WRKY transcription factor family in lettuce plant (*Lactuca sativa*): Genome-wide characterization, chromosome location, phylogeny, structures and expression patterns

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WRKY transcription factors (TF) have been identified in many plant species and play critical roles in multiple stages of growth and development and under various stress conditions. Because of its high nutrient content, asparagus lettuce plays an important role in balancing people's diets. However, identifying WRKY TFs family in asparagus lettuce is limited. With the publication of the lettuce (*Lactuca sativa* L.) genome, we identified 76 WRKY TFs and constructed the analysis of structural characteristics, phylogenetic relationships, chromosomal distribution and their expression profiles in growth and development regulation as well as the stress response. The 76 LasaWRKY TFs were phylogenetically classified as Groups I, II (IIa-IIe) and III. *Cis* element analysis of the promoter region revealed complex regulatory relationships between LasaWRKY TFs in response to abiotic stresses and phytohormones. Interaction network analysis revealed that LasaWRKY TFs could interact with other proteins, such as SIB (sigma factor binding protein), WRKY TFs, MPK and NPR proteins. The expression patterns of LasaWRKY TFs were analyzed at different stages of lettuce stem enlargement. According to RT-qPCR analysis, abiotic stresses (drought, salt, low/high temperature) could induce specific LasaWRKY genes. LasaWRKY gene expression was also affected by phytohormone treatment. The findings provide systematic and comprehensive information on LasaWRKY TFs and lay the foundation for further clarification of the regulatory mechanism of LasaWRKY TFs involved in stress response and the progression of plant growth and development.

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ABSTRACT

WRKY transcription factors (TF) have been identified in many plant species and play critical roles in multiple stages of growth and development and under various stress conditions. Because of its high nutrient content, asparagus lettuce plays an important role in balancing people's diets. However, identifying WRKY TFs family in asparagus lettuce is limited. With the publication of the lettuce (*Lactuca sativa* L.) genome, we identified 76 WRKY TFs and constructed the analysis of structural characteristics, phylogenetic relationships, chromosomal distribution and their expression profiles in growth and development regulation as well as the stress response. The 76 LasaWRKY TFs were phylogenetically classified as Groups I, II (IIa-IIe) and III. *Cis* element analysis of the promoter region revealed complex regulatory relationships between LasaWRKY TFs in response to abiotic stresses and phytohormones. Interaction network analysis revealed that LasaWRKY TFs could interact with other proteins, such as SIB (sigma factor binding protein), WRKY TFs, MPK and NPR proteins. The expression patterns of LasaWRKY TFs were analyzed at different stages of lettuce stem enlargement. According to RT-qPCR analysis, abiotic stresses (drought, salt, low/high temperature) could induce specific LasaWRKY genes. LasaWRKY gene expression was also affected by phytohormone treatment. The findings provide systematic and comprehensive information on LasaWRKY TFs and lay the foundation for further clarification of the regulatory mechanism of LasaWRKY TFs involved in stress response and the progression of plant growth and development.

Keywords: Asparagus lettuce, WRKY TF, Expression patterns, Genome-wide

INTRODUCTION

Long-term domestication and directional selection of lettuce have resulted in the development of a variety of cultivars to meet various needs, including oil lettuce, stem lettuce (also known as asparagus lettuce) and various varieties of leaf lettuce. Asparagus lettuce (*Lactuca sativa* L. 2n=2x=18), an annual or biennial variety of lettuce that can form fleshy tender stems, is a member of the Lactuca genus of Compositae. In 2016, the world's lettuce production (including chicory) and cultivation area were 26.78 million tons and 1.223 million hectares, respectively (http://www.fao.org/faostat/en/). China has the highest yield and cultivation area globally, accounting for 56% and 51% of the total, respectively. Asparagus lettuce is widely cultivated and consumed throughout the year in China's North and South. It contains various vitamins, proteins, fats and phytochemicals (flavonoids and terpenoids). As an economically important vegetable, asparagus lettuce plays an important role in balancing people's diets due to its high nutrient content (*Cui et al., 2014*). The diameter of the stems and their freshness and tenderness influence the quality and quantity of asparagus lettuce. Due to advancements in molecular biology techniques, more and more technologies, such as whole-genome analysis, cell activity analysis and linkage map analysis, can be used to investigate the molecular mechanism of stem enlargement (*Li et al., 2020*). However, the regulatory mechanism of stem expansion remains a mystery.

Plant transcription factors (TFs) have been found to play essential regulatory roles in stem enlargement. MADS-box, ABF/AREB and homeo-box TFs were discovered to be involved in the formation of roots and tubers (*Pernisova et al., 2011*). MADS-box TFs including *IbMADS1*,

IbMADS3, *IbMADS4* and *IbMADS79* were mainly expressed in the root tubers of sweet potato (*Kim et al., 2002*; *Kim et al., 2005*; *Cheng et al., 2013*). ABF4 (ABF-binding factor) regulates potato tuber induction positively. The expression of *ABF4* increased the number and weight of tubers. Overexpression of *ABF4* in *Arabidopsis* improved potato production as well as salt and drought tolerance (*Garcia et al., 2018*). The silencing of *StNAC103*, which was discovered in potato tuber periderm, increased the total load of suberin and wax in the periderm (*Verdaguer et al., 2016*). The homeo-box TFs KNOX and POTH1 were found to be related to the development of both sweet and normal potato tubers. Further studies found that the interaction of POTH1 and StBELs jointly regulated gibberellin synthesis and affected potato tuber development (*Chen et al., 2004*). However, the role of WRKY TFs in plant root and tuber development remains unknown.

WRKY proteins which contain the conserved WRKY domain in N terminus and zinc finger motif in C terminus (C2H2 or C2HC), can recognize and bind to the W-box element (TTGAC/T) in the promoter region of target genes. WRKY TFs are classified into three types: groups I (two WRKY domains with C2H2 motif), II (one WRKY domain with C2H2 motif), and III (one WRKY domain with C2HC motif (*Eulgem et al., 2000*). Group II is subdivided into five subgroups IIa-IIe. WRKY TFs are involved in the regulation of multiple physiological processes (*Li et al., 2020b*; *Liu et al., 2021a*; *Wei et al., 2021*). WRKY TFs in *Arabidopsis* and rice were involved in biotic and abiotic stresses (*Dong et al., 2003*; *Rengasamy et al., 2008*). Drought stress elicited 88 WRKYs in *Phaseolus vulgaris* and 58 WRKYs in maize (*Wu et al., 2016*; *Zhang et al., 2017*). *Brachypodium distachyon BdWRKY38* has been identified as a participant in

response to *Rhizoctonia solani* by mediating SA signaling (*Kouzai et al., 2020*). Tomato *SlWRKY81* inhibited plant drought tolerance by suppressing SlRBOH1-derived H_2O_2 accumulation (*Ahammed et al., 2020*).

WRKY TFs play important roles in physiological processes and are also involved in developmental programs, for example, seed germination, reproductive processes, senescence and plant organ development (*Chen et al., 2017*). The flowering process was upregulated by *AtWRKY71* by regulating expression of flowering genes, while *AtWRKY6* plays an important role in leaf senescence by regulating the enzyme SIRK (*Robatzek & Somssich 2002*; *Yanchong & Yu 2016*). *Zhang et al.* (*2011*) found that rice *OsWRKY78* could regulate stem elongation; the expression pattern of *OsWRKY78* in the elongated stem was most abundant, and inhibition of *OsWRKY7*8 expression resulted in the shortening of somatic cell length. Cotton *GhWRKY15* improved not only resistance to virus and fungal infection but also stem elongation (*Yu et al., 2012*). *Li et al.* (*2016*) found that WRKY TFs were involved in carrot root development. As a result of systematic studies, many WRKY TF family members have been identified in different plant species, such as 72 in *Arabidopsis*, 81 in tomato, 95 in carrot, 55 in cucumber, 59 in grape, 45 in *Eucommia ulmoides* and 64 in *Isatis indigotica* (*Ishiguro & Nakamura 1994*; *Yang et al., 2020*; *Liu et al. 2021b*; *Qu et al., 2021*). However, members of WRKY TFs have yet to be identified in asparagus lettuce. In this study, 76 WRKY TFs were identified in asparagus lettuce through genome-wide analysis. Exon-intron structure, phylogenetic relationships, motif compositions, collinearity of *WRKY* genes and chromosome distribution analysis were identified. We also investigated the different levels of WRKY gene expression at different stages of stem

expansion. Our results will provide the basis of WRKY TFs in asparagus lettuce and will highlight the role of WRKY TFs in stem expansion.

MATERIAL AND METHODS

Sequence retrieval and identification of WRKY TFs in lettuce

The lettuce gene and protein sequence were obtained from the lettuce's genome (V7, https://lgr.genomecenter.ucdavis.edu/). The amino acid sequences of *Arabidopsis* WRKY TFs were used as query sequences to search for the homologous LasaWRKY TFs sequences. Subsequently, the conserved WRKY domain was identified by SMART (http://smart.embl-heidelberg.de/), Pfam database (http://pfam.janelia.org/), SMART (http://smart.embl-heidelberg.de/) and NCBI CDD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The molecular weight (Mw) and theoretical isoelectric point (pI) of LasaWRKY TFs were identified by the ExPASY server (http://www.expasy.ch/tools/pi_tool.html).

