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The molecular characteristics of soybean ARR-B transcription factors

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Abstract: The Type-B authentic response regulator (ARR-Bs) gene family is one of the important plant-specific transcription factor families involved in variety of physiological processes. However, study of ARR-Bs gene family in soybean is limited. Genome-wide analysis and expression profiling of the ARR-Bs gene family were performed in the soybean genome. 31 ARR-Bs genes (namely GmARR-B1-31) were identified, containing conserved catalytic domains with protein lengths and molecular weights ranging from 246 to 699 amino acids (aa) and 28.30 to 76.86 kDa, respectively. Phylogenetic analysis grouped ARR-Bs genes into three clusters—Cluster I, Cluster II, and Cluster III—which included 15, 12, and 4 genes, respectively, and were asymmetrically distributed on 17 chromosomes. Tissue specific expression analysis of GmARR-Bs family revealed a high transcription level in flowers, roots and seeds. The subcellular localization of GmARR5, GmARR14 were observed in the nucleus, and the promoter region of them included low-temperature responsive element (LTR), the C-repeat/dehydration responsive element (DRE), MBS, ABRE, MYB, MEJA and TCA-elements, which possibly participate in abiotic stress and hormones responses. qRT-PCR analysis showed that the expression of GmARR-B genes were affected by different abiotic stresses, especially cold stress and salt stress, and GmARR-B5 and GmARR-B14 were significantly induced by cold stress. This suggested that ARR-Bs genes were involved in multiple abiotic stress response pathways and acted as a positive factor under cold stress.

Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the significant cash crops worldwide. It can not only be an excellent source of protein, oil, and specialized metabolites such as isoflavonoids and saponins, but also improve soil by microbial nitrogen fixation (Abdelghany *et al.*, 2020; Hina *et al.*, 2018). Therefore, it is necessary to promote soybean yield. However, soybean often suffers from a variety of adverse environments, including salt, drought, cold, heat and pathogens, which limits the yield of soybean (Shu *et al.*, 2015). Transcription factors are molecular switches that control gene expression, so they play an important role in plant response to abiotic stress (Kidokoro *et al.*, 2014).

In the past decades, a large number of identified and characterized transcription factors (VOZ (Li *et al.*, 2020), bZIP (Zhang *et al.*, 2020b), WRKY (Yu *et al.*, 2016), GRF (Chen *et al.*, 2019), etc.) in soybean, which are widely associated with improving abiotic stress resistance, can effectively promote the growth and development of soybean and increase yield (Hwang and Back, 2019; Tolosa and Zhang, 2020). In previous studies, It has been demonstrated that the type-B authentic response regulators (type-B ARRs) gene family is one of the large plant-specific transcription factors family, which play an essential roles in response to abiotic stresses (D'Agostino, 2000). There are 11 genes and a potential pseudogene in the *Arabidopsis* genome encoding type-B ARR proteins (ARR 1, 2, 10–14, 18–21, 23) (Takatoshi *et al.*, 2004; Horák *et al.*, 2008; Pils and Heyl, 2009). Each of type-B ARRs contains the two common and characteristic domains, namely, "receiver domain" and "GARP motif", respectively. The receiver domain is implicated in phosphorylation, the C-terminal extension carrying a highly conserved phospho-accepting REC domain that was responsible for the regulation of transcription of cytokinin-related genes (Sakai *et al.*, 2001). The C-terminal region is highly variable, but they all have a small conserved motif

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composed of 60 amino acids, which is closely related to the Myb repeat of real mammals (Safi *et al.*, 2017), whereas the GARP motif serves as a specific DNA binding domain (Hosoda *et al.*, 2002). The 11 type-B ARRs of *Arabidopsis* share a common structural but fall into three subfamilies based on phylogenetic analysis, Subfamily I (7 members, AtARR 1, 2, 10–12, 14, 18), Subfamily II (3 members, AtARR 13, 21, 23) and Subfamily III (2 members, AtARR 19, 20) response regulators (Tajima *et al.*, 2004). The Subfamily 1 has been most extensively characterized.

The expression pattern in *Arabidopsis* and genetic analysis indicate that at least five members, ARR 1 (Sakai *et al.*, 2010), ARR2 (Lohrmann *et al.*, 2001), ARR 10 (Yokoyama *et al.*, 2007), ARR 11, and ARR 12, of Subfamily I mediate cytokinin signaling, acting as transcriptional regulators (Mason *et al.*, 2005). Cytokinin signal transduction is mediated by a multistep phosphorelay two-component signal transduction system (TCS), including cytokinin receptors, phosphotransferases, and type-B response regulators (Kakimoto, 2003; Werner and Schmülling, 2009). According to this model of Argyros (Argyros *et al.*, 2008) and Ishida (Ishida *et al.*, 2008), the transcription of many genes that response to cytokinin is positive mediated by the type-B ARRs, for the last step of the TCS process, and type-B ARRs plays a role at the top of the transcription cascade. In addition to participating in cytokinin response, previous studies have shown that type-B ARR gene family is also involved in cell response to ethylene and environmental stress through TCS (Schaller *et al.*, 2011; Hutchison, 2002; Hwang *et al.*, 2002). In *Arabidopsis*, ARR 1, ARR 10 and ARR 12 have been shown to play a role in cold and drought signaling, and their mutants showed dwarf characteristics (Nguyen *et al.*, 2016; Kang *et al.*, 2013); ARR 2 functions in ethylene signal transduction, and induces plant immunity to a bacterial pathogen via TGA 1/NPR dependent salicylic acid signaling pathway (Carella *et al.*, 2015; Shi *et al.*, 2012). It was also found that type-A ARRs played a role in abiotic stresses resistance in rice, ABA, dehydration, salt and cold could induce the expression of OsRR 6 gene (Jain *et al.*, 2006). What's more interesting is that type-B ARRs has been revealed to promote the expression of type-A ARR directly through phosphorylation, or rapidly and briefly induce type-A ARRs through cytokinin treatment (Birkenbihl *et al.*, 2005; Buechel *et al.*, 2010). And type-A ARRs was regulated by type-B ARRs to improve crop cold tolerance (Jeon *et al.*, 2010; Jeon and Kim, 2013; Kim and Jeon, 2013). In agreement with this, drought-responsive expression of ARR 22, type-C ARR, occurs in cytokinin receptor-dependent and receptor-independent pathways. overexpression of ARR 22 can also increase cold stress resistance (Kang *et al.*, 2013; Wallmeroth *et al.*, 2019). To sum up, type-B ARRs positively participated in plant growth and respond to environmental stress directly or indirectly.

Because of their apparent multifunctionality, type-B ARR genes are promising targets for developing crop varieties that are better adapted to abiotic stresses. Type-B ARRs have been identified and applied to enhance abiotic stress resistance in many plants, including *Arabidopsis thaliana*, *Populus*, rice, pear, and peach (Schaller *et al.*, 2008; Yu *et al.*, 2020; Huang *et al.*, 2019; Ni *et al.*, 2017; Zeng *et al.*, 2017). Here, we also performed a genome-wide search of type-B ARR gene family members in soybean. Identification and characterization of type-B ARRs in soybean could provide a new insight for further study on stress resistance function and regulation mechanism of soybean type-B ARRs from the perspective of functional differentiation and evolution.

Materials and Methods

Identification and nomenclature of the type-B authentic response regulator genes in soybean

To identify type-B ARR family members of soybean from Soybean Database, *Arabidopsis* ARR-Bs gene sequence was acquired from TAIR database (http://www.arabidopsis.org/), which was used as a comparative sequence to search type-B ARR family members of soybean from NCBI (http://www.ncbi.nlm.nih.gov/), phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#) and plantTFDB (http://planttfdb.cbi.pku.edu.cn/). The type-B ARR domain of the ARR proteins (PF 00072 and PF 00049) (http://pfam.xfam.org) as queries to Hmmer software searched for GmARR genes at E-value ≤ 1×10^{-10}. The sequence with $E < 1 \times 10^{-10}$ and amino acid number >200 aa was selected. Using the unique gene name and the characteristic functional domains of ARRs, the potential GmARR-B genes were confirmed on the SMART (http://smart.embl-heidelberg.de) website (Ivica *et al.*, 2015). A total of 31 non-redundant candidate GmARR-B genes were retained for further analysis. For the nomenclature of each putative gene, the prefix "Gm" (for *Glycine max* (L.) Merrill) was added to ARR followed by type "B" and Arabic numbers. 31 type-B GmARR genes were named from GmARR-B 1 to GmARR-B 31 according to their physical locations (from top to bottom) on chromosomes 1–20.

Phylogenetic tree of GmARR-B genes, conserved motif and exon/intron structure analysis

The full-length amino acid sequences of *Arabidopsis* (*Arabidopsis thaliana*) type-B ARRs were acquired using NCBI BLASTp tools from the NCBI database (https://www.ncbi.nlm.nih.gov/). To identify orthologs of the type-B ARR genes in soybean genome, the amino acid sequence of *Arabidopsis* type-B ARRs were used to search the publicly available databases at NCBI (http://www.ncbi.nlm.nih.gov/) and Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html). The protein sequences were imported into Mega v7.0 software, multiple sequence alignment was implemented by clusalw method, and phylogenetic analysis was carried out by neighbor connection (NJ) algorithm. For this, a Poisson model was applied, with bootstrapping (1000 replicates) and pairwise deletion for gaps/missing data (Luo *et al.*, 2017), divided GmARR-B genes into different subgroups. The conserved functional motifs within GmARR-B gene domains were identified using the MEME v4.9 program (http://meme.sdsc.edu) (Bailey *et al.*, 2009). The length of the motif was fixed to 6–100 amino acids. To detect motifs ZOOPs model was used, which considers that the motif occurrence can be zero or 1 in a sequence. Maximum 10 motifs were searched (Bailey *et al.*, 2009). The exon/intron

structures of individual GmARR-B family genes were obtained through the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) (Guo *et al*., 2007) by aligning the coding or cDNA sequences with their corresponding genomic DNA sequences from Phytozome v9.1 (http://www.phytozome.net/) (Goodstein *et al*., 2012).

Analysis of chromosome distribution, Cis-regulatory elements and gene duplication and synteny

The chromosome distribution of soybean ARR-B genes was obtained from Phytozome, GmARR-B genes were mapped onto the 17 soybean chromosomal linkage groups according to their physical position (bp). MapInspect was used to map chromosome location. The 1,500 bp upstream of the transcription start site of all GmARR-B genes insoybean was obtained from Phytozome v9.1 (http://www.phytozome.net/), and the cis-regulatory elements were identified using the PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al*., 2002).

