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

Ping Du [1], **Qinglian Wu** [1], **Yihua Liu** [1], **Xue Cao** [1], **Wenyi Yi** [1], **Tikun Jiao** [1], **Mengqi Hu** [1], **Ying Huang** [Corresp. 1]

[1] Linyi University, Linyi, China

Corresponding Author: Ying Huang
Email address: hyhappy1314@163.com

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. As one of the most popular vegetable crops, asparagus lettuce has important medicinal and nutritional value. However, identifying the WRKY TFs family in asparagus lettuce is limited. With the lettuce (*Lactuca sativa* L.) genome publication, we identified 76 WRKY TFs and analyzed structural characteristics, phylogenetic relationships, chromosomal distribution, interaction network, and expression profiles. The 76 LsWRKY TFs were phylogenetically classified as Groups I, II (IIa-IIe), and III. *Cis* element analysis revealed complex regulatory relationships of LsWRKY genes in response to different biological progresses. Interaction network analysis indicated that LsWRKY TFs could interact with other proteins, such as SIB (sigma factor binding protein), WRKY TFs, and MPK. The WRKYIII subfamily genes showed different expression patterns during the progress of asparagus lettuce stem enlargement. According to RT-qPCR analysis, abiotic stresses (drought, salt, low temperature, and high temperature) and phytohormone treatment could induce specific LsWRKYIII gene expression. These results will provide systematic and comprehensive information on LsWRKY TFs and lay the foundation for further clarification of the regulatory mechanism of LsWRKY, especially LsWRKYIII TFs, involved in stress response and the progress of plant growth and development.

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

Ping Du*, Qinglian Wu*, Yihua Liu, Xue Cao, Wenyi Yi, Tikun Jiao, Mengqi Hu, Ying Huang

College of Agriculture and Forestry Sciences, Linyi University, Linyi, Shandong 276000, China

*These authors contributed equally to this work

Please address all correspondence to: Ying Huang (hyhappy1314@163.com)

Dr. Ying Huang

College of Agriculture and Forestry Sciences,

Linyi University,

Linyi, 276000, China

Email: hyhappy1314@163.com

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. As one of the most popular vegetable crops, asparagus lettuce has important medicinal and nutritional value. However, identifying the WRKY TFs family in asparagus lettuce is limited. With the lettuce (*Lactuca sativa* L.) genome publication, we identified 76 WRKY TFs and analyzed structural characteristics, phylogenetic relationships, chromosomal distribution, interaction network, and expression profiles. The 76 LsWRKY TFs were phylogenetically classified as Groups I, II (IIa-IIe), and III. *Cis* element analysis revealed complex regulatory relationships of LsWRKY genes in response to different biological progresses. Interaction network analysis indicated that LsWRKY TFs could interact with other proteins, such as SIB (sigma factor binding protein), WRKY TFs, and MPK. The WRKYIII subfamily genes showed different expression patterns during the progress of asparagus lettuce stem enlargement. According to RT-qPCR analysis, abiotic stresses (drought, salt, low temperature, and high temperature) and phytohormone treatment could induce specific LsWRKYIII gene expression. These results will provide systematic and comprehensive information on LsWRKY TFs and lay the foundation for further clarification of the regulatory mechanism of LsWRKY, especially LsWRKYIII TFs, involved in stress response and the progress of plant growth and development.

Keywords: Asparagus lettuce, WRKY TF, Abiotic stress, Expression patterns

INTRODUCTION

Long-term domestication and directional selection of lettuce have resulted in the development of various cultivars (oil lettuce, stem lettuce (also known as asparagus lettuce), and various varieties of leaf lettuce) to meet various needs. 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 family. 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 a vital role in balancing people's diets (*Cui et al., 2014*). The freshness and tenderness of stems influence the quality 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., 2020a*). However, the regulatory mechanism of lettuce stem expansion remains unclear.

Plant transcription factors (TFs) have been found to play essential regulatory roles in stem enlargement. MADS-box, ABF/AREB, and homeobox TFs were discovered to be involved in forming 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

potatoes (*Kim et al., 2002*; *Kim et al., 2005*; *Cheng et al., 2013*). *ABF4* (ABF-binding factor) positively regulates potato tuber induction. 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*). However, the roles of WRKY TFs in plant root and stem development remain unclear.

Containing the conserved WRKY domain in the N terminus and zinc finger motif in the C terminus (C2H2 or C2HC), WRKY proteins can recognize and bind to the W-box element (TTGAC/T) existed in the promoter region of target genes. WRKY TFs are classified into three types: Group 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 further subdivided into five subgroups, i.e., IIa-IIe. The WRKY TFs regulate multiple physiological processes (*Dong et al., 2003*; *Rengasamy et al., 2008*; *Li et al., 2020b*; *Liu et al., 2021a*; *Wei et al., 2021*). It has been observed that 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 WRKYIII subfamily gene *SlWRKY81* inhibited plant drought tolerance by suppressing SlRBOH1-derived H_2O_2 accumulation (*Ahammed et al., 2020*).

WRKY TFs are also involved in plant development such as seed germination, reproductive processes, senescence, and plant organ development (*Chen et al., 2017*). The flowering process was up-regulated by *AtWRKY71* by regulating the expression of flowering genes, while

78 *AtWRKY6* plays a vital role in leaf senescence by regulating the enzyme SIRK (*Robatzek &*
79 *Somssich 2002*; *Yanchong & Yu et al., 2016*). *Zhang et al.* (*2011*) found rice *OsWRKY78* could
80 regulate stem elongation; the expression pattern of *OsWRKY78* in the elongated stem was most
81 abundant, and inhibition of *OsWRKY78* expression resulted in the shortening of somatic cell
82 length. Cotton *GhWRKY15* improved resistance to the virus, fungal infection, and stem
83 elongation (*Yu et al., 2012*). *Li et al.* (*2016*) found WRKY TFs were involved in carrot root
84 development.

85 As a result of systematic studies, many WRKY TF family members have been identified in
86 different plant species, such as 72 in Arabidopsis, 81 in tomato, 95 in carrot, 55 in cucumber, 59
87 in grape, 45 in *Eucommia ulmoides*, and 64 in *Isatis indigotica* (*Ishiguro & Nakamura 1994*;
88 *Yang et al., 2020*; *Liu et al. 2021b*; *Qu et al., 2021*). However, systematic and comprehensive
89 information of WRKY TFs in asparagus lettuce were unclear.

90 In this study, 76 WRKY TFs were identified in asparagus lettuce through genome-wide
91 analysis. Exon-intron structure, phylogenetic relationships, motif compositions, collinearity
92 analysis, and chromosome distribution of WRKY genes were identified. WRKYIII subfamily
93 TFs have been identified to involve in different biological processes. So, we investigated the
94 expression levels of WRKYIII genes at different stages of stem expansion, various abiotic
95 stresses, and plant hormones. Our results will provide the basis of WRKY TFs in asparagus
96 lettuce and will highlight the role of WRKY TFs, especially WRKYIII TFs, in stem expansion
97 and stress response.

98

MATERIAL AND METHODS

Sequence retrieval and identification of WRKY TFs in lettuce

The lettuce WRKY TFs were obtained from the lettuce's genome (https://lgr.genomecenter.ucdavis.edu/). Amino acid sequences of *Arabidopsis* WRKY TFs were used as query sequences to search for the homologous LsWRKY TFs sequences. Subsequently, the conserved WRKY domain was identified by SMART (http://smart.embl-heidelberg.de/), Pfam database (http://pfam.janelia.org/), and NCBI CDD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The molecular weight (Mw) and theoretical isoelectric point (pI) of LsWRKY TFs were identified by 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 LsWRKY TFs. MEME online program (https://meme-suite.org/meme/tools/meme) was used to identify the conserved motif of LsWRKY TFs (*Bailey et al., 2009*). The result was visualized by TBtools software (*Chen et al., 2020*). To investigate *cis*-elements, the promoter region of the 2000 bp genomic DNA upstream sequence was submitted to the PlantCARE database (*Lescot et al., 2002*).

Multiple sequence alignment and phylogenetic tree of LsWRKY TFs

Multiple sequence alignment of LsWRKY TFs was performed using the DNAMAN software. The WRKY TFs of *Arabidopsis thaliana* and tomato were downloaded from TAIR

120 (https://www.arabidopsis. org/) and SGN (https://solgenomics.net/), respectively. The
121 phylogenetic tree was constructed by using MEGA 7.0 software with the neighbor-joining
122 method with 1000 bootstrap replicates (*Kumar et al., 2008*).
123
124 **Chromosomal distribution and syntenic relationship of LsWRKY TFs**
125 MapChart software was used to draw the chromosomal distribution of WRKY TFs from lettuce's
126 genome, while STRING software was used to conduct the interaction network (*Franceschini et*
127 *al., 2013*). McscanX was used to identify the orthologous and paragons genes of WRKY TFs in
128 *L. sativa* and willow-leaf lettuce *L. saligna* to elucidate LsWRKY TFs' origin. The symbiotic
129 relationships were displayed using Circos software (*Krzywinski et al., 2009*).
130
131 **Plant materials, stress, and phytohormone treatments**
132 Seeds of the cultivated asparagus lettuce 'Yonganhong' were sown in a controlled environment
133 chamber for 12 h photoperiod at 22 and 18 ˚C (day vs. night) with a light intensity of 20,000
134 μmol/m²/s (lux). Asparagus lettuce seedlings were used in subsequent experiments once they
135 reached the four-leaf stage. Seedlings were treated with 200 mmol/L NaCl (salt), 20% PEG6000
136 (drought), 4 ˚C (low temperature), and 37 ˚C (high temperature) for abiotic stress treatment,
137 respectively. Salicylic acid (SA, 0.5 mmol/L), abscisic acid (ABA, 75 μmol/L), and gibberellin
138 (GA, 50 μmol/L) were sprayed on them and placed for different duration of time. The expression
139 patterns of LsWRKY TFs were also analyzed at different stages of asparagus lettuce stem
140 development (S1: the transverse diameter length is 1 cm, S2: the transverse diameter length is 2

cm, S3: the transverse diameter length is 3 cm, and S4: the transverse diameter length is 4 cm) (Fig. 1). All samples were frozen in liquid nitrogen and stored in a -80 ˚C refrigerator. Total RNA was isolated from 4 stem swelling stages (S1, S2, S3, and S4), abiotic stress (drought, salt, low temperature, and high temperature), and hormone treatment (ABA, SA, and GA) using a plant total RNA isolation kit (Vazyme, Nanjing, China) and first-strand cDNA was synthesized using a 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China).

Quantitative transcript analysis and qRT-PCR validation

Four stem enlargement stages (S1, S2, S3, and S4) of 'Yonganhong' were chosen to conduct an RNA sequence to identify the differentially expressed genes during different progress of lettuce stem enlargement. PCR amplified the purified double-stranded cDNA in 4 stages, and the products were further purified to obtain libraries for Illumina HiSeq 4000 sequencing (Biomarker Technologies Co., Ltd., Beijing, China). The transcript abundance of LsWRKY genes in different development stages was counted by HTSeq and FPKM (fragments per kilobase exon per million fragments mapped) to estimate the expression level. The transcriptome sequencing data have been submitted to a public database (NCBI: PRJNA844256).

RT-qPCR was conducted to verify the expression patterns of LsWRKY genes further. For RT-qPCR, SYBR Green I (TaKaRa, Dalian, China) and the Roche LightCycler 96 were used. *LsTIP41* (Lsat_1_v5_gn_5_116421) was used to normalize and calculate the expression levels of each LsWRKYIII gene (*Borowski et al., 2014*). The relative expression levels of LsWRKYIII genes were calculated using the $2^{-\Delta\Delta CT}$ methods based on the mean value of three technical

repeats. SPSS statistics 17.0 software was used to analyze the significant difference. The primer pairs were designed by Primer Premier 6.0 and are listed in Supplemental Table S1.

RESULTS

Identification of LsWRKY TFs in lettuce

Guo et al. (*2019*) identified 74 WRKY TFs in lettuce. In this study, a total of 76 LsWRKY TFs (denoted as LsWRKY01 to LsWRKY76) were identified, which increased two TFs i.e., LsWRKY02 (Lsat_1_v5_gn_0_3061.1) and LsWRKY38 (Lsat_1_v5_gn_6_220.3). The coding sequence (CDS) lengths of LsWRKY TFs ranged from 546 bp (LsWRKY02) to 2232 bp (LsWRKY42), with corresponding amino acid (aa) numbers ranging from 181 aa to 743 aa. The MWs and pI values of the identified LsWRKY TFs ranged from 20.7 kDa (LsWRKY02) to 81.7 kDa (LsWRKY42) and from 5.19 (LsWRKY44) to 9.98 (LsWRKY35), respectively. 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 LsWRKY proteins are hydrophilic (Supplemental Table S2).

Multiple sequence alignment and phylogenetic analysis of LsWRKY TFs

As shown in Fig. S1, sequence alignment of LsWRKY TFs was conducted. Two WRKY domains with the conserved WRKYGQK were present in Group I, which contained C2H2-type zinc-finger domains. All 46 LsWRKY TFs in Group II, such as LsWRKY03, LsWRKY05, LsWRKY10, and LsWRKY13, had one WRKY domain and a C2H2-type zinc-finger. All

183 members in Group III had one complete WRKY domain and a C2HC zinc finger. However, the
184 WRKYGQK sequence was found changed in some LsWRKY TFs, i.e., WRKYGKK in
185 LsWRKY50 and WKKYGEK in LsWRKY61.

186 To investigate the phylogenetic relationship of LsWRKY TFs, the phylogenetic tree was
187 also constructed using MEGA7.0 software. All 76 LsWRKY TFs were divided into three groups
188 (I, II, and III). Group II had the most members (46), but the distribution was uneven among the
189 five subgroups IIa (3), IIb (9), IIc (17), IId (9), and IIe (8). Group I contained 17 LsWRKY TFs,
190 while group III formed the smallest group with 13 LsWRKY TFs (Fig. 2). A total of 949 WRKY
191 TFs from 10 different plant species were chosen to analyze the classification of the WRKY TFs
192 family (Fig. S2). *Glycine max* had the most WRKY TFs (176), followed by *Zea mays* (131),
193 *Oryza sativa* (100), and *Daucus carota* (95); *Vitis vinifera* had the fewest WRKY TFs (59).
194 Among the three groups, WRKY TFs were mainly classified into Group II. For instance, 45 of
195 72 *A. thaliana* WRKY TFs belonged to Group II, while Groups I and III contained 13 and 14
196 members. The distribution of WRKY TFs in *Solanum lycopersicum* was 15 (Group I), 52 (Group
197 II), and 11 (Group III). The number of WRKY TFs in Groups I and III among the ten plant
198 species was similar, except for *V. vinifera*, which had 12 Group I members and six Group III
199 members, respectively (Fig. S2).
200
201 **Gene structure, conserved motif, and *cis*-elements analysis of LsWRKY TFs**
202 The TBtools program was used to explore the gene structure to analyze the introns and exons of
203 LsWRKY TFs. The numbers of introns in 76 LsWRKY TFs ranged from one to six. The

majority of members (33 of 76 LsWRKY TFs) had two introns and three exons, followed by three introns (17) and four introns (10). The LsWRKY52 had the highest number of introns (6) and exons (7), while LsWRKY73, LsWRKY29, LsWRKY12, LsWRKY30, and LsWRKY58 each had only one intron (Fig. 3B). The losses and gains of LsWRKY TFs may be related to the functional diversity during the evolution of LsWRKY TFs.

Despite the gene structure of LsWRKY TFs differed, some conserved motifs were found in all LsWRKY TFs. MEME program identified ten conserved motifs to illustrate the similarity and diversity of motif composition. The conserved motifs in 76 LsWRKY TFs ranged from two to seven. Motif 1 and motif 2 existed in all 76 LsWRKY TFs. There were only two motifs in 9 LsWRKY TFs (LsWRKY51, 60, 69, 26, 07, 44, 66, 67, and 73). Motif 9 and motif 10 mainly existed in Group III and II, respectively. Motif 3 and motif 5 were unique in Group I, such as LsWRKY65, 32, 24, 53, 74, 06, 59, 42, and 48 (Fig. 3A). The results indicated that LsWRKY TFs from the same group have similar conserved motifs. But differences also existed in these LsWRKY TFs, indicating the functional diversity of LsWRKY TFs (*Rose 2004*).

To conduct the *cis*-elements analysis, 2.0 kb DNA sequences upstream from 76 LsWRKY TF codons were chosen. As shown in Fig. 4. 10 types of *cis*-elements contained hormone-related (GA-responsive element TCTGTTG, SA responsiveness element CCATCTTTTT, ABA responsiveness element ACGTG, auxin-responsive element), stress-related (defense and stress responsiveness element, low-temperature responsiveness element) and plant growth and development-related *cis*-elements were found in 47 LsWRKY TFs. MYB binding site (CAACAG) was found in 47 LsWRKY TFs. GA-responsive, SA-responsive, and ABA-

responsive elements were found in 70, 32, and 56 LsWRKY, respectively. Low-temperature responsiveness element LTR (CCGAAA) was found in 23 LsWRKY TFs.

Chromosomal distribution and syntenic relationship of LsWRKY TFs

LsWRKY TFs were investigated according to the lettuce genome database to evaluate the chromosomal distribution. Except for LsWRKY01 and LsWRKY02, 74 LsWRKY TFs were found on nine lettuce chromosomes (Fig. 5). The LsWRKY TFs were mainly found on chromosome 09 (14), followed by chromosome 07 (13), chromosome 04 (11), and chromosome 08 (10). The number of LsWRKY TFs was seven on both chromosomes 3 and 5. Six LsWRKY TFs were found on chromosome 6. Only 3 LsWRKY TFs were mapped on chromosome 01. *L. saligna*, which also belonged to the genus *Lactuca*, was chosen to construct the comparative analysis to identify the paralogs and orthologs with *L. sativa*. As shown in Fig. 6, 75 and 70 pairs of paralogs were identified in *L. sativa* and *L. saligna*, respectively. Moreover, 75 pairs of orthologs between *L. sativa* and *L. saligna* were identified (Supplemental Table S3).

Interaction network analysis of LsWRKY TFs

To analyze the regulation mechanism, STRING software was used to construct an interaction network of LsWRKY TFs based on the orthologs in *A. thaliana*. As shown in Fig. 7, 49 LsWRKY TFs were identified as the orthologs of *A. thaliana* WRKY TFs. For example, six lettuce LsWRKY TFs (LsWRKY14/30/31/53/65/68) were identified as the homology to WRKY6; four lettuce LsWRKY TFs (LsWRKY02/14/19/54) showed high similar to WRKY75.

LsWRKY TFs showed complex interaction with other proteins such as WRKY TFs, MPK4, and sigma factor binding protein (SIB). For Group III, WRKY TFs, WRKY53 (LsWRKY39/51/58) and WRKY33 (LsWRKY14/30/31/53/65/68) showed interactions with 11 or 14 stress related-proteins, including MPK proteins (MPK4 and MPK3), SIB proteins, and ACS6 respectively, indicating the critical roles in the regulation of transcription and biological processes of lettuce stem. LsWRKY8/69/75 (WRKY70) and LsWRKY14/30/31/53/65/68 (WRKY33) interacted with other proteins in a similar manner. They both interacted with SIB1, SIB2, MEK1, and LsWRKY28, indicating that their regulatory networks were similar. MEK1, MPK3, and MPK4 could interact with the LsWRKY8/69/75 (WRKY70) and LsWRKY25/47 TFs (WRKY22). LsWRKY TFs with co-expression relationships with other WRKY TFs included LsWRKY13 (WRKY18), LsWRKY13 (WRKY40), and LsWRKY34/27 (WRKY60), which indicated the auto-regulation or cross-regulation with each other.

Gene expression analysis

Expression patterns of LsWRKYIII subfamily genes in response to abiotic stress

To investigate the role of WRKYIII genes during abiotic stress, ten genes (*LsWRKY9*, *LsWRKY12*, *LsWRKY39*, *LsWRKY51*, *LsWRKY58*, *LsWRKY69*, *LsWRKY70*, *LsWRKY71*, *LsWRKY72*, and *LsWRKY75*) were chosen to identify the expression patterns during abiotic stresses (salt, drought, low temperature, and high temperature) by RT-qPCR (Fig. 8).

Salt stress

Expression levels of *LsWRKY58* were increased about two times (12 h) and ten times (24 h) after NaCl treatment. Five WRKYIII genes (*LsWRKY09*, *LsWRKY70*, *LsWRKY71*, *LsWRKY72*, and *LsWRKY69*) showed down-regulation expression patterns after 12 h and 24 h. Expression profiles of *LsWRKY12*, *LsWRKY39*, *LsWRKY51*, and *LsWRKY75* were increased at 12 h but decreased at 24 h.

Drought stress

The expression profiles of *LsWRKY12*, *LsWRKY39*, *LsWRKY51*, and *LsWRKY75* were similar under drought stress; these four genes showed increased expression levels not only at 12 h but also at 24 h. Compared with contrast check, the expression levels of *LsWRKY12* and *LsWRKY39* increased about two times (12 h) and five times (12 h), respectively. In contrast, *LsWRKY71* showed decreased expression profiles under drought treatment, indicating that *LsWRKY71* may play a negative regulatory role. *LsWRKY09*, *LsWRKY70*, and *LsWRKY72* showed increased expression at 12 h but decreased expression at 24 h. There were no apparent changes in the mRNA levels of *LsWRKY58* at 24 h, although the expression level increased about three times at 12 h.

***Low temperature* (4 ˚C)**

Under different treatment times, low temperature significantly induced the expression of 2 LsWRKY genes, i.e., *LsWRKY58* and *LsWRKY39*. The *LsWRKY58* and *LsWRKY39* showed 5-fold (24 h) and 9-fold (12 h) increase, respectively. In contrast, five genes, *LsWRKY12*, *LsWRKY69*, *LsWRKY70*, *LsWRKY72*, and *LsWRKY75*, were expressed up-regulated at 12 h and

down-regulated at 24 h, respectively. *LsWRKY09*, *LsWRKY51*, and *LsWRKY71* showed decreased expression profiles throughout the treatment period.

***High temperature* (*37 ˚C*)**

Compared with control check, 4 WRKYIII genes, including *LsWRKY09*, *LsWRKY51*, *LsWRKY72*, and *LsWRKY71,* showed a significantly decreased expression at 12 h and 24 h, whereas the expression of *LsWRKY75* increased about three times at 12 and 24 h (Fig. 8). No significant changes were detected in the expression of *LsWRKY12* (24 h) and *LsWRKY58* (12 h). The expression profiles of *LsWRKY39*, *LsWRKY69*, and *LsWRKY70* decreased at 12 h but increased at 24 h.

Expression patterns of LsWRKYIII subfamily genes under treatment with phytohormone

As important phytohormones, ABA, GA, and SA play critical roles in plant growth and development and various stresses by participating in various signal transduction pathways. Interestingly, most LsWRKY, including LsWRKYIII gene promoters, contained one or more phytohormone response elements (ABA, GA, SA, and auxin) as determined by *cis*-elements analysis (Fig. 4). The results indicated that LsWRKY TFs might respond to different biological progress by participating in plant hormone signaling. To investigate possible response mechanisms, we examined expression profiles of LsWRKYIII genes in response to exogenous ABA, SA, and GA.

As shown in Fig. 9, exogenous ABA, SA, and GA significantly induced the expression of *LsWRKY09*, *LsWRKY69*, and *LsWRKY75* except for ABA treatment at 24 h, which showed no

significant change in expression. The expression levels of *LsWRKY69* and *LsWRKY75* peaked under SA treatment at 24 h. *LsWRKY70* and *LsWRKY72* increased significantly under GA and SA treatment, while the expression levels were decreased under ABA treatment. *LsWRKY12* and *LsWRKY39* were induced by SA treatment, but they both showed insensitive expression patterns under ABA treatment. In construct, the expression profiles of *LsWRKY58* were increased only under GA treatment.

Tissue-specific expression patterns of LsWRKYIII subfamily genes

To investigate the potential functions of LsWRKY TFs during the development of *L. sativa*, the expression patterns of 10 LsWRKYIII genes in different organs (root, stem, and leaf) were identified (Fig. 10). The expression patterns of 3 LsWRKY genes (*LsWRKY39*, *LsWRKY58*, and *LsWRKY71*) were similar; these genes showed the highest expression levels in root as compared with stem and leaf. In contrast, the expression pattern of *LsWRKY12*, *LsWRKY72*, *LsWRKY51*, *LsWRKY69*, and *LsWRKY70* in the leaf and stem increased compared with in the root. *LsWRKY09* showed the highest expression in the leaf, while *LsWRKY75* showed the highest expression in the stem. The preferential expression patterns of LsWRKYIII genes in different organs indicated that each LsWRKYIII genes might play a unique role in organ development or function.

Transcript abundance analysis of LsWRKY genes in lettuce

The developmental expression profiles of LsWRKY genes were conducted to explore the WRKY TFs function involved in the progression of stem development. As shown in Fig. 11, 43 of 76 LsWRKY TFs showed different expressions. Several genes, including *LsWRKY53*, *LsWRKY49*, *LsWRKY21*, *LsWRKY28*, *LsWRKY39*, and *LsWRKY58*, showed up-regulated expression profiles as lettuce stem enlargement progressed. While, the expression levels of some genes such as *LsWRKY17*, *LsWRKY72*, *LsWRKY66*, and *LsWRKY08* were decreased, indicating that LsWRKY genes may play negative regulatory roles in lettuce stem enlargement. Some genes showed wavy expression patterns, including *LsWRKY60, LsWRKY14*, and *LsWRKY02*, while the LsWRKYIII genes showed different expressions in the progression of stem development. For WRKYIII subfamily genes, 6 (*LsWRKY39*, *LsWRKY58*, *LsWRKY69*, *LsWRKY70*, *LsWRKY71*, and *LsWRKY72*) of 13 WRKYIII genes were detected the different expression. The expression levels of *LsWRKY39*, *LsWRKY70*, *LsWRKY71*, and *LsWRKY58* were significantly induced, while, *LsWRKY72* and *LsWRKY69* showed decreased expression patterns.