Gene structure, conserved motif and cis-elements analysis of promoter

GSDS (http://gsds.gao-lab.org/) was used to analyze the structure of LasaWRKY TFs, whereas, MEME online program (https://meme-suite.org/meme/tools/meme) was used to identify the conserved motif of LasaWRKY TFs (*Bailey et al., 2009*). The result was visualized by TBtools software (*Chen et al., 2020*). To investigate the *cis*-elements, the promoter region of the 2000 bp genomic DNA upstream sequence was submitted to the PlantCARE database (*Lescot et al., 2020*).

122

123 **Multiple sequence alignment and phylogenetic tree of LasaWRKY TFs**

124 Multiple sequence alignment of LasaWRKY TFs was performed using the DNAMAN software.
125 After ClustalX aligned the amino acid sequences of LasaWRKY TFs, a phylogenetic tree was
126 constructed using the neighbor-joining method with 1000 bootstrap replicates using MEGA 7.0
127 (*Kumar et al., 2008*).

128

129 **Chromosomal distribution and gene duplication of LasaWRKY TFs**

130 TBtools was used to draw the chromosomal distribution of each LasaWRKY TF from lettuce's
131 genome. STRING software was used to conduct the interaction network (*Franceschini et al.,*
132 *2013*). McscanX was used to identify the orthologous and paragons genes of WRKY TFs in *L.*
133 *sativa* and *Lactuca saligna*. The symbiotic relationships were displayed using Circos software
134 (*Krzywinski et al., 2009*).

135

136 **Plant materials, stress and phytohormone treatments**

137 Seeds from the cultivated asparagus lettuce 'Yonganhong' were sown in a controlled environment
138 chamber for 12 h photoperiod at 22 and 18 ^{o}C (day vs. night) with light intensity of 20,000.
139 Asparagus lettuce seedlings were used in subsequent experiments once they reached the four-leaf
140 stage. Seedlings were treated with 200 mmol/L NaCl (salt), 20% PEG6000 (drought), 4 ^{o}C (low
141 temperature) and 37 ^{o}C (high temperature) for abiotic stress treatment, respectively. To treat
142 seedlings with hormones, salicylic acid (SA, 0.5 mmol/L), abscisic acid (ABA, 75 μmol/L) and

143 gibberellin (GA, 50 μmol/L) were sprayed on them and placed for different duration of time. The
144 expression patterns of LasaWRKY TFs were also analyzed using different stages of asparagus
145 lettuce stem development (S1: diameter length is 1cm, S2: diameter length is 2 cm, S3: diameter
146 length is 3 cm, and S4: diameter length is 4 cm). For each treatment, three biological replicates
147 were collected. All samples were frozen in liquid nitrogen and stored in a -80 ^{o}C refrigerator.
148 Total RNA was isolated from four stem swelling, abiotic stress and hormone treatment using a
149 plant total RNA isolation kit (Vazyme, Nanjing, China) and first-strand cDNA was synthesized
150 using a 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China).
151

152 **Quantitative transcript analysis and qRT-PCR validation**

153 The transcriptome sequencing completed by our lab according to the FPKM value yielded the
154 expression abundance of LasaWRKY TFs in asparagus lettuce during different developmental
155 stages of stem swelling (S1, S2, S3 and S4). For qRT-PCR, SYBR Green I (TaKaRa, Dalian,
156 China) and the Roche LightCycler 96 were used. *LasaTIP41* (Lsat_1_v5_gn_5_116421) was
157 used to normalize and calculate the expression levels of each LasaWRKY TFs (*Borowski et al.,*
158 *2014*). The relative expression levels of LasaWRKY TFs were calculated using the $2^{-\Delta\Delta CT}$
159 methods based on the mean value of three technical repeats. The primer pairs were designed by
160 Primer Premier 6.0 and are listed in Supplemental Table S1.
161

162 **RESULTS**

163 **Identification of LasaWRKY TFs in *L. sativa***

The asparagus lettuce Yonganhong was planted in the teaching base of Linyi University. Although the root system of asparagus lettuce is straight, some dense lateral roots form after transplantation. It has very large basal and lower leaves. During the course of plant growth, the shortened stem gradually elongates and thickens (Fig. 1).

From the lettuce genome, 76 LasaWRKY TFs were identified, denoted as LasaWRKY01 to LasaWRKY76. LasaWRKY TFs had coding sequences (CDS) lengths ranging from 546 bp (LasaWRKY02) to 2232 bp (LasaWRKY42), with corresponding amino acid (aa) numbers ranging from 181 aa to 743 aa. The MWs and pI values of the identified LasaWRKY TFs ranged from 20.7 kDa (LasaWRKY02) to 81.7 kDa (LasaWRKY42), and from 5.19 (LasaWRKY44) to 9.98 (LasaWRKY35). On average, the polypeptide was composed of 59.90% aliphatic amino acids and 7.50% aromatic amino acids. The GRAVY values ranged from -1.274 to -0.46, indicating that LasaWRKY proteins are hydrophilic in nature (Supplemental Table S2).

Multiple sequence alignment and phylogenetic analysis of LasaWRKY TFs

Multiple sequence alignment of LasaWRKY TFs was identified, as shown in Fig. S1. Two WRKY domains with the conserved WRKYGQK were present in Group I, which contained C2H2-type zinc-finger domains. All 43 LasaWRKY TFs in Group II include LasaWRKY03, LasaWRKY05, LasaWRKY10 and LasaWRKY13 had one WRKY domain and a C2H2-type zinc-finger. All the members in Group III had one complete WRKY domain and a C2HC zinc finger. However, the WRKYGQK sequence has changed in some LasaWRKY TFs, for example, WRKYGKK in LasaWRKY50 and WKKYGEK in LasaWRKY61.

185 To investigate the phylogenetic relationship of LasaWRKY TFs, the phylogenetic tree was
186 also constructed using MEGA7 software. All 76 LasaWRKY TFs were divided into three groups
187 (I, II, and III). Group II had the most members (43), but the distribution is uneven among the five
188 subgroups IIa (3), IIb (9), IIc (15), IId (8), and IIe (8). Group I contained Ninteen LasaWRKY
189 TFs. Group III formed the smallest group with 14 LasaWRKY TFs (Fig. 2). To analyze the
190 classification of WRKY TFs family, a phylogenetic tree was constructed using 949 WRKY TFs
191 from 10 different plant species (Fig. S2). *Glycine max* had the most WRKY TFs, with 176,
192 followed by *Zea mays* (131), *Oryza sativa* (100) and *Daucus carota* (95); *Arabidopsis thaliana*
193 had the fewest WRKY TFs (72). Among the three groups, WRKY TFs were mainly classified
194 into Group II. For instance, 45 of the 72 *Arabidopsis thaliana* WRKY TFs belonged to Group II;
195 while, Groups I and III contained 13 and 14 members, respectively. The distribution of WRKY
196 TFs in *Solanum lycopersicum* was 15 (Group I), 52 (Group II) and 11 (Group III). The number
197 of WRKY TFs among the 10 plant species in Groups I and III was similar, except *Vitis vinifera*,
198 which was classified to Group I and Group III, with 12 and 6 members, respectively (Fig. S2).
199
200 **Gene structure, conserved motif and *cis*-elements analysis of LasaWRKY TFs**
201 The TBtools program was used to explore the gene structure to analyze the introns and exons of
202 LasaWRKY TFs. There were introns in all 76 LasaWRKY TFs ranging from 1 to 6. The
203 majority (33 of 76 LasaWRKY TFs) had 2 introns and 3 exons, followed by 3 introns (17) and 4
204 introns (10). LasaWRKY52 had the highest number of introns (6) and exons (7), while,
205 LasaWRKY73, LasaWRKY29, LasaWRKY12, LasaWRKY30 and LasaWRKY58 each had only

206 1 intron (Fig. 3A). The losses and gains of LasWRKY TFs may be related to the functional
207 diversity during the evolution of LasWRKY TFs.

208 Despite the fact that the gene structure of LasaWRKY TFs differed, some conserved motifs
209 were found in all LasaWRKY TFs. MEME program identified Ten conserved motifs to illustrate
210 the similarity and diversity of motif composition. The conserved motifs in all 76 LasaWRKY
211 TFs ranged from 2 to 7. Motifs 1 and motif 2 existed in all 76 LasaWRKY TFs. There were only
212 two motifs in nine LasaWRKY TFs (LasaWRKY51, 60, 69, 26, 07, 44, 66, 67 and 73). Motifs 9
213 and 10 mainly existed in Group III and Group II, respectively. Motifs 3 and 5 were unique in
214 Group I, such as LasaWRKY65, 32, 24, 53, 74, 06, 59, 42 and 48 (Fig. 3B). The results indicated
215 that LasaWRKY TFs from the same group have similar conserved motifs. The difference also
216 existed in LasaWRKY TFs, belonging to the same subgroup, indicating the functional diversity
217 of LasaWRKY genes (*Rose 2004*).

218 The *cis*-elements of the promoter region, a sequence with 2.0 kb DNA sequences upstream
219 from the codons of the 76 LasaWRKY TF, were identified as shown in Fig. 3C. Sixteen types
220 contained hormone-related, stress-related and plant growth and development-related *cis*-elements,
221 such as 4 types of hormone-related *cis*-elements gibberellin-responsive element, SA
222 responsiveness element, ABA responsiveness element, auxin-responsive element), stress-related
223 elements (defense and stress responsiveness element, low-temperature responsiveness element).
224 MYB binding site (CAACAG) was found in 47 LasaWRKY TFs. TCTGTTG (gibberellin-
225 responsive element), CCATCTTTTT (SA responsiveness element) and ACGTG (ABA
226 responsiveness element) were found in 70, 32 and 56 LasaWRKY, respectively. MYB binding

227 site (CAACAG) was detected in 47 LasaWRKY TFs, and low-temperature responsiveness
228 element LTR (CCGAAA) was found in 23 LasaWRKY TFs.
229
230 **Chromosomal distribution and duplication of LasaWRKY TFs**
231 Each LasaWRKY TF was investigated according to the lettuce genome database to evaluate the
232 chromosomal distribution. Except for LasaWRKY01 and LasaWRKY02, a total of 74
233 LasaWRKY TFs were found on 9 lettuce chromosomes (Fig. 4). The LasaWRKY TFs were
234 mostly found on chromosome 09 (14), followed by chromosome 07 (13), chromosome 04 (11)
235 and chromosome 08 (10). The number of LasaWRKY TFs on chromosome 3 and chromosome 5
236 was the same (7). Six LasaWRKY TFs were found on chromosome 6. Only 3 LasaWRKY TFs
237 were mapped on chromosome 01. *L.saligna*, which also belonged to the genus Lactuca, was
238 chosen to construct the comparative analysis to identify the paralogs and orthologs. As shown in
239 Fig. 5, a total of 75 and 70 pairs of paralogs were identified in *L. saliva* and *L.saligna*,
240 respectively. Moreover, 75 pairs of orthologs between *L. saliva* and *L.saligna* were identified
241 (Supplemental Table S3).
242
243 **Interaction network analysis of LasaWRKY TFs**
244 STRING software was used to construct interaction network of LasaWRKY TFs in order to
245 analyze the regulation mechanism. As shown in Fig. 6, 49 LasaWRKY TFs showed complex
246 interaction with other proteins such as WRKY TFs, MPK4, and Sigma factor binding protein
247 (SIB). LasaWRKY8/69/75 (WRKY70) and LasaWRKY14/30/31/53/65/68 (WRKY33)

interacted with other proteins in a similar manner. They both interacted with SIB1, SIB2, MEK1, NPR1 and LasaWRKY28, indicating that their regulatory networks were similar. MEK1, MPK3, MPK4 could interact with the LasaWRKY8/69/75 (WRKY70) and LasaWRKY25/47 (WRKY22) TFs. LasaWRKY TFs with co-expression relationships included LasaWRKY13 (WRKY18), LasaWRKY13 (WRKY40) and LasaWRKY34/27 (WRKY60).

Gene expression analysis

Expression of LasaWRKY TFs in response to abiotic stress

Five LasaWRKY TFs (*LasaWRKY16*, *LasaWRKY32*, *LasaWRKY39*, *LasaWRKY55* and *LasaWRKY58*), with relatively high expression levels across the stem developmental stages, were chosen for RT-qPCR analysis to identify the expression patterns of abiotic stresses (high salt, drought, low and high temperature).

Salt stress

After NaCl treatment, the expression level of *LasaWRKY16* and *LasaWRKY39* increased at different times. The expression level of *LasaWRKY16* increased approximately 32 times (12 h), 3 times (24 h) and 26 times (48 h) (Fig. 7). Compared to CK, *LasaWRKY39* increased by about 4 times, 3 times and 10 times increase after 12 h, 24 h and 48 h, respectively. *LasaWRKY58* also showed increased expression after NaCl treatment for 24 h (40 times) and 48 h (20 times). *LasaWRKY55* showed an insensitive response to salt treatment.

Drought stress

The expression profiles of *LasaWRKY16* and *LasaWRKY39* were similar under drought stress. *LasaWRKY16* and *LasaWRKY39* showed the highest expression (32 folds and 10 folds, respectively), as shown in Fig. 7. There were no obvious changes in the mRNA levels of *LasaWRKY32*, *LasaWRKY55* and *LasaWRKY58*.

***Low temperature* (4 °C)**

Under different treatment times, low temperature significantly induced the expression of four LasaWRKY genes (*LasaWRKY16*, *LasaWRKY32*, *LasaWRKY58* and *LasaWRKY39*). *LasaWRKY16*, *LasaWRKY32*, *LasaWRKY58* and *LasaWRKY39* showed 30-fold (48 h), 15-fold (24 h), 10-fold (48 h) and 8-fold (12 h) increase, respectively. In contrast, the expression of *LasaWRKY55* was upregulated by 9 times (24 h) and downregulated by 0.11 times (12 h) and 0.03 times (48 h), respectively.

***High temperature* (*37 °C*)**

The transcription level of 2 LasaWRKY genes (*LasaWRKY16* and *LasaWRKY32*) was significantly increased by high temperatures. As shown in Fig. 7, the expression of *LasaWRKY16* and *LasaWRKY32* increased continuously as treatment time was increased. At 48 h, the expression levels of *LasaWRKY16* and *LasaWRKY32* increased the most, 32-fold and 27-fold, respectively. *LasaWRKY58*, *LasaWRKY39* and *LasaWRKY55* all had similar expression patterns. At 24 h, the expression levels of *LasaWRKY58*, *LasaWRKY39* and *LasaWRKY55* increased 10-fold, 3-fold and 2-fold, respectively; however, after 12 and 48 hours of treatment, the expression levels of *LasaWRKY58*, *LasaWRKY39* and *LasaWRKY55* were markedly decreased.

289 **Expression levels of LasaWRKY TFs under treatment with phytohormone**

290 Several hormone-responsive elements, such as GA, ABA and SA, were found in the promoter
291 regions of LasaWRKY TFs (Fig. 3C), indicating that LasaWRKY TFs may respond to hormone
292 treatment. The expression levels of 5 LasaWRKY TFs involved in different hormones (SA, ABA,
293 and GA) were investigated (Fig. 8). The expression levels of both *LasaWRKY16*, *LasaWRKY32*
294 and *LasaWRKY58* were in response to ABA, GA and SA treatment at different times. After ABA
295 treatment for 48 h, the expression levels of *LasaWRKY16* and *LasaWRKY32* increased about 40
296 and 14 times, respectively. The expression levels of *LasaWRKY39* increased about 3.5 folds (24
297 h) in response to SA treatment. The expression level of *LasaWRKY39* showed insensitive
298 expression patterns after ABA and GA treatment. *LasaWRKY55* showed insensitive response to
299 ABA, SA, and GA treatment (Fig. 8).

300

301 **Tissue-specific expression patterns of LasaWRKY TFs**

302 To investigate the potential functions of LasaWRKY TFs during the development of *L. sativa*,
303 the expression patterns of five genes in different organs (root, stem and leaf) were identified (Fig.
304 9). The expression patterns of four LasaWRKY TFs (*LasaWRKY16*, *LasaWRKY58*,
305 *LasaWRKY39* and *LasaWRKY55*) were similar. These 4 genes had the highest expression levels
306 in root as compared with stem and leaf. While the expression pattern of *LasaWRKY32* in the leaf
307 increased about 3.5 times more than the expression in stem and leaf. The preferential expression
308 patterns of LasaWRKY TFs in different organs indicated that each LasaWRKY TFs might play a
309 unique role in organ development or function.

Expression profile of LasaWRKY TFs at different stages of stem development

We used a heat map to analyze the expression patterns in different stages of *L. sativa* stem enlargement based on transcriptome data to explore the function of LasaWRKY TFs involved in the progression of stem development. As shown in Fig. 10, 43 of the 76 LasaWRKY TFs showed different expression levels. Several genes including *LasaWRKY53*, *LasaWRKY49*, *LasaWRKY21*, *LasaWRKY28* and *LasaWRKY58*, showed upregulated expression profiles as lettuce stem enlargement progressed. While, the expression levels of some genes such as *LasaWRKY17*, *LasaWRKY72*, *LasaWRKY66* and *LasaWRKY08* were decreased, indicating that they may play negative regulatory roles in lettuce stem enlargement. Some genes, including *LasaWRKY60*, *LasaWRKY14* and *LasaWRKY02*, showed wavy expression patterns.

To explore the function of LasaWRKY TFs involved in lettuce development, RT-qPCR was used to examine the expression profiles of these 5 LasaWRKY genes (*LasaWRKY16*, *LasaWRKY32*, *LasaWRKY58*, *LasaWRKY39* and *LasaWRKY55*) (Fig. 11). The heat map analysis revealed that the relative expression levels of these five genes except *LasaWRKY55* increased, indicating that these genes were upregulated in the later stages of development. The results of RT-qPCR revealed that the expression patterns of *LasaWRKY32* and *LasaWRKY16* increased continuously during the progress of stem developmental stages. At the S4 stage, the expression of *LasaWRKY32* and *LasaWRKY16* increased about 10-fold and 27-fold, respectively. Although the expression level of *LasaWRKY55* was reduced at the S2 stage, it increased by about 3-fold and 5.5-fold at S3 and S4 stages, respectively. At the S1 and S2 stages, no obvious fluctuations

were observed for *LasaWRKY58*, but at the S4 stage, the expression level increased about 12-fold.

DISCUSSION

Identification of WRKY TFs family in lettuce

As we know, the WRKY TFs family has been confirmed to take part in a variety of biological processes, including various environmental stresses, plant growth and development. Because of high-throughput sequencing technology advancement, the WRKY TFs family has been identified in numerous higher plants (*Ishiguro & Nakamura 1994*; *Yang et al., 2020*; *Qu et al., 2021*). However, the analysis of WRKY TFs family Asterales plant order was limited. Plants of this order, such as sunflower (*Helianthus annuus* L.), globe artichoke (*Cynara cardunculus* var. scolymus L.) and lettuce, are rich in vitamins, proteins, fats, and phenolic compounds. *Guo et al. (2019)* analyzed WRKY TFs in the plants of Asteraceae. There were 112, 60 and 74 WRKY TFs found in sunflower, globe artichoke and lettuce, respectively. In our study, 76 LasaWRKY TFs were identified in lettuce, and the difference could be attributed to the different E values used while screening the WRKY domain. Comparative analysis revealed that the number of WRKY TFs is unrelated to plant genome size. The genome size of Arabidopsis, tomato and lettuce was 125 Mb, 900 Mb and 2.5 Gb, respectively, with a similar number of WRKY TFs (72 in *Arabidopsis*, 78 in tomato and 76 in lettuce) (*Reyes-Chin-Wo et al., 2017*) (Fig. S2). The number of WRKY TFs in both potato and *Hevea brasiliensis* was 81, but the potato genome size was 844 Mb, and the *Hevea brasiliensis* genome size was 2.15 Gb. These results indicated that the plants'

genome size could not determine the numbers of WRKY TFs.

Cis element and interaction network analysis of LasaWRKY TFs

Dehais et al. (1999) and *Lescot et al. (2002)* have found the *cis*-regulation element in the promoter region could regulate gene expression. The *cis*-elements found in the promoter region of 76 LasaWRKY TFs were investigated to analyze the potential function. As shown in Fig. 5, 10 *cis*-elements were identified, including hormone-responsive element (SA, GA, ABA, auxin) and abiotic stress, indicating that the function of LasaWRKY TFs was related to the phytohormone-regulation pathway or stress-regulation pathway (*Chaemyeong et al., 2021*; *Negi & Khurana, 2021*). MYB binding sites were also found in the promoter regions of many WRKY TFs, such as LasaWRKY71, LasaWRKY74 and LasaWRKY62. The WRKY TFs binding site W-box element (C/TTGACT/C) was found in many LasaWRKY TFs, including LasaWRKY03 and LasaWRKY06 LasaWRKY14, LasaWRKY20, LasaWRY36. The results suggested that LasaWRKY TFs could participate in various biological processes through self-regulation or cross-regulating with other genes.

The interaction network revealed that LasaWRKY TFs could interact with other proteins such as WRKY TFs, MPK and SIB to regulate different biological processes (Fig. 6). MAPK cascades, as an important signal transduction pathway, play vital roles in the progression of plant disease resistance (*Horak 2020*; *Yao et al. 2020*). WRKY TFs can be phosphorylated and activate MAPK, triggering downstream signaling pathways (*Chi et al., 2013*; *Yao et al., 2020*). After being directly phosphorylated by MPK3 and MPK6, AtWRKY33 played a major role in

the progression of fungus-induced camalexin accumulation (*Mao et al., 2011*). *Yao et al. (2020)* confirmed that WRKY TFs induced important defense response in tobacco resistance to whitefly after being phosphorylated by MAPK. VQ proteins (containing VQ motif FxxhVQxhTG) as a class of plant-specific transcriptional regulators could fine-tune the regulatory networks of plant-growth or plant-stress by cooperating with their interacting partners, including WRKY TFs (*Lai et al., 2011*; *Hu et al., 2013*). *Lai et al. (2011)* found VQ proteins SIB1 and SIB2 could serve as transcriptional activators of WRKY33 in response to *Botrytis cinerea*. *Hu et al. (2013)* identified VQ9, in collaboration with WRKY8, can regulate the plant-salt stress response. All of the results showed a complex relational mechanism of LasaWRKY TFs during various biological growths, which may form complexes with other proteins such as SIB, MPK and WRKY TFs.

The functions of LasaWRKY TFs

A large number of studies have confirmed the role of WRKY TFs in plant growth and development, pathogen defense, and abiotic stress (*Dong et al., 2003*; *Rengasamy et al., 2008*; *Kouzai et al., 2020*; *Wei et al., 2021*). The regulatory mechanisms of WRKY TFs in plant biological progresses are complex, because WRKY TFs can effectively combine with W-box found in the promoter regions of downstream target genes to regulate the expression of target genes or bind other acting elements to form protein complexes. Because the W-box element is present in many TFs, including the majority of WRKY TFs, WRKY TFs can combine with the W-box element in other WRKY TFs to form self-regulation or cross-regulation networks (Li et al., 2020; *Zentgraf et al., 2010*).

Some valuable clues have revealed the roles of WRKY TFs in plant development, including seed development, senescence, seed dormancy, and germination (*Sun et al., 2003*; *Luo et al., 2005*; *Zhou et al., 2011*). TTG2, as one of the WRKY TFs, was identified to play a role in organ development for the first time, including trichome outgrowth and seed coat morphogenesis (*Johnson et al., 2002*). Gene expression profiles are linked to gene function (*Xu et al., 2015*). Rice *OsWRKY78* was confirmed to promote seed development and stem elongation (*Zhang et al., 2011*). *AtWRKY23* and *AtWRKY12* were identified to regulate embryo development and secondary cell wall formation, respectively (*Wang et al., 2010*; *Grunewald et al., 2013*). According to RT-qPCR analysis, AcWRKY TFs may also play a role in specific pineapple physiological processes (*Xie et al., 2018*). Transcriptome data analysis revealed that 43 of 76 LasaWRKY TFs had different expression patterns (Fig. 10). The expression levels of five LasaWRKY genes (*LasaWRKY16*, *LasaWRKY32*, *LasaWRKY37*, *LasaWRKY39* and *LasaWRKY55*) were analyzed in different stages of stem development (Fig. 11), indicating that differently expressed WRKY TFs may be the key regulators of lettuce stem development.

The regulatory roles of WRKY TFs in response to abiotic stresses were also inferred. In Arabidopsis, 26 WRKY TFs responded to abiotic stress, and one WRKY TF participated in multiple stresses (*Jiang & Deyholos, 2006*). *AtWRKY30* improved resistance to salt stress and oxidative stress (*Scarpeci et al., 2013*). *OsWRKY76* improved the resistance of rice to cold stress (*Naoki et al., 2013*). WRKY TFs may improve tolerance to various abiotic stresses by increasing some material accumulation. For instance, the overexpression of *Boea hygrometrica BhWRKY1* in *Nicotiana tabacum* improved the seedling drought resistance by inducing the accumulation of

raffinose family oligosaccharides (*Wang et al., 2009*). WRKY TFs can improve tolerance to various abiotic stresses by directly regulating the expression of stress resistance-related genes. By binding W-box elements in the promoter of drought-resistant gene *RD29A* and *NCED3, AtWRKY57* played a positive role in drought stress response (*Zheng et al., 2020*). Similarly, *SbWRKY50* from *Sorgnum bicolor* participated in salt response by directly binding the promoters of SOS1 and HKT1 (*Song et al., 2020*). While, *AtWRKY34* played negative roles in the CBF-mediated cold response pathway (*Zou et al., 2010*).

The function of WRKY TFs in abiotic stress is often related to defense-associated phytohormones such as JA, SA, and ABA. As a major phytohormone, ABA has been shown to increase salt and drought tolerance (*Yin et al., 2017*). ABA could improve drought tolerance by attenuating the inhibition of OsWRKY5 to its downstream gene, such as OsMYB2 (*Chaemyeong et al., 2021*). *Shang et al. (2010)* found that AtWRKY18, AtWRKY40 and AtWRKY60 were involved in the ABA signaling pathway. *Chrysanthemum morifolium* CmWRKY1 participated in drought response by an ABA-mediated pathway (*Fan et al., 2016*). In addition to ABA, WRKY TFs play an important role in the SA signaling pathway. *AtWRKY39* responded to high temperatures by collaboratively participating in SA and JA signaling pathways (*Li et al., 2010*). According to *Kim et al. (2008), AtWRKY38* and *AtWRKY62* inhibited the expression of the SA responsive gene *AtPR1* and decreased tolerance to pathogens. The comprehensive expression analysis of LasaWRKY TFs revealed that LasaWRKY TFs could respond to different abiotic stresses (high salt, drought, low temperature, high temperature) by participating in the phytohormone signaling pathway.

436

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449 **Competing Interests**

450 All authors declare they have no other competing interests.

451

452 **Author Contributions**

453 - Ping Du conceived and designed the experiments, prepared figures and/or tables, authored or
454 reviewed drafts of the paper, and approved the final draft.
455 - Qinglian Wu conceived and designed the experiments, performed the experiments, analyzed
456 the data, authored or reviewed drafts of the paper, and approved the final draft.

- Yihua Liu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Xu Cao analyzed the data, prepared figures and/or tables, and approved the final draft.
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- Tikun Jiao performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Mengqi Hu analyzed the data, prepared figures and/or tables, and approved the final draft.
- Ying Huang conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplementary Files.

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659 **Figure Legends**

660 **Fig. 1:** Growth of asparagus lettuce plants (A) and the cross section of asparagus lettuce stem (B).

661

662 **Fig. 2:** Phylogenetic analysis of LasaWRKY TFs among lettuce, Arabidopsis, tomato and tea
663 plants by MEGA7.0.