We attempted to identify the duplication event and analyzed the synteny relationships among the ARR-Bs of *A. thaliana*, and soybean. And duplicated genes were obtained from Plant genome duplication database (PGDD) (http://chibba.agtec.uga.edu/duplication/) by downloading the dataset of duplicated blocks in soybean genome (Lee *et al*., 2013). Duplicated GmARR-B gene pairs were searched in the dataset. Similarly, syntenic blocks between soybean and *A. thaliana* were also searched. A total number of 19 chromosomes (*A. thaliana*-5, soybean-14) with a total number of 29 ARR-Bs (*A. thaliana*-22, soybean-7) were used to map the synteny relationships. TBtools (Chen *et al*., 2020) was used to map gene replication.

Physical and chemical properties and subcellular localization of GmARR-Bs protein

Physico-chemical features of the GmARR-B proteins, including length, molecular weight, theoretical isoelectric point, instability index were predicted using the protein identification and analysis tools in ExPASy Server10 (https://web.expasy.org/protparam/) (Panu *et al*., 2012). All selected proteins were subjected to sub-cellular localization prediction using pSORT web server (http://www.genscript.com/psort/wolf_psort.html) to know the relative abundance of these proteins in different sub-cellular compartments.

Secondary structure analysis of proteins

Prediction of secondary structure of GmARR-B proteins by NPS (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl page=/NPSA/npsa_sopma.html) online website (Akbudak and Filiz, 2020).

RNA seq data analysis for soybean expression in diverse tissues

To dissect the expression patterns of GmARR-B genes in the tissue and organ, we used all the genes as obtained for the soybean genomes. The RNA seq data for the expression of GmARR-Bs in different tissues at various developmental stages were obtained from Phytonome (https://phytozome.jgi.doe.gov/pz/portal.html#) databases (Debbie *et al*., 2007). The RNA sequencing reads have been listed in Suppl. Table S1. The heat maps were constructed using heat maps of the R Packages.

Plant material and treatments

Soybean (*Glycine max*) seeds with uniform colors and sizes were selected and disinfected with 95% ethyl alcohol and washed with sterile water five times before they were sown. The soybean seeds were sowed with the soil at 25°C (16 h light/8 h dark cycle) in a greenhouse. The treatments for abiotic stress were: (1) cold stress: the seedlings of V 1 stage (The first trefoil stage of vegetative growth period) were transferred to the incubator at 4°C for 8 days. (2) heat stress: the seedlings of V 1 stage were transferred to the incubator at 30°C for 8 days. (3) salt stress: 20 mL of 300 mmol/L NaCl solutions was irrigated into V 1 seedlings for 4 days. (4) alkaline stress: 20 mL of 75 mmol/L $NaHCO_3$ solutions was irrigated into V 1 seedlings for 4 days. (5) ABA stress: 100 μmol/L ABA solution was sprayed on both sides of the leaves of seedlings at V1 stage to drip with the leaves. All experiment involved three biological repeats and each repetition has six soybean plants. The samples were collected from each treatment at 0, 6, 12, 24, 48, and 96 h after stresses and frozen in liquid nitrogen, and stored at −80°C.

RNA extraction and quantitative PCR analysis

We investigated the expression of the 10 ARR genes under ABA, cold, heat, salt, and alkali stress conditions, which are closest to *Arabidopsis* in the evolutionary tree, in order to confirm if different abiotic stress treatments influenced the expression of type-B GmARR genes (Fig. 1). Using a sterile mortar and pestle, frozen soybean leaves were crushed to a fine powder under liquid nitrogen. TriZol reagent was used to extract total RNA from 30 mg fresh weight of frozen tissue. The cDNA was synthesized using the ReverTra Ace qPCR RT Kit (TAKARA) as directed by the manufacturer. The GmActin1 gene was used as a control, and relative expression levels of genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Each sample was assayed in three repeats, the significance of the differences between the samples was analyzed using paired *T*-test, with *P*-values of $^{*}P < 0.05$ and $^{**}P < 0.01$ being considered significant.

qRT-PCR primers were designed using Primer 5.0 (Suppl. Table S2).

Plasmid construction and subcellular localization

Two genes with high expression under abiotic stress were selected for subcellular localization test to further clarify whether the protein encoded by the GmARR-B family is embedded within the nucleus or not. Therefore, two genes were subsequently chosen to make a GFP-Gm.05G115400/GFP-Gm.08G100900 (ARR-B 5&14), fusion vector (pYBA1132-Gm_05G115400/Gm.08G100900 (ARR-B 5&14). The free GFP was used as the control. For subcellular localization study, GmARR-Bs were obtained by gene cloning, and cloned into the vector GFP-pYBA 1132 with the target fragment by homologous recombination, transformed into *Escherichia coli* DH5α. Then the plasmid was transferred into *Agrobacterium* GV3101 by freeze-thaw

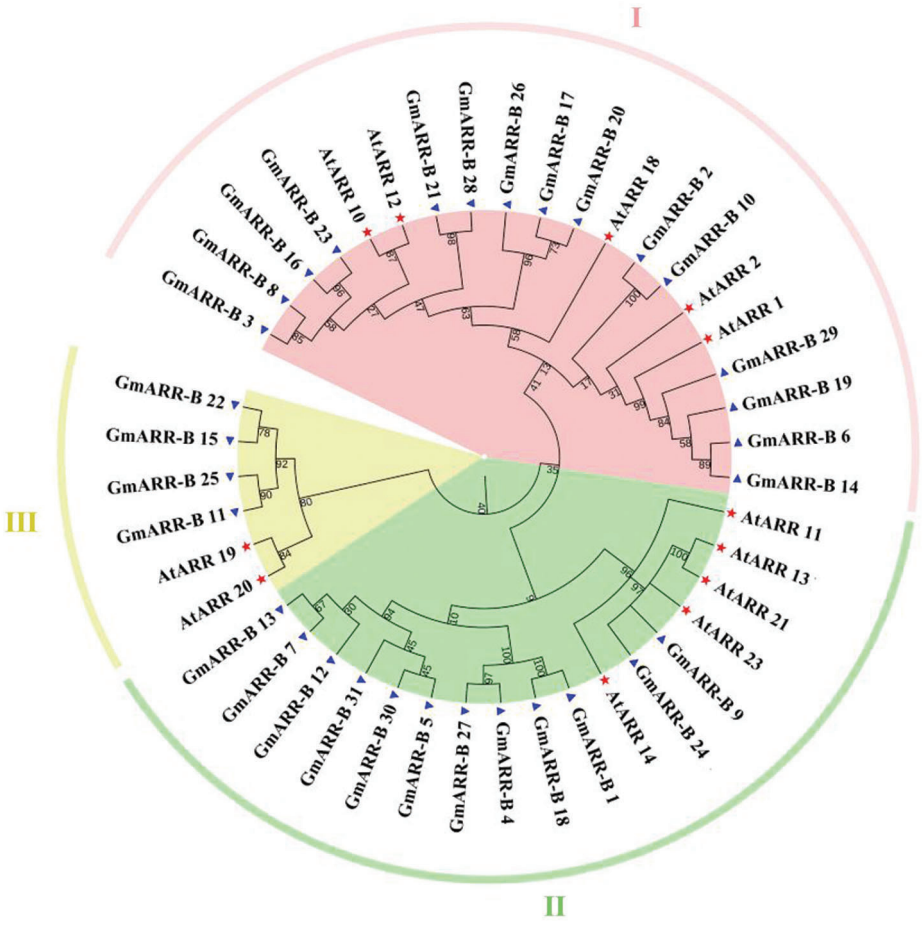

FIGURE 1. Phylogenetic relationships of the type B authentic response regulators (ARRs). Phylogenetic relationships of the ARR-Bs from soybean (Blue triangle) and *Arabidopsis* (Red Pentagram). MEGA 7.0 was used to create the phylogenetic tree. The 43 ARR-Bs from two plant species may be classified into three groups, with distinct colored arcs (Note: At stands for *Arabidopsis thaliana*, while Gm stands for *Glycine max*.).

method. *Agrobacterium tumefaciens* were cultured overnight for 24 h, gently centrifuged and diluted in osmotic buffer (10 mM $MgSO_4{\cdot}7H_2O$ with 100 μM acetosyringone; both from Sigma (Sigma GmbH, Erlangen, Germany) until optical density (OD) 0.1 and used for infiltration by syringe on the *Nicotiana benthamiana* leaf surface. Transient expression was visualized through fluorescence microscope 48 h later. An excitation wavelength of 434 nm was used and the emissions were collected between 460 and 490 nm to visualize GFP

Results

Identification and phylogenetic analysis of type-B GmARR genes in soybean

31 soybean type-B ARRs were identified in the soybean genome by queries to conduct sequence homology searches of type-B AtARRs, which is nearly three times as many as *Arabidopsis* (12). 31 type-B GmARR genes were named from GmARR-B 1 to GmARR-B 31 on the basis of their physical locations (from top to bottom) on chromosomes 1–20. We compared the amino acid sequences of all 31 putative type B-GmARR with each other to determine whether these genes contain key amino acid residues required for ARR function, as shown in Suppl. Fig. S1.

To understand the evolution history of type-B ARR genes and assist in the classification of the type-B ARR gene family in soybean, a phylogenetic tree was constructed from aligned soybean and *Arabidopsis* ARR protein sequences. A total of 43 type-B ARR genes from soybean (31), *Arabidopsis* (12) were used for phylogenetic analysis. The maximum likelihood phylogeny indicated that all of the 31 newly uncovered type-B GmARR genes and 12 type-B AtARR genes were divided into three subgroups by distinctive protein structures (Type I, Type B II, Type III; Fig. 1). The Subgroup I contained 15 GmARR-Bs and 5 AtARR-Bs, which was the largest group in this phylogenetic tree; Subgroup II included 12 GmARR-Bs and 5 AtARR-Bs; Subgroup III consisted of 4 GmARR-Bs and 2 AtARR-Bs. Among them, the distribution of GmARR-Bs in soybean was mostly concentrated in the I subgroup, and less in the II and III subgroups (Fig. 1). In addition, we also predicted the secondary protein structures of the type-B GmARR genes family in Suppl. Fig. S2.

Analysis of type-B GmARR proteins is shown in Table 1. All of these 31 type-B GmARR genes contained the domains PF00072 and PF00049 based on Pfam and SMART tests. The GmARR-B genes coded 246–699 amino acids, with calculated molecular weights from 28.30 to 76.86 kDa, average molecular weight of 63.13 kDa. At the same time, we also found that the isoelectric points of these nucleic acids ranged from 4.87 to 8.59, and most of these proteins were negatively charged, while only 4 proteins were positively charged (Table 1). Further bioinformatics investigations demonstrated that GmARR-Bs were possibly

TABLE 1

The Physicochemical and subcellular localization of GmARR-Bs proteins

Name	Reference genetic names	Gene accession number	Chromosome	Amino acid number /aa	Molecular weight /Da	Isoelectric point	Subcellular localization
GmARR-B1	APRR2	Glyma.01G200800	Chr01	559	62434.4	6.4695	Nucl
GmARR-B2	ARR2	Glyma.02G085900	Chr02	634	69759.6	6.727	Nucl
GmARR-B3	ARR12	Glyma.04G062500	Chr04	657	72493.5	6.8476	Nucl
GmARR-B4	APRR2	Glyma.05G070200	Chr05	572	63972.5	6.7627	Nucl/Cyto
GmARR-B5	ARR14	Glyma.05G115400	Chr05	382	43099.1	8.5805	Chlo/Cyto/ Nucl/ Mito
GMARR-B6	ARR11	Glyma.05G144500	Chr05	636	71711.9	5.3077	Chlo/Nucl /Mito
GmARR-B7	ARR2	Glyma.05G239800	Chr05	246	28301.4	8.4601	Nucl/Cyto
GmARR-B8	ARR12	Glyma.06G063500	Chr06	697	76774.2	7.0028	Nucl
GmARR-B9	ARR2	Glyma.07G079000	Chr07	484	54082	5.5405	Nucl/Cyto
GmARR-B10	ARR2	Glyma.07G171200	Chr07	634	69792.6	6.6105	Nucl/Chlo
GmARR-B11	ARR2	Glyma.07G243300	Chr07	680	74250.5	5.9887	Nucl/Chlo/E.R.
GmARR-B12	ARR14	Glyma.08G046700	Chr08	402	45440	5.9133	Nucl
GmARR-B13	ARR2	Glyma.08G046800	Chr08	493	54810.6	7.6784	Nucl/Chlo
GmARR-B14	ARR11	Glyma.08G100900	Chr08	511	57545.2	4.8686	Nucl
GmARR-B15	ARR2	Glyma.09G040000	Chr09	674	73845	6.5522	Nucl/Chlo
GmARR-B16	ARR12	Glyma.09G098600	Chr09	699	76222.1	5.6535	Nucl/Mito
GmARR-B17	ARR12	Glyma.10G101000	Chr10	286	32856	8.5869	Cyto/Nucl
GmARR-B18	APRR2	Glyma.11G041300	Chr11	571	63668.9	6.8168	Nucl
GmARR-B19	ARR11	Glyma.11G246400	Chr11	605	68074.7	5.4658	Nucl/Cyto/Mito
GmARR-B20	ARR12	Glyma.13G155400	Chr13	681	75246.4	6.2911	Nucl/Cyto
GmARR-B21	ARR12	Glyma.14G110600	Chr14	673	74317.5	6.0617	Nucl/vacu
GmARR-B22	ARR1	Glyma.15G145200	Chr15	673	73650.7	6.3622	Nucl/Chlo
GmARR-B23	ARR12	Glyma.15G206200	Chr15	702	76856.1	6.1364	Nucl
GmARR-B24	ARR2	Glyma.16G198800	Chr16	670	73975.8	8.2171	Nucl/Cyto
GmARR-B25	ARR2	Glyma.17G030600	Chr17	678	73855.2	6.1466	Nucl/Cyto/vacu/E.R.
GmARR-B26	ARR12	Glyma.17G076000	Chr17	682	75164.2	5.1623	Nucl/Cyto
GmARR-B27	APRR2	Glyma.17G152800	Chr17	577	64279.9	6.7623	Nucl
GmARR-B28	ARR12	Glyma.17G217100	Chr17	668	73691.4	6.3288	Nucl
GmARR-B29	ARR11	Glyma.18G010800	Chr18	456	51483.7	6.6936	Nucl/Cyto
GmARR-B30	ARR2	Glyma.19G047900	Chr19	386	43672.3	7.2319	Chlo/Cyto/Nucl/Mito
GmARR-B31	ARR2	Glyma.19G049400	Chr19	367	41962.1	6.0951	Cyto/Nucl

Abbreviations: Nucl, nucleus; Chlo, Chloroplast; Cyto, Cytoplasm; Mito, Mitochondrion; ER, Endoplasmic reticulum.

localized in a few organelles such as the nucleus, cytoplasm, chloroplasts, and mitochondria (Table 1). And most of the type-B GmARRs were located in the nucleus.

Analysis of the structure and conserved domain of type-B GmARR genes

In order to reveal the diversity of the gene sequence of the ARR-B gene family in soybean, exon-intron distribution and 10 different conserved motifs of each gene were analyzed (Fig. 2). The motifs with a size range of 15–20 amino acids were selected for identification, and other details of amino acid sequence conservation and the length of 10 conservative motifs were shown in Suppl. Table S3. The specific motifs of subfamily lead to the difference of gene function of type-B GmARR subfamily to some extent. The motif analysis showed that each group of genes classified by phylogenetic analysis has the similar conserved motif composition in its own group clearly, however, motif conservation differed slightly within each subgroup and was more dramatically across subgroups. We identified ten conserved motifs in all putative 31 type-B GmARRs and named them as Motifs 1 to 10. Motifs 1–7 were present in all of the type-B GmARRs. We also observed motifs that were specific to a subgroup, including Motif 9 and Motif 10

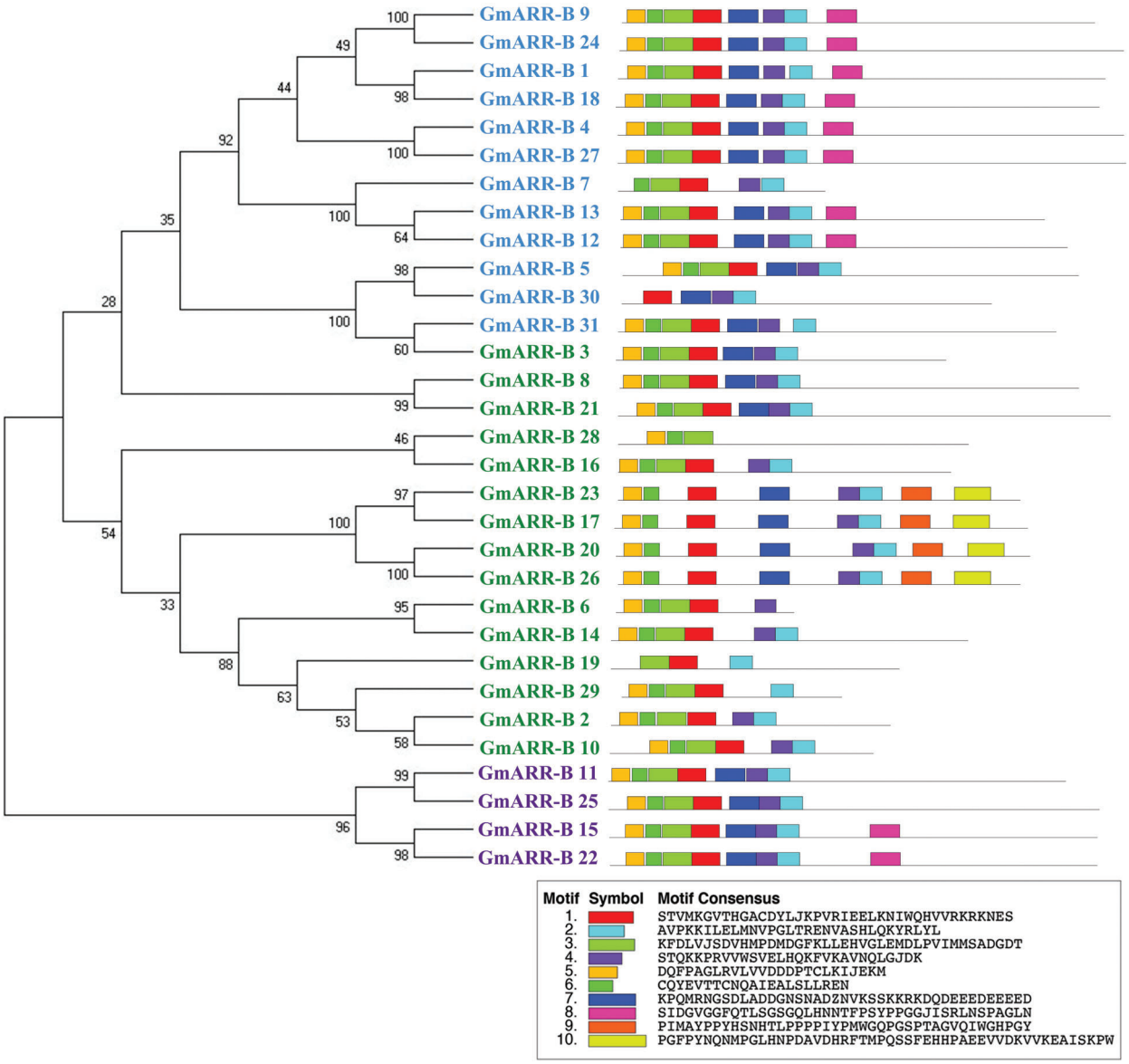

FIGURE 2. (Continued)

