Identification and analysis of Verbena bonariensis WRKY transcription factor gene family members

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Abstract

Background: The *WRKY* transcription factor gene family is one of the unique gene families in plants, it plays an important role in response to abiotic stresses such as cold and drought, hormone signal transduction, regulation of biosynthesis, leaf senescence and seed germination etc. However, little information is available about WRKY transcription factors in Verbena bonariensis.

Results: In this study, 70 members of *VbWRKY* were identified by bioinformatics tool analysis and named *VbWRKY1-VbWRKY70*. The phylogenetic analysis of the *WRKY*gene family in Verbena bonariensis and Arabidopsis shows that the *WRKY* genes in Verbena bonariensis can be divided into three groups: I, II, and III, which contains 13, 47 and 10 members respectivelys. Group II can be further divided into five subclasses: IIa (5), IIb (10), IIc (18), IId (6), and IIe (8). Conservative motif analysis showed that 64 proteins encoded by *VbWRKY* gene had conserved motifs 1,2 and 3, and the same subclass motif elements were approximately the same. The collinearity analysis showed that there were 44 homologous gene pairs among the *VbWRKRs*, and these homologous gene pairs may have the same function. Promoter sequence analysis showed that the *VbWRKY* gene has multiple cis acting elements, including not only cis acting elements related to low temperature and light responses, but also cis acting elements related to hormone regulation, Among them, most *VbWRKY* genes contain response elements related to low temperature, 30 *VbWRKY* genes contain low temperature response elements (LTR) , and 61 *VbWRKY* genes contain abscisic acid response elements (ABRE), indicating that *VbWRKY*plays a crucial role in plant growth and abiotic stress. According to the expression of *VbWRKY* in the cold stress transcriptome, *52 VbWRKYs*showed significant differences in expression, and 37 *VbWRKY* genes were up-regulated under stress

Conclusions: Ultimately, the present study provides a comprehensive analysis of predicted Verbena bonariensis *WRKY* family genes, which provided a theoretical basis for the study of low temperature resistance and growth and development of Verbena bonariensis.

Background

The *WRKY* transcription factor gene family is a unique gene family in plants, and it is also one of the largest transcription factor families in plants[1], named after the highly conserved WRKY conserved domain. The WRKY conserved domain contains approximately 60 amino acids and consists of an N-terminal WRKYGQK heptapeptide domain and a C-terminal zinc finger structure [2]. According to the number of WRKY domains and the type of zinc finger structure, the *WRKY* family can be divided into three main groups: I, II and III. Group I contains two WRKY heptapeptide domains and one C2H2 zinc finger structure; group II contains one WRKY heptapeptide domain and one C2H2 zinc finger structure; group III contains a WRKY heptapeptide domain and a C2HC type zinc finger structure. The Group II can be subdivided into five subfamilies based on evolutionary relationships, namely IIa, IIb, IIc, IId, and Iie[3]. Systematic evolutionary data analysis shows that the WRKY transcription factor family can be more accurately divided into groups I, IIa+IIb, IIc, IId+IIe, and III in higher plants[4].

The first WRKY transcription factor was identified from sweet potatoes in 1994[5], and subsequently identified in potatoes[6], tobacco[7], wheat and barley[8], Arabidopsis[9-11], rice[12, 13], poplar[14], rapeseed[15], cucumber[16], cotton[17], etc. Numerous studies have shown that WRKY transcription factors have rich biological functions and are closely related to plant growth and development. In plants, WRKY transcription factors mediate defense regulatory functions, mainly responding to various biotic and abiotic stresses[18, 19]. At the same time, abiotic stress can induce a large number of WRKY transcription factors to regulate plant tolerance to stress and obtain corresponding resistance capabilities[20]. In addition, it also participates in regulating the physiological development process of plants, involving multiple aspects such as hormone signal transduction, biosynthesis regulation, leaf senescence, embryo formation, and seed germination[21, 22]. In Arabidopsis, *AtWRKY33*, *AtWRKY46*, and *AtWRKY57* can enhance the tolerance of Arabidopsis to drought and salt stress by regulating the ABA signaling network[23-25]. Chen et al. found that *SlWRKY12*, *SlWRKY13*, *SlWRKY23*, *SlWRKY50*, and *SlWRKY51* were significantly upregulated under cold stress, indicating that they may be involved in the response mechanism of tomato to low temperature stress[26]. Wang et al[27]. found in their study on Gossypium hirsutum that the *GhWRKY22* gene can participate in pollen development through transcriptional regulation. Wheat (Triticum aestivum) WRKY7 is an important regulatory factor for leaf senescence, with its expression continuously increasing during the process of natural leaf senescence[28]. *TaWRKY2* and *TaWRKY19* enhanced their tolerance to

