Genome-wide identification and expression analysis of *CaARRs-B* transcription factor gene family in pepper (*Capsicum annuum* L.) under salinity stress

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In plants, *ARRs-B* transcription factors play a crucial role in regulating cytokinin signal transduction, abiotic stress resistance, and plant development. A number of adverse environmental conditions have caused severe losses for the pepper (*Capsicum annuum* L.) - a significant and economically important vegetable. Among the transcription factors of type *B-ARRs* family, multiple members have different functions. In pepper, only a few members of the *ARRs-B* family have been reported and characterized. The current study aimed to characterize *ARRs*-B transcription factors in *C. annuum*, including phylogenetic relationships, gene structures, protein motif arrangement, and RT-qPCR expression analyses and their role in salinity stress. In total, ten genes encode *CaARRs-B* transcription factors (*CaARR1* to *CaARR10*) from the largest subfamily of type-*B ARRs* were identified in *C. annum*. The genome-wide analyses of the *CaARRs-B* family in *C. annuum* were performed based on the reported *ARRs-B* genes in Arabidopsis. An analysis of homologous alignments of candidate genes, including their phylogenetic relationships, gene structures, conserved domains, and qPCR expression profiles, was conducted. In comparison with other plant ARRs-B proteins, *CaARRs-B* proteins showed gene conservation and potentially specialized functions. In addition, tissue-specific expression profiles showed that *CaARRs-B* genes were differentially expressed, suggesting functionally divergent. *CaARRs-B* proteins had a typical conserved domain, including AAR-like (pfam: PF00072) and Myb DNA binding (pfam: PF00249) domains. Ten of the *CaARRs-B* genes were asymmetrically mapped on seven chromosomes in Pepper. Additionally, the phylogenetic tree of *CaARRs-B* genes from *C. annuum* and other plant species revealed that *CaARRs-B* genes were classified into four clusters, which may have evolved conservatively. Moreover, the expression analysis using quantitative real-time qRT-PCR revealed nine *CaARRs-B* genes in both examined tissues that were differentially expressed, including roots, and leaves, exhibit various expression patterns as highly responsive to salinity stress. In particular, the expression of *CaARR3, CaARR5, CaARR6, CaARR7*, and *CaARR8* showed significant expression levels in roots. The

results could expand our understanding of the roles of *CaARRs-B* genes in pepper and set a good foundation for further characterization in various growth development for salinity stress processes in the *Capsicum annum L*.

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Abstract

In plants, *ARRs-B* transcription factors play a crucial role in regulating cytokinin signal transduction, abiotic stress resistance, and plant development. A number of adverse environmental conditions have caused severe losses for the pepper (*Capsicum annuum* L.) - a significant and economically important vegetable. Among the transcription factors of type *B-ARRs* family, multiple members have different functions. In pepper, only a few members of the *ARRs-B* family have been reported and characterized. The current study aimed to characterize *ARRs*-B transcription factors in *C. annuum*, including phylogenetic relationships, gene structures, protein motif arrangement, and RT-qPCR expression analyses and their role in salinity stress. In total, ten genes encode *CaARRs-B* transcription factors (*CaARR1* to *CaARR10*) from the largest subfamily of type-*B ARRs* were identified in *C. annum*. The genome-wide analyses of the *CaARRs-B* family in *C. annuum* were performed based on the reported *ARRs-B* genes in Arabidopsis. An analysis of homologous alignments of candidate genes, including their phylogenetic relationships, gene structures, conserved domains, and qPCR expression profiles, was conducted. In comparison with other plant ARRs-B proteins, *CaARRs-B* proteins showed gene conservation and potentially specialized functions. In addition, tissue-specific expression profiles showed that *CaARRs-B* genes were differentially expressed, suggesting functionally divergent. *CaARRs-B* proteins had a typical conserved domain, including AAR-like (pfam: PF00072) and Myb DNA binding (pfam: PF00249) domains. Ten of the *CaARRs-B* genes were asymmetrically mapped on seven chromosomes in Pepper. Additionally, the phylogenetic tree of *CaARRs-B* genes from *C. annuum* and other plant species revealed that *CaARRs-B* genes were classified into four clusters, which may have evolved conservatively. Moreover, the expression analysis using quantitative real-time qRT-PCR revealed nine *CaARRs-B* genes in both examined tissues that were differentially expressed, including roots, and leaves, exhibit various expression patterns as highly responsive to salinity stress. In particular, the expression of *CaARR3, CaARR5, CaARR6, CaARR7*, and *CaARR8* showed significant expression levels in roots. The results could expand our understanding of the roles of *CaARRs-B* genes in pepper and set a good foundation for further characterization in various growth development for salinity stress processes in the *Capsicum annum L*.

Keywords: *Capsicum annuum*, *CaARRs*-type B transcription factor, Gene structure, Phylogenetic analysis, Expression analysis, and Salinity stress.

1. Introduction

Peppers (*Capsicum annuum* L.) are important and widely grown vegetables belonging to the family Solanaceae, which includes eggplants, tomatoes, potatoes, etc. In temperate and subtropical regions worldwide, *C. annuum* is commonly cultivated as a seasoning vegetable. Peppers distribution, growth, and development are influenced by abiotic stresses such as drought, high salinity, and temperature extremes. Therefore, it is crucial to investigate the mechanisms behind Pepper's tolerance to such stresses (Chen et al., 2015).

Cytokinins are adenine derivatives, which are important plant hormones that regulate nearly all aspects of plant growth and development; they regulate cell division and metabolism, stimulate chloroplast development, modulate shoot and root development, and delay senescence (Mok & Mok, 1994; Haberer & Kieber, 2002; Kakimoto, 2003; Werner et al., 2003). Signals are transduced using two-component phosphorelay systems, and elements of these systems act in response to cytokinin and ethylene hormones, as well as to red light and osmosis (Schaller, 2000; Hutchison & Kieber, 2002; Hwang, Chen & Sheen, 2002; Mason et al., 2005). Initially, the two-component systems were discovered in bacteria, which are controlled by His sensors kinase and response regulators (Mizuno, 1997; Stock, Robinson & Goudreau, 2000). In addition, it has been suggested that endogenous cytokinin levels are closely correlated with pleiotropic developmental alteration (Ferreira & Kieber, 2005).

There are two types of Arabidopsis response regulators, type-A and type-B, according to their domain structure and sequence (Imamura et al., 1999). Numerous studies indicate that type-B *ARRs* contain DNA binding and receiver domains (Sakai, Aoyama & Oka, 2000; Lohrmann et al., 2001; Hosoda et al., 2002). Analysis of the Arabidopsis genome reveals 23 Authentic response regulators *ARRs*, classified into four types based on their core receiver domain structure and C-terminal domain sequence, including type A, type B, type C, and pseudo (Schaller et al., 2007). Type-*B ARRs* have a DNA-binding domain followed by a receiver domain, thus serving as transcription factors (Lohrmann et al., 2001; Hosoda et al., 2002). Interestingly, type-*A ARR* genes appear to be the direct targets of *ARR-B* regulators, which can be induced by the phosphorylated *ARR-Bs* (Hwang, Chen & Sheen, 2002; Imamura et al., 2003). *ARR-Bs* have also been reported to function as positive regulators of cytokinin signaling (Mizuno, 2004). The type-*B ARR* is unified as the type-B authentic response regulator *ARR-B* in this study.

Lately, the pepper genome has been sequenced (Kim et al., 2014; Qin et al., 2014; Magdy et al., 2019; Magdy & Ouyang, 2020). In addition, many RNA sequencing reads derived from several tissues such as root, shoot, leaf, flower, and fruit are also available. These data sets provide a framework for identifying and functionalizing a gene family from a global view for pepper improvement and basic research. This study aimed to identify all potential *ARRs-B* genes encoded in the pepper genome. Additionally, chromosomal distribution, phylogenetic analysis, and gene structure analyses were carried out by using routine bioinformatics analyses. As well as functional predictions based on gene expression analysis during different developmental stages and salinity stress responses. The results will provide an essential foundation for future studies on gene family and functional characterization of *ARRs-B* in Pepper.

2. Materials and Methods

2.1 Identification of members of the *CaARRs-B* gene family in Pepper

The *Arabidopsis thaliana* containing the structural domain of *ARRs-B* genes were retrieved from the TAIR.org database. The obtained sequences were used as probes for homology searches using BLASTp to prevent the erroneous loss of sequences with low similarity to the probes (Wang et al., 2020a). BLASTP searches selected the sequences of *CaARRs-B* genes to the online pepper genomics database (http://peppersequence.genomics.cn/), where the genome database of the *C. annuum* cultivar Znula was selected (Qin et al., 2014). The search yielded Pepper *ARRs-B* candidate genes. The physicochemical properties of the *ARRs-B* family genes were estimated using the online tool Expasy (http://web.expasy.org/) (Gasteiger et al., 2005). Finally, the subcellular localization analysis of the pepper *CaARRs-B* gene family was performed using the online tool Cell-Ploc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). The *CaARRs-B* gene family protein sequences were aligned with *A. thaliana ARR-B* genes by MAFFT aligner (Katoh & Standley, 2013) using the embedded algorithms in Geneious Prime. Subsequently, the rooted phylogenetic trees were generated using the maximum likelihood methods (ML). The ML tree was computed using FastTree V2 (Kumar et al., 2018), embedded in Geneious Prime.

2.2 Chromosomal location, Gene structure, domain annotation, and secondary structure analysis

The chromosomal location of *CaARRs-B* genes throughout the pepper genome was investigated using the MG2C V2 online tool (Chao et al., 2021). GSDS was used to identify *CaARRs-B* gene exon-intron structures by comparing their cDNA sequences to their genomic sequences (http://gsds.cbi.pku.edu.cn/) (Marchler-Bauer et al., 2015). The putative function of these motifs was predicted through InterProScan (Wang et al., 2022). Finally, the online software SPOMA was used to predict the secondary Structures of *CaARRs-B* proteins (Sapay, Guermeur & Deléage, 2006).

2.3 Potential cis-element analysis in promoter regions

BLASTn search over pepper genome cultivar Znula utilized as query sequences for *CaARRs-B* genes. The 1.5 kb of genomic sequences upstream of the initiation codon of each gene was retrieved for The PlantCARE database and has been analyzed for potential cis-elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.4 Plant materials and salinity stress treatment

Seeds of the pepper cultivar Gedeon F1 (*Capsicum annuum* L.; https://www.syngenta.com.eg/) were sterilized with 1 % sodium hypochlorite for 30 min, washed with sterile water, and then sown in perlite beds at 28º C following (Qin et al., 2014). The germinated seeds were sown in pots and grown under 16h/light at 25 °C and eight h/night at 18 °C with a relative humidity of 60 % until the seedlings developed six leaves. Plants were irrigated with Hoagland solution at half-strength pH 5.6. Leaves and roots were harvested from the seedlings with three biological replicates as control. For the salt stress experiment, five-week-old plants were irrigated with 200 mM NaCl (Wang et al., 2022). Each sample (containing three to four leaves) with three biological replicates was collected after 12H and 24H of treatment. Liquid nitrogen was used to rapidly freeze samples, followed by -80°C storage until RNA extraction.

2.5 RNA expression and qPCR analysis

According to the kit manufacturer's instructions, total RNA was extracted from pepper tissues (leaves and roots) at different salt stress treatments using EasyPure® Plant RNA Kit (TransGen Biotech, Beijing, China). The extracted RNA was assessed by electrophoresis on 2% agarose gels and Quantus™ Fluorometer (Promega, USA) for the quality and quantity assessment. The cDNA synthesis performed by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and adjusted to 100 ng $\mu L^{-}1$. A range of 108 to 147 bases were amplified in the PCR by avoiding the conserved region (Table 1).

Quantitative real-time RT-PCR (qRT-PCR) was performed using TransStart® Green qPCR SuperMix (TransGen Biotech, Beijing, China). The amplification reactions were performed following: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s. Melting curve analysis, performed by increasing the temperature from 55 to 95 °C (0.5 °C per 10 s), and a gel electrophoresis of the amplified fragments confirmed that the product contained single amplicons. In each experiment, the relative fold differences were calculated using the $^{\Delta\Delta}$ Ct. Normalization was conducted using GAPDH as the reference gene (Arce-Rodríguez & Ochoa-Alejo, 2015). In this experiment, three biological replicates per sample were used.

3. Results

3.1 Identification of the *CaARRs-B* family genes members in Pepper

The identification of the *CaARRs-B* family genes in the *C. annuum* genome (cultivar Gedeon F1) based on the pepper genome sequences database was performed using BLASTp database search to query *Arabidopsis thaliana ARRs-B* genes. In total, ten sequences were putative as pepper *ARRs-B* genes (*CaARRs*). The chromosome location, exon number, and genomic and physiochemical characteristics of each gene are in Table (2). In the current study, the shortest putative open reading frame (ORF) was 1374, while the longest was 2025, with the amino acid length of *CaARRs* proteins ranging from 389 to 684, a molecular weight (Mw) of 43.09 to 75.16, and a theoretical isoelectric point (PI) varying from 5.39 to 9.47. In addition, the presence of the ARR-like domain was verified using the Pfam database, and its position was unfixed among all copies, while all copies were sublocalized in the nucleus.

3.2 Alignment and Phylogenetic Analysis of *CaARRs-B*

Alignment and phylogenetic analyses were conducted to confirm the identified *CaARRs* copies and determine the evolutionary relationships of the *CaARRs-B* genes of Pepper and *ARRs-B* genes of Arabidopsis. With full-length amino acid sequences, the alignment of the *ARRS-B* domain was conducted and used to perform an unrooted phylogenetic tree with a bootstrap test. The genes were further submitted to CDD, Pfam, and SMART to confirm the *CaARRs-B* domain, and the presence of the Myb DNA binding domain was confirmed that distinguish the *ARR-A* from *ARR-B* genes. The analyzed *ARRs-B* proteins were grouped into five distinct clusters. The *CaARRs-B* proteins in

Pepper were clustered into four of the five subclusters with strong bootstrap support (Fig. 1). The presence or absence of species-specific *CaARRs-B* could have resulted in functional divergence.

3.3 Chromosomal location and duplication event of *CaARRs-B* genes

As indicated by the starting and ending positions of *CaARRs-B* genes on the chromosomes in Table (1), the genomic DNA sequences of each *CaARRs-B* gene were mapped to the chromosomal location (Fig. 2). With few exceptions, *CaARRs-B* genes were mainly found at the extremities of their respective chromosomes. The *CaARRs-B* genes were asymmetrically distributed on chromosomes 1, 5, 6, 7, 9, 11, and 12, and none were found on chromosomes 2, 3, 4, 8, and 10. The *CaARR* 1 and 2 genes were located on chromosome 1, *CaARR* 3 and 4 were located on chromosome 5 with a separation of 10 Mb, and *CaARR5* was on chromosome 6. In addition, *CaARR* 6 and 7 take place on chromosome 7 with a separation of <1 Mb, while chromosome 9 had *CaARR8*, chromosome 11 had *CaARR9*, and *CaARR10* was in chromosome 12 (Fig. 2).

3.3 Predicted secondary structures of *CaARRs-B* proteins

The prediction of the secondary structure of *CaARRs-B* proteins was conducted. The primary constituent forms of the secondary structure of the pepper *ARRs-B* protein, with α-helices, β-turns, and extended chains, were investigated and measured. According to structural predictions, pepper *ARRs-B* gene family members contain α-helices, irregularly coiled, extended chains, and β-turns. The most average proportion for alpha-helices was around 34.1 ± 8%; the highest percentage was from *CaARR6* (84.7%) the lowest was from *CaARR2* (26.3%). Most genes have averaged around 21.6 ± 4% β-turns; the highest percentage was from *CaARR3* (26.3%) the lowest was from *CaARR5* (13.8%). The results show that the irregular coiling average record was 23.3 ± 6%, as the highest proportion was for *CaARR7* (30.3%), while the lowest was for *CaARR6* (13.7%; Table 3).

3.4 Gene structure *CaARRs-B* proteins

In order to examine the *CaARRs-B* gene family structural characteristic further, exon-intron distribution and conserved motifs were analyzed for each gene. For each gene, several isoforms were detected with the exception for *CaARR2* and *CaARRs* 5 – 10 (unique isoform). The *CaARR1* recorded two isoforms where one was complete and a shorter isoform (x_2: 1431 bp and 477 aa). The *CaARR3* recorded three isoforms: one complete and two shorter isoforms (x_2: 1992 bp and 664 aa; x_3: 1956 bp and 652 aa). Finally, the *CaARR4* recorded five isoforms where one was complete, and five shorter isoforms (x_2: 1785 bp and 595 aa; x_3: 1896 bp and 632 aa; x_4: 1899 bp and 633 aa; x_5: 1908 bp and 636 aa).

Exonic and intronic regions varied across the identified 10 *CaARRs-B*. The number of introns ranged from 4 to 10, whereas the number of exons ranged from 5 to 11. Based on the CDS, the *CaARRs-B* genes were clustered into three groups according to their sequence homology, exhibiting similar gene structures within each cluster. Regardless of their chromosomal location, one cluster included *CaARR6 and CaARR8*, another included *CaARR7*, *CaARR1*, and *CaARR5*, and the other included *CaARR2, CaARR4,* and *CaARR9* (Fig. 3).

3.5 Potential *cis*-element analysis in promoter regions of *CaARRs-B* genes

To further characterize the potential regulatory mechanisms of *CaARRs-B*, 1500 bp upstream sequences from the translation start sites were analyzed. Based on their biological significance, cis-elements in *CaARRs-B* were identified and categorized. A total of 146 putative cis-elements in the promoter sequences were identified for 10 *CaARRs-B* genes. The results from promoter prediction showed that *CaARRs-B* promoters included abscisic acid responsiveness, auxin responsiveness, binding site of ATBP 1, defense and stress responsiveness, endosperm expression, enhancer-like involved in anoxic specific inducibility, Gibberellin responsiveness, light responsiveness, low temperature responsiveness, MeJA responsiveness, meristem expression, MYB binding site involved in drought inducibility, MYB binding site involved in flavonoid biosynthetic genes regulation, MYB binding site involved in light responsiveness, MYBHv1 binding site, Salicylic acid responsiveness, and zein metabolism regulation (Fig. 4). The cis-acting elements were classified into three main groups: hormones, environmental stress, and photoresponse. According to the cis-component, CaARRs-B genes can respond to different abiotic stresses.

3.6 *CaARRs-B* gene expression profiles in response to salinity stress

The expression level of the *CaARRs-B* was measured with qRT-PCR for seedlings exposed to salt stress. The expression patterns of *CaARRs-B* genes varied among stress levels and showed considerable variation in expression patterns of the *CaARRs-B* genes over time in the roots of plants more than in leaves. A heat map was generated to show the expression profiles while the significant differences were highlighted (Fig. 5). In response to salinity stress, the expression of patterns of *CaARR2* and *CaARR9* in the roots were up-regulated after 12 and 24 h; additionally, *CaARR5* and *CaARR6* were up-regulated in roots after 24 and 12 h, respectively. Meanwhile, the *CaARR3*, *CaARR4*, *CaARR7*, and *CaARR8* were highly expressed in control samples and were downregulated after 12 and 24 h of treatments. In the leaves, the expression level of *CaARR1* was the only expressed *CaARR* gene, and only in control samples, followed by *CaARR5,* which showed slight expression after 24 h of salinity treatment. The expression of *CaARR2, CaARR6,* and *CaARR9* increased slightly after 24 h of salinity treatment. The expression of *CaARR3*, *CaARR4*, *CaARR7*, and *CaARR8* was downregulated in 24h. The expression level on the leaves of *CaARR6*, *CaARR5*, and *CaARR67* was decreased, while *CaARR3* was induced after 12 h and 24 h of stress. The *CaARR10* was the only copy that showed no amplification curves during the qPCR.

4. Discussion

ARRs were initially recognized as essential components of prokaryotic His-Asp phosphorelay signal transduction pathways (Suzuki et al., 1998). Based on the phylogeny of the *ARR-B* gene family, it was determined that they originated from nonvascular plants (bryophytes) like phylostratum, which showed that *ARR-B* genes evolved from nonvascular plants. Several orthologous genes exist in the Plantae kingdom (Cuming et al., 2007). The *ARR-B* genes have remained evolutionary conserved in each of the selected species. Their expansion in higher plants reflects this; *ARR-B* is a transcription factor as they have a receiver domain at the C-terminal region and a DNA-binding domain at the N-terminal region (Ishida et al., 2008). Based on their conserved domains, ARRs have been classified as types A and B (Imamura et al., 1999). A key component of cytokinin signaling pathways, type *B-ARRs* genes have been linked to plant response to various environmental stresses; in response to different stress conditions, superoxide anion and hydrogen peroxide contents were measured as antioxidant enzyme activity (Nakamichi et al., 2009). There are *ARRs-B* transcription factors identified in Arabidopsis genomes (Mason et al., 2004; Ramírez-Carvajal, Morse & Davis, 2008), soybean (He et al., 2022), fragrant rice (Rehman et al., 2022), rice (Schaller et al., 2007), pear (Ni et al., 2017), and peach (Zeng et al., 2017). Despite this, little is known about *ARRs-B* in Pepper. Studies have shown that gene organization plays an important role in how multiple gene families evolve (Xu et al., 2012; Ullah et al., 2019; Wang et al., 2020; Arce-Rodríguez, Martínez & Ochoa-Alejo, 2021; Ahiakpa et al., 2022). In the current study, ten *CaARRs-B* genes in pepper have been identified and characterized by their structure, cis-elements in the promoter regions, chromosomal location, gene duplication, and phylogeny. In addition, salinity stress affects the expression profile of different tissues. To characterize the biological function of the *CaARRs-B* gene family, a comprehensive analysis of their gene family was conducted.

From the public genomic data, we derived the genomic sequences, protein sequences, and chromosomal locations of the identified *CaARRs-B* genes (Chao et al., 2021); and SMART for protein-conserved domain annotation (Marchler-Bauer et al., 2015). The protein sequences were analyzed with the software SPOMA to predict the secondary Structures of *CaARRs-B* proteins and obtain the number of amino acids and molecular weight (Mw) (Sapay, Guermeur & Deléage, 2006). Based on InterProScan predictions, these motifs are thought to serve a functional purpose (Wang et al., 2022). A total of ten predicted *CaARRs-B* genes were found on seven chromosomes, suggesting the family consists of segmental repetitions. During plant genome evolution, duplication or large-scale segmental duplication is thought to produce gene families (Cannon et al., 2004). Many transcription factor families have been reported in gene duplication events, including *C2H2-ZF, NAC, SlARR-B, MYB,* and *HD-ZIP* (Liu et al., 2015; Chen et al., 2015; Arce-Rodríguez, Martínez & Ochoa-Alejo, 2021).

All the *CaARRs-B* homologous gene pairs identified using the pepper genome *versus* the Arabidopsis genome showed tight phylogenetic clustering; these homologous gene pairs have more closely related topologies, indicating they are more closely related. In addition, intron numbers were related to *CaARRs*-B gene classifications. The duplication of genes is crucial to genomic expansion and realignment (Kumar, Tyagi & Sharma, 2011). Based on the phylogenetic tree and synteny analysis, the results were consistent. The variation in the *CaARRs-B* family gene could be caused by gene or genome duplication events, which have been considered the primary source of genetic evolution. The result showed that segmental duplication events promote the evolution *of CaARRs-B* genes (Yang, Tuskan & Cheng, 2006; Yang et al., 2008; Gillis et al., 2009).

Further clarifying the roles of *CaARRs-B* promoter regions in response to abiotic stresses, we also identified several conserved cis-regulatory elements. An analysis of cis-acting elements in pepper type-*B ARRs* genes showed a close relationship between these genes and growth, hormonal signal transduction, and abiotic stress resistance. Several cis-elements involved in drought resistance are found in the promoter region of *ARR-B*, and a triple mutant lacking all three of these genes was reported in Arabidopsis (Nguyen et al., 2016). Further, *ARR-B* belongs to the helix-loop-helix family and are nuclear-localized transcription factors, as evidenced by their helix-loop-helix structure in the *CaARRs-B* domain (Hosoda et al., 2002). The *ARRs-B* regulators target type-A *ARRs* genes directly, which are activated by phosphorylated *ARRs-B* (Hwang & Sheen, 2001; Imamura et al., 2003). There is a close relationship between the expression profiles of genes and their biological functions. Despite this, *ARR-B* expression patterns in different tissues have rarely been detected in Pepper. It has been shown that *ARR*-B transcription factors play a role in plant growth, vascular development, and cytokinin signal transduction; in addition, they may play a role in root development and drought- and salinity-tolerance (Garay-Arroyo et al., 2012; Kiryushkin et al., 2019; Seo et al., 2020). Based on the qPCR analysis, we found that these genes had broad expression profiles in Pepper, *CaARR2*, *CaARR5*, *CaARR6,* and *CaARR9* were highly expressed in the roots. while *CaARR2*, *CaARR4*, *CaARR6*, and *CaARR9* where downregulated on the shoots. Interestingly, the Arabidopsis ortholog of *SlARR-B1* regulates sodium accumulation in tomato shoots (Mason et al., 2010). Several gene families have been identified, and their expression profiles have been characterized according to the stress responses and phytohormone responses of different plant species (Ye et al., 2009; Zhu et al., 2013; Zhao et al., 2016; Xia et al., 2017; Wang et al., 2017; Liu et al., 2020; Zhang et al., 2020; He et al., 2020).

Conclusion

In order to conduct functional studies, it is essential first to characterize and classify gene families. During the present study, 10 *CaARRs-B* genes were identified and classified. Seven of the twelve chromosomes of *C. annuum* contained uneven distributions of genes. According to the phylogenetic analysis, most *CaARRs*-B presented possible orthologs in Arabidopsis, indicating a common evolutionary origin. Moreover, different salinity stress levels induced different expression levels of *CaARRs-B* genes in leaves and roots, supporting the theory that *CaARRs-B* has functionally divergent functions. By integrating our results, we identified *CaARRs-B* candidates that might contribute to regulating salt stress resistance and shed new light on *CaARRs-B* transcription factors' role in secondary metabolism. To better understand how *CaARRs-B* function and how they are regulated in *Capsicum* spp., more interspecific functional characterization of *CaARRs-B* genes is required.

Conflict of Interest

The author declares no Conflict of Interest.

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Figure Legend

Figure 1. (**A**) Alignment of the *ARR* domain sequences from 10 putative *ARRs-B* genes in pepper and 21 *ARRs-B* genes from *Arabidopsis thaliana*. *ARRs-B* and Myb DNA binding motifs on amino acid sites are marked at the top, and sequence identities are shown below. (**B**) An unrooted phylogenetic tree displays *CaARRs-B* genes' relationships in *C. annuum* and *A. thaliana*. Different colors indicate the five different groups. Numbers at nodes represent bootstrap values based on 1000 replicates.

Figure 2. Chromosomal distribution of *CaARRs-B* gene genes in Pepper. The scale is in million bases (Mb). Chromosomes without *CaARRs* genes are not shown.

Figure 3. A schematic diagram of the 10 *CaARRs*-B gene structures showing exons and introns structures.

Figure 4. The number of various cis-elements on the promoters of each *CaARRs-B* gene. Promoter sequences (-1500 bp) of 10 *CaARRs-B* genes were analyzed.