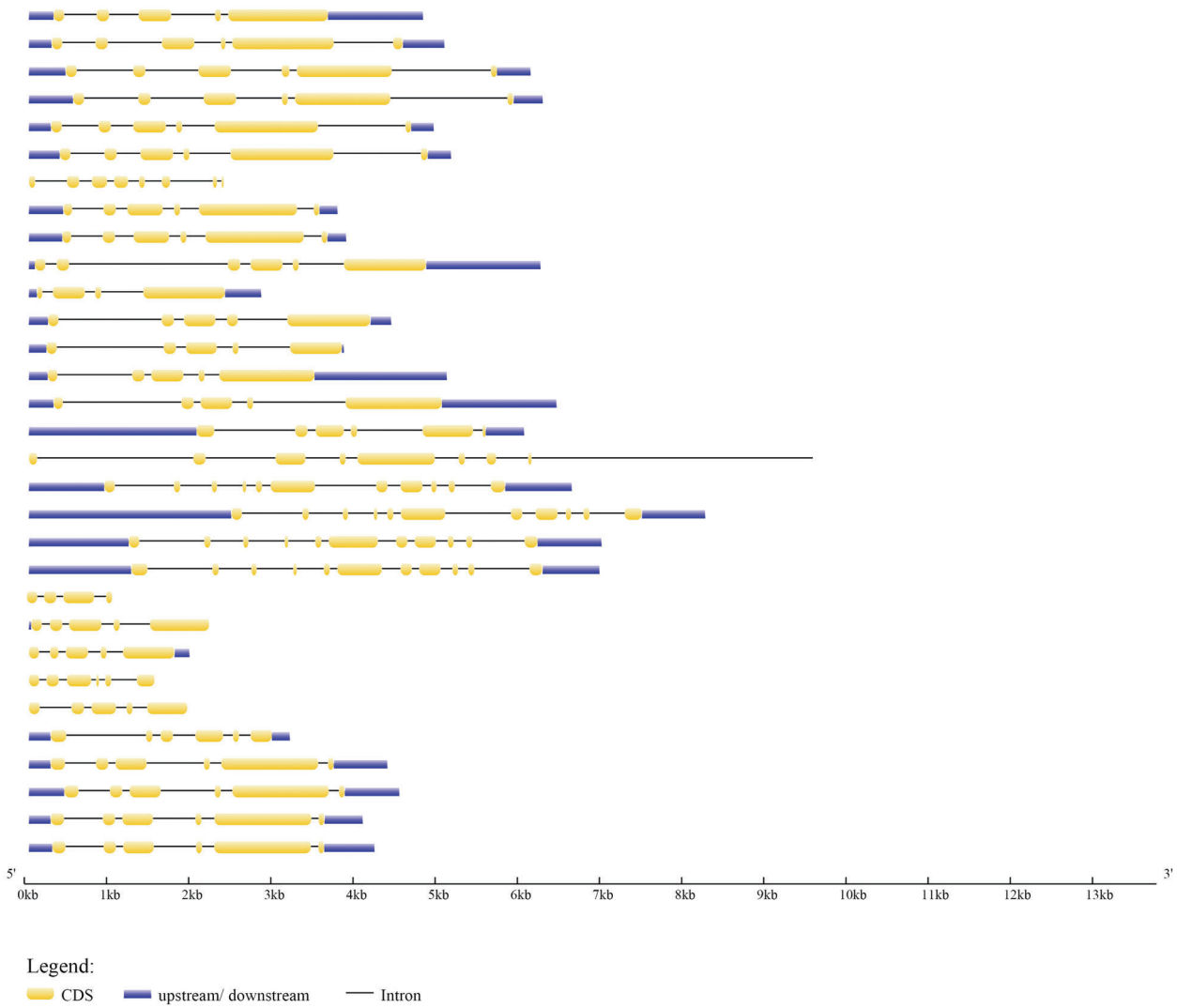

FIGURE 2. GmARR-Bs phylogenetic connections, conserved domains, and gene structures on the left side of the picture, the three subgroups are represented by different colors. Green is Subgroup 1, blue is Subgroup 2, and purple is Subgroup 3. The schematic representation of motif analysis in the center: The upper panel indicates predicted motifs in type-B GmARRs, represented in different colour using MEME suite v5.1.0. Whereas the lower panel shows the signature of each motif with conserved amino acid residues. Structural elucidation of 31 identified type-B GmARR genes on the right. The structural features of type-B GmARRs are represented in different colors, where Orange indicates an exonic region, blue indicates upstream/downstream region, black indicates intronic region.

that were only discovered in Group II genes. Similarly, motif 8 was found to exist in Groups I and III genes. In GmARR-Bs, the similarity of conservative motifs was high, and the homology was relatively close, which suggested that the function was relatively conserved.

The evolution of the genome and the functional divergence of multigene family members are both linked to gene structure. Exonic and introic regions differed among the 31 type-B GmARRs found (Fig. 2). In type-B GmARRs, the number of introns varied from three to ten. Genes were grouped into three categories based on sequence homology, with common gene structural characteristics within each cluster. It was consistent with the results of evolutionary tree analysis. One cluster, which was comprised of Type I genes, all shared 5 homologous exons and 4 homologous introns, except GmARR-B 17. Be the same with Type I, the Type III gene cluster (GmARR-B 11, GmARR-B 15, GmARR-B 25 and GmARR-B 22) all shared 5 homologous exons and 4 homologous introns. However, Type II genes did not show such clear exon-intron arrangements across the subgroup, among them, GmARR-B 1, GmARR-B 4, GmARR-B 18 and GmARR-B 27 had the highest number of exons (11), while GmARR-B 7 had the least number of exons. The different gene structures existing in the different phylogenetic groups suggested that genes evolved into diverse exon-intron structures to carry out different functions in the soybean genome, indicating that in the variable cutting process, these genes may be from the same transcript to possess similar characteristics.

Chromosomal location, collinearity, and cis-element analysis of type-B GmARRs

The chromosomal locations and gene structures of all GmARR-Bs were downloaded from soybean genome database. The cross sections of chromosome location and

gene structure of all GmARR-B are shown in Suppl. Fig. S3. The 31 GmARR-B genes were widely distributed on 17 chromosomes except chromosome 3, 12, 20. (Fig. 3 and Suppl. Fig. S3). Chromosome 5 and chromosome 17 had the maximum of GmARR-B genes, with 4 members. Three GmARR-B genes were discovered on chromosomes 7 and 8, while two genes were discovered on chromosomes 9, 11, 15 and 19, and the other chromosomes (1, 2, 4, 6, 10, 13, 14, 16 and 18) had only one type-B GmARR gene (Fig. 3).

The generation and maintenance of gene families are mainly rely on gene tandem duplication and segmental genome duplication. We have reason to believe that two homologous genes that are physically closely linked may be produced by tandem replication. As shown in Fig. 3, synteny was used to analyze GmARR-B gene duplication. There were 10 GmARR-Bs including GmARR-B 1, 2, 4-8, 11, 14 and GmARR-B 17 showed collinearity with interior of soybean. There were 7 GmARR-Bs including GmARR-B 5, 9, 11, 14 and GmARR-B 15-17 that showed collinearity with the ARRs of dicots, *Arabidopsis*.

Each GmARR-B gene was extracted 1.5 kb genomic sequences upstream of its transcriptional start codon for cis-regulatory elements in order to further expound the biological functions and regulation network of GmARR-Bs, and 13 cis-acting regulatory elements related to stress, hormone, and development were found in the upstream sequences. Many elements linked to stress response and plant hormones were found in the promoter region of this GmARR-B family gene. The number of cis-acting regulatory elements on each ARR-B genes varied widely, from 3 to 13. As shown in Fig. 4, cold resistance related LTR, was found in the promoter region of GmARR-B 6, 8, 11, 19–22, 25, 29 and 30; the drought-induced element MYB and MBS were found in the promoter region of GmARR-B 2, 5, 6, 11, 28, 30 and GmARR-B 1, 16, 17, 23. Except for abiotic stress, hormone-responsive elements were predicted, almost all GmARR-B genes contained hormone responsive element ARE, except GmARR-B 8, 9, 16. ABA-responsive elements ABREs were found in the promoter region of GmARR-B 4, 6-10, 16-19, 27 and 31. MeJA-responsive elements were revealed in the promoter region of GmARR-B 2, 3, 5, 9, 10-12, 15, 17 and GmARR-B 19. SA-acting elements were explored in GmARR-B 1, 4, 6, 13, 14, 18, 20 and GmARR-B 24. And there were seven type-B GmARR genes (GmARR-B 1, 4, 9, 15, 16, 20, 23, 26), which were identified as auxin homeostatic elements and five type B GmARR genes (GmARR-B 2, 7, 17, 23, 25) were identified as gibberellin homeostatic elements. In addition, as light-responsive elements, MRE was found in the promoter region of GmARR-B genes.

Tissue and organ specific expression of GmARR-B genes in soybean

To dissect the expression patterns of GmARR-B genes in the tissue and organ, the transcription level of GmARR-Bs in eleven diverse tissues were analyzed (Suppl. Table S1). The heat map (Fig. 5) revealed that the expression of 31 GmARR-Bs was high in the apical meristem, seed, root, leaf, nodules, and flower, with the expression patterns largely grouped into three Groups (A–C) based on the hierarchical

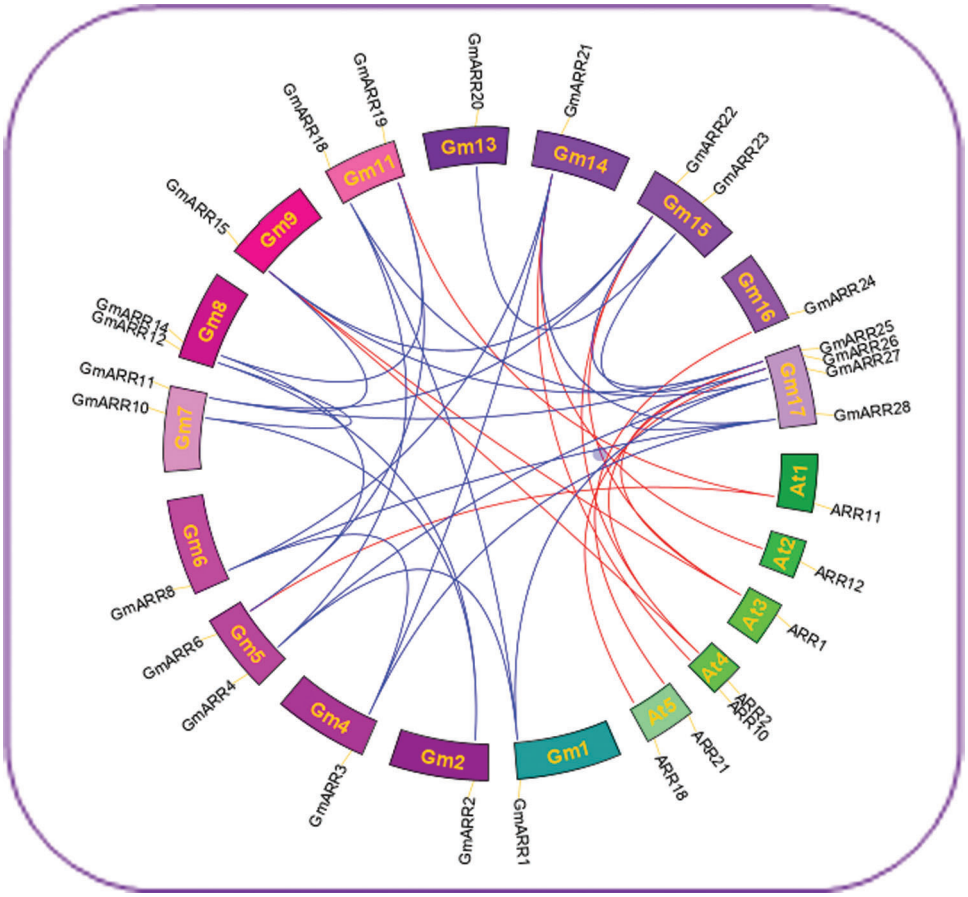

FIGURE 3. The Circos plot representation for synteny relationship of ARR-B genes in soybean and *Arabidopsis* using CIRCOS v0.52. Each chromosome is represented by a color box, with the chromosome number shown in each chromosomal box. Megabase is the scale. A short blue line indicates the approximate position of each soybean ARR-B gene. The linkage group with segmentally duplicated ARR-B gene pairs is indicated by red lines in the circle, and segmental duplication areas were found using the Plant Genome Duplication Database.