Validation of expression profile of LsWRKYIII subfamily genes at different stages of stem enlargement

To explore the function of LsWRKY genes involved in lettuce stem development, RT-qPCR was used to examine the expression profiles of 10 LsWRKYIII genes (*LsWRKY09*, *LsWRKY12*, *LsWRKY39*, *LsWRKY51*, *LsWRKY58*, *LsWRKY69*, *LsWRKY70*, *LsWRKY71*, *LsWRKY72*, and *LsWRKY75*) (Fig. 12). *LsWRKY69* and *LsWRKY72* showed decreased expression profiles during lettuce stem development. The results were consistent with the results of RNA-Seq. Four WRKY

348 genes (*LsWRKY58*, *LsWRKY70*, *LsWRKY39*, and *LsWRKY71)* showed the highest expression
349 levels at the S3 stage. Interestingly, *LsWRKY09*, *LsWRKY12*, *LsWRKY39*, and *LsWRKY51*,
350 which RNA-Seq did not detect, also showed the response to asparagus lettuce stem enlargement
351 by RT-qPCR. As shown in Fig. 12, the expression profiles of *LsWRKY12* and *LsWRKY75* both
352 showed the highest expression levels at the S3 stages; the expression at S2 and S4 stages were
353 decreased compared with the S1 stage. *LsWRKY09* and *LsWRKY51* showed opposite expression
354 patterns. The expression levels of *LsWRKY09* increased continuously during the progress of stem
355 developmental stages. In contrast, compared with the S1 stage, *LsWRKY51* showed decreased
356 expression levels at S2, S3, and S4 stages.
357

358 **DISCUSSION**

359 **Identification of WRKY TFs family in lettuce**

360 The WRKY TFs have been confirmed to participate in various biological processes, including
361 various environmental stresses, plant growth, and development (*Dong et al., 2003*; *Rengasamy et*
362 *al., 2008*; *Kouzai et al., 2020*; *Wei et al., 2021*). Because of high-throughput sequencing
363 technology advancement, WRKY TFs family has been identified in numerous higher plants
364 (*Ishiguro & Nakamura, 1994*; *Yang et al., 2020*; *Qu et al., 2021*). *Guo et al.* (*2019*) analyzed the
365 WRKY TFs family from Asterales plant orders such as sunflower (*Helianthus annuus*) and globe
366 artichoke (*Cynara cardunculus*), and lettuce. There were 112, 60, and 74 WRKY TFs found in
367 sunflower, globe artichoke, and lettuce, respectively. Furthermore, apart from 74 LsWRKY TFs
368 identified by *Guo et al.* (*2019*), 2 TFs (LsWRKY02 and LsWRKY38) were also identified as

WRKY TFs in lettuce in our study. 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 members was 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 in *Arabidopsis* (72), tomato (78), and lettuce (76) (*Reyes-Chin-Wo et al., 2017*) (Fig. S2). The number of WRKY TFs in both potato and *Hevea brasiliensis* was 81, but the potato and *H. brasiliensis* genome sizes were 844 Mb and 2.15 Gb, respectively. These results indicated that the plants' genome size could not determine the numbers of WRKY TFs.

Phylogenetic analysis of LsWRKY TFs

Many studies showed that genes that belonged to the same subfamily play similar roles (*Ding et al., 2015*; *Ma et al., 2017*; Yang et al., 2021). The *CaWRKY30*, a homolog of *AtWRKY30*, showed similar roles to *AtWRKY30*; these 2 WRKY genes positively regulate biotic and abiotic stresses (*Scarpeci et al., 2013*; *El-Esawi et al., 2019*; *Hussain et al., 2021*). To analyze the possible function of LsWRKY TFs, WRKY TFs from Arabidopsis, tomato, and lettuce were chosen to conduct the phylogenetic relationship. As shown in Fig. 2, 76 LsWRKY TFs were classified into seven subfamilies (I, IIa-IIe, and III). *AtWRKY23* and *AtWRKY12* were identified to regulate embryo development and secondary cell wall formation (*Wang et al., 2010*; *Grunewald et al., 2013*). As the homology of *AtWRKY23* and *AtWRKY12*, *LsWRKY46* and *LsWRKY05* might play similar roles in the progress of embryo development. *Chen et al. (2010)* found that WRKYII subfamily TFs (*AtWRKY18*, *AtWRKY40*, and *AtWRKY60*) were involved in

the ABA signaling pathway. Thus, it is envisaged that the homology of *AtWRKY18*, *AtWRKY40*, and *AtWRKY60*, 3 WRKYII TFs (*LsWRKY13*, *LsWRKY34*, and *LsWRKY28*) in lettuce may also be involved in ABA signaling pathways. However, the function still needs further verification. Compared with WRKYI and WRKYII, WRKYIII subfamily TFs were proved as the most adaptable and advanced in monocot. The proportion of WRKYIII TFs ranged from 10% to 36% (Fig. S2). The 14 WRKYIII members in *Arabidopsis* participate in different plant defense signaling pathways, indicating that WRKYIII evolution needed increasing biological requirements (*Kalde et al., 2003*).

Roles of LsWRKY TFs in abiotic stress

Many 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*). A variety of abiotic factors such as drought and salt could induce the expression of WRKY genes. In *Arabidopsis*, 26 WRKY TFs were identified to respond to abiotic stress (*Jiang & Deyholos, 2006*). Similarly, most WRKYIII subfamily genes participate in abiotic stress. The *AtWRKY30* improved resistance to salt stress and oxidative stress (*Scarpeci et al., 2013*), while *AtWRKY46* overexpression plants showed more sensitivity to drought and salt stress (*Ding et al., 2015*). Similar results were found in wheat. *TaWRKY146*, the homology of *AtWRKY46*, also negatively regulated drought and salt stresses (*Ma et al., 2017*). *OsWRKY76* improved the resistance of rice to cold stress (*Naoki et al., 2013*). Our results showed that different expression patterns of LsWRKYIII genes responded to abiotic stresses. Drought could induce the expression

411 of *LsWRKY12*, *LsWRKY39*, *LsWRKY51*, *LsWRKY75*. The expression levels of most LsWRKYIII
412 genes, except *LsWRKY58*, decreased the homology of *AtWRKY30* and showed increased
413 expression levels under salt treatment, indicating that increased expression levels of *LsWRKY58*
414 might play a similar function to *AtWRKY30* (*Scarpeci et al., 2013*).
415
416 **Roles of LsWRKY TFs in plant' growth and development**
417 Some valuable clues have revealed the roles of WRKY TFs in plant development, including seed
418 development, senescence, seed dormancy, and germination (*Sun et al., 2003*; *Luo et al., 2005*;
419 *Zhou et al., 2011*). TTG2, one of the WRKY TFs, was identified to play a role in organ
420 development for the first time (*Johnson et al., 2002*). Gene expression profiles are linked to gene
421 function (*Xu et al., 2015*). Rice *OsWRKY78* was confirmed to promote seed development and
422 stem elongation (*Zhang et al., 2011*). AcWRKY TFs may also play a role in specific pineapple
423 physiological processes (*Xie et al., 2018*). *Ding et al.* (*2015*) found that *AtWRKY46* positively
424 regulated the lateral root development. WRKYIII gene *GhWRKY53*, an orthologous gene of
425 *AtWRKY46,* could significantly increase the density of trichomes in *A. thaliana* and increase the
426 yield of cotton fiber (*Yang et al., 2021*). These studies cemented the critical role of WRKYIII
427 subfamily TFs in plant's growth and development. RNA-Seq analysis revealed that 43 of 76
428 LsWRKY TFs had different expression patterns in different stages of stem enlargement (Fig. 11).
429 The expression levels of the WRKYIII subfamily in response to stem enlargement were
430 identified by RT-qPCR. Most genes except *LsWRKY51*, *LsWRKY69*, and *LsWRKY72*, showed
431 increased expression during the progress of stem enlargement, indicating that differently

expressed WRKYIII TFs may be the key regulators of lettuce stem development (Fig. 12).

Regulation mechanism of WRKY TFs involved in different biological signs of progress

The regulatory mechanisms of WRKY TFs in plant biological progress are complex. WRKY TFs can effectively combine to W-box existing in the promoter regions of downstream target genes to regulate the expression of target genes or bind other acting elements to form protein complexes. In brief, WRKY TFs could work in coordination or independently in response to different biological signs of progress.

W-box elements exist in many TFs, including WRKY TFs. Hence WRKY TFs can combine with the W-box element in other WRKY TFs to form self-regulation or cross-regulation networks (Li et al., *2020b*; *Zentgraf et al., 2010*). For example, *AtWRKY57* played a positive role in drought stress response by binding W-box elements that existed in the promoter of drought-resistant gene *RD29A* and *NCED3* (*Jiang et al., 2012*). Similarly, *SbWRKY50* from *Sorghum 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 WRKY TFs binding site W-box element (C/TTGACT/C) was found in many LsWRKY TFs, including *LsWRKY03*, *LsWRKY06*, *LsWRKY14*, *LsWRKY20*, and *LasaWRY36*, which indicated these genes might participate in different biological signs of progress by self-regulation or cross-regulation with other WRKY TFs. Similarly, WRKY TFs also could improve tolerance to various abiotic stresses by increasing some material accumulation. For instance, the overexpression of *Boea hygrometrica BhWRKY1* in *Nicotiana*

453 *tabacum* improved the seedling drought resistance by inducing the accumulation of raffinose
454 family oligosaccharides (*Wang et al., 2009*).

455 WRKY TFs may have crosstalk with plant hormones to avoid different stress conditions
456 (*Negi & Khurana, 2021*; *Lim et al., 2022*). The function of WRKY TFs in abiotic stress is often
457 related to defense-associated phytohormones such as JA, SA, and ABA. As a major
458 phytohormone, ABA has been shown to increase salt and drought tolerance (*Yin et al., 2017*).
459 The ABA could improve drought tolerance by attenuating the inhibition of *OsWRKY5* to its
460 downstream gene, such as *OsMYB2* (*Lim et al., 2022*). *Chrysanthemum morifolium CmWRKY1*
461 participated in drought response by an ABA-mediated pathway (*Fan et al., 2016*). In addition to
462 ABA, WRKY TFs play an essential role in the SA signaling pathway. *AtWRKY39* responded to
463 high temperatures by collaboratively participating in SA and JA signaling pathways (*Li et al.,*
464 *2010*). According to *Kim et al. (2008)*, *AtWRKY38* and *AtWRKY62* inhibited the expression of
465 the SA responsive gene *AtPR1* and decreased tolerance to pathogens. Our study identified
466 hormone-responsive elements (SA, GA, ABA, and auxin) in LsWRKY TFs. The GA-responsive
467 element existed in the promoter of 9 LsWRKYIII TFs except for *LsWRKY39*; the ABA-
468 responsive element also existed in the promoter of 9 LsWRKYIII TFs except for *LsWRKY51*; the
469 promoter region of *LsWRKY09*, *LsWRKY51*, and *LsWRKY75* had SA-responsive element. As
470 shown in Fig. 9, most LsWRKYIII TFs could participate in plant hormone responses (ABA, SA,
471 and GA) with different expression patterns. All the results indicated that LsWRKY TFs,
472 including WRKYIII TFs, may interplay with plant hormones to enhance the adaptation to stress
473 conditions.

LsWRKY TFs could interact with other proteins such as WRKY TFs, MPK, and SIB to regulate different biological processes (Fig. 7). 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 significant role in the progression of fungus-induced camalexin accumulation (*Mao et al., 2011*). *Yao et al. (2020)* confirmed that WRKY TFs induced a critical defense response in tobacco resistance to whitefly after being phosphorylated by MAPK. The VQ proteins (containing VQ motif FxxhVQxhTG) as a class of plant-specific transcriptional regulators could fine-tune plant growth or stress regulatory networks by cooperating with their interacting partners, including WRKY TFs (*Lai et al., 2011*; *Hu et al., 2013*). *Lai et al. (2011)* found that VQ proteins SIB1 and SIB2 could serve as transcriptional activators of WRKY33 in response to *Botrytis cinerea*. *Hu et al. (2013)* identified that VQ9, in collaboration with WRKY8, can regulate the plant-salt stress response. The results showed a complex regulation mechanism of LsWRKY TFs during various growth conditions, which may form complexes with other proteins such as SIB, MPK, and TFs.

CONCLUSION

In general, 76 WRKY TFs were identified in lettuce in the present study. A comprehensive analysis of LsWRKY TFs was conducted, including structural characteristics, phylogenetic

relationships, chromosomal distribution, interaction network, and expression profiles. RT-qPCR analysis indicated that the LsWRKYIII genes could respond to abiotic stress, hormone treatment, and stem enlargement. This study provides a theoretical basis for enriching WRKY TFs to regulate stem enlargement and for further exploration of the function of plant WRKY members.

REFERENCES

Ahammed GJ, Li X, Yang YX, Liu CC, Zhou GZ, Wan HJ, Cheng Y. 2020. Tomato WRKY81 acts as a negative regulator for drought tolerance by modulating guard cell H2O2-mediated stomatal closure. *Environmental and Experimental Botany* 171 DOI 10396010.1016/j.envexpbot.2019.103960.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren JY, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Research* 37:W202-W208 DOI 10.1093/nar/gkp335.

Borowski JM, Galli V, Messias Rda S, Perin EC, Buss JH, dos Anjos e Silva SD, Rombaldi CV. 2014. Selection of candidate reference genes for real-time PCR studies in lettuce under abiotic stresses. *Planta* 239:1187-1200 DOI 10.1007/s00425-014-2041-2.

Chen CJ, Chen H, Zhang Y, Thomas HR, Frank MH, He YH, Xia R. 2020. TBtools: an integrative toolkit developed for interactive analyses of big biological data. *Molecular Plant* 13(8):1194-1202 DOI 10.1016/j.molp.2020.06.009.

Chen F, Hu Y, Vannozzi A, Wu KC, Cai HY, Qin Y, Mullis A, Lin ZG, Zhang LS. 2017. The WRKY transcription factor family in model plants and crops. *Critical Reviews in Plant Sciences* 36:311-335 DOI 10.1080/07352689.2018.1441103.

Chen H, Wang Y, Liu J, Zhao T, Yang C, Ding Q, Zhang Y, Mu J, Wang D. 2021. Identification of WRKY transcription factors responding to abiotic stresses in *Brassica napus* L. *Planta* 255(1):3 DOI 10.1007/s00425-021-03733-x.

Cheng LB, Li SY, Yin JJ, Li LJ, Chen XH. 2013. Genome-wide analysis of differentially expressed genes relevant to rhizome formation in lotus root (*Nelumbo nucifera* Gaertn). *PLoS One* 8 DOI e6711610.1371/journal.pone.0067116.

Chi Y, Yang Y, Zhou Y, Zhou J, Fan B, Yu JQ, Chen Z. 2013. Protein-protein interactions in the regulation of WRKY transcription factors. *Molecular Plant* 6:287-300 DOI 10.1093/mp/sst026.

Cui D, Zhang P, Ma YH, He X, Li YY, Zhang J, Zhao YC, Zhang ZY. 2014. Effect of cerium oxide nanoparticles on asparagus lettuce cultured in an agar medium. *Environmental Science* 7(3):177-179. DOI 10.1039/c4en00025k.

Dong J, Chen C, Chen Z. 2003. Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Molecular Biology* 51:21-37 DOI 10.1023/a:1020780022549.

Ding ZJ, Yan JY, Li CX, Li GX, Wu YR, Zheng SJ. 2015. Transcription factor WRKY46 modulates the development of Arabidopsis lateral roots in osmotic/salt stress conditions via regulation of ABA signaling and auxin homeostasis. *Plant Journal* 84:56-69 DOI 10.1111/tpj.12958.

El-Esawi MA, Al-Ghamdi AA, Ali HM, Ahmad, M. 2019. Overexpression of *AtWRKY30* transcription factor enhances heat and drought stress tolerance in wheat (*Triticum aestivum* L.). *Genes* 10(2):163 DOI 10.3390/genes10020163.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE. 2000. The WRKY superfamily of plant transcription factors. *Trends in Plant Science* 5:199-206 DOI 10.1016/S1360-1385(00)01600-9.

Fan Q SA, Jiang J, Zhang T, Sun H, Wang Y, Chen S, Chen F. 2016. CmWRKY1 enhances the dehydration tolerance of chrysanthemum through the regulation of ABA-associated genes. *PLoS One*:11: e0150572 DOI 10.1371/journal.pone.0150572.

Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, Lin JY, Minguez P, Bork P, Mering C, Jensen LJ. 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Research* 41:808-815 DOI 10.1093/nar/gks1094.

Garcia MNM, Cortelezzi JI, Fumagalli M, Capiati DA. 2018. Expression of the Arabidopsis ABF4 gene in potato increases tuber yield, improves tuber quality and enhances salt and drought tolerance. *Plant Molecular Biology* 98:137-152 DOI 10.1007/s11103-018-0769-y.

Grunewald W, De Smet I, De Rybel B, Robert HS, van de Cotte B, Willemsen V, Gheysen G, Weijers D, Friml J, Beeckman T. 2013. Tightly controlled WRKY23 expression mediates Arabidopsis embryo development. *EMBO Reports* 14:1136-1142 DOI 10.1038/embor.2013.169.

Guo H, Zhang Y, Wang Z, Lin L, Cui M, Long Y, Xing Z. 2019. Genome-wide identification of WRKY transcription factors in the Asteranae. *Plants (Basel)* 8 DOI 10.3390/plants8100393.

Horak H. 2020. Defense, Fast and Slow: Activation of different MAPK pathways in response to wounding. *Plant Cell* 32:1788-1789 DOI 10.1105/tpc.20.00282.

Hu Y, Chen L, Wang H, Zhang L, Wang F, Yu D. 2013. Arabidopsis transcription factor WRKY8 functions antagonistically with its interacting partner VQ9 to modulate salinity stress tolerance. *Plant Journal* 74:730-745 DOI 10.1111/tpj.12159.

Hussain A, Khan MI, Albaqami M, Mahpara S, Noorka IR, Ahmed MAA, Aljuaid BS, El-Shehawi AM, Liu Z, Farooq S, Zuan ATK. 2021. CaWRKY30 positively regulates pepper immunity by targeting CaWRKY40 against *Ralstonia solanacearum* inoculation through modulating defense-related genes. *International Journal Molecular Science* 22(21):12091 DOI 10.3390/ijms222112091.

Ishiguro S, Nakamura K. 1994. Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5′ upstream regions of genes coding for sporamin and β-amylase from sweet potato. *Molecular General Genetics* 244:563-571 DOI 10.1007/BF00282746.

Jiang Y, Deyholos MK. 2006. Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. *BMC Plant Biology* 6:25 DOI 10.1186/1471-2229-6-25.

Jiang Y, Liang G, Yu D. 2012. Activated expression of WRKY57 confers drought tolerance in Arabidopsis. *Molecular Plant* 5(6):1375-88 DOI 10.1093/mp/sss080

Johnson CS, Kolevski B, Smyth DR. 2002. TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. *Plant Cell* 14:1359-1375 DOI 10.1105/tpc.001404.

Kalde M, Barth M, Somssich IE, Lippok B. 2003. Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. *Molecular Plant Microbe Intercation* 16(4):295-305 DOI 10.1094/MPMI.2003.16.4.295.

572 Kim KC, Lai Z, Fan B, Chen Z. 2008. Arabidopsis WRKY38 and WRKY62 transcription factors interact with
573 histone deacetylase 19 in basal defense. *Plant Cell* 20:2357-2371 DOI 10.1105/tpc.107.055566.
574 Kim SH, Hamada T, Otani M, Shimada T. 2005. Isolation and characterization of MADS box genes possibly related
575 to root development in sweetpotato (*Ipomoea batatas* L. Lam.). *Journal of Plant Biology* 48:387-393 DOI
576 10.1007/Bf03030580.
577 Kim SH, Mizuno K, Fujimura T. 2002. Isolation of MADS-box genes from sweet potato (*Ipomoea batatas* (L.) lam.)
578 expressed specifically in vegetative tissues. *Plant and Cell Physiology* 43:314-322 DOI 10.1093/Pcp/Pcf043.
579 Kouzai Y, Shimizu M, Inoue K, Uehara-Yamaguchi Y, Takahagi K, Nakayama R, Matsuura T, Mori IC, Hirayama
580 T, Abdelsalam S. 2020. BdWRKY38 is required for the incompatible interaction of *Brachypodium distachyon*
581 with the necrotrophic fungus *Rhizoctonia solani*. Plant Journal 104(4):995-1008 DOI 10.1111/tpj.14976.
582 Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. 2009. Circos: an
583 information aesthetic for comparative genomics. *Genome Research* 19:1639-1645 DOI 10.1101/gr.092759.109.
584 Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA
585 and protein sequences. *Briefings in Bioinformatics* 9:299-306 DOI 10.1093/bib/bbn017.
586 Lai Z, Li Y, Wang F, Cheng Y, Fan B, Yu JQ, Chen Z. 2011. Arabidopsis sigma factor binding proteins are
587 activators of the WRKY33 transcription factor in plant defense. *Plant Cell* 23:3824-3841 DOI
588 10.1105/tpc.111.090571.
589 Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S. 2002. PlantCARE, a
590 database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter
591 sequences. *Nucleic Acids Research* 30:325-327 DOI 10.1093/nar/30.1.325.
592 Li M, He Q, Zhang Y, Sun B, Luo Y, Zhang Y, Chen Q, Wang Y, Zhang F, Zhang Y, Lin YX, Wang X, Tang H.
593 2020a. New insights into the evolution of the SBP-box family and expression analysis of genes in the growth
594 and development of *Brassica juncea*. *Biotechnology & Biotechnological Equipment* 34:810-824.
595 Li MY, Xu ZS, Tian C, Huang Y, Wang F, Xiong AS. 2016. Genomic identification of WRKY transcription factors
596 in carrot (*Daucus carota*) and analysis of evolution and homologous groups for plants. *Scientific Reports*
597 6:23101 DOI 10.1038/srep23101.
598 Li S, Zhou X, Chen L, Huang W, Yu D. 2010. Functional characterization of *Arabidopsis thaliana* WRKY39 in heat
599 stress. *Molecular Cells* 29:475-483. DOI 10.1007/s10059-010-0059-2.
600 Li W, Pang S, Lu Z, Jin B. 2020b. Function and mechanism of WRKY transcription factors in abiotic stress
601 responses of plants. *Plants (Basel)* 9 DOI 10.3390/plants9111515.
602 Lim C, Kang K, Shim Y, Yoo SC, Paek NC. 2022. Inactivating transcription factor OsWRKY5 enhances drought
603 tolerance through abscisic acid signaling pathways. *Plant Physiology* 188(4):1900-1916 DOI
604 10.1093/plphys/kiab492.
605 Liu HH, Wang LP, Jing XJ, Chen Y, Hu FR. 2021a. Functional analysis of *CgWRKY57* from *Cymbidium goeringii*
606 in ABA response. *Peerj* 9 DOI e1098210.7717/peerj.10982.
607 Liu J, Wang XY, Chen YL, Liu Y, Wu YS, Ren SS, Li L. 2021b. Identification, evolution and expression analysis
608 of WRKY gene family in *Eucommia ulmoides*. *Genomics* 113:3294-3309 DOI 10.1016/j.ygeno.2021.05.011.
609 Luo M, Dennis ES, Berger F, Peacock WJ, and Chaudhury A. 2005. MINISEED3 (MINI3), a WRKY family gene,
610 and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis.
611 *Proceedlings Natlional Academy Sciences* 102:17531-17536 DOI 10.1073/pnas.0508418102.
612 Ma J, Gao X, Liu Q, Shao Y, Zhang D, Jiang L, Li C. 2017. Overexpression of TaWRKY146 increases drought

tolerance through inducing stomatal closure in *Arabidopsis thaliana*. *Frontier Plant Science* 8:2036 DOI 10.3389/fpls.2017.02036.

Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S. 2011. Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *Plant Cell* 23:1639-1653 DOI 10.1105/tpc.111.084996.

Naoki Y, Yuko S, Shigeru T, Tetsuya C, Takafumi S, Kazunori O, Hisakazu Y, Masaki S, Shoji S, Hiroshi T. 2013. WRKY76 is a rice transcriptional repressor playing opposite roles in blast disease resistance and cold stress tolerance. *Journal of Experimental Botany* 64(16):5085-97 DOI 10.1093/jxb/ert298.

Negi N, Khurana P. 2021. A salicylic acid inducible mulberry WRKY transcription factor, Mi WRKY53 is involved in plant defence response. *Plant Cell Reports* 40(11):2151-2171 DOI 10.1007/s00299-021-02710-8.

Pernisova M, Kuderova A, Hejatko J. 2011. Cytokinin and auxin interactions in plant development: metabolism, signalling, transport and gene expression. *Current Protein Peptide Science* 12:137-147. DOI 10.2174/138920311795684887.

Qu RJ, Cao YW, Tang XQ, Sun LQ, Wei L, Wang KC. 2021. Identification and expression analysis of the WRKY gene family in *Isatis indigotica*. *Gene* 783 DOI 14556110.1016/J.Gene.2021.145561.

Rengasamy R, Jiang SY, Nadimuthu K, Nori VP, Srinivasan R. 2008. A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments. *Plant & Cell Physiology* 865-879 DOI 10.1093/pcp/pcn061.

Reyes-Chin-Wo S, Wang Z, Yang X, Kozik A, Arikit S, Song C, Xia L, Froenicke L, Lavelle DO, Truco MJ, Xia R, Zhu S, Xu C, Xu H, Xu X, Cox K, Korf I, Meyers BC, Michelmore RW. 2017. Genome assembly with in vitro proximity ligation data and whole-genome triplication in lettuce. *Nature Communications* 8:14953 DOI 10.1038/ncomms14953.

Robatzek S, Somssich IE. 2002. Targets of AtWRKY6 regulation during plant senescence and pathogen defense. *Genes & Development* 16:1139-1149 DOI 10.1101/gad.222702.

Rose AB. 2004. The effect of intron location on intron-mediated enhancement of gene expression in Arabidopsis. *Plant Journal* 40:744-751 DOI 10.1111/j.1365-313X.2004.02247.x.

Scarpeci TE, Zanor MI, Mueller-Roeber B, Valle EM. 2013. Overexpression of AtWRKY30 enhances abiotic stress tolerance during early growth stages in *Arabidopsis thaliana*. *Plant Molecular Biology* 83:265-277 DOI 10.1007/s11103-013-0090-8.

Song YS, Li JL, Sui Y, Han GL, Zhang Y, Guo SJ, Sui N. 2020. The sweet sorghum SbWRKY50 is negatively involved in salt response by regulating ion homeostasis. *Plant Molecular Biology* 102:603-614 DOI 10.1007/s11103-020-00966-4.

Sun C, Palmqvist S, Olsson H, Boren M, Ahlandsberg S, Jansson C. 2003. A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. *Plant Cell* 15:2076-2092 DOI 10.1105/tpc.014597.

Verdaguer R, Soler M, Serra O, Garrote A, Fernandez S, Company-Arumi D, Antico E, Molinas M, Figueras M. 2016. Silencing of the potato *StNAC103* gene enhances the accumulation of suberin polyester and associated wax in tuber skin. *Journal of Experimental Botany* 67:5415-5427 DOI 10.1093/jxb/erw305.

Wang H, Avci U, Nakashima J, Hahn MG, Chen F, Dixon RA. 2010. Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants. *Proceedlings Natlional Academy Sciences* 107:22338-22343 DOI 10.1073/pnas.1016436107.

Wang Z, Zhu Y, Wang L, Liu X, Liu Y, Phillips J, Deng X. 2009. A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (BhGolS1) promoter. *Planta* 230:1155-1166 DOI 10.1007/s00425-009-1014-3.

Wei ZP, Ye JF, Zhou ZQ, Chen G, Meng FJ, Liu YF. 2021. Isolation and characterization of PoWRKY, an abiotic stress-related WRKY transcription factor from *Polygonatum odoratum*. *Physiology and Molecular Biology of Plants* 27:1-9 DOI 10.1007/s12298-020-00924-w.

Wu J, Chen JB, Wang LF, Wang SM. 2016. Genome-wide investigation of WRKY transcription factors involved in terminal drought stress response in common bean. *Frontiers in Plant Science* 8 DOI 38010.3389/Fpls.2017.00380.

Xie T, Chen C, Li C, Liu J, Liu C, He Y. 2018. Genome-wide investigation of WRKY gene family in pineapple: evolution and expression profiles during development and stress. *BMC Genomics* 19:490 DOI 10.1186/s12864-018-4880-x.

Xu Z, Sun L, Zhou Y, Yang W, Cheng T, Wang J, Zhang Q. 2015. Identification and expression analysis of the SQUAMOSA promoter-binding protein (SBP)-box gene family in *Prunus mume*. *Molecular Genetics Genomics* 290:1701-1715 DOI 10.1007/s00438-015-1029-3.

Yu Y C, Liu Z H, Wang L, Kim S G, Seo P J, Qiao M, Wang N, Li S, Cao X F, Park C M, Xiang F N. 2016. WRKY71 accelerates flowering via the direct activation of FLOWERING LOCUS T and LEAFY in *Arabidopsis thaliana*. *Plant Journal* 85(1):96-106 DOI 10.1111/tpj.13092.

Yang Y, Liu J, Zhou XH, Liu SY, Zhuang Y. 2020. Identification of WRKY gene family and characterization of cold stress-responsive WRKY genes in eggplant. *PeerJ* 8 DOI E877710.7717/Peerj.8777.

Yang DJ, Liu YY, Cheng HL, Wang QL, Lv LM, Zhang YP, Song GL, Zuo DY. 2021. Identifification of the group III WRKY subfamily and the functional analysis of GhWRKY53 in *Gossypium hirsutum* L. *Plants* 10:1235 DOI 10.3390/plants10061235.

Yao DM, Zou C, Shu YN, Liu SS. 2020. WRKY transcription factors in *Nicotiana tabacum* modulate plant immunity against whitefly via interacting with MAPK cascade pathways. *Insects* 12 DOI 10.3390/insects12010016.

Yin X, Cui Y, Wang M, Xia X. 2017. Overexpression of a novel MYB-related transcription factor, OsMYBR1, confers improved drought tolerance and decreased ABA sensitivity in rice. *Biochemical and Biophysical Research Communications* 490:1355-1361 DOI 10.1016/j.bbrc.2017.07.029.

Yu FF, Huaxia YF, Lu WJ, Wu CG, Cao XC, Guo XQ. 2012. GhWRKY15, a member of the WRKY transcription factor family identifi *Fan* ed from cotton (*Gossypium hirsutum* L.), is involved in disease resistance and plant development. *BMC Plant Biology* 12 DOI 14410.1186/1471-2229-12-144.

Zentgraf U, Laun T, Miao Y. 2010. The complex regulation of WRKY53 during leaf senescence of *Arabidopsis thaliana*. *European Journal Cell Biology* 89:133-137 DOI 10.1016/j.ejcb.2009.10.014.

Zhang CQ, Xu Y, Lu Y, Yu HX, Gu MH, Liu QQ. 2011. The WRKY transcription factor OsWRKY78 regulates stem elongation and seed development in rice. *Planta* 234:541-554 10.1007/s00425-011-1423-y.

Zhang T, Tan D, Zhang L, Zhang X, Han Z. 2017. Phylogenetic analysis and drought-responsive expression profiles of the WRKY transcription factor family in maize. *Agri Gene* 3:99-108 DOI 10.1016/j.aggene.2017.01.001.

Zhou X, Jiang Y, Yu D. 2011. WRKY22 transcription factor mediates dark-induced leaf senescence in Arabidopsis. *Moelularl Cells* 31:303-313 DOI 10.1007/s10059-011-0047-1.

Zou C, Jiang W, Yu D. 2010. Male gametophyte-specific WRKY34 transcription factor mediates cold sensitivity of

695 mature pollen in Arabidopsis. *Journal of Experimental Botany* 61:3901-3914 DOI 10.1093/jxb/erq204.

696 **Figure Legends**

697 **Fig 1:** The cross-sections of the asparagus lettuce stem development stages. S1: the transverse
698 diameter is 1 cm, S2: the transverse diameter is 2 cm, S3: the transverse diameter length is 3 cm,
699 S4: the transverse diameter length is 4 cm. Scale bars: 0.5 cm (S1); 1 cm (S2, S3, S4).

700

701 **Fig. 2:** Phylogenetic analysis of WRKY TFs among lettuce, Arabidopsis, and tomato plants.
702 MEGA 7.0 software was used to conduct the phylogenetic analysis using a neighbor-joining
703 method with 1000 bootstrap replicates. Different colors represent different subfamily WRKY
704 TFs.

705

706 **Fig. 3:** Phylogenetic relationship, exon-intron structure, and conserved motifs analysis of WRKY
707 TFs in lettuce. (a) The phylogenetic tree created by MEGA 7.0 and conserved motifs predicted in
708 WRKY protein. The MEME program identified the ten motifs, with each number of the colored
709 box representing a different motif. (b) Exon-intron structures from online software GSDS.
710 Yellow boxes and lines represent exons and introns, respectively; blue boxes represent the UTR.
711

712 **Fig. 4:** *Cis*-element analysis of LsWRKY genes in lettuce. Boxes with different colors represent
713 different cis-element identified by the PlantCARE program, with each number of the colored box
714 representing a different motif.

715

716 **Fig. 5:** Chromosomal distribution of LsWRKY TFs in the lettuce chromosomes. Red represented
717 the distribution of WRKYIII subfamily TFs in the lettuce chromosomes.

718

719 **Fig. 6:** Comparative analysis of synteny between *Lactuca sativa* and *Lactuca saligna* (least
720 lettuce, willow-leaf lettuce). Red line represented the identified the identified gene pair.

721

722 **Fig. 7:** An interaction network analysis of LsWRKY TFs. Edges represents protein-protein
723 associations. Different colored horizontal lines represent different interactions (known
724 interactions, Predicted Interactions, and others).
725

726 **Fig. 8:** Relative expression of LsWRKYIII genes under different abiotic stresses at 12 h (A) and
727 24 h (B). Bars with different lowercase letters were significantly different by Duncan's multiple
728 range tests (p=0.05).

729

Fig. 9: Relative expression of LsWRKYIII genes under hormone treatment at 12 h (A) and 24 h (B). Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels.

Fig. 10: Relative expression of LsWRKYIII genes at root, stem, and leaf. Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels.

Fig. 11: Expression profiles of LsWRKY genes by the transcriptome data analysis at different lettuce stem enlargement periods. S1: diameter length is 1 cm, S2: diameter length is 2 cm, S3: diameter length is 3 cm, S4: diameter length is 4 cm. a b c represents three biological replicates of each developmental stage. FPKM values of LsWRKY genes were transformed by $\log10^{(FPKM+1)}$, and the heatmap was constructed with TBtools software. The red box represents lettuce WRKYIII genes.

Fig. 12: Expression profiles of LsWRKYIII genes at different lettuce stem enlargement periods. Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels. S1: diameter length is 1 cm, S2: diameter length is 2 cm, S3: diameter length is 3 cm, S4: diameter length is 4 cm.

Supplementary Files

Supplementary File 1

Fig. S1: Alignment of the amino acid sequence of LsWRKY TFs. The red box represented the conservative domain WRKYGQK of WRKY TFs; amino acids of different colors indicated different degrees of similarity.

Supplementary File 2

Fig. S2: WRKY family TFs members among different plant species. Different colored boxes represented different WRKY subfamily TFs.

Supplementary File 3

Fig. S3: Phylogenetic analysis of WRKY TFs lettuce and Arabidopsis by MEGA7.0. Different colors represent different subfamily WRKY TFs.

Supplementary File 4

Table S1: Primer sequences used in the text.

Table S2: Characteristic features of LsWRKY TFs. The red box represented the newly identified

764 WRKY transcription factor.

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

766 **Table S4:** Proteins interacting with LsWRKY transcription factors.

767

768 **Table S5:** *Cis* elements analysis of LsWRKY genes in lettuce. The red represents lettuce
769 WRKYIII genes.

770

771 **Table S6:** Amino acids of 76 WRKY transcription factors in lettuce.

772

773 **Table S7:** Cq data of RT-qPCR experiment in analyzing responses of lettuce
774 LsWRKYIII genes to different abiotic stresses.

775

776 **Table S8:** Cq data of RT-qPCR experiment in analyzing responses of lettuce
777 LsWRKYIII genes to different hormone treatments.

778

779 **Table S9:** Cq data of LsWRKY TFs involved in different tissues by RT-qPCR analysis.

780

781 **Table S10:** Expression profiles of LsWRKY genes by the transcriptome data analysis at
782 different lettuce stem enlargement periods.

783

784 **Table S11:** Cq data of LsWRKY TF involved in different stem enlargement periods by RT-
785 qPCR analysis.

786

Figure 1

The cross-sections of the asparagus lettuce stem development stages.

S1: the transverse diameter is 1 cm, S2: the transverse diameter is 2 cm, S3: the transverse diameter length is 3 cm, S4: the transverse diameter length is 4 cm. Scale bars: 0.5 cm (S1); 1 cm (S2, S3, S4).

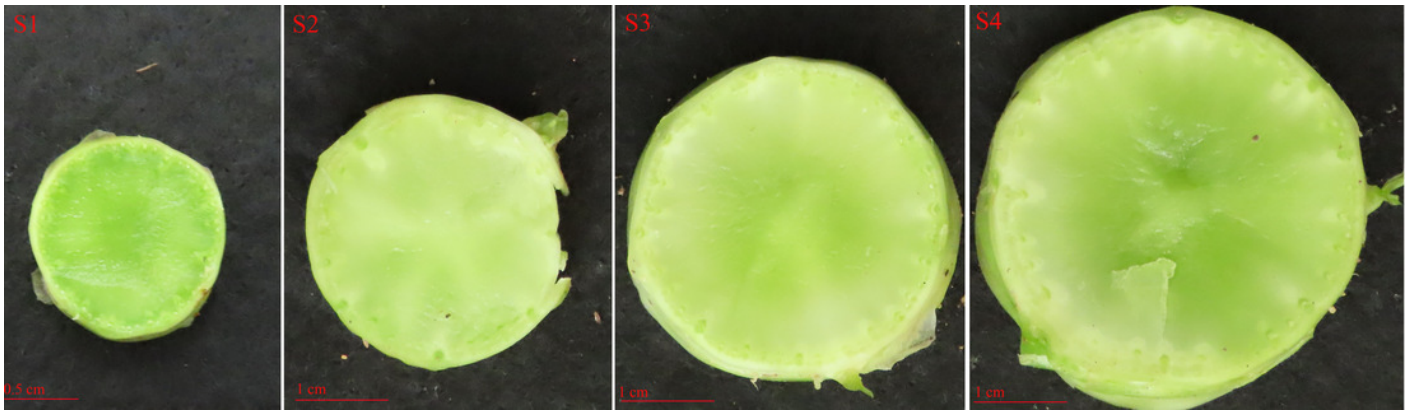

Figure 2

Phylogenetic analysis of WRKY TFs among lettuce, Arabidopsis, and tomato plants.

MEGA 7.0 software was used to conduct the phylogenetic analysis using a neighbor-joining method with 1000 bootstrap replicates. Different colors represent different subfamily WRKY TFs.

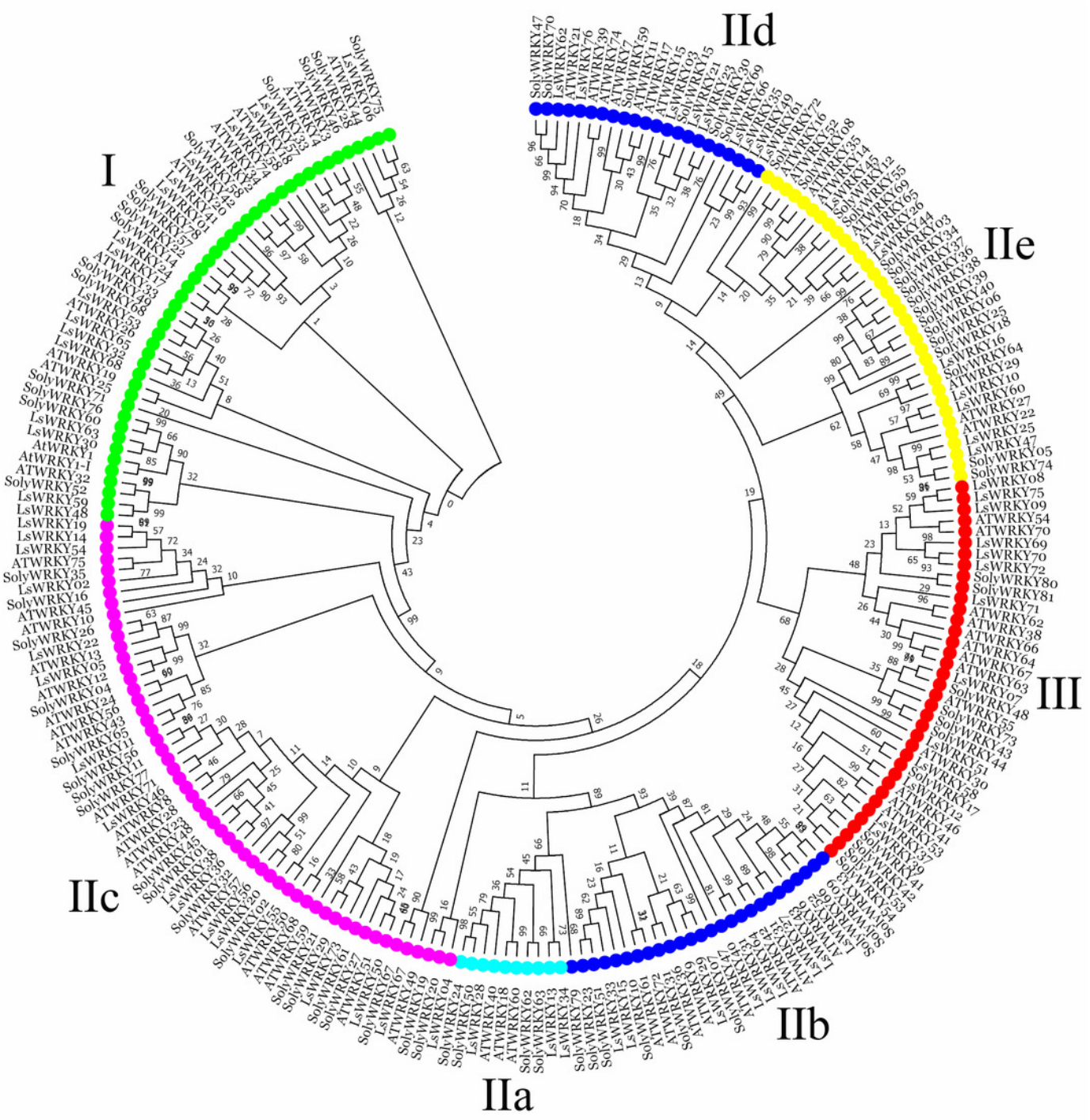
I
IIa
IIb
IIc
IId
IIe
III

Figure 3

Phylogenetic relationship, exon-intron structure, and conserved motifs analysis of WRKY TFs in lettuce.

(a) The phylogenetic tree created by MEGA 7.0 and conserved motifs predicted in WRKY protein. The MEME program identified the ten motifs, with each number of the colored box representing a different motif. (b) Exon-intron structures from online software GSDS. Yellow boxes and lines represent exons and introns, respectively; blue boxes represent the UTR.

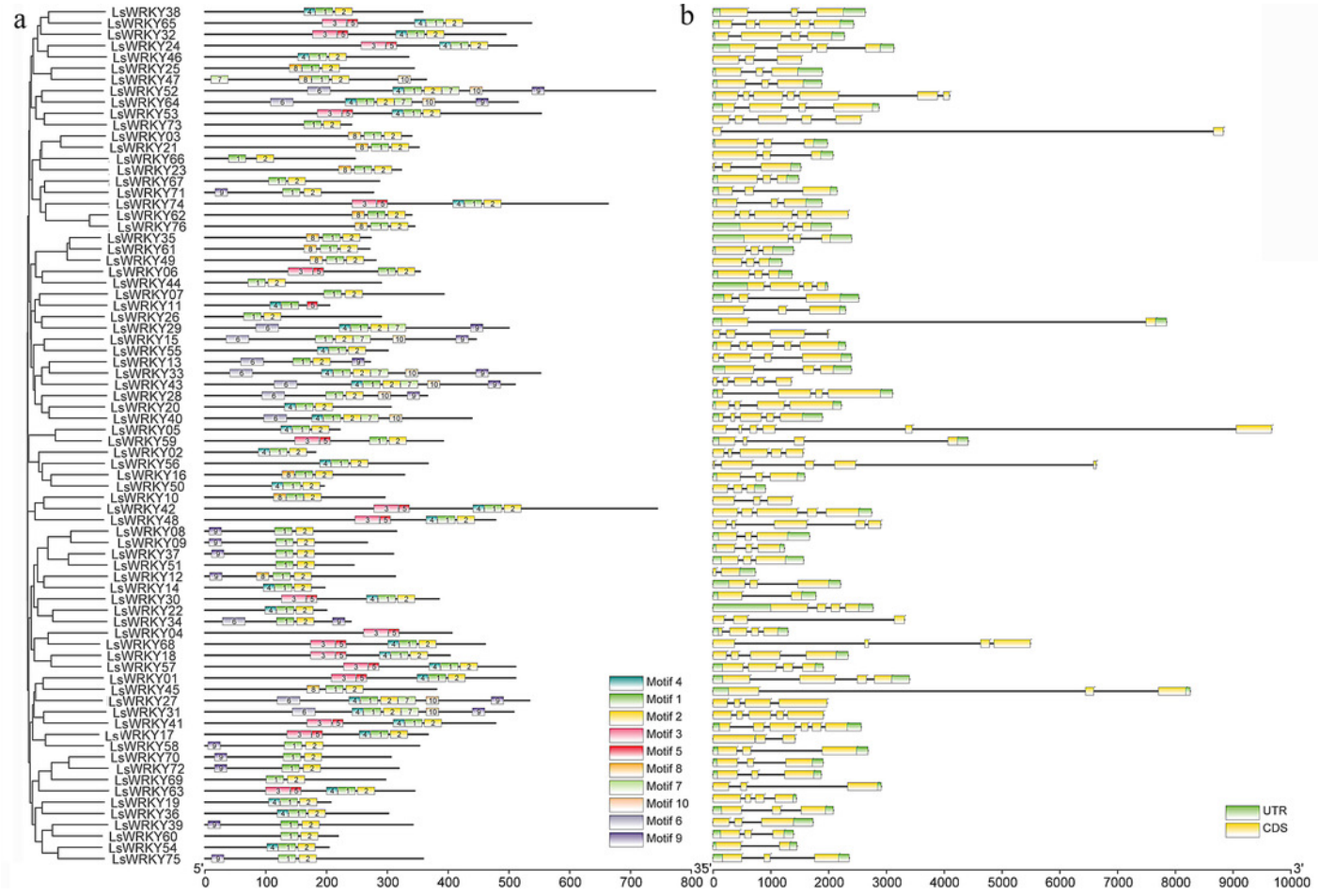

Figure 4

Cis-element analysis of LsWRKY genes in lettuce.

Boxes with different colors represent different cis-element identified by the PlantCARE program, with each number of the colored box representing a different motif.

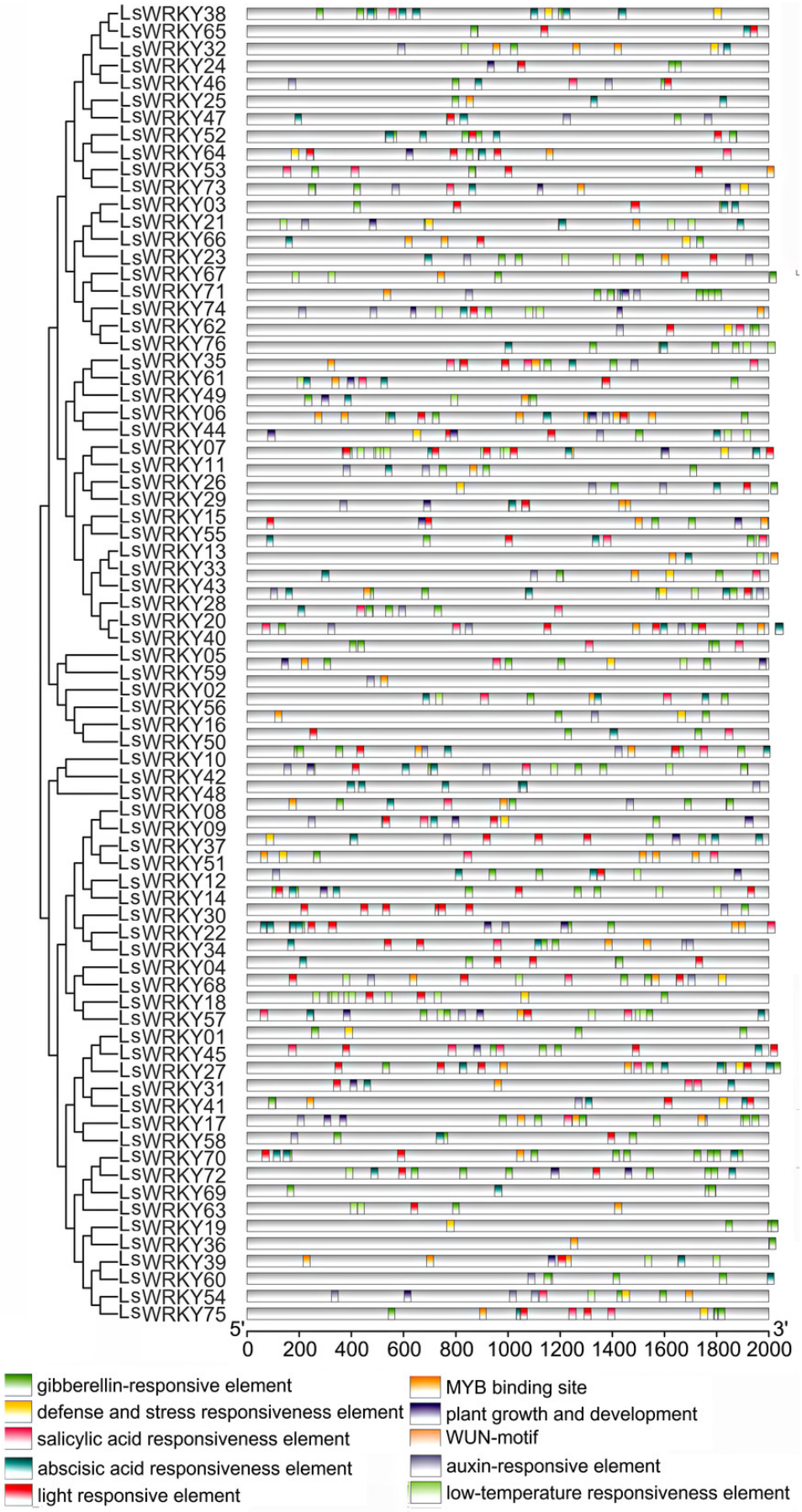
LsWRKY38
LsWRKY65
LsWRKY32
LsWRKY24
LsWRKY46
LsWRKY25
LsWRKY47
LsWRKY52
LsWRKY64
LsWRKY53
LsWRKY73
LsWRKY03
LsWRKY21
LsWRKY66
LsWRKY23
LsWRKY67
LsWRKY71
LsWRKY74
LsWRKY62
LsWRKY76
LsWRKY35
LsWRKY61
LsWRKY49
LsWRKY06
LsWRKY44
LsWRKY07
LsWRKY11
LsWRKY26
LsWRKY29
LsWRKY15
LsWRKY55
LsWRKY13
LsWRKY33
LsWRKY43
LsWRKY28
LsWRKY20
LsWRKY40
LsWRKY05
LsWRKY59
LsWRKY02
LsWRKY56
LsWRKY16
LsWRKY50
LsWRKY10
LsWRKY42
LsWRKY48
LsWRKY08
LsWRKY09
LsWRKY37
LsWRKY51
LsWRKY12
LsWRKY14
LsWRKY30
LsWRKY22
LsWRKY34
LsWRKY04
LsWRKY68
LsWRKY18
LsWRKY57
LsWRKY01
LsWRKY45
LsWRKY27
LsWRKY31
LsWRKY41
LsWRKY17
LsWRKY58
LsWRKY70
LsWRKY72
LsWRKY69
LsWRKY63
LsWRKY19
LsWRKY36
LsWRKY39
LsWRKY60
LsWRKY54
LsWRKY75
5'
3'
0 200 400 600 800 1000 1200 1400 1600 1800 2000
gibberellin-responsive element
defense and stress responsiveness element
salicylic acid responsiveness element
abscisic acid responsiveness element
light responsive element
MYB binding site
plant growth and development
WUN-motif
auxin-responsive element
low-temperature responsiveness element

Figure 5

Chromosomal distribution of LsWRKY TFs in the lettuce chromosomes.

Red represented the distribution of WRKYIII subfamily TFs in the lettuce chromosomes.

