participate in the interaction between soybean and salicylic acid, which requires further confirmation. Furthermore, *GLR* CAREs belonged to many categories, indicating the diversity of GLR functions. Moreover, although the CAREs of the three species were not identical, they were conservative, indicating that *GLR* was functionally conservative. Additionally, the number of photoreactive elements was the highest, indicating that *GLRs* may be more sensitive to light regulation.

In plants, *GLRs* play crucial roles in growth, development, and many physiological and biochemical processes. For example, in rice, *GLR3.1* is involved in cell proliferation and survival in the root apical meristem, and its mutation can lead to a short-root phenotype [52]; additionally, the *GLR* mutant in mosses can inhibit sperm chemotaxis, causing a significant decrease in spores [53]. Furthermore, in *A. thaliana*, the functional deletion or *AtGLR3.6* overexpression can reduce or induce the growth of the main and lateral roots [54]; mutations in *AtGLR3.2*, *AtGLR3.4*, or *AtGLR3.2/AtGLR3.4* could result in overproduction and aberrant placement of lateral root primordia [55]; *GLR 3.7* regulates root hair elongation [56], and *GLR1.2* regulates pollen tube growth and morphogenesis [57]; five of the nine Clade II members showed specific expression in 8-week-old roots, indicating their essential function [5]. Our study showed that in the R6 stage of *G.max*, *GmGLR 2.1a*, *GmGLR 2.1b*, and *GmGLR 2.2b* of the Clade II member were weakly expressed in the root, *GmGLR2.9* and *GmGLR2.10* were relatively strongly expressed, and *GmGLR3.6* and *GmGLR 3.7* of the Clade III member were moderately expressed in the root, and they were not expressed in other tissues at the same phase. These findings suggest that these genes have specific functions in root development. Additionally, Clade III member expression levels in the tissues, especially *GmGLR3.1*, *GmGLR3.5*, *GmGLR3.8*, *GmGLR3.11*, *GmGLR3.13*, *GmGLR3.15*, *GmGLR3.16*, and *GmGLR3.18*, were generally higher than those of Clade II members, consistent with the findings in crops, such as rice and sugarcane [7,31]. This result indicates that they might be widely involved in some basic biological processes in tissues; hence, it is important to investigate their specific functions.

GLRs are essential for stress adaptation and plant hormone signal transduction. Previous evidence suggested that *GLRs* are closely related to beneficial host traits. *OsGLR1* and *OsGLR2* overexpression significantly improves drought tolerance in rice and *Arabidopsis* [58]. Additionally, *GLR3.7*-S860A mutant overexpression can significantly reduce the sensitivity of primary roots to salt stress [59]. Furthermore, transgenic *Arabidopsis* seedlings overexpressing small radish *RsGluR* showed upregulated expression of JA biosynthesis-related genes and inhibited the growth of the pathogenic fungus *Botrytis cinerea* [60]. Interestingly, a single nucleotide mutant of *GhGLR4.8* (from *GhGLR4.8C* to *GhGLR4.8A*) can confer cotton resistance to *Fusarium oxysporum* f. sp. *Vasinfectum* [6]. These results highlight the beneficial phenotypes that can be enhanced by *GLRs* overexpression, heterogeneous expression, and genetic engineering modification. In our study, the expression levels of the *GmGLR* family members changed to varying degrees in response to salt stress and JA treatment. JA significantly induced *GmGLR3.5* and *GmGLR3.9*, and *GmGLR2.5* expression under salt treatment

increased significantly. This suggests that they may play different roles in JA signal transduction and salt stress. Furthermore, these results indicate that these genes are potential targets for cultivating fine soybean varieties.

5. Conclusion

We identified and characterized 105 members of the GLR family in soybeans (67 in *G. soja* and 38 in *G. max*). They were classified into two large groups according to their phylogenetic relationships, and the motif and genetic structure of most members in each subgroup were conserved. Furthermore, tandem duplication was the primary method for *GsGLR* gene amplification, which was not evident in *GmGLRs*. Additionally, the GO and KEGG analyses and the predictions of *cis*-acting elements illustrated the diversity of *GLR* functions in soybeans and their possible regulation by various factors. We further investigated *GmGLRs* expression patterns and found that Clade III members had more general expressions. Moreover, *GmGLR3.5* and *GmGLR3.9* responded to the signaling pathway of JA, and *GmGLR2.5* showed significant differences in expression under salt stress. Overall, given the significance of *GLRs* in adaptive innovation and their potential applications in crop breeding and improvement, functional validation and genetic engineering to obtain beneficial phenotypes will be a significant future research direction. Our research provides useful information for further biological studies on *GLRs* in soybeans.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Miao Zhu: Data curation, Funding acquisition, Methodology, Visualization, Writing – original draft. **Tianhao Zhu:** Data curation, Methodology, Software, Visualization. **Xuying Wang:** Conceptualization, Writing – original draft. **Xinran Li:** Conceptualization, Data curation, Methodology, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21655.

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