Figure 5. Expression profiles of 9 pepper *ARRs-B* genes in different tissues, a case-oriented PCA based on the complete qPCR profile (left), and a gene-oriented heatmap (right) generated using the heat mapper tool were shown. Blue, white, and red colors correspond to low, moderate, and high fold change levels.

Figure 1

Alignment of the *ARR* domain sequences

(**A**) Alignment of the *ARR* domain sequences from 10 putative*ARRs-B*genes in pepper and 21 *ARRs-B* genes from *Arabidopsis thaliana*. *ARRs-B* and Myb DNA binding motifs on amino acid sites are marked at the top, and sequence identities are shown below. (**B**) An unrooted phylogenetic tree displays *CaARRs-B* genes' relationships in*C. annuum* and*A. thaliana*. Different colors indicate the five different groups. Numbers at nodes represent bootstrap values based on 1000 replicates

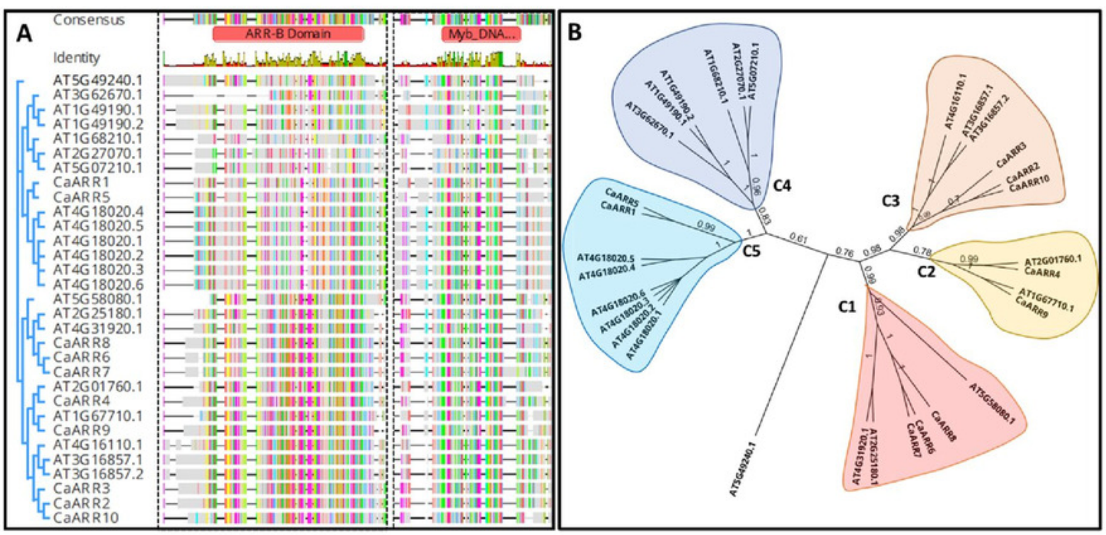

A
Consensus
Identity
ARR-B Domain
Myb_DNA...
AT5G49240.1
AT3G62670.1
AT1G49190.1
AT1G49190.2
AT1G68210.1
AT2G27070.1
AT5G07210.1
CaARR1
CaARR5
AT4G18020.4
AT4G18020.5
AT4G18020.1
AT4G18020.2
AT4G18020.3
AT4G18020.6
AT5G58080.1
AT2G25180.1
AT4G31920.1
CaARR8
CaARR6
CaARR7
AT2G01760.1
CaARR4
AT1G67710.1
CaARR9
AT4G16110.1
AT3G16857.1
AT3G16857.2
CaARR3
CaARR2
CaARR10
B
C1
C2
C3
C4
C5

Figure 2

Chromosomal distribution of*CaARRs-B*genegenes in Pepper

Chromosomal distribution of*CaARRs-B*genegenes in Pepper. The scale is in million bases (Mb). Chromosomes without *CaARRs* genes are not shown.

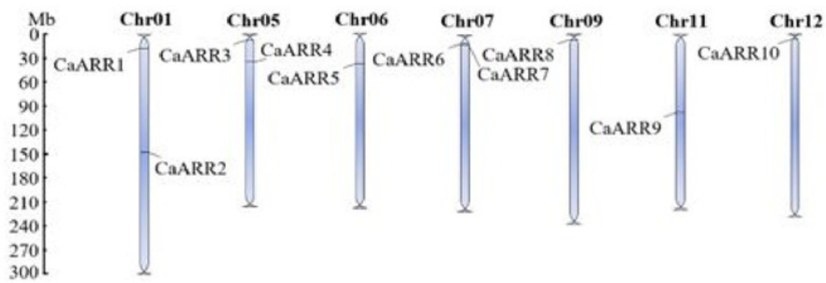
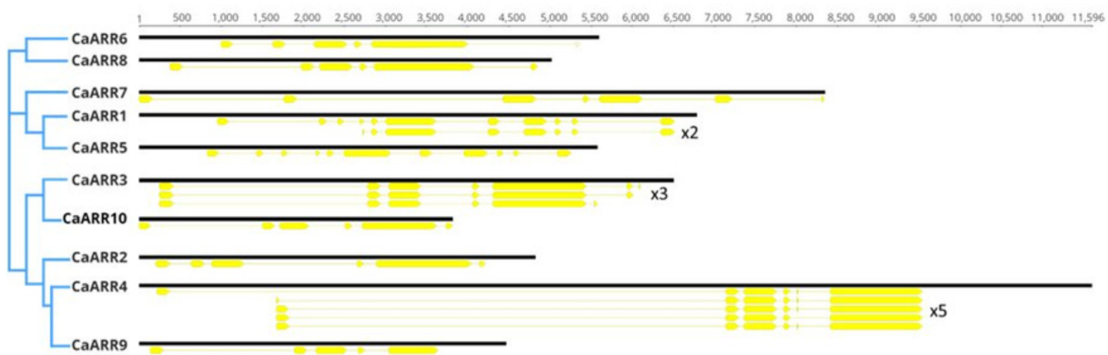