drought, salt, and cold stress in transgenic Arabidopsis. Transgenic wheat overexpressing *TaWRKY2* and *TaWRKY19* has improved salt tolerance, drought resistance, and frost resistance[29]. Compared with the wild-type, *CsWRKY46* enhances cold resistance in cucumber by regulating a series of regulated cold response genes and overexpressing them[25]. *OsWRKY71* plays a positive role in cold resistance by regulating downstream target genes in rice[30]. Under cold stress, the induction of *VbWRKY32* in Verbena bonariensis leaves was greater than that in the stems and roots. Compared with the wild-type (WT), overexpression (OE) in Verbena bonariensis improved cold resistance[31].

Verbena bonariensis is a perennial herbaceous plant of the Verbena genus in the Verbenaceae family. It is native to Brazil, Argentina, and other regions in South America and is distributed in most parts of East, South, Northwest, and Southwest China. The optimum temperature for the growth of Verbena bonariensis is 20-30 °C. It enjoys light and has strong drought resistance. Seedlings can be obtained by sowing or cuttings. The flowering period is mostly in summer and autumn, and the peak flowering period can reach 3 months. It is an excellent ornamental variety for gardens. The Verbena bonariensis also has the functions of detoxification, detumescence and spasmolysis, mainly treating symptoms such as dysmenorrhea, vaginal infections, and traumatic swelling and pain. However, in the cultivation of Verbena bonariensis, the Verbena bonariensis is not tolerant to the cold and grows slowly when the temperature is below 10 ℃. The low temperature environment in winter seriously affects the yield and ornamental value of Verbena bonariensis[31]. Many studies have shown that when plants are subjected to cold stress, they can improve tolerance by regulating the expression of a series of genes. Many transcription factors (TFS), including WRKY, ERF, and MYB, are important regulators associated with cold stress[32, 33]. The transcriptomic data of Verbena bonariensis plants under low temperature stress indicate that WRKY-TFs play a crucial role in helping plants to cope with low temperature stress.

In this study, genome-wide identification of members of the Verbena bonariensis *WRKY* gene family was performed using genomic data measured by our research team, and analyzed their phylogeny, subgroup types, chromosome distribution, conserved motifs, gene structure, and cis acting elements. Additionally, transcriptome sequencing data and related physiological indicators under low temperature stress were used to systematically analyze the regulatory mechanism of the low temperature response of this gene family, and to provide a theoretical basis for the study of Verbena bonariensis growth and low temperature resistance.

Results

Identification and Physicochemical Property Analysis of *VbWRKYs*

Using the Hidden Markov model of the WRKY conserved domain in the Pfam database, HMMER3.0 was used to search for the genome protein sequence of Verbena bonariensis. Further conserved domain identification by NCBI-CDD and Pfam, finally, 70 members of the *VbWRKY* gene family were identified. Based on the arrangement order of these genes on chromosomes, they were renamed as *VbWRKY1*~*VbWRKY70* (Table 1). The results of physicochemical property analysis showed that the length of Verbena bonariensis WRKY protein varied from 98(*VbWRKY18*) to 1012(*VbWRKY47*) amino acids, and the number of amino acids varied significantly; The relative molecular weight ranges from 11299.6 (*VbWRKY18*) to 112752.07 (*VbWRKY47*) Da; The isoelectric point is between 4.54(*VbWRKY9*) and 9.84(*VbWRKY58*). The predicted subcellular localization showed that 70 WRKY members of Verbena bonariensis are located in the nucleus.

Chromosome Mapping of *VbWRKYs*

Using TBtools software, the *VbWRKY* gene was mapped on the chromosomes of Verbena bonariensis. The results showed that the 70 Verbena bonariensis *WRKY* genes were distributed unevenly on all seven chromosomes. Among them, Chr1 chromosome is the most distributed, with 14; Chr2 and Chr5 take second place, each with 12 *WRKY* genes distributed in Verbena bonariensis; Chr7 has 10 *WRKY* genes distributed in Verbena officinalis; There are 9 *WRKY* genes distributed on Chr3, 8 *WRKY* genes distributed on Chr4, and the least distributed on Chr6, with only 5 *WRKY* genes. According to Holub[34], chromosomal regions containing two or more genes within 200 KB can be defined as gene clusters. In Verbena bonariensis, a total of 16 *VbWRKY* genes are clustered into 8 gene clusters, marked in blue in the figure (Figure 1). The chromosome distribution of gene cluster was irregular, except for Chr7 chromosome, the other 6 chromosomes all had gene cluster. Further analysis of tandem repeats revealed that only one pair of tandem repeats, *VbWRKY10* and *VbWRKY11*, were located on Chr1.

phylogenetic analysis and multiple sequence alignment of *VbWRKY* gene family

Perform phylogenetic analysis on the identified 70 WRKY protein sequences of Verbena bonariensis and 72 known WRKY protein sequences in Arabidopsis. The results showed that the identified 70 Verbena bonariensis *WRKY* transcription factors could be classified into three major groups (Figure 2) , which are in line with the definitions of group I, group II, and group III in Arabidopsis by Eulgem et al.[1]. There are 13 WRKY proteins belonging to group I, 47 WRKY proteins belonging to group II, and 10 WRKY proteins belonging to group III. WRKY members in group II can be further divided into five subclasses: IIa, IIb, IIc, IId, and IIe, with 5, 10, 18, 6, and 8 members, respectively. According to evolutionary relationships, the WRKY family II of higher plants can be divided into three subclasses: IIa+IIb, IIc, and IId+IIe, which is consistent with the results presented by the phylogenetic tree.

The WRKY domain protein sequence of the Verbena bonariensis *WRKY* gene was analyzed by DNAMAN sequence analysis software (Figure 3, Table 1) , The results showed that group I contained two WRKY domains, located at the N and C ends of the sequence, respectively. The WRKY heptapeptide domain and zinc finger structure types were WRKYGQK and C2H2, with VbWRKY38 and VbWRKY69 having only one WRKY domain. Group II contains one WRKY domain, with the WRKY heptapeptide domain and zinc finger structure being WRKYGQK and C2H2. Among them, *VbWRKY47* of IIc has no conserved WRKY domain and zinc finger structure, *VbWRKY18* has a missing zinc finger structure, and WRKYGQK at the conserved sites of *VbWRKY2*, *VbWRKY18*, and *VbWRKY35* has become WRKYGKK. Group III contains one WRKY domain, with WRKY heptapeptide domain and zinc finger structure being WRKYGQK and C2HC. Among them, *VbWRKY57* and *VbWRKY58* have no conserved WRKY domain and zinc finger structure.

Gene structure and conserved motif analysis of *WRKY* family in Verbena bonariensis

Using MEME online software to analyze 70 VbWRKY protein sequences, 10 conserved motifs were found (Figure 4). The frequency of Motif in the WRKY protein determines its importance in the sequence, Among them, motif 1 and motif 8 are conserved sequences of the WRKYGQK heptapeptide segment, motif 2 and motif 5 are zