Genome-wide identification of *CaARR-Bs* transcription factor gene family in pepper and their expression patterns under salinity stress

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Abstract

In plants, *ARR-Bs* 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 *ARR-Bs* family have been reported and characterized. The current study aimed to characterize *ARR-Bs* transcription factors in *C. annuum* at genomic and proteomic levels and investigate their regulation under salinity stress. A total of ten genes, named *CaARR1* to *CaARR10*, belonging to the largest subfamily of type-B ARRs, were discovered in *C. annum*. To investigate the *CaARR-Bs* family in *C. annuum* on a genome-wide scale, the analysis relied on the known *ARR-Bs* 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 *ARR-Bs* proteins, *CaARR-Bs* proteins showed gene conservation and potentially specialized functions. Furthermore, tissue-specific expression profiles revealed that CaARR-Bs genes are differentially expressed, suggesting they are functionally distinct. *CaARR-Bs* proteins had a typical conserved domain, including AAR-like (pfam: PF00072) and Myb DNA binding (pfam: PF00249) domains. Ten of the *CaARR-Bs* genes were asymmetrically mapped on seven chromosomes in Pepper. Additionally, the phylogenetic tree of *CaARR-Bs* genes from *C. annuum* and other plant species revealed that *CaARR-Bs* genes were classified into four clusters, which may have evolved conservatively. Furthermore, the utilization of quantitative real-time qRT-PCR for expression analysis indicated that nine *CaARR-Bs* genes displayed differential expression in both examined tissues, namely roots and leaves. These genes exhibited diverse expression patterns and were found to be highly responsive to salinity stress. In particular, the expression of *CaARR3, CaARR5, CaARR6, CaARR7*, and *CaARR8* showed significant expression levels in roots. These findings enhance our comprehension of the functions performed by *CaARR-Bs* genes in pepper plants and establish a solid groundwork for future investigations into their involvement in growth and development processes under salinity stress conditions.

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. Consequently, it is imperative to understand the mechanisms that contribute to Pepper's tolerance of such conditions (Chen et al., 2015).

Cytokinins are adenine derivatives, which are important plant hormones that affect nearly every aspect of plant development and growth. Among their functions, they promote chloroplast growth, delay senescence and regulate shoot and root development (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). A close correlation has also been found between endogenous cytokinin levels and pleiotropic developmental dysregulation (Ferreira & Kieber, 2005). A two-component signaling pathway is mediated by CKs through histidine kinases (HKs) and response regulators (RRs).

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). Based on their core receiver domain structures and C-terminal domain sequences, Arabidopsis' genome contains 23 authentic response regulators (ARRs), divided into four types: A, B, C, and pseudo (Schaller et al., 2007). DNA-binding and receiver domains distinguish Type-B ARRs as transcription factors (Lohrmann et al., 2001; Hosoda et al., 2002). There is compelling evidence that type-A ARR genes are direct targets of phosphorylated *ARR-B* regulators (Hwang, Chen & Sheen, 2002; Imamura et al., 2003). The *ARR-Bs* have also been shown to positively regulate cytokinin signaling (Mizuno, 2004). In this study, the type-B authentic response regulator is unified as *ARR-Bs*. ARRs are phosphorylated by HKs upon perception of a stress signal, leading to changes in their DNA-binding activity and subsequent regulation of target genes. Several studies have demonstrated the involvement of ARRs in plant responses to salt stress. For instance, in Arabidopsis thaliana, ARR2 and ARR12 were found to be upregulated in response to salt stress, and loss-of-function mutants of ARR2 displayed increased sensitivity to salt stress, indicating the importance of ARR2 in salt stress tolerance (Mizuno, 2004). Similarly, in rice, the overexpression of OsRR22, a member of the ARR family, enhanced salt stress tolerance by upregulating the expression of stress-responsive genes (Zhang, 2009).

Lately, the pepper genome has been sequenced (Kim et al., 2014; Qin et al., 2014; Magdy et al., 2019; Magdy & Ouyang, 2020). A wide range of RNA molecules derived from several tissues, including root, shoot, leaf, flower, and fruit are also available. Using these data sets, pepper improvement and basic research can identify and functionalize a gene family from a global perspective. The purpose of this study was to identify all potential ARR-B genes encoded in the pepper genome. Routine bioinformatic approaches were conducted to examine the chromosomal

81 distribution, phylogenetic relationships, and gene structure of the identified *CaARR-Bs* genes.
82 Additionally, functional predictions were made based on gene expression analysis across various
83 developmental stages and in response to salinity stress. These comprehensive results serve as a
84 crucial basis for future investigations focused on gene family exploration and the functional
85 characterization of *ARR-Bs* in pepper plants.

86 2. Materials and Methods

87 2.1 Identification of members of the *CaARR-Bs* gene family in Pepper

88 Using the TAIR.org database, we were able to retrieve Arabidopsis thaliana structural domains for
89 *ARR-Bs* genes. The obtained sequences were used as probes for homology searches using BLASTp
90 (Wang et al., 2020a). BLASTP searches selected the sequences of *CaARR-Bs* genes to the online
91 pepper genomics database (http://peppersequence.genomics.cn/), where the genome database of
92 the *C. annuum* cultivar Znula was selected (Qin et al., 2014). The search yielded Pepper *ARR-Bs*
93 candidate genes. The physicochemical properties of the *ARR-Bs* family genes were estimated using
94 Expasy (http://web.expasy.org/) (Gasteiger et al., 2005). Finally, the subcellular localization
95 analysis of the pepper *CaARR-Bs* gene family was performed using Cell-Ploc 2.0
96 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). The *CaARR-Bs* gene family protein sequences
97 were aligned with *A. thaliana ARR-B* genes by MAFFT aligner (Katoh & Standley, 2013) using
98 the embedded algorithms in Geneious Prime. Subsequently, the rooted phylogenetic trees were
99 generated using the maximum likelihood methods (ML). The ML tree was constructed using
100 FastTree V2 (Kumar et al., 2018), embedded in Geneious Prime.

101 2.2 Chromosomal location, Gene structure, domain annotation, and secondary structure
102 analysis

103 The chromosomal location of *CaARR-Bs* genes throughout the pepper genome was investigated
104 using the MG2C V2 online tool (Chao et al., 2021). GSDS was used to identify *CaARR-Bs* gene
105 exon-intron structures by comparing the genomic sequences *vs* cDNA sequences
106 (http://gsds.cbi.pku.edu.cn/). The *CaARR-Bs* and the Myb DNA binding domains (a domain that
107 distinguish the *ARR-A* from *ARR-B* genes) were detected using CDD (Marchler-Bauer et al., 2015),
108 SMART, pfam and InterProScan databases (Wang et al., 2022). SPOMA was used to predict
109 CaARR-Bs secondary structures (Sapay, Guermeur & Deléage, 2006).

110 2.3 Potential *cis*-element analysis in promoter regions

111 A BLASTn search was conducted on the pepper genome using the cultivar Znula as the query
112 sequences to identify *CaARR-Bs* genes. For each gene, a 1500 bp upstream the initiation codon
113 was retrieved and analyzed for *cis*-elements using PlantCARE database
114 (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

115 2.4 Plant materials and salinity stress treatment

116 Seeds of the pepper cultivar Gedeon F1 (*Capsicum annuum* L.; https://www.syngenta.com.eg/)
117 were sterilized with 1 % sodium hypochlorite for 30 min, washed with sterile water, and then sown
118 in perlite beds at 28° C (Qin et al., 2014). Seedlings were grown under 16 h/light at 25 °C and eight

119 h/night at 18 °C with a relative humidity of 60% until they had six leaves. Plants were irrigated
120 with Hoagland solution at half-strength pH 5.6. Leaves and roots were harvested from the
121 seedlings with three biological replicates as control. For the salt stress experiment, five-week-old
122 plants were irrigated with 200 mM NaCl (Wang et al., 2022). After 12 hours and 24 hours of
123 treatment, each sample (3-4 leaves) was collected in three biological replicates. Liquid nitrogen
124 was used to rapidly freeze samples, followed by -80°C storage until RNA extraction.