664

665 **Fig. 3:** Phylogenetic relationship, exon-intron structure, conserved motifs and cis-element
666 analysis of LasaWRKY genes in lettuce. (a) The phylogenetic tree created by MEGA 7.0 and
667 exon-intron structures from online software GSDS. (b) Conserved motifs predicted in
668 LasaWRKY protein. Ten motifs were identified by the MEME program, with each number of
669 colored boxes representing a different motif. (c) Cis-element analysis in the promoter region of
670 LasaWRKY genes.

671

672 **Fig. 4:** Chromosomal distribution of LasaWRKY TFs.

673

674 **Fig. 5:** Comparative analysis of synteny between *L. saliva* and *L.saligna*.

675

676 **Fig. 6:** Interaction network analysis of LasaWRKY TFs.

677

678 **Fig. 7:** Relative expression of five LasaWRKY genes under different abiotic stresses.

679

680 **Fig. 8:** Relative expression of five LasaWRKY genes under hormone treatment.

681

682 **Fig. 9:** Relative expression of five LasaWRKY genes at different tissues.

683

684 **Fig. 10:** Expression profiles of LasaWRKY genes by the transcriptome data analysis at different
685 L. sativa stem enlargement periods.

686

687 **Fig. 11:** Expression profiles of five LasaWRKY genes at different L. sativa stem enlargement
688 periods.

689

690 **Supplementary Files**

691 **Supplementary File 1**

692 **Fig. S1:** Alignment of the amino acid sequence of LasaWRKY TFs.

693

694 **Supplementary File 2**

695 **Fig. S2:** WRKY family TFs members among different plant species.

696

697 **Supplementary File 3**

698 **Table S1:** Primer sequences used in the text.

699 **Table S2:** Characteristic features of LasaWRKY TFs.

700 **Table S3:** The paralogs and orthologs of WRKY TFs between *L. saliva* and *L.saligna*.

701

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703

704

Figure 1

Growth of asparagus lettuce plants (A) and the cross section of asparagus lettuce stem (B).

Figure 2

Phylogenetic analysis of LasaWRKY TFs among lettuce, Arabidopsis, tomato and tea plants by MEGA7.0.

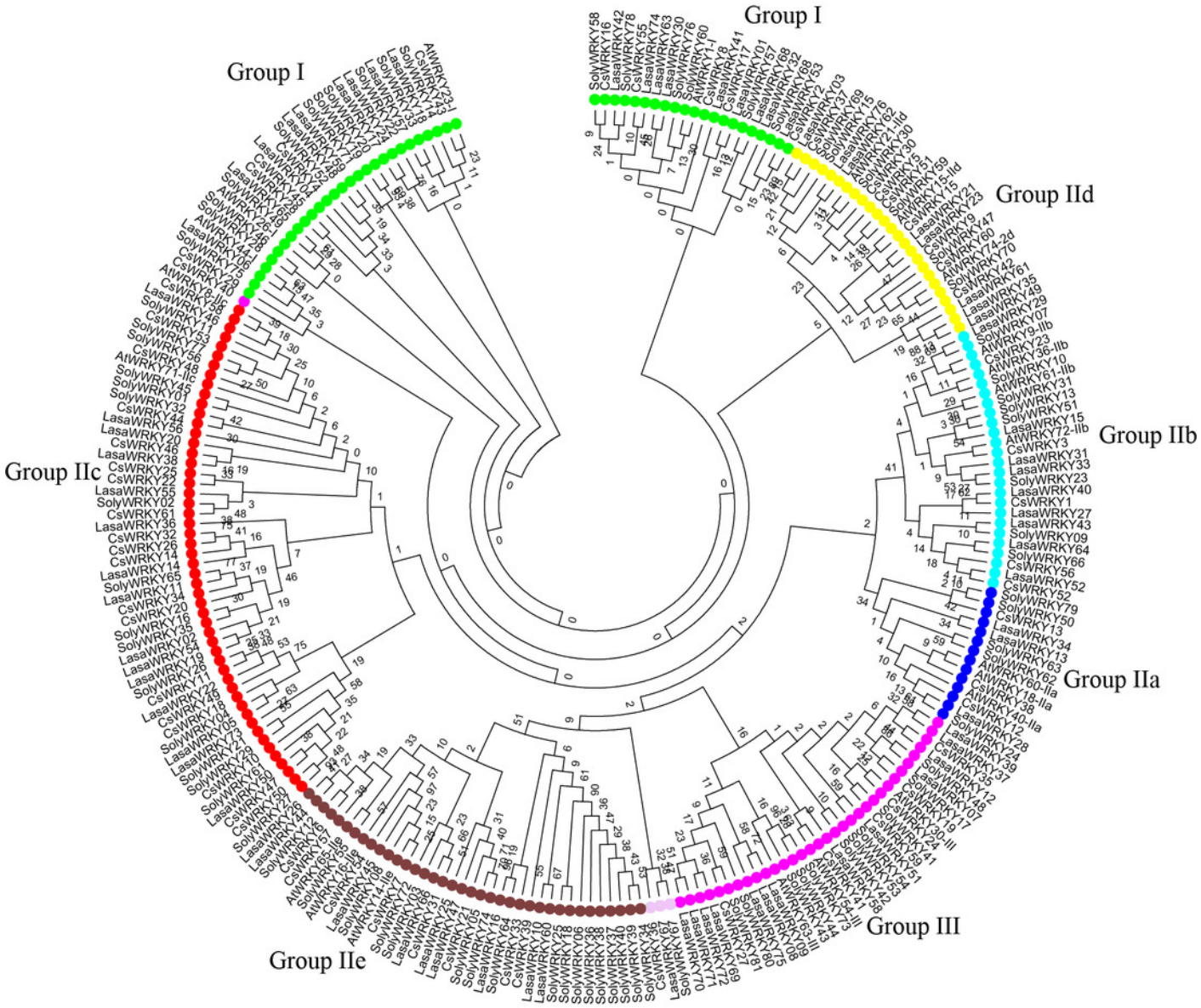


Figure 3

Phylogenetic relationship, exon-intron structure, conserved motifs and cis-element analysis of LasaWRKY genes in lettuce.

(a) The phylogenetic tree created by MEGA 7.0 and exon-intron structures from online software GSDS. (b) Conserved motifs predicted in LasaWRKY protein. Ten motifs were identified by the MEME program, with each number of colored boxes representing a different motif. (c) Cis-element analysis in the promoter region of LasaWRKY genes.

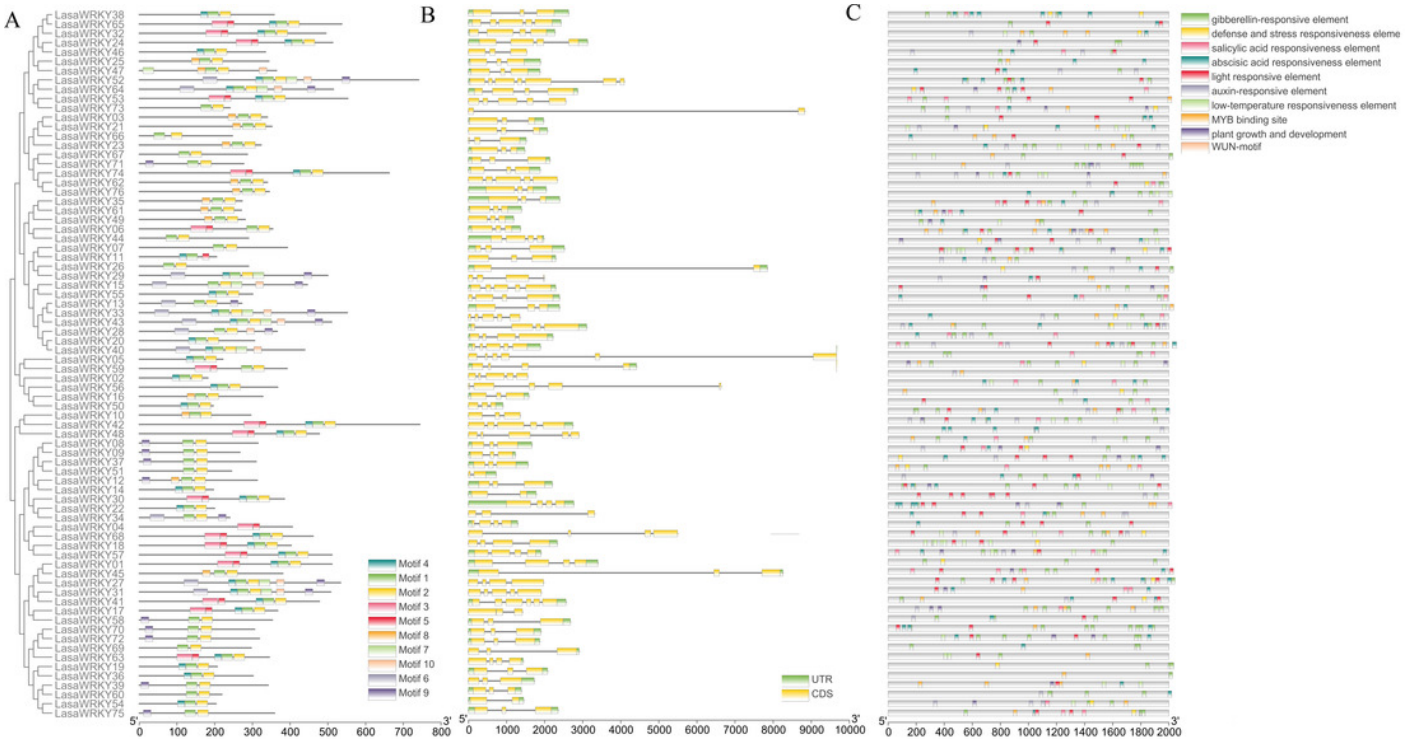


Figure 4

Chromosomal distribution of LasaWRKY TFs.

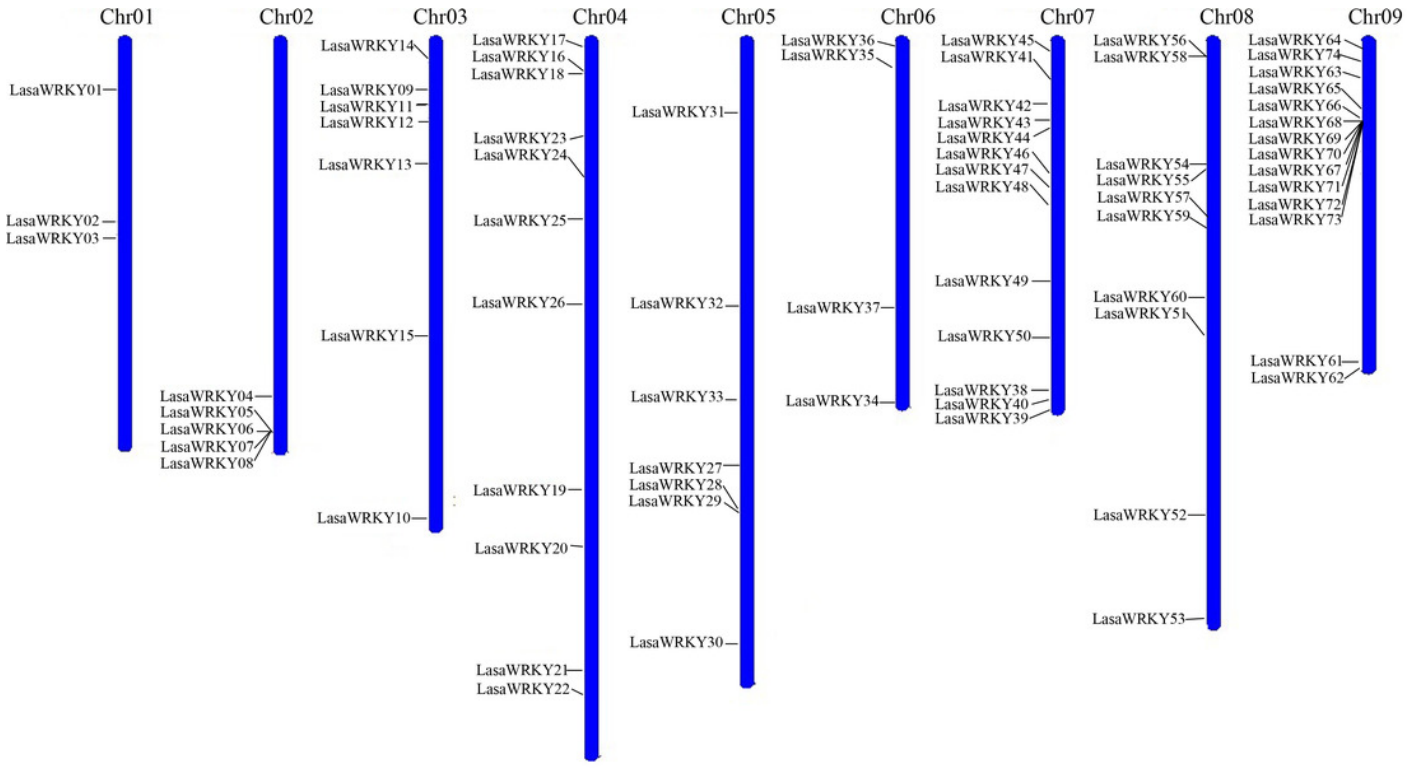


Figure 5

Comparative analysis of synteny between *L. saliva* and *L.saligna*.

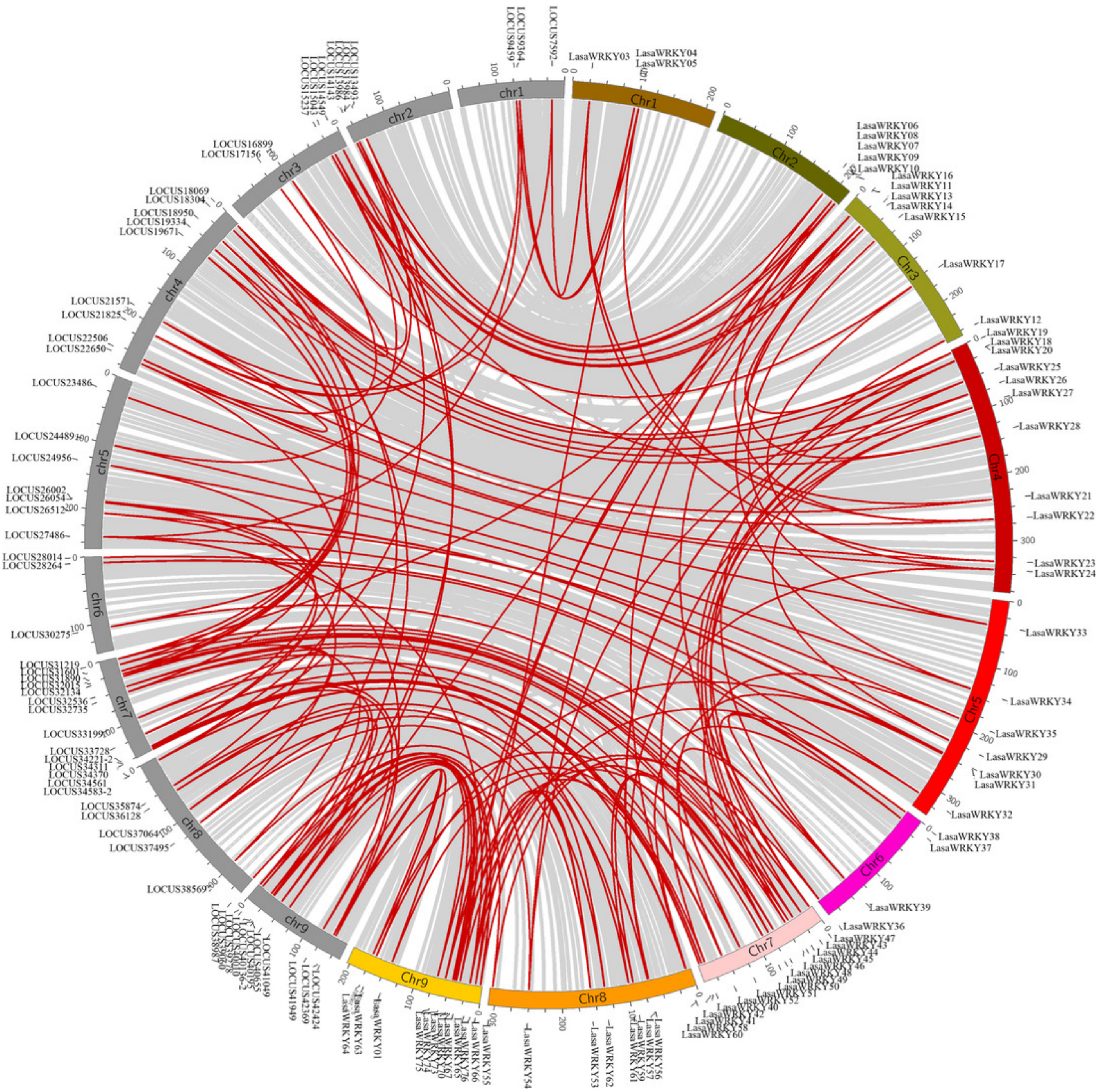
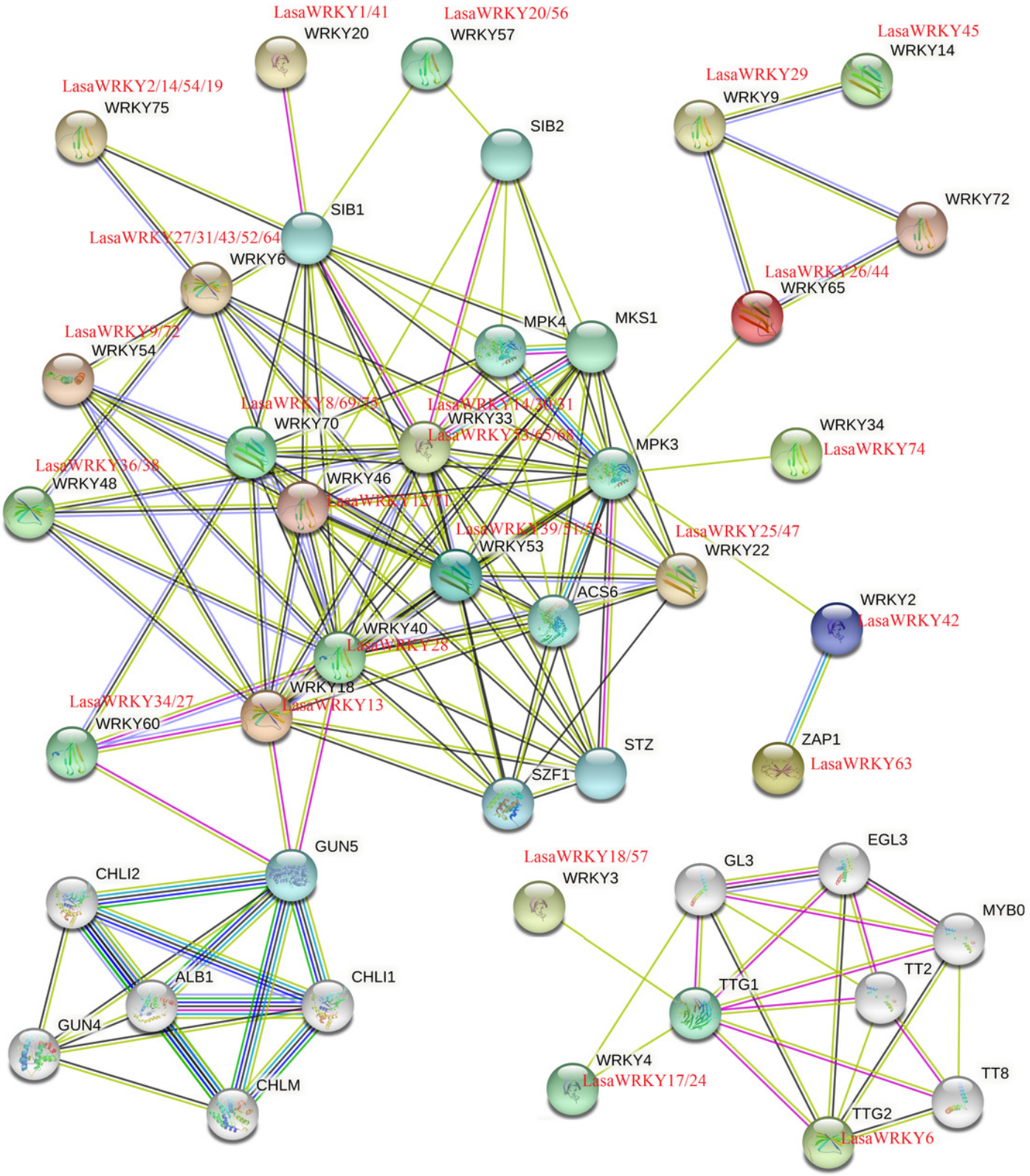