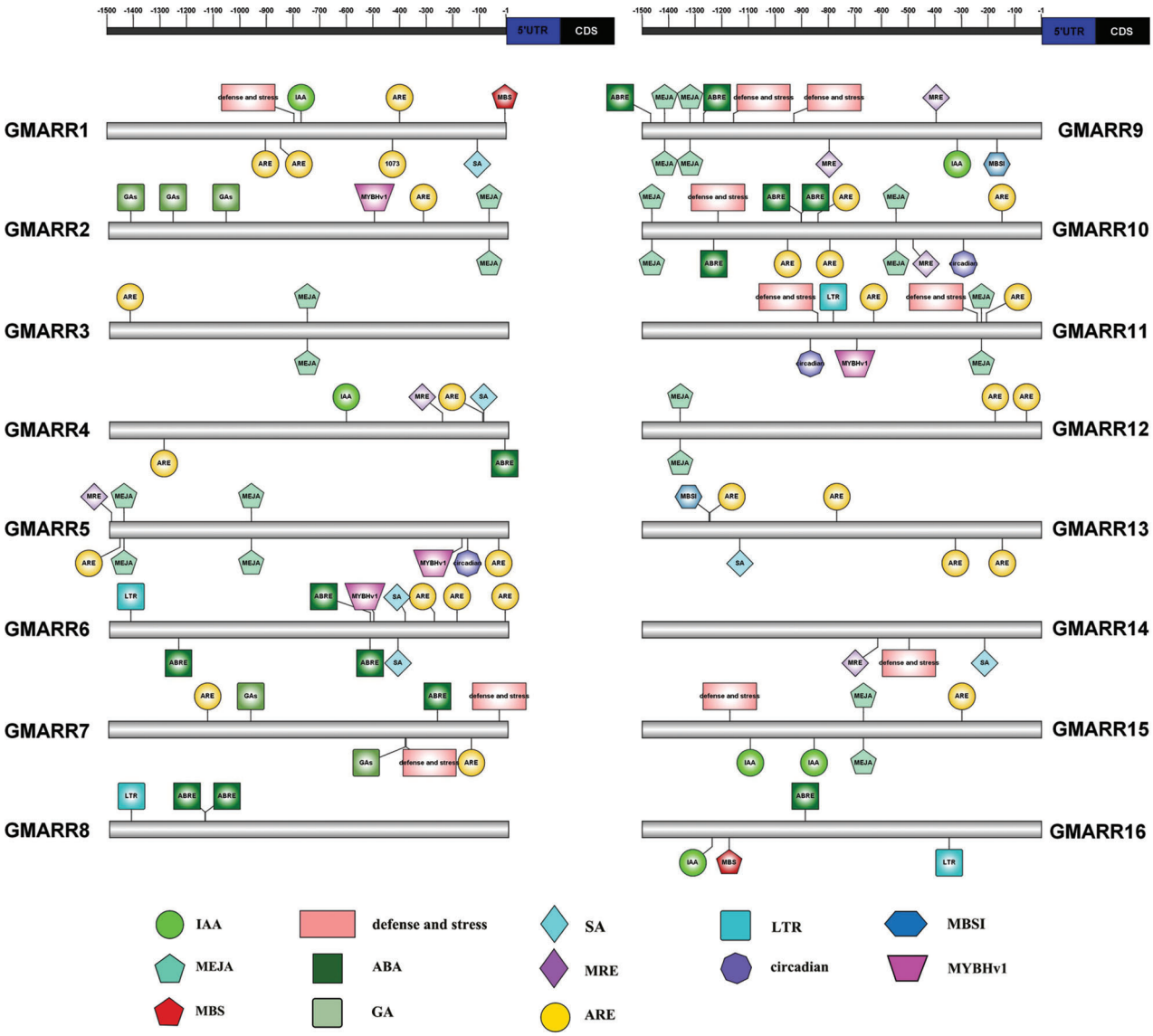

FIGURE 4. (Continued)

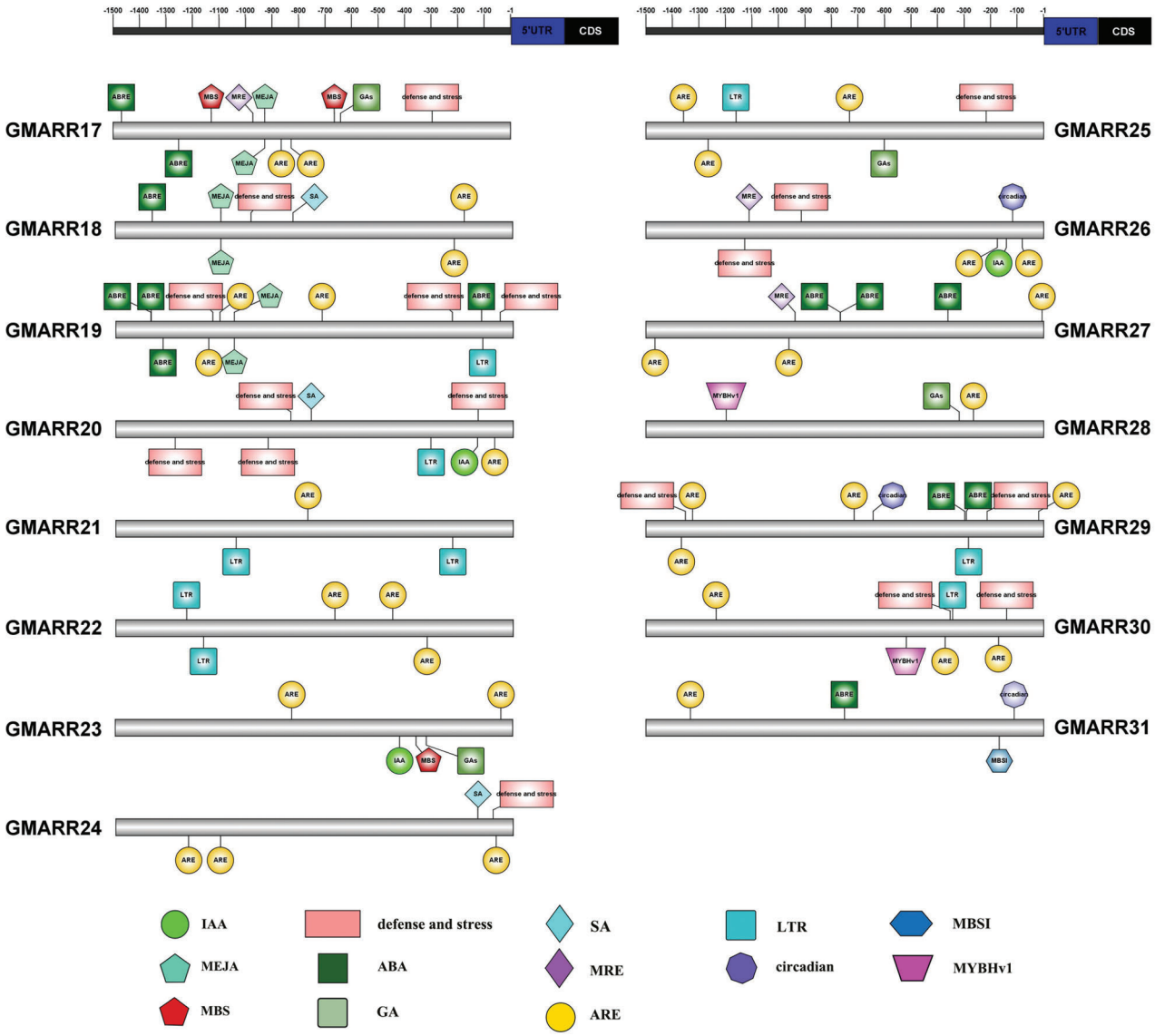

FIGURE 4. Anticipated cis-elements within the promoter districts (1,500 bp upstream from the transcription start location) of the soybean GmARR-B genes. Each functional element is represented by a different surname, and the corresponding ruler position in the 1.5 kb area before CDS is marked with a line. The action elements corresponding to different figures are shown in the lower part of the figure.

clustering analysis. Group A contained 11 soybean GmARR-B members (GmARR-B 3, 6, 8, 14, 15, 19, 21–23, 28, 29), and 9 genes of them were members of the Type II subgroup and 2 genes (GmARR-B 15, 22) of them were members of the type III subgroup. All these members showed highly expressed in root and nodules. Interestingly, Group B included 13 soybean GmARR-B members, which were all Type-I members, and expressed in seed, pod skin and flower. Among them, the expression of GmARR-B 20 was high in pod, while the transcription level of GmARR-B 1 and GmARR-B 12 were high in seed. Group C was consisted of 7 GmARR-B members (GmARR-B 4, 11, 17, 25, 27, 30, 31), most of which showed consistent expression pattern in the majority of tissues. GmARR-B 11 and GmARR-B 17 had significant levels of transcription in the root. The expression of four GmARR-B genes (GmARR-B 4, 27, 30, and 31) was also significantly increased in the shoot apical meristem. Tissue expression patterns particular to subfamilies may be linked to gene functions. The above results show that ARR-B transcription factors may play diverse roles in the growth and developmental regulation of soybean.

GmARR-Bs were involved in abiotic stress responses

Under ABA, cold, heat, salt and alkali stress conditions, the leaf spots of NBT, DAB and Evans blue staining indicated that, with the extension of stress time, the accumulation of reactive oxygen species and H_2O_2 in leaves and the degree of cell damage gradually increased (Fig. 6). In addition, compared with the control, the reactive oxygen species content and antioxidant enzyme activity of soybean under ABA, cold, heat, salt and alkali stress increased to some extent with the extension of stress time, and the most significant increase of all indexes was observed after 24 h treatment, which further reflected the stress degree of soybean (Fig. 7).