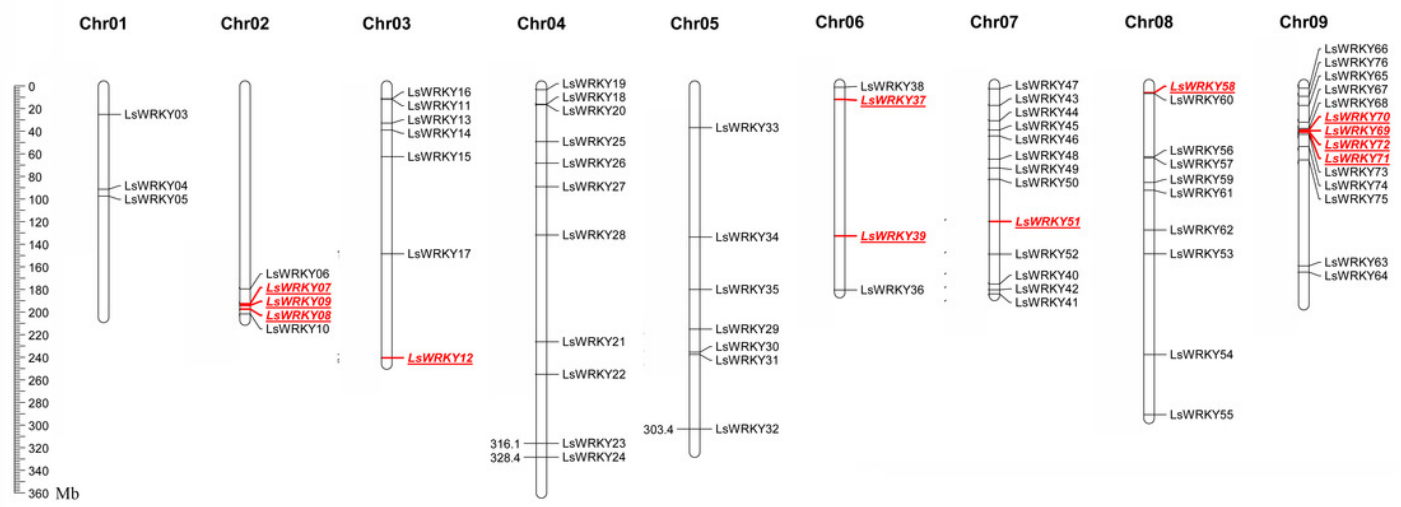

Figure 6

Comparative analysis of synteny between *Lactuca sativa* and *Lactuca saligna* (least lettuce, willow-leaf lettuce).

Red line represented the identified the identified gene pair.

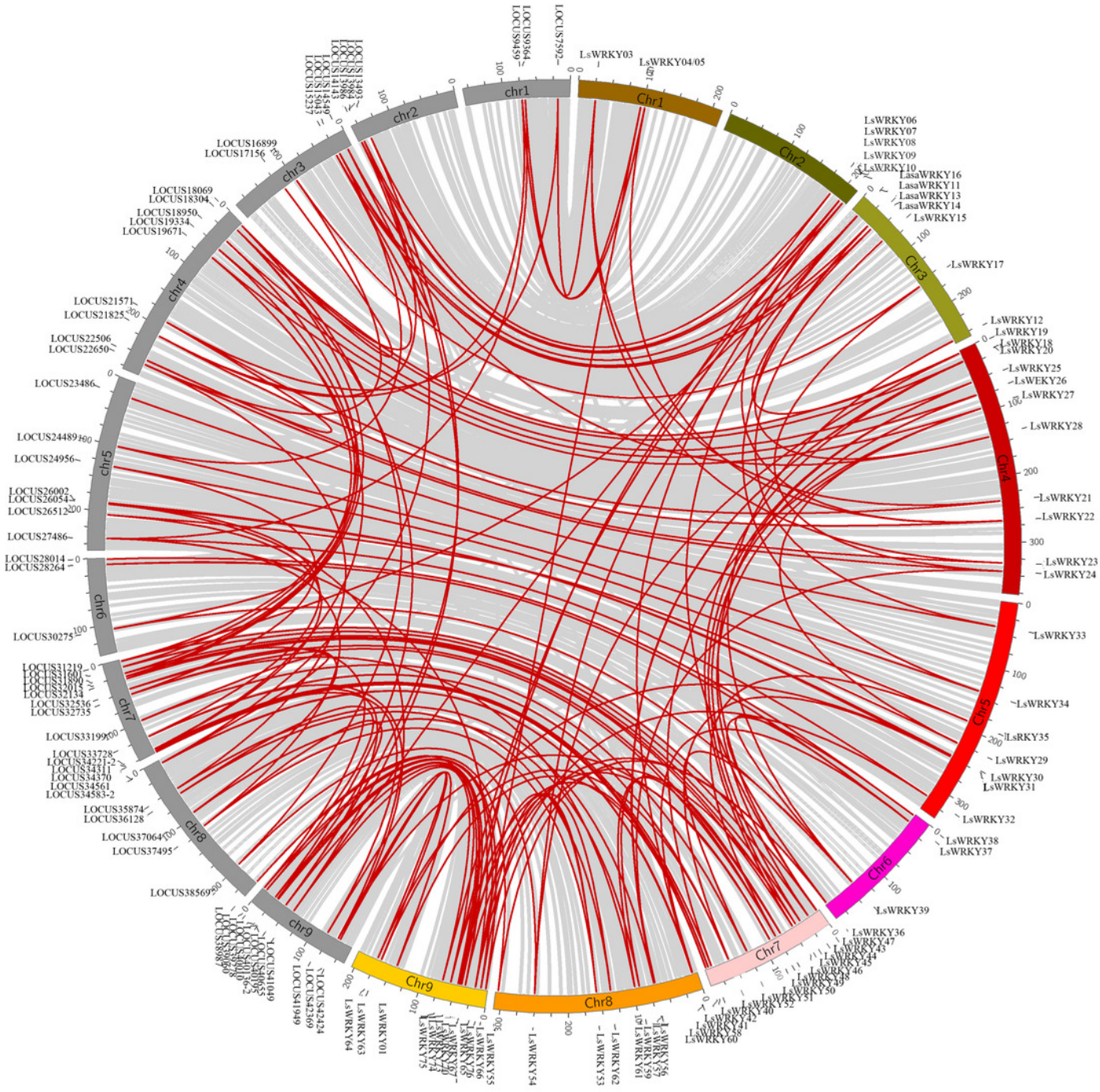

Figure 7

An interaction network analysis of LsWRKY TFs.

Edges represents protein-protein associations. Different colored horizontal lines represent different interactions (known interactions, Predicted Interactions, and others).

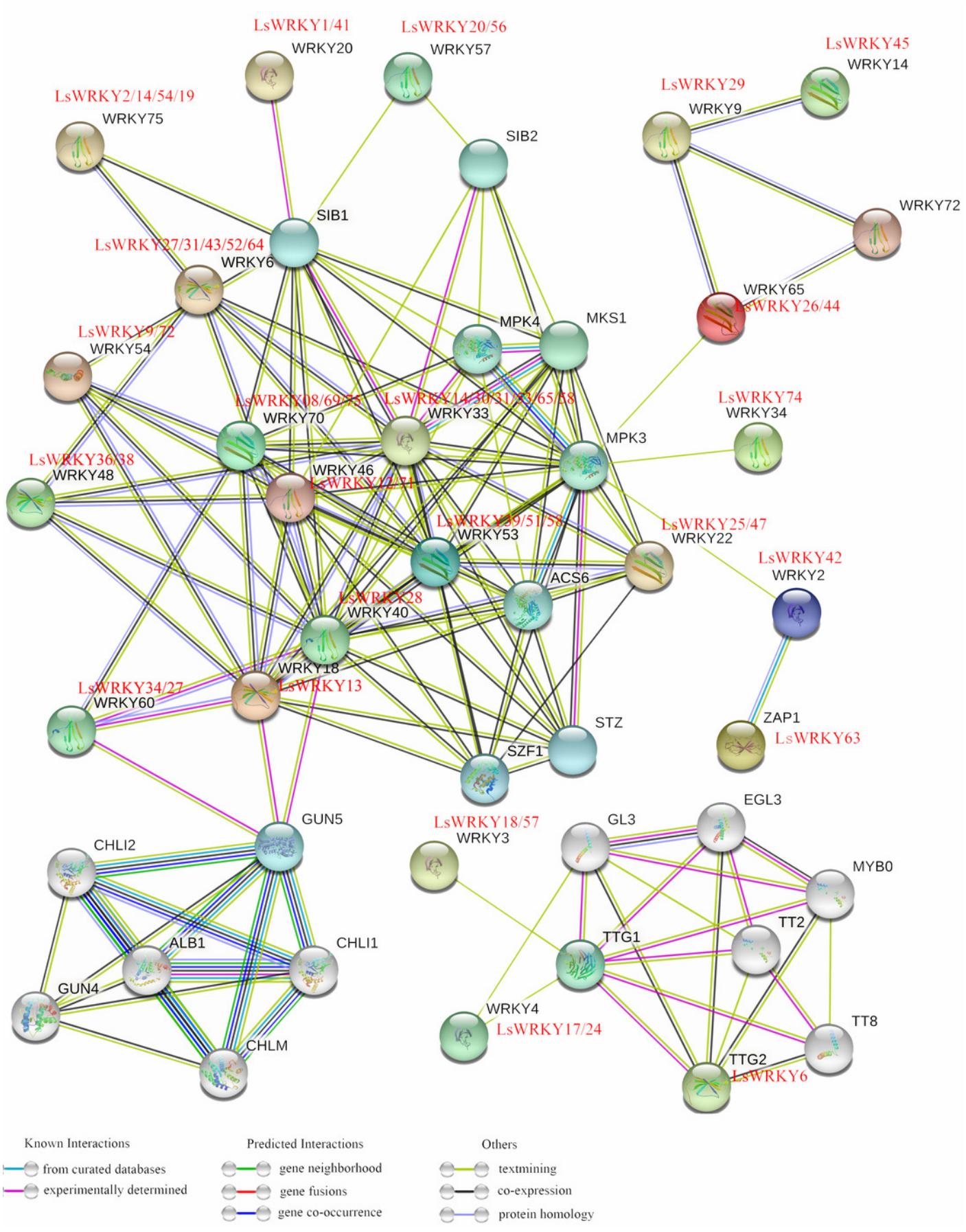
LsWRKY1/41
WRKY20
LsWRKY20/56
WRKY57
LsWRKY45
WRKY14
LsWRKY29
WRKY9
LsWRKY2/14/54/19
WRKY75
SIB2
WRKY72
SIB1
LsWRKY27/31/43/52/64
WRKY6
WRKY65
LsWRKY26/44
MPK4
MKS1
LsWRKY9/72
WRKY54
LsWRKY74
WRKY34
LsWRKY08/69/75
WRKY70
LsWRKY14/30/31/53/65/68
WRKY33
MPK3
LsWRKY36/38
WRKY48
WRKY46
LsWRKY12/71
LsWRKY39/51/58
WRKY53
LsWRKY25/47
WRKY22
LsWRKY42
WRKY2
ACS6
LsWRKY28
WRKY40
WRKY18
LsWRKY13
LsWRKY34/27
WRKY60
STZ
ZAP1
LsWRKY63
SZF1
EGL3
GUN5
LsWRKY18/57
WRKY3
GL3
MYB0
CHLI2
TT2
TTG1
ALB1
CHLI1
GUN4
WRKY4
LsWRKY17/24
TT8
CHLM
TTG2
LsWRKY6
Known Interactions
from curated databases
experimentally determined
Predicted Interactions
gene neighborhood
gene fusions
gene co-occurrence
Others
textmining
co-expression
protein homology

Figure 8

Relative expression of LsWRKYIII genes under different abiotic stresses.

Bars with different lowercase letters were significantly different by Duncan's multiple range tests (p=0.05).

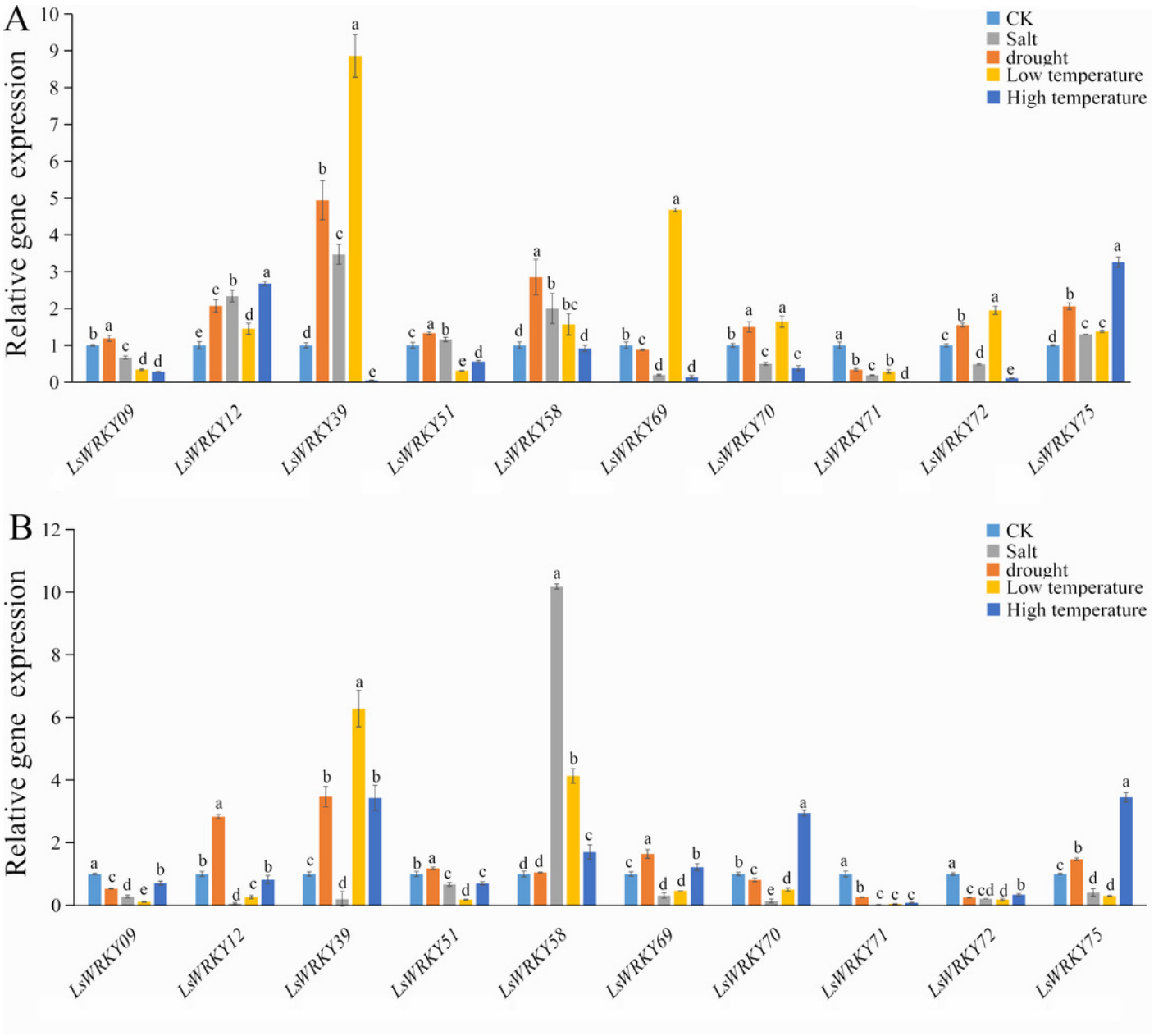

Figure 9

Relative expression of LsWRKYIII genes under hormone treatment.

Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels.

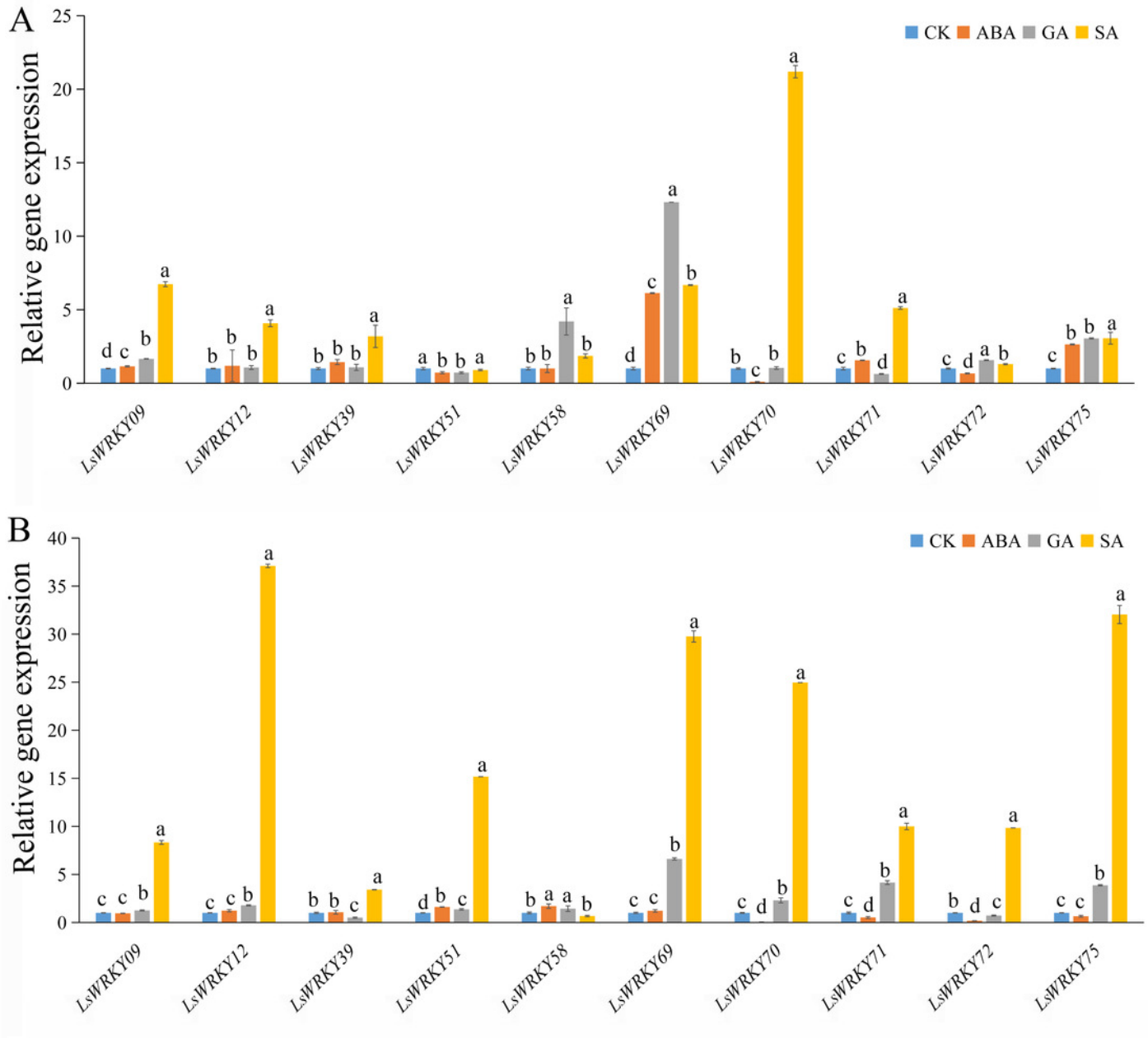

Figure 10

Relative expression of LsWRKYIII genes at root, stem, and leaf.

Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels.

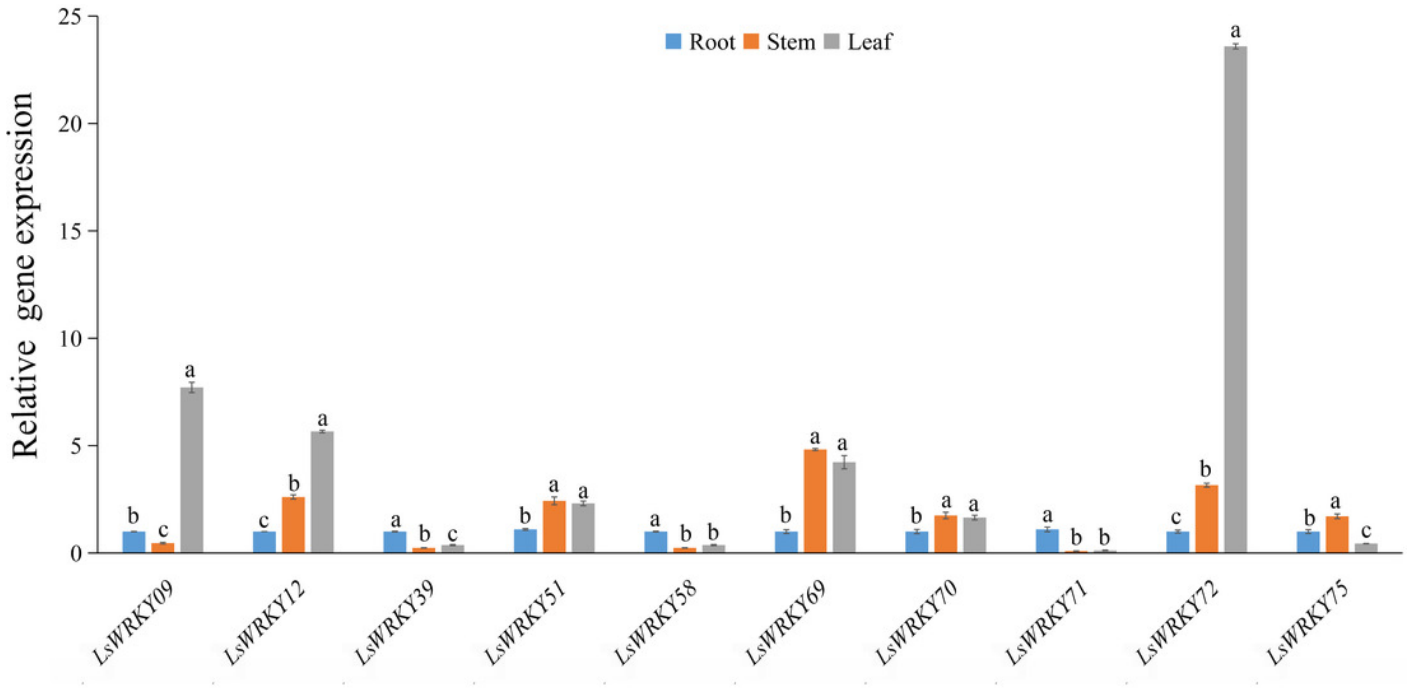

Figure 11

Expression profiles of LsWRKY genes by the transcriptome data analysis at different lettuce stem enlargement periods.

S1: diameter length is 1 cm, S2: diameter length is 2 cm, S3: diameter length is 3 cm, S4: diameter length is 4 cm. a b c represents three biological replicates of each developmental stage. FPKM values of LsWRKY genes were transformed by $\log10^{(FPKM+1)}$, and the heatmap was constructed with TBtools software. The red box represents lettuce WRKYIII genes.

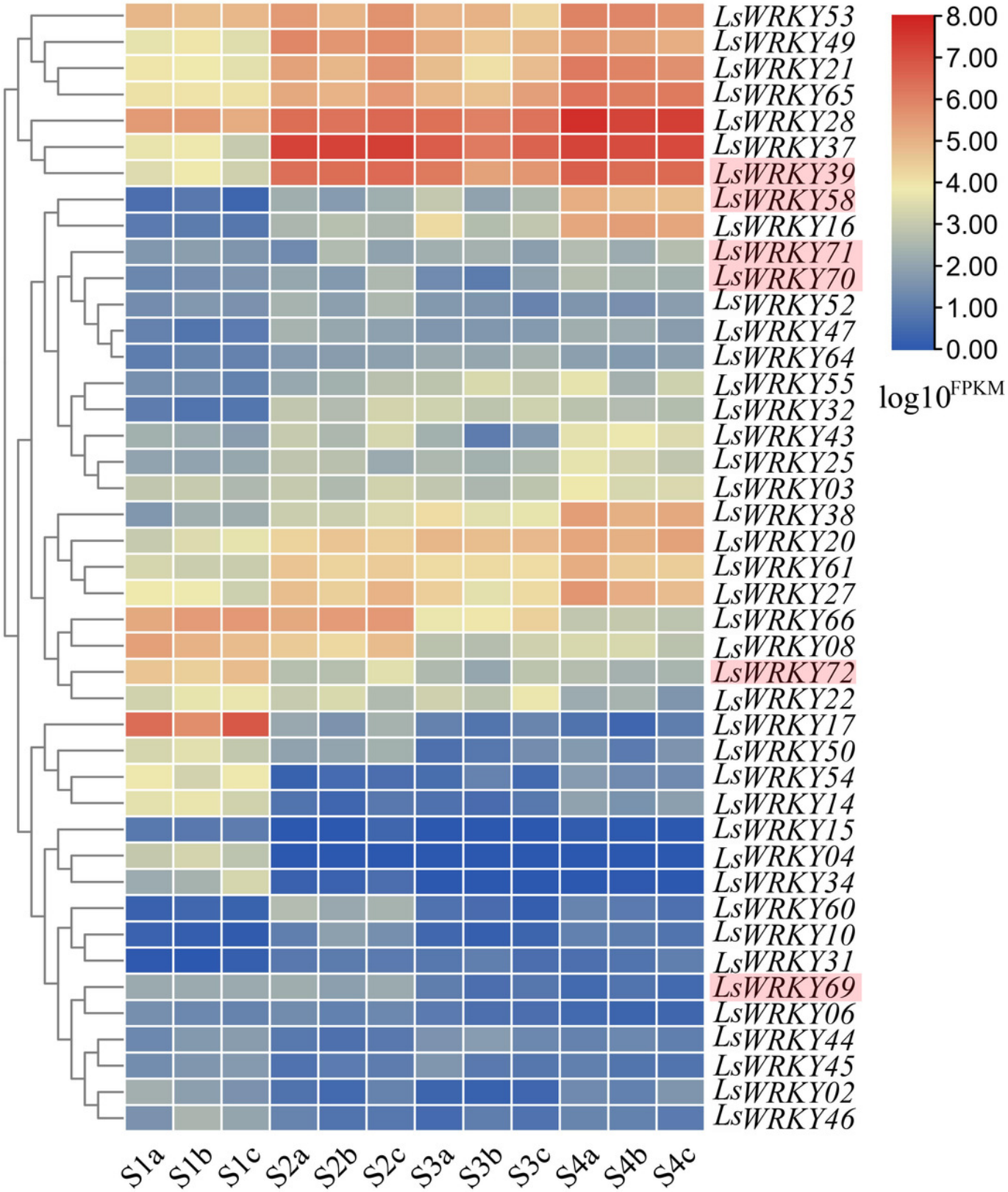
LsWRKY53
LsWRKY49
LsWRKY21
LsWRKY65
LsWRKY28
LsWRKY37
LsWRKY39
LsWRKY58
LsWRKY16
LsWRKY71
LsWRKY70
LsWRKY52
LsWRKY47
LsWRKY64
LsWRKY55
LsWRKY32
LsWRKY43
LsWRKY25
LsWRKY03
LsWRKY38
LsWRKY20
LsWRKY61
LsWRKY27
LsWRKY66
LsWRKY08
LsWRKY72
LsWRKY22
LsWRKY17
LsWRKY50
LsWRKY54
LsWRKY14
LsWRKY15
LsWRKY04
LsWRKY34
LsWRKY60
LsWRKY10
LsWRKY31
LsWRKY69
LsWRKY06
LsWRKY44
LsWRKY45
LsWRKY02
LsWRKY46
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
log10FPKM

Figure 12

Expression profiles of LsWRKYIII genes at different lettuce stem enlargement periods.

Bars with different lowercase letters were significantly different by Duncan's multiple range tests at 0.05 levels. S1: diameter length is 1 cm, S2: diameter length is 2 cm, S3: diameter length is 3 cm, S4: diameter length is 4 cm.