Figure 6

Interaction network analysis of LasaWRKY TFs.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WRKY20
LasaWRKY20/56
WRKY57
LasaWRKY45
WRKY14
LasaWRKY29
WRKY9
LasaWRKY2/14/54/19
WRKY75
SIB2
WRKY72
SIB1
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WRKY6
LasaWRKY26/44
WRKY65
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WRKY60
STZ
ZAP1
LasaWRKY63
SZF1
GUN5
LasaWRKY18/57
WRKY3
EGL3
GL3
MYB0
CHLI2
TT2
TTG1
ALB1
CHLI1
GUN4
WRKY4
LasaWRKY17/24
TT8
CHLM
TTG2
LasaWRKY6

Figure 7

Relative expression of five LasaWRKY genes under different abiotic stresses.

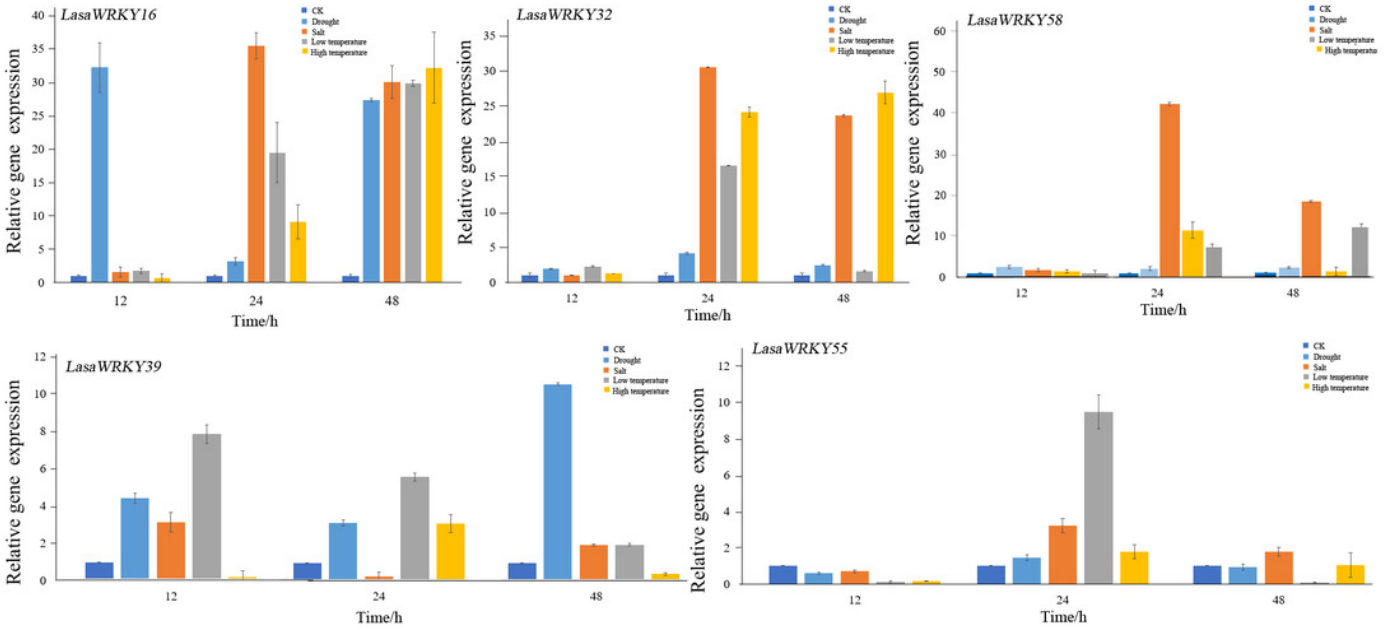


Figure 8

Relative expression of five LasaWRKY genes under hormone treatment.

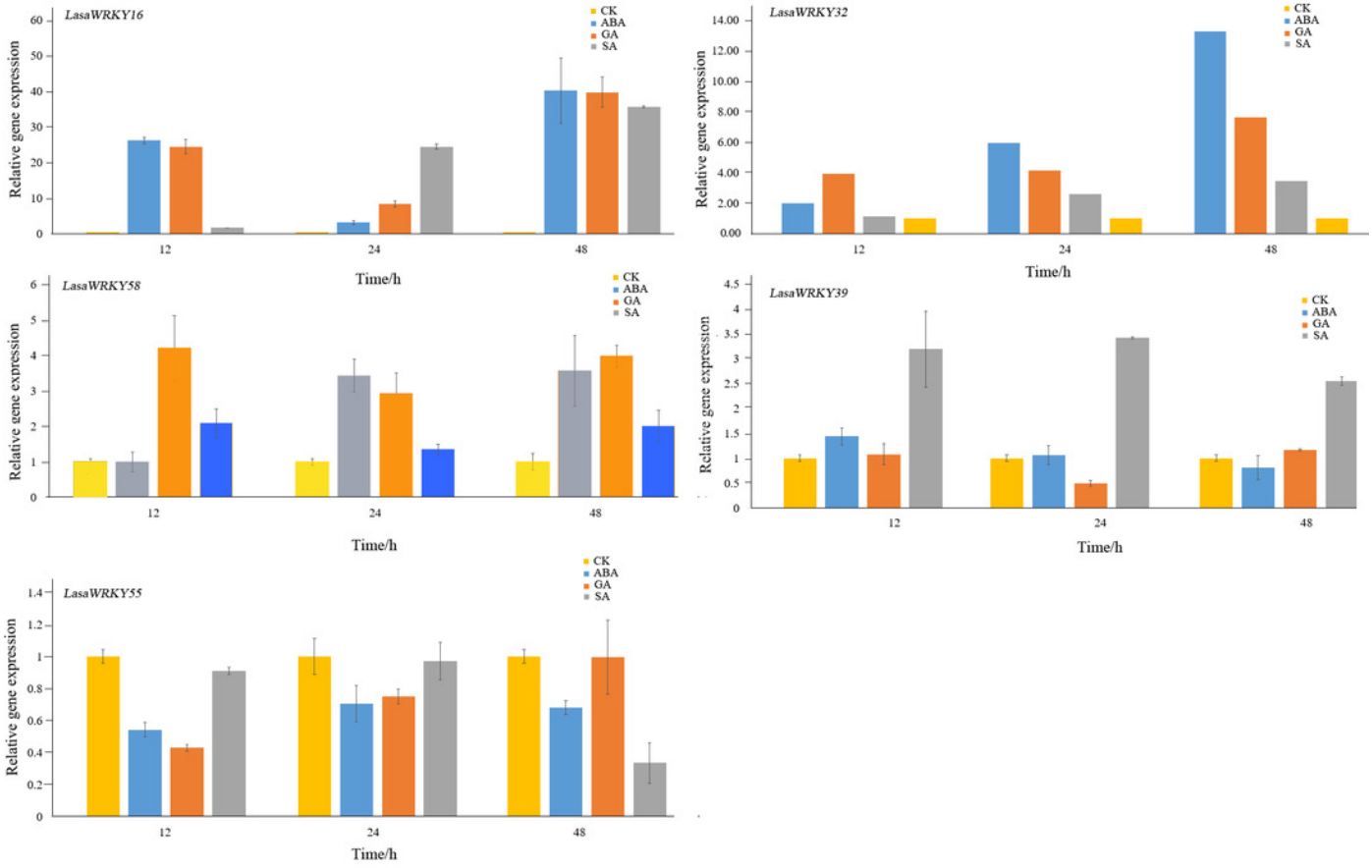


Figure 9

Relative expression of five LasaWRKY genes at different tissues.