125 **2.5 RNA expression and qPCR analysis**

126 Total RNA was extracted from pepper tissues (leaves and roots) subjected to different salt stress
127 treatments using the EasyPure® Plant RNA Kit (TransGen Biotech, Beijing, China) following the
128 instructions provided by the kit manufacturer. The quality and quantity of the extracted RNA were
129 evaluated using electrophoresis on 2% agarose gels and a Quantus™ Fluorometer (Promega,
130 USA). The cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen,
131 Carlsbad, CA, USA) and adjusted to a concentration of 100 ng µL-1. During the PCR
132 amplification, a range of 108 to 147 base pairs was targeted while avoiding the conserved region
133 (Table 1).

134 Quantitative real-time RT-PCR (qRT-PCR) was performed using TransStart® Green qPCR
135 SuperMix (TransGen Biotech, Beijing, China). The amplification reactions were performed
136 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
137 30 s. Melting curve analysis, performed by increasing the temperature from 55 to 95 °C (0.5 °C
138 per 10 s), and a gel electrophoresis of the amplified fragments confirmed that the product contained
139 single amplicons. In each experiment, the relative fold differences were calculated using the $^{\Delta\Delta}$ Ct.
140 Normalization was conducted using GAPDH Ct values, amplified using 5'-
141 ATGATGATGTGAAAGCAGCG-3' and 5'-TTTCAACTGGTGGCTGCTAC-3' as a reference
142 gene (Arce-Rodríguez & Ochoa-Alejo, 2015). In this experiment, three biological replicates per
143 sample were used.

144 # 3. Results

145 **3.1 Identification of the *CaARR-Bs* family genes members in Pepper**

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

156 **3.2 Alignment and phylogenetic analysis of *CaARR-Bs***

157 Phylogenetics were conducted to confirm the identified *CaARRs* copies and determine the
158 evolutionary affinity of the *CaARR-Bs* genes and *ARR-Bs* genes of Arabidopsis. With full-length
159 amino acid sequences, the alignment of the *ARR-BS* domain was conducted and used to perform
160 an unrooted phylogenetic tree with a bootstrap test. The analyzed *ARR-Bs* proteins were grouped
161 into five distinct clusters. The *CaARR-Bs* proteins in Pepper were clustered into four of the five
162 subclusters with strong bootstrap support (Fig. 1). Functional divergence could have resulted from
163 the presence or absence of species-specific *CaARR-Bs*.

164 **3.3 Chromosomal location and duplication event of *CaARR-Bs* genes**

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

174 **3.4 Predicted secondary structures of *CaARR-Bs* proteins**

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

185 **3.5 Gene structure *CaARR-Bs* proteins**

186 For each gene in the CaARR-Bs gene family, exon-intron distribution and conserved motifs were
187 analyzed. For each gene, several isoforms were detected with the exception for *CaARR2* and
188 *CaARRs* 5 – 10 (unique isoform). The *CaARR1* recorded two isoforms where one was complete
189 and a shorter isoform (x_2: 1431 bp and 477 aa). The *CaARR3* recorded three isoforms: one
190 complete and two shorter isoforms (x_2: 1992 bp and 664 aa; x_3: 1956 bp and 652 aa). Finally, the
191 *CaARR4* recorded five isoforms where one was complete, and five shorter isoforms (x_2: 1785 bp
192 and 595 aa; x_3: 1896 bp and 632 aa; x_4: 1899 bp and 633 aa; x_5: 1908 bp and 636 aa).