Mb
0
30
60
90
120
150
180
210
240
270
300
Chr01
Chr05
Chr06
Chr07
Chr09
Chr11
Chr12
CaARR1
CaARR2
CaARR3
CaARR4
CaARR5
CaARR6
CaARR8
CaARR7
CaARR9
CaARR10

Figure 3

A schematic diagram of the 10 *CaARRs*-B gene structures

A schematic diagram of the 10 *CaARRs*-B gene structures showing exons and introns structures


1
500
1,000
1,500
2,000
2,500
3,000
3,500
4,000
4,500
5,000
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7,000
7,500
8,000
8,500
9,000
9,500
10,000
10,500
11,000
11,596
CaARR6
CaARR8
CaARR7
CaARR1
x2
CaARR5
CaARR3
x3
CaARR10
CaARR2
CaARR4
x5
CaARR9

Figure 4

The number of variouscis-elements on the promoters of each*CaARRs-B*gene

The number of variouscis-elements on the promoters of each*CaARRs-B*gene. Promoter sequences (-1500 bp) of 10*CaARRs-B* genes were analyzed

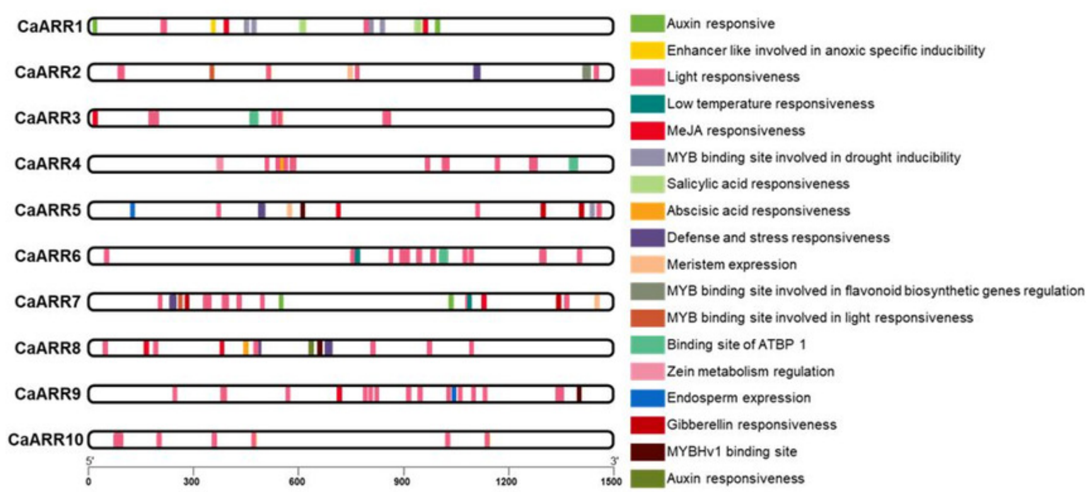
CaARR1
CaARR2
CaARR3
CaARR4
CaARR5
CaARR6
CaARR7
CaARR8
CaARR9
CaARR10
5'
3'
0
300
600
900
1200
1500
Auxin responsive
Enhancer like involved in anoxic specific inducibility
Light responsiveness
Low temperature responsiveness
MeJA responsiveness
MYB binding site involved in drought inducibility
Salicylic acid responsiveness
Abscisic acid responsiveness
Defense and stress responsiveness
Meristem expression
MYB binding site involved in flavonoid biosynthetic genes regulation
MYB binding site involved in light responsiveness
Binding site of ATBP 1
Zein metabolism regulation
Endosperm expression
Gibberellin responsiveness
MYBHv1 binding site
Auxin responsiveness

Figure 5

Expression profiles of 9 pepper *ARRs-B* genes in different tissues

Expression profiles of 9 pepper *ARRs-B* genes in different tissues, a case-oriented PCA based on the complete qPCR profile (left), and a gene-oriented heatmap (right) generated using the heat mapper tool were shown. Blue, white, and red colors correspond to low, moderate, and high fold change levels.

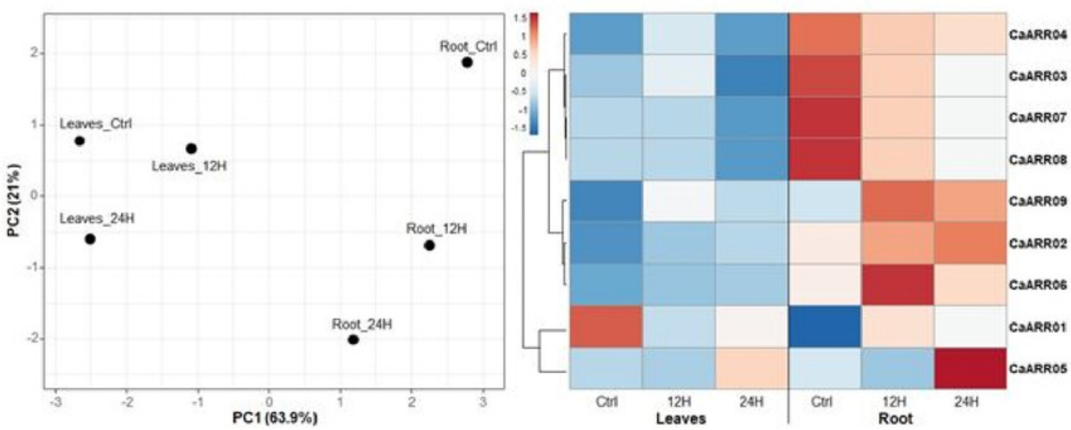
Leaves_Ctrl
Leaves_12H
Leaves_24H
Root_Ctrl
Root_12H
Root_24H
PC1 (63.9%)
PC2 (21%)
CaARR04
CaARR03
CaARR07
CaARR08
CaARR09
CaARR02
CaARR06
CaARR01
CaARR05
Ctrl
12H
24H
Leaves
Root

Table 1(on next page)

New primer list designed to quantify *CaARRs-B* genes in Pepper. The list includes details on the position start (min) and end (max) of each primer, along with the sequence and expected size in base pair (bp).

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Table 1. New primer list designed to quantify *CaARRs-B* genes in Pepper. The list includes details on the position start (min) and end (max) of each primer, along with the sequence and expected size in base pair (bp).

GeneID	Direction	Min	Max	5'-Seq-3'	Exp. size (bp)
CaARR1	F	1282	1301	TCTAATCATGTCGCCCCAGC	133
	R	1395	1414	GCTTCCAGTGCCAAGTCTCT	
CaARR2	F	738	757	ACTGATGAACGTTCCCGGAC	147
	R	865	884	CAAAAGGTTGTGTCTGGGCG	
CaARR3	F	1307	1326	TTTCTCAGCCTCCGTTGTCC	128
	R	1415	1434	AGATGCTGGGGAGACTGGAT	
CaARR4	F	813	832	AGTTCAACAACAGGGTGGCA	148
	R	941	960	CTCGGCATGAAGAGCTGTCA	
CaARR5	F	1212	1231	CATGGCCTTCCCGACCTATC	143
	R	1335	1354	AATTCTCGGGTGGTTGCCAT	
CaARR6	F	980	999	TTCGCAACCTGACAGCATCT	147
	R	1107	1126	ATGCGACGTGGACAATGACT	
CaARR7	F	774	793	AGTCGCAAGCCATCTTCAGA	110
	R	864	883	TGACATAGTGCCCTGGAGGA	
CaARR8	F	832	851	GCTGCTGCATTAGGGGGTAA	108
	R	920	939	CTGACCCTGACCGAACCTTC	
CaARR9	F	333	352	AAGCAGGGTGATGAAGGGTG	133
	R	446	465	ATTTCCAACGTCCCTTGCCT	
CaARR10	F	571	590	CGTGTACTTTGGTCACCGGA	145
	R	696	715	TCTGAAGGTGGCTAGCAACG	

Table 2 (on next page)

List of *CaARRs-B* family genes identified in Pepper includes genomic and physiochemical characteristics of each gene.

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Table 2. List of *CaARRs-B* family genes identified in Pepper includes genomic and physiochemical characteristics of each gene.

Name	Gene ID	Genomic location	ORF[a]	AA[b]	pI[d]	Mw[e]	ARR-B[c]	Localization predicted
CaARR1	Capana01g000809	Chr01:16748515:16754075: +	1737	579	5.70	64.34	20-126	nucleus
CaARR2	Capana01g002340	Chr01:148777192:148781202: -	2013	671	5.72	74.35	34-142	nucleus
CaARR3	Capana05g000373	Chr05:8121795:8127559: +	1992	664	5.39	73.47	32-140	nucleus
CaARR4	Capana05g000907	Chr05:35121771:35129633: -	1908	636	6.17	69.38	28-136	nucleus
CaARR5	Capana06g001571	Chr06:38165007:38169434: -	1674	558	6.19	61.80	20-127	nucleus
CaARR6	Capana07g000239	Chr07:10638104:10640334: -	1167	389	9.48	43.09	22-131	nucleus
CaARR7	Capana07g000240	Chr07:18343788:18352137: +	2010	670	6.76	73.59	22-136	nucleus
CaARR8	Capana09g000064	Chr09:1430274:1434744: +	2052	684	5.84	75.16	26-134	nucleus
CaARR9	Capana11g001030	Chr11:98813212:98816708: -	1347	449	7.42	50.05	27-136	nucleus
CaARR10	Capana12g000157	Chr12:2698224:2702038: +	1695	565	5.61	63.33	23-130	nucleus

[a] Open reading frame (bp), [b] Amino acid, [c] ARR-B domain, [d] Theoretical isoelectric point, [e] Molecular weight (kDa)

Table 3(on next page)

Predicted secondary structure of the *CaARRs-B* family proteins

Predicted secondary structure of the *CaARRs-B* family proteins

Table 3. Predicted secondary structure of the *CaARRs-B* family proteins.

GeneID	Alpha Helix (%)	Beta Strand (%)	Coil (%)	Turn (%)
CaARR1	28.8	19.1	24.0	28.1
CaARR2	26.3	24.0	27.3	22.4
CaARR3	32.9	26.2	18.7	22.1
CaARR4	34.2	21.4	27.9	16.4
CaARR5	43.3	13.8	20.8	22.1
CaARR6	48.7	18.5	13.7	19.2
CaARR7	26.6	22.7	30.3	20.3
CaARR8	27.5	20.6	29.6	22.4
CaARR9	40.3	24.3	17.8	17.6
CaARR10	31.9	25.0	23.2	20.0
Average±SD	34.1±8	21.6±4	23.3±6	21.1±3