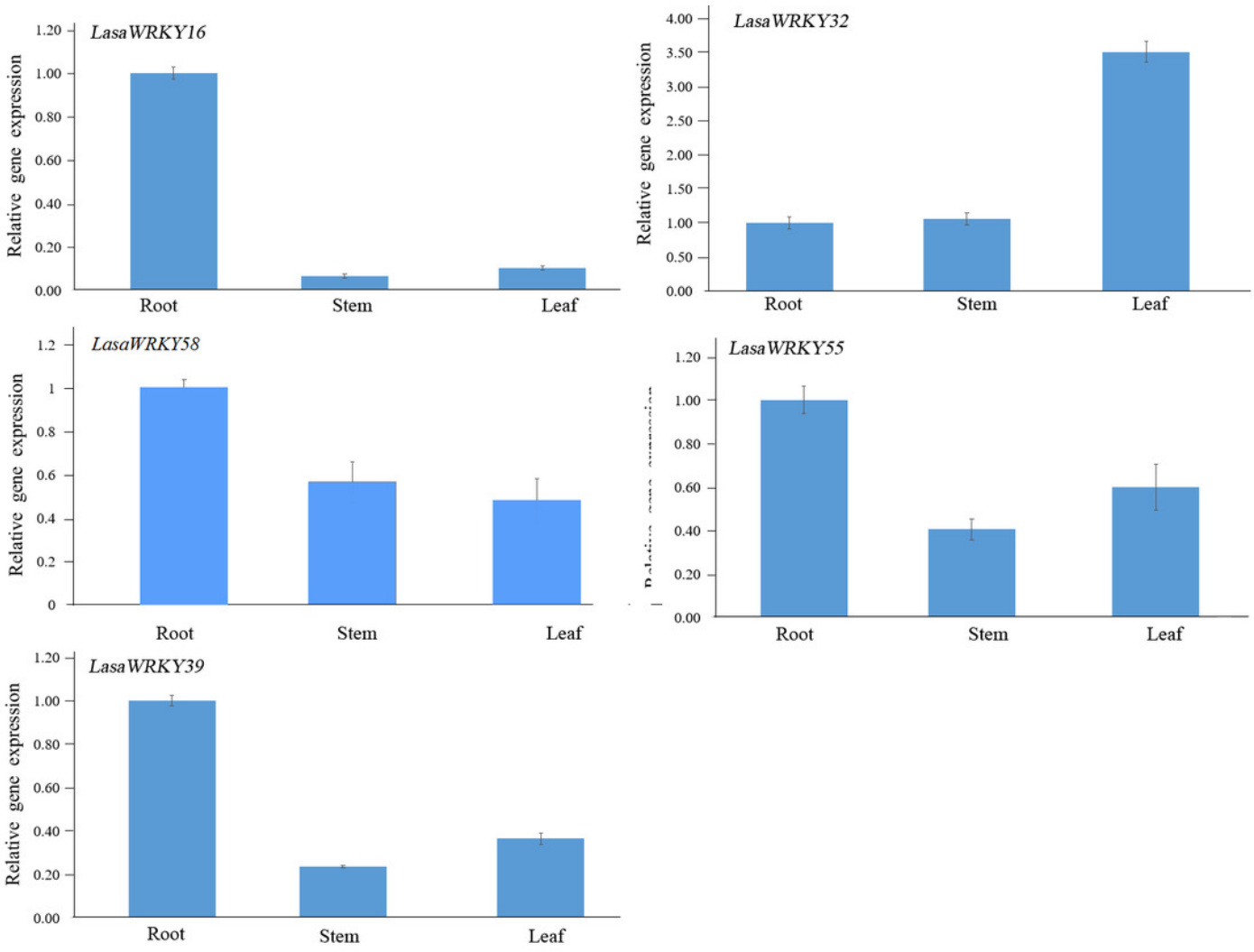
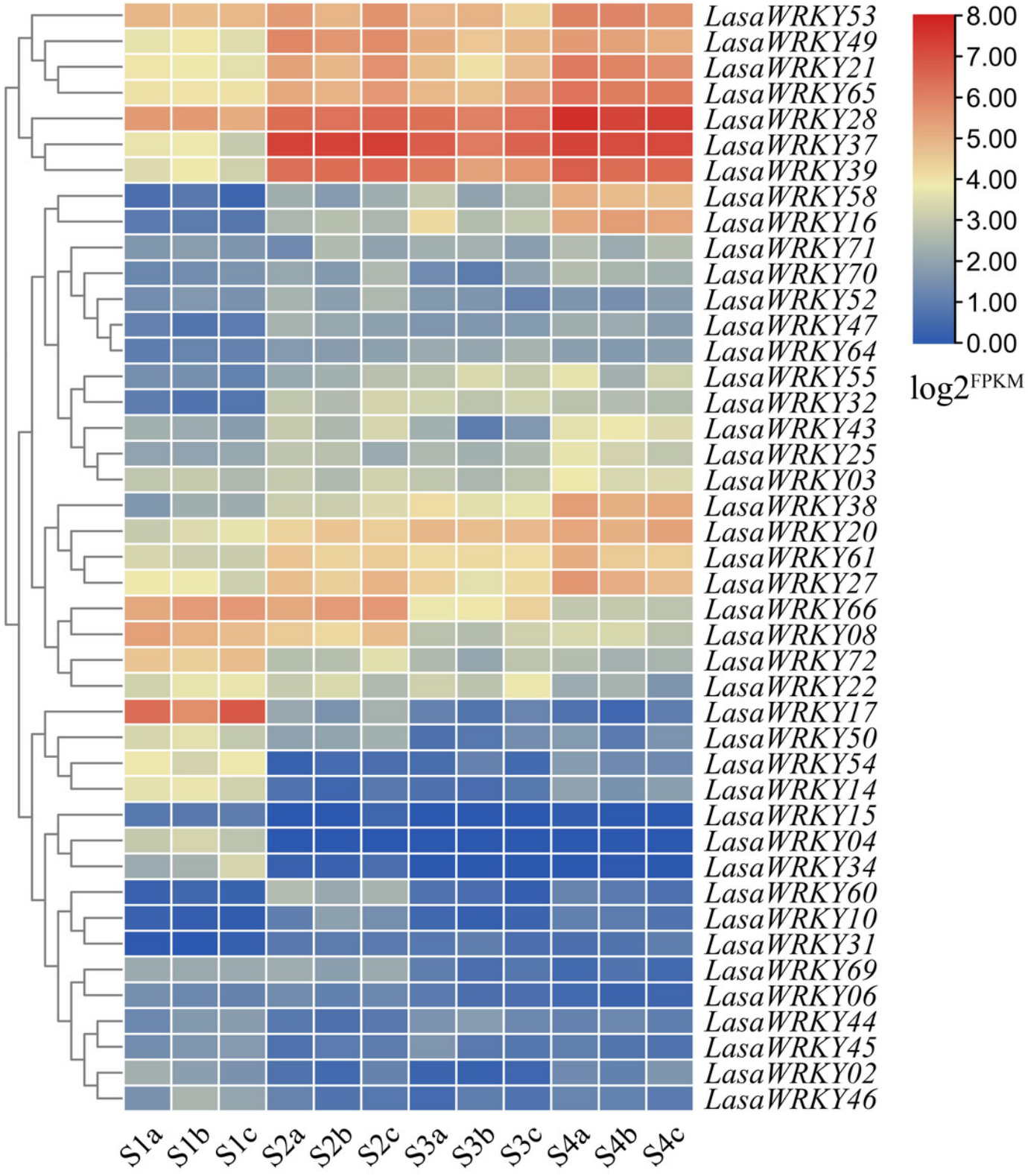


Figure 10

Expression profiles of LasaWRKY genes by the transcriptome data analysis at different L. sativa stem enlargement periods.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LasaWRKY37
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LasaWRKY16
LasaWRKY71
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LasaWRKY52
LasaWRKY47
LasaWRKY64
LasaWRKY55
LasaWRKY32
LasaWRKY43
LasaWRKY25
LasaWRKY03
LasaWRKY38
LasaWRKY20
LasaWRKY61
LasaWRKY27
LasaWRKY66
LasaWRKY08
LasaWRKY72
LasaWRKY22
LasaWRKY17
LasaWRKY50
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LasaWRKY14
LasaWRKY15
LasaWRKY04
LasaWRKY34
LasaWRKY60
LasaWRKY10
LasaWRKY31
LasaWRKY69
LasaWRKY06
LasaWRKY44
LasaWRKY45
LasaWRKY02
LasaWRKY46
S1a
S1b
S1c
S2a
S2b
S2c
S3a
S3b
S3c
S4a
S4b
S4c
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
log2FPKM

Figure 11

Expression profiles of five LasaWRKY genes at different L. sativa stem enlargement periods.