193 There were differences in exonic and intronic regions between the 10 *CaARR-Bs*. Exons ranged
194 from 5 to 11 while introns ranged from 4 to 10. The CaARR-Bs genes were clustered according to
195 their sequence homology into three groups, showing similar gene structures within each group.
196 Regardless of their chromosomal location, one cluster included *CaARR6 and CaARR8*, another

197   included *CaARR7*, *CaARR1*, and *CaARR5*, and the other included *CaARR2, CaARR4,* and *CaARR9*
198   (Fig. 3).

3.6 Potential *cis*-element analysis in promoter regions of *CaARR-Bs* genes

200   To further characterize the potential regulatory mechanisms of *CaARR-Bs* , 1500 bp upstream
201   sequences from the translation start sites were analyzed. Based on their biological significance,
202   *cis*-elements in *CaARR-Bs* were identified and categorized. The promoter sequences of 10
203   CaARR-Bs genes contained 146 possible cis-elements. Three main groups of cis-acting elements
204   were identified: hormones, environmental stress, and photoresponses. The *CaARR-Bs* promoters
205   included transcription factors binding sites related to abscisic acid responsiveness, auxin
206   responsiveness, defense and stress responsiveness, endosperm expression, enhancer-like involved
207   in anoxic specific inducibility, light responsiveness, low temperature responsiveness, meristem
208   expression, zein metabolism regulation, MeJA responsiveness, Gibberellin responsiveness, ATBP
209   1 transcription factor, MYB binding site involved in drought inducibility, MYB binding site
210   involved in flavonoid biosynthetic genes regulation, MYB binding site involved in light
211   responsiveness, MYBHv1 binding site, and Salicylic acid responsiveness (Fig. 4). According to
212   the *cis*-component, *CaARR-Bs* genes can respond to different abiotic stresses.

3.7 *CaARR-Bs* gene expression profiles in response to salinity stress

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

4. Discussion

230   The His-Asp phosphorelay signaling pathway in prokaryotic cells is controlled by ARRs (Suzuki
231   et al., 1998). On the basis of their phylogeny, the ARR-B gene family evolved from nonvascular
232   plants (bryophytes) such as phylostratum, while several orthologous genes exist in the plants
233   (Cuming et al., 2007). The ARR-B genes have exhibited evolutionary conservation across all the
234   selected species. This conservation is evident in their expansion within higher plants. ARRs have
235   been classified as types A and B based on their conserved domains (Imamura et al., 1999). An N-
236   terminal DNA-binding domain and a receiver domain indicate that ARR-B functions as a
237   transcription factor (Ishida et al., 2008). A key component of cytokinin signaling pathways, type

238 *B-ARRs* genes have been linked to plant response to various environmental stresses; in response
239 to different stress conditions, superoxide anion and hydrogen peroxide contents were measured as
240 antioxidant enzyme activity (Nakamichi et al., 2009). There are *ARR-Bs* transcription factors
241 identified in Arabidopsis genomes (Mason et al., 2004; Ramírez-Carvajal, Morse & Davis, 2008),
242 rice (Schaller et al., 2007), peach (Zeng et al., 2017), pear (Ni et al., 2017), soybean (He et al.,
243 2022), and fragrant rice (Rehman et al., 2022). Despite this, little is known about *ARR-Bs* in
244 Pepper. Studies have shown that gene organization plays an important role in how multiple gene
245 families evolve (Xu et al., 2012; Ullah et al., 2019; Wang et al., 2020; Arce-Rodríguez, Martínez
246 & Ochoa-Alejo, 2021; Ahiakpa et al., 2022). In the current study, ten *CaARR-Bs* genes in pepper
247 have been identified and characterized by their structure, *cis*-elements in the promoter regions,
248 chromosomal location, gene duplication, and phylogeny. In addition, salinity stress affects the
249 expression profile of different tissues. Thus, in this study, the CaARR-Bs genes were
250 comprehensively analyzed to determine their biological function.