Quantitative Real-Time PCR was conducted to examine the expression level of 10 GmARR-B qualities beneath abiotic stresses, including ABA, cold, heat, salt, alkali. All

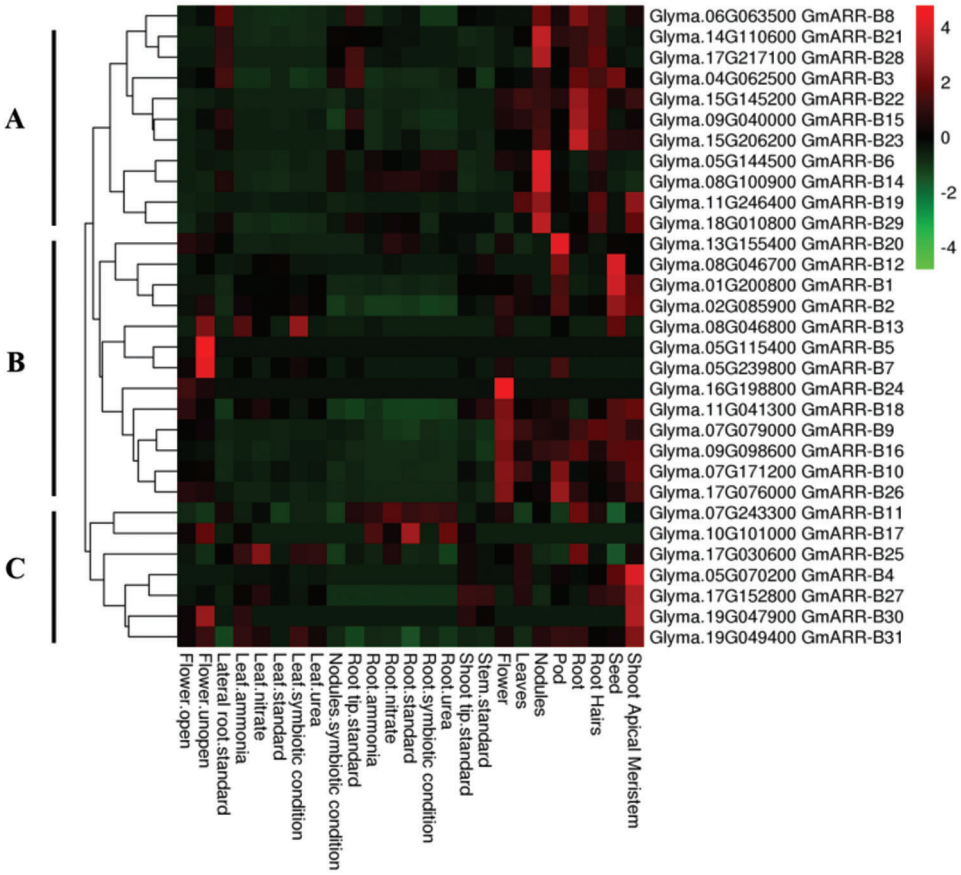

FIGURE 5. GmARR-B expression profiles in various tissues. The degree of expression is shown by the color bar to the right of the graph, which ranges from light blue to red, suggesting that the amount of expression is growing.

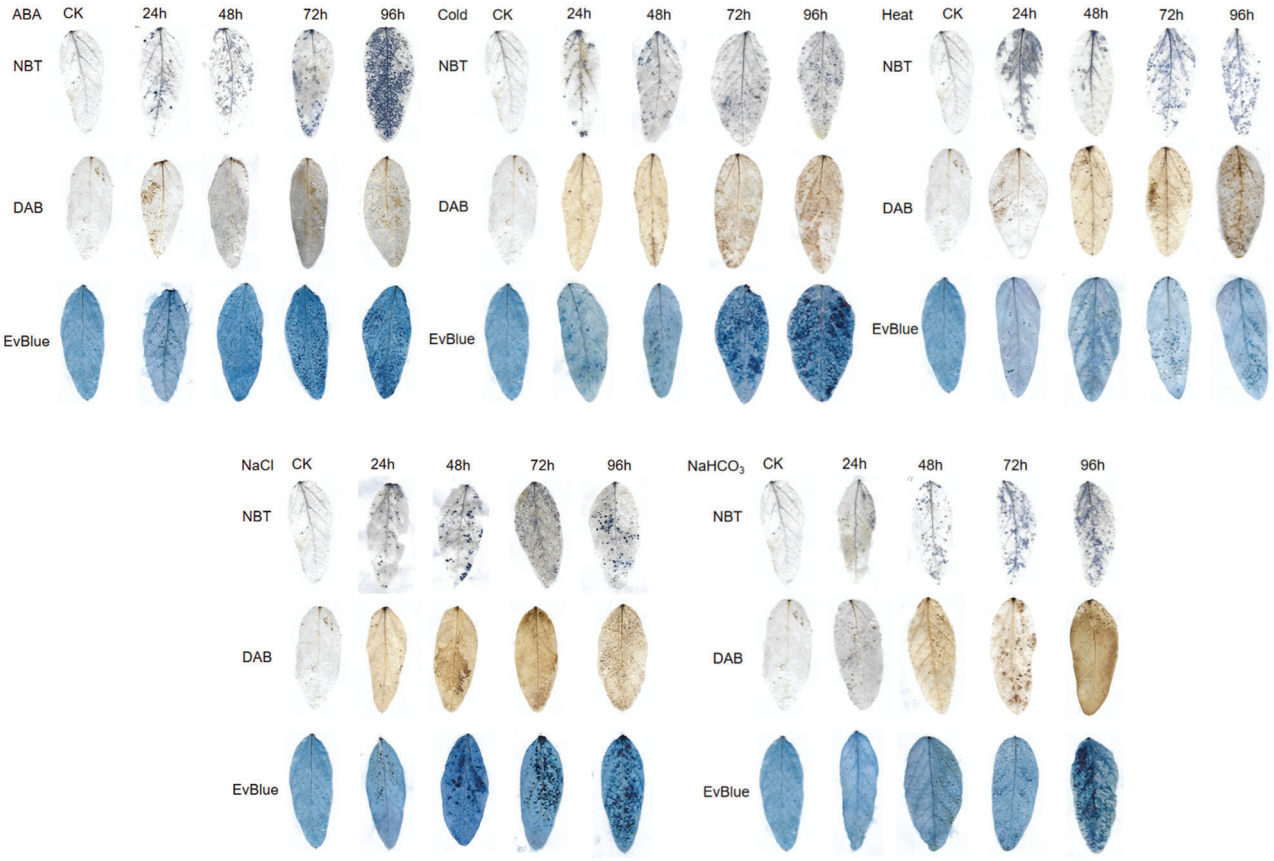

FIGURE 6. Reactive oxygen species staining of soybean leaves under different abiotic stress.

the 10 GmARR-B genes responded to the five stresses, but the reaction speeds and intensities were different. It could be seen from Fig. 8, under ABA stress (40 μmol/L), compared with the control, the expression of 10 genes were inhibited after treatment 24 h. However, partially restored to the control level at 48 h, and the transcription level of GmARR-B 5 was significantly induced. For the cold treatment, the expression of GmARR-B 9 and GmARR-B 13 was significantly up-regulated at 12 h, almost all individuals were unequivocally actuated at 24 h and 48 h, except GmARR-B 7, GmARR-B 17 and GmARR-B 30 at 24 h. Under heat conditions, the expression of 10 GmARR-B genes were inhibited at 6 h exposed to heat stress, except for GmARR-B 14, while the transcription of tested GmARR-B genes were induced, and reached the highest level at 24 h, except for GmARR-B 17. Salt stress unequivocally induced the expression of ARR genes, and most GmARR-B members individuals appeared an awfully critical reaction to salt stress, compared with control. All individuals were strongly induced between 6 h and 24 h after treatment, but for the expression of GmARR-B 30. The expression patterns of the GmARR-B qualities amid alkali base treatment displayed self-evident contrasts, showing the utilitarian differences of these genes. For GmARR-B 7, 9, 12, 30 and GmARR-B 31, the gene expression was enhanced and reached the peak at 6 h, but the transcription level of GmARR-B 13 was induced between 24 h and 48 h. And the expression of GmARR-B 17 got the highest transcription level at 24 h. Additionally, the transcription of several GmARR-B genes decreased, such as GmARR-B 5, GmARR-B 14 and GmARR-B 29.

Subcellular localization of GmARR-B 5 and GmARR-B 14

Prediction analysis indicated that GmARR-Bs exhibited various patterns of subcellular localization (Table 1), although mostly concentrated in the nucleus. GmARR-B 5 and GmARR-B 14 with high expression under abiotic stress were selected to determine if the protein encoded were embedded within the nucleus or not. The free GFP was used as the control. We transiently expressed the GFP fusion protein with these two genes in tobacco leaves. Green fluorescence signals of both fusion proteins with target gene were localized in the nucleus (Fig. 9), consistent with the predicted localization using bioinformatics programs.

Discussion

Soybean is a major leguminous trim; it is of extraordinary centrality of breeding high-yield varieties of this important crop in our changing environment. In order to adapt to the changing environment, a full understanding of the key regulators of stress resistance is essential. As important components of cytokinin signaling pathways, the type-B ARR gene family has been appeared to play imperative parts in plant reaction to different environment stresses

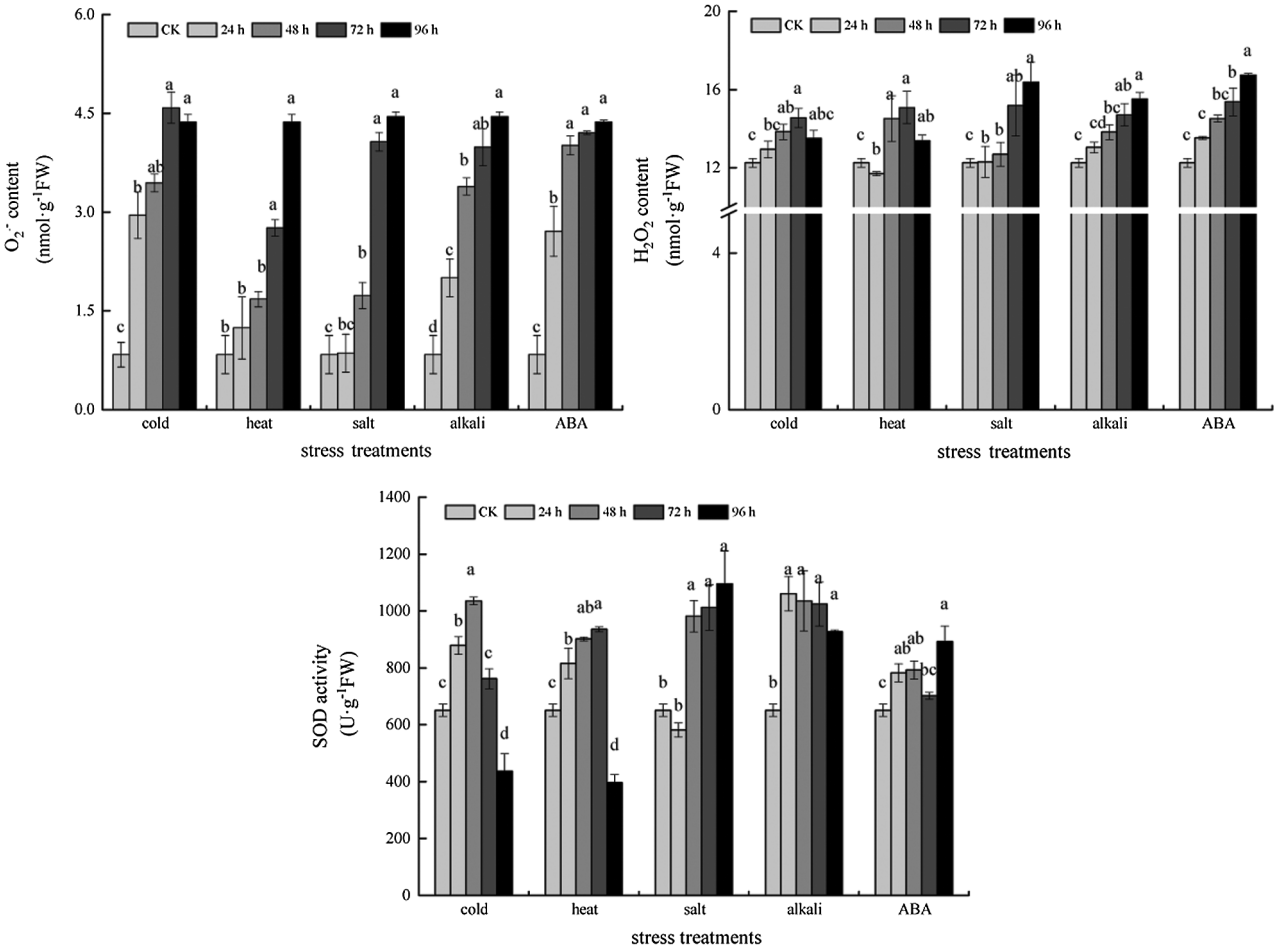

FIGURE 7. Superoxide anion, hydrogen peroxide content and antioxidant enzyme activity under different stress treatments.

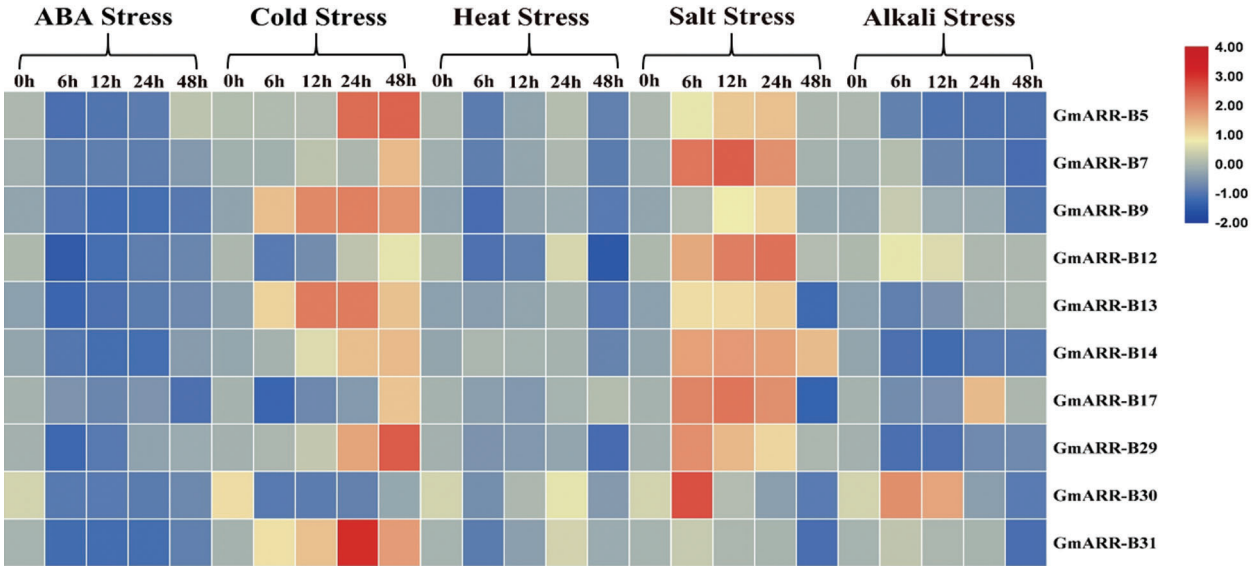

FIGURE 8. Heat map of the expression patterns of 10 GmARR-Bs under ABA, cold, heat, salt and alkali treatments by quantitative real-time PCR (qRT-PCR) analysis of ARR-B genes in soybean seedlings in response at different treatmenttime points (0, 6, 12, 24 and 48 h). The data comes from three separate biological investigations, and the relative transcription levels of GmARR-B genes were normalized to the expression of GmActin 1.

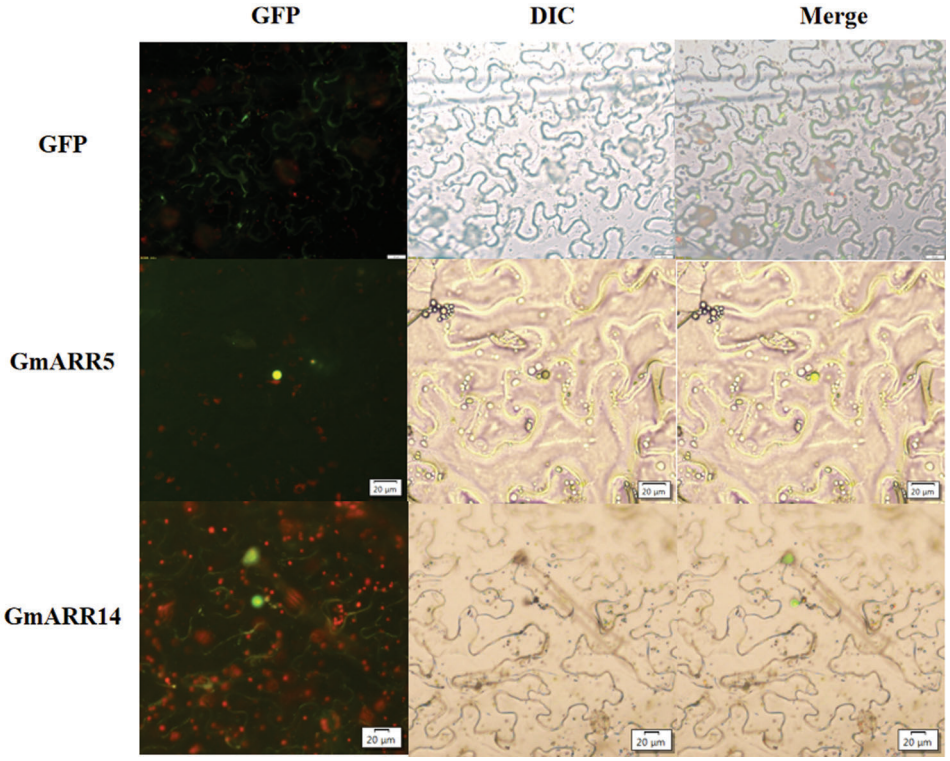

FIGURE 9. Subcellular localization of GmARR-Bs. GmARR-Bs-GFP fusion constructs were utilized to setting-out the subcellular localization of GmARR-B 5 and GmARR-B 14. Images of GFP were captured with fluorescence microscope and are shown in green. Scale bars, 20 μm).

(Nakamichi *et al.*, 2009). This paper has systematically analyzed the type-B ARR gene family in soybean, assigned the GmARR-B genes family. The results revealed that theGmARR-B genes family consisted of 31 gene members in soybean, which was largely more than those identified in other species, such as *Arabidopsis* (12) (Ramírez-Carvajal *et al.*, 2008), rice (13) (Schaller *et al.*, 2007), pear (23) (Ni *et al.*, 2017), and peach (11) (Zeng *et al.*, 2017). In previous studies on TCS system of soybean, 15 type-B response regulatory factors were also found, which were inconsistent with the results of this experiment. This difference may be due to the following reasons: In the process of identification of GmARR-B family members, the former scholars focused on TCS system, and performed similarity search among known.

TCS members' protein sequence data sets. In this study, we used *Arabidopsis* ARR-B protein sequence to query soybean proteome data set (Keiichi *et al.*, 2010). Through phylogenetic analysis, we also found that the GmARR-B genes family could be classified into three clades within the

neighbor-joining phylogenetic tree (Fig. 1). The results were consistent with B-ARR gene family in *Arabidopsis thaliana*.

The type-B ARR gene family is an old gene family with developmental inertia. As the ARR genes identified with *Arabidopsis* (AtARR) and rice (OsRR), in addition to a highly conserved phospho-accepting REC domain, type-B ARR gene family in soybean contained the B motifs (a phosphorylated Asp residue at the center and a short stretch of conserved amino acids) (Hosoda *et al*., 2002), which was consistent with previous studies in *A. thaliana*. Genetic structure analysis showed that the exon distribution and location differed across genes in the soybean type-B ARR genes family, but a much higher conservation of motifs (Fig. 2). We also discovered that the expression of GmARR-Bs was conserved in the clade, conserved domain and gene structures between homologous genes. Gene duplication could be a common wonder in eukaryotes, and contributes to biological diversity during evolution (Magadum *et al*., 2013; Taylor and Raes, 2004). To explore type-B GmARR gene duplication occasions, the location of ARR-B genes within the soybean genome was determined (Fig. 7), and homologous genes of soybean and *A. thaliana* was compared (Fig. 3). Co-expression analysis, which was a pointer of functional correlation between genes, recommended that a majority of the GmARR-B genes remained functionally correlated, in spite to their sequence divergence. Although it is not uncommon to know that there is functional redundancy in ARR genes of *A. thaliana* (Nguyen *et al*., 2016). There were 10 GmARR-Bs (GmARR-B 1, 2, 4–8, 11, 14, 17) showed collinearity with interior of soybean and 7 GmARR-Bs (GmARR-B 5, 9, 11, 14, 15–17) showed collinearity with the ARRs of *Arabidopsis* (dicot). To sum up, type-B ARR genes might have arisen from a common ancestor, these patterns are almost similar with other species, suggesting that these genes occurred in a common ancestor before Chlamydomonas and exhibited partial or complete divergence from one another between the ARR-B gene family (Pickett and Meeks-Wagner, 1995; Zhang *et al*., 2020a; Yu *et al*., 2012).

The subcellular distribution of protein is closely related to its function, which is one of the important characteristics of a protein. The accuracy of distinguishing different proteins by the major subcellular localization of proteins is over 90%. Previous studies suggest that the nucleus is integral in the coordination of various cellular activities, some of which geared at improving the performance under abiotic stress conditions (Hosoda *et al*., 2002). In this study, we identified 31 genes encoding putative nuclear type-B ARRs in the soybean genome (Table 1), similar with *Arabidopsis*, nuclear ARRs constitute the largest fraction of the ARR-B gene family in soybean (Tajima *et al*., 2004). Meanwhile, we transiently expressed GmARR-B 5 and GmARR-B 14 genes with GFP fusion protein in tobacco leave to further to verify this prediction. Green fluorescence signals of both genes fusion proteins were localized in the nucleus (Fig. 9), consistent with the predicted localization using bioinformatics programs, which suggested to function in cellular regulation in relation to stress tolerance. Furthermore, we also identified various types of conserved cis-regulatory elements in the promoter regions of GmARR-Bs to further clarify the roles in response to abiotic stresses.