251 From the public genomic data, we derived the genomic sequences, protein sequences, and
252 chromosomal locations of the identified *CaARR-Bs* genes (Chao et al., 2021). Following the
253 standard protocol, the secondary structures of *CaARR-Bs* proteins were putatively predicted
254 (Sapay, Guermeur & Deléage, 2006) and functionally characterized with proper tools (Wang et
255 al., 2022). Resulting in a total of ten predicted *CaARR-Bs* genes were found on seven
256 chromosomes, suggesting that the gene family show a segmental repetition. During plant genome
257 evolution, duplication or large-scale segmental duplication is thought to produce gene families
258 (Cannon et al., 2004). Many transcription factor families have been reported in gene duplication
259 events, including *HD-ZIP, C2H2-ZF, SlARR-B, MYB,* and *NAC* (Liu et al., 2015; Chen et al., 2015;
260 Arce-Rodríguez, Martínez & Ochoa-Alejo, 2021).

261 All the *CaARR-Bs* homologous gene pairs identified using the pepper genome *versus* the
262 Arabidopsis genome showed tight phylogenetic clustering; their topologies are more closely
263 related, suggesting they are more closely related. In addition, intron numbers were related to
264 *CaARR-Bs* gene classifications. The duplication of genes is crucial to genomic expansion and
265 realignment (Kumar, Tyagi & Sharma, 2011). Based on the phylogenetic tree and synteny analysis,
266 the results were consistent. Genetic evolution has been attributed to gene or genome duplication
267 events as the primary source of variation in the CaARR-Bs family gene. The result showed that
268 segmental duplication events promote the evolution *of CaARR-Bs* genes (Yang, Tuskan & Cheng,
269 2006; Yang et al., 2008; Gillis et al., 2009).

270 Further clarifying the roles of *CaARR-Bs* promoter regions in response to abiotic stresses, we also
271 identified several conserved *cis*-regulatory elements. An analysis of *cis*-acting elements in pepper
272 type-*B ARRs* genes showed a close relationship between these genes and growth, hormonal signal
273 transduction, and abiotic stress resistance. Several *cis*-elements involved in drought resistance are
274 found in the promoter region of *ARR-B*, and a triple mutant lacking all three of these genes was
275 reported in Arabidopsis (Nguyen et al., 2016). Further, *ARR-B* belongs to the helix-loop-helix
276 family and are nuclear-localized transcription factors, as evidenced by their helix-loop-helix
277 structure in the *CaARR-Bs* domain (Hosoda et al., 2002). The *ARR-Bs* regulators target type-A
278 *ARRs* genes directly, which are activated by phosphorylated *ARR-Bs* (Hwang & Sheen, 2001;
279 Imamura et al., 2003).

280 In Capsicum, the involvement of two-component response regulators (ARRs) has been identified
281 as key components in these signaling pathways. Salt-responsive genes are regulated by ARRs,
282 which mediate the plant's adaptive response. The involvement of ARRs in salt stress signaling
283 pathways can be attributed to their ability to regulate downstream stress-responsive genes. ARRs
284 function by interacting with other transcription factors and *cis*-acting elements in the promoter
285 regions of target genes, thereby modulating their expression. This regulatory mechanism enables
286 ARRs to orchestrate the activation of stress-responsive genes involved in ion homeostasis, osmotic
287 regulation, antioxidative defense, and other adaptive processes that contribute to salt stress
288 tolerance in Capsicum (Urao et al., 2000).

289 There is a close relationship between the expression profiles of genes and their biological
290 functions. Despite this, *ARR-B* expression patterns in different tissues have rarely been studied in
291 Pepper. Studies have shown that ARR-B transcription factors are involved in cytokinin signal
292 transduction; in addition, they may play a role in root development and drought- and salinity-
293 tolerance (Garay-Arroyo et al., 2012; Kiryushkin et al., 2019; Seo et al., 2020). Based on the qPCR
294 analysis, we found that these genes had broad expression profiles in Pepper, *CaARR2*, *CaARR5*,
295 *CaARR6,* and *CaARR9* were highly expressed in the roots. while *CaARR2*, *CaARR4*, *CaARR6*, and
296 *CaARR9* were down-regulated in the shoots. Interestingly, the Arabidopsis ortholog of *SlARR-B1*
297 regulates sodium accumulation in tomato shoots (Mason et al., 2010). Several gene families have
298 been identified, and their expression profiles have been characterized according to the stress
299 responses and phytohormone responses of different plant species (Ye et al., 2009; Zhu et al., 2013;
300 Zhao et al., 2016; Xia et al., 2017; Wang et al., 2017; Liu et al., 2020; Zhang et al., 2020; He et
301 al., 2020).

302 **Conclusion**

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

314 **Conflict of Interest**

315 The author declares no Conflict of Interest.

316 **Funding**

317 The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of
318 Education in Saudi Arabia for funding this reseach work through the project number MoE-IF-UJ-
319 22-04103022-X.

320 **Acknowledgments**

321 The authors would like to express their deepest gratitude to the University of Jeddah, for the
322 technical and financial support for this study.

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Figure Legend

549

550   **Figure 1.** (**A**) Alignment of the *ARR* domain sequences from 10 putative *ARRs-B* genes in pepper
551   and 21 *ARRs-B* genes from *Arabidopsis thaliana*. *ARRs-B* and Myb DNA binding motifs on amino
552   acid sites are marked at the top, and sequence identities are shown below. (**B**) An unrooted
553   phylogenetic tree displays *CaARRs-B* genes' relationships in *C. annuum* and *A. thaliana*. Different
554   colors indicate the five different groups. Numbers at nodes represent bootstrap values based on
555   1000 replicates.

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

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

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

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

Figure 1

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*.

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.
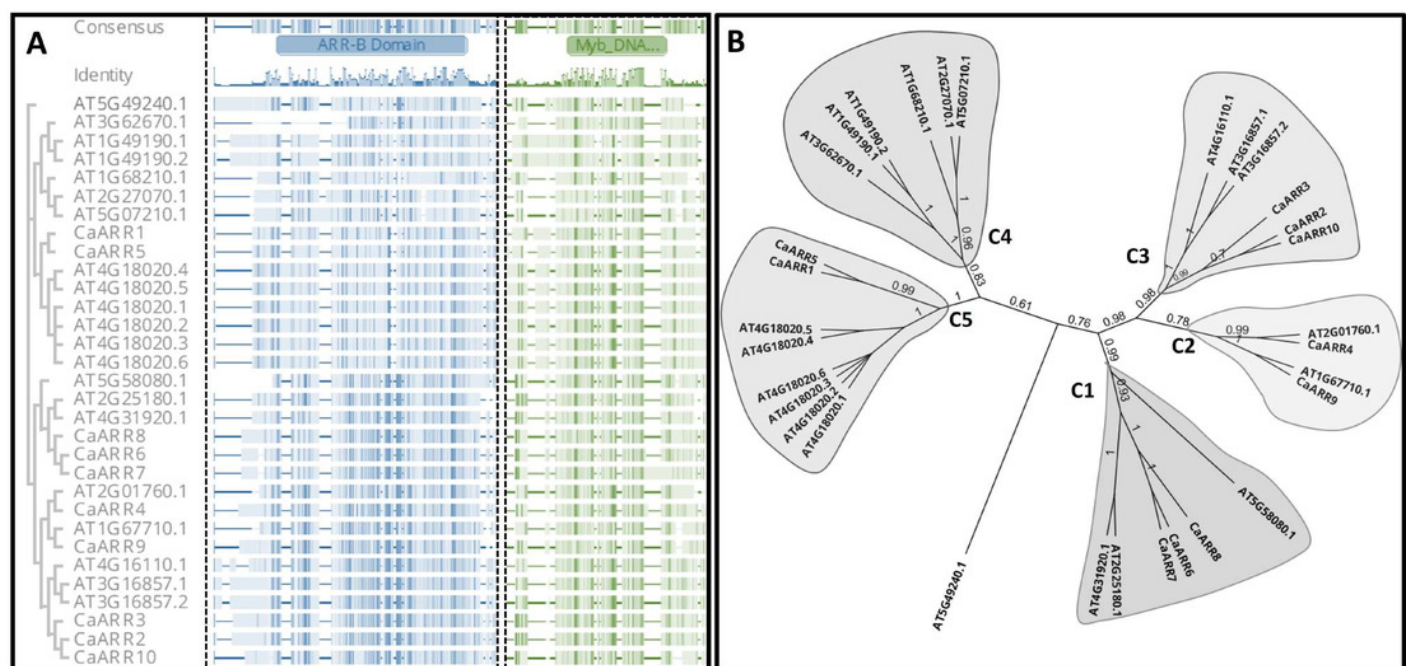
Figure 2

Chromosomal distribution of*CaARRs-B*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.
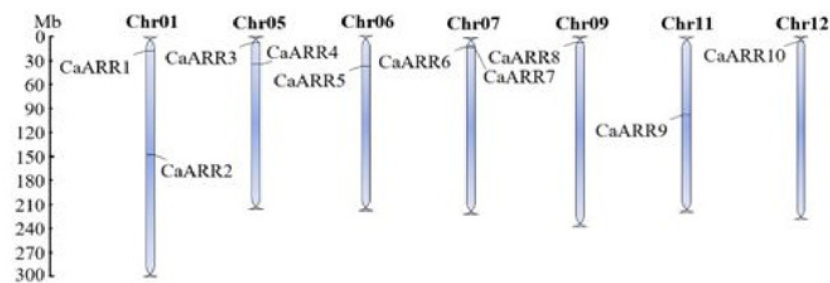
Figure 3

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
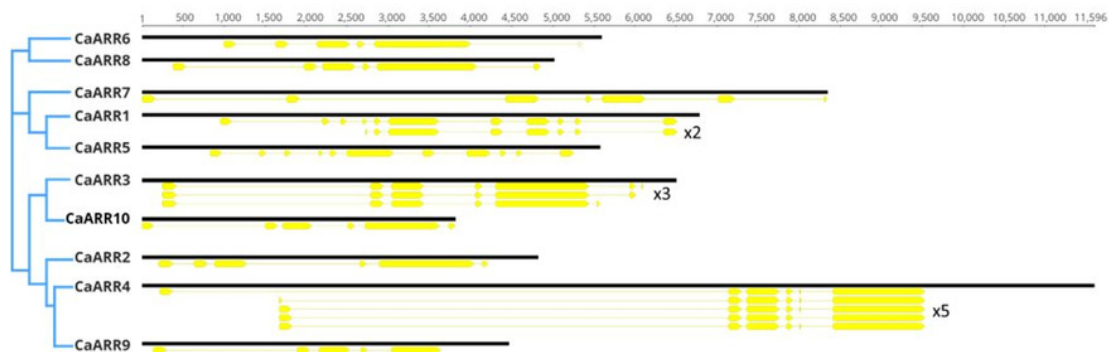
Figure 4

Figure 4. The number of various*cis*-elements on the promoters of each*CaARRs-B*gene.

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Promoter sequences (-1500 bp) of 10*CaARRs-B* genes were analyzed.