Cis-acting element analysis of soybean type-B ARR genes showed that these genes were closely related to growth, hormone signal transduction and abiotic stress resistance. The expression pattern of GmARR-B genes in different tissues (Fig. 5) showed that all these members largely grouped into three Groups (A–C) based on the hierarchical clustering analysis. The distinct GmARR-B genes groups exhibited high variation in their expression profiles, dependent on tissue/organ types and developmental stages. It show that ARR-B transcription factors may play diverse roles in the growth and developmental regulation of soybean. Nguyen *et al*. (2016) reported that the promoter regions of ARR-B are rich in cis-element involved in CTK and drought resistance response, and an *Arabidopsis* triple loss-of-function mutant of ARR 1, ARR 10, and ARR 12 exhibited reduced stature, enhanced seed size, altered chlorophyll and anthocyanin content, and an aborted primary root with protoxylem but no metaxylem. It further indicated that GmARR-B family was related to the growth and development of plants. In addition, in *Arabidopsis*, the result of transient assay demonstrated that the ARR 1, ARR 2, and ARR 10 of type-B ARRs were not only involved in cytokinin signaling, but also regulated the mRNA expression of type-A ARRs (Argyros *et al*., 2008), and in Chinese cabbage, the promoter regions of BrRR 3 and BrRR 17 are rich in cis-element involved in ABA response, BrRR 3 and BrRR 17 strongly and instantly suppressed by ABA treatment (Liu *et al*., 2014), possibly related to various hormones-related cis-elements in its promoter region (Zhang *et al*., 2016; Zhang and Huang, 2010) (i.e., ABA, MeJA, IAA, and SA) (Fig. 4). The presence of these cis-regulatory elements including ABRE, MeJA, SA implicates GmARR-B genes in regulating various hormonal signaling pathways related to stress response. This result is consistent with those of the type-B ARR genes obtained in other plant species. What's more interesting is that the presence of various abiotic stress-specific cis-regulatory elements, including LTR, MYB and MBS, in the promoter region of identified GmARR-Bs could suggest that these genes were involved in stresses tolerance of soybean (Fig. 4). ARR 1, ARR 10, and ARR 12 negatively regulated plant responses to drought, and ARR 8 and ARR 9, as negative regulators, were also involved in plant responses to water stress stimulated by sorbitol (Wohlbach *et al*., 2008). Low temperature inhibits root growth by reducing auxin accumulation via ARR 1/12 (Zhu *et al*., 2015; Shi *et al*., 2012), while overexpression of ARR 5, ARR 7, and ARR 15 enhanced the freezing tolerance of plants (Kieber and Schaller, 2014). In order to further confirm the function of type-B GmARRs, we analyzed 10 ARR-B genes distributed in different parts of cells under different abiotic stresses with qRT analysis, containing ABA, cold, heat, salt and alkali stress. As shown in Fig. 8, all 10 GmARR-Bs responded to the five stress conditions, but the response speeds and intensities were different. The expression of GmARR-Bs reached the peak at 48 h after ABA and cold stress treatment. While the expression of GmARR-Bs was significantly up-regulated at 24 h after cold, heat and salt

stress. Genes expression levels coped with different treatments, especially GmARR 5 and GmARR 14, were significantly induced. Our results showed that type-B ARRs might have a positive effect on abiotic stresses tolerance. These results were consistent with the result reported previously about the type-B ARRs involved in environmental stress response of crops such as *Arabidopsis* and Chinese cabbage.

Finally, based on prior studies and the evidence we gathered in this study, we propose a model of ARR response to stresses, depicting a responsive transcriptional network (Fig. 10). Plants have developed their own self-regulation mechanism through physiological and metabolic reactions to deal with different environmental stresses. Extensive studies on the type-B ARR gene family members, *in vivo* or *in vitro*, have recommended to be involved in many biological processes, counting cytokinin signaling, plant growth, and stress responses through a chaperone or by isomerization of proline residues during protein folding. Previous studies have shown that cytokinin signaling pathway plays a significant role in plant growth and development (Kieber and Schaller, 2018). Additional recent studies have revealed the role of cytokinins and the cytokinin TCS in abiotic stress responses, including cold and drought stress. As shown in Fig. 10, on the one hand, when plants are exposed to abiotic stresses, AHKs, as stress receptor in cytoplasm, will be stimulated and regulate GmARR-B 5, 13, 30 predicted in cytoplasm and mitochondria, and GmARR-B 5, 7, 9, 17, 29, 30, 31 predicted in chloroplast through phosphorylation. The expression of GmARR-B 5, 9, 17, 29, 31 filled in cytoplasm, was significantly up-regulated under abiotic stress, and the expression of 5 in chloroplast and mitochondria was also significantly increased, especially under low temperature stress. These genes were significantly up-regulated under cold and salt stress to improve stress resistance. On the other hand, type-B ARRs expression will be affected in some degrees by CTK, ABA and SA signaling. Abiotic stress stimulates cytokinin synthesis by binding to the AHK 2/AHK 3/AHK 4 cytokinin receptors within the ER lumen, activating the transmitter domain, which autophosphorylates on a His residue (indicated by p). The phosphate is subsequently transferred to an AHP protein, which travels between the cytoplasm and the nucleus, the mitochondrion, and the chloroplast. The AHPs transfer phosphate to type-B ARRs in the nucleus, and abiotic stress, particularly cold and salt stress, dramatically up-regulated the expression of type-B ARR genes such as GmARR-B 5, 9, 13, 14, and 31, which controlled the expression of numerous target genes, including the type-A ARRs. Plant growth and development related genes, cytokinin response genes and abiotic stress related genes. Then, the type-A ARRs, which are phosphorylated by the AHPs, then feedback to block cytokinin signaling (indicated by ⊥) (Kieber and Schaller, 2014; Xie *et al.*, 2019; Nishiyama *et al.*, 2011). Meanwhile, SnRK2s interacted with ARR in the nucleus, revealing the ABA and CK signaling pathways' cross-talk. By interacting with type-B ARRs gene in the nucleus, TGA in the SA pathway controls soybean growth and abiotic stress response (Hass *et al.*, 2014). This work adds to the growing body of data suggesting the GmARR-B gene family is involved in plant abiotic stress response.

Conclusions

In this study, 31 type-B ARR genes were identified with soybean Phylogenetic analysis. Gene and protein structure analysis showed that 31 B-type GmARRs could be divided into three subfamilies and mainly distributed in the nuclear region. Exon/intron and motif analysis showed that GmARR-B genes structures and conserved motifs were

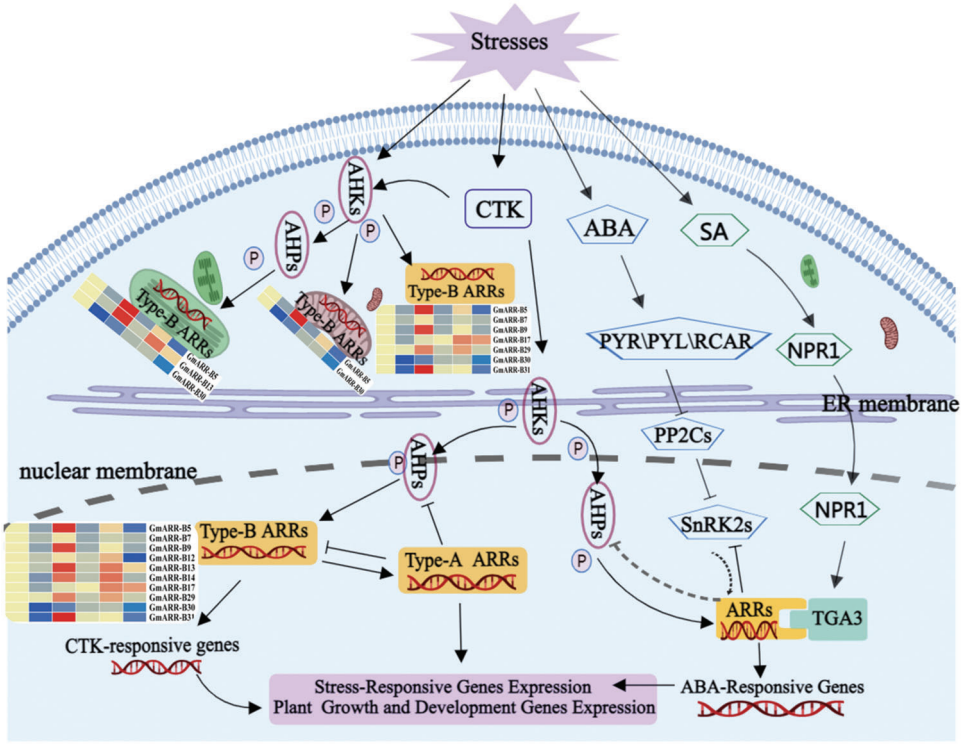

FIGURE 10. Proposed model for response regulators responding to stress and hormone signals through different regulatory pathways. (Abiotic stressors change phytohormone synthesis and distribution, which then mediates stress responses via hormone signaling components and type-B ARRs. The activation and repression effects are indicated by arrows and bar ends, respectively. MedPeer (http://www.medpeer.cn/) was used to produce this image).

similar within group but diverse between groups. It was further confirmed that the expansion of type-B GmARR gene family might mainly come from chromosome replication. Expression profiling revealed variable regulation models of GmARR-B genes in different parts of tissues, and the possible transcriptional regulatory networks of GmARR-Bs were further explored. The qRT-PCR analysis revealed that all 10 GmARR-Bs responded to five stresses, although the response speeds and intensities were different, especially for GmARR 5 and GmARR 14, which were significantly induced by cold stress, due to the presence of LTR, ABRE and other stress response elements in the promoter region of the gene family. These findings not only contribute to a better understanding of the *Glycine max* ARR-B gene family's complicated regulation, but also give useful information for future soybean functional genomics research.

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Author Contribution: Conceptualization, GY, YZ; methodology, HL, RC; software, JL; formal analysis, HL; investigation, RC, HL and XJ; resources, YZ; data curation, QC, FC, CR and ZC; writing-original draft preparation, HL; writing-review and editing, YZ; funding acquisition, YZ. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

Supplementary Figure 1 Sequence alignment of GmARR-Bs.
Supplementary Figure 2 Prediction of secondary structure of GmARR-Bs gene family proteins.
Supplementary Figure 3 Chromosome location of GmARR-Bs.
Supplementary Table 1 The RNA sequencing reads of GmARR-B genes in the tissues and organs.
Supplementary Table 2 The qRT-PCR primers.
Supplementary Table 3 Details and length of 10 conserved motifs.