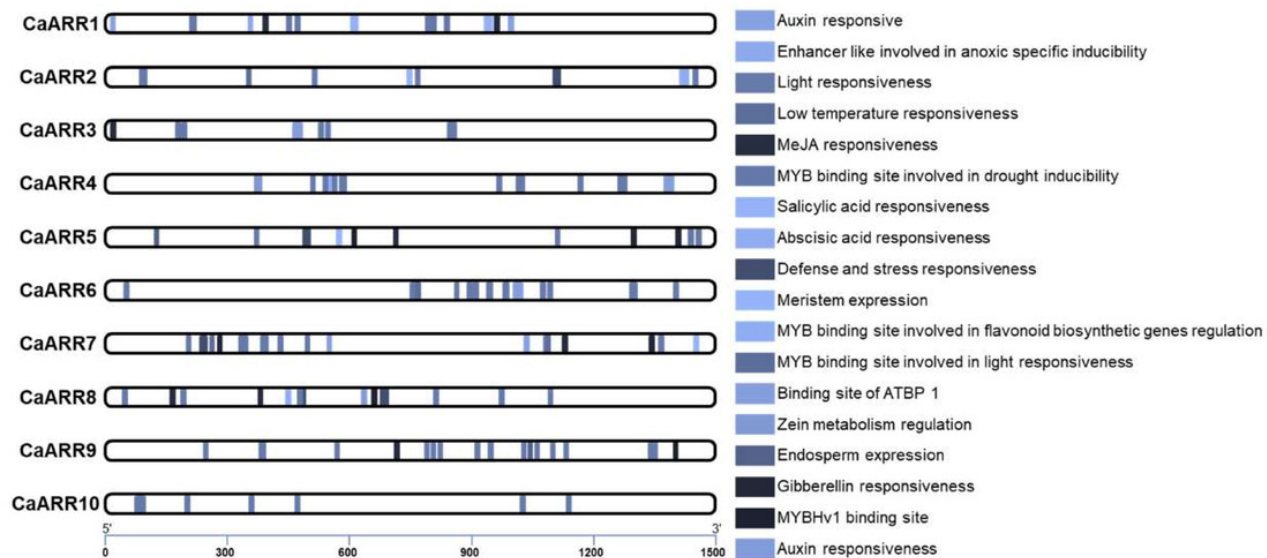
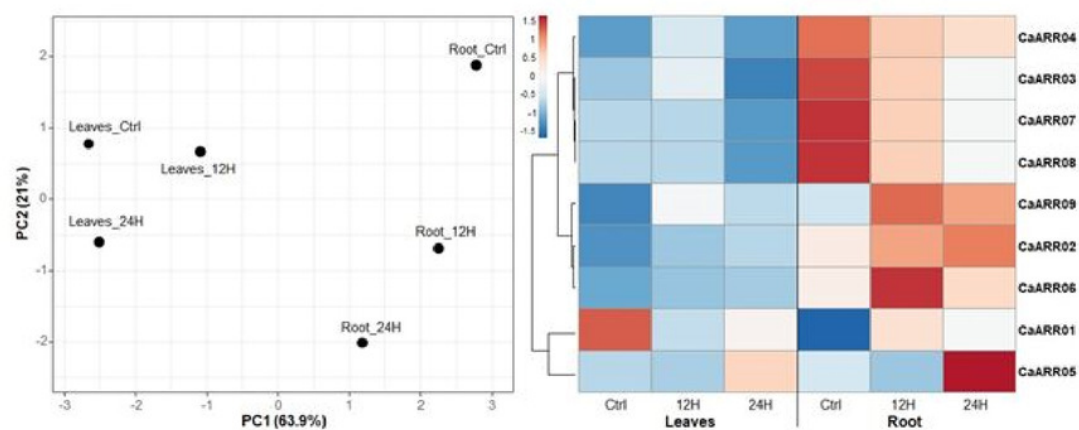



Figure 5

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. Z-score is the normalization for the heatmap values.

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).

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).

1 **Table 1.** New primer list designed to quantify *CaARRs-B* genes in Pepper. The list includes details
2 on the position start (min) and end (max) of each primer, along with the sequence and expected
3 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	

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

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

1 **Table 2.** List of *CaARRs-B* family genes identified in Pepper includes genomic and
2 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

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

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

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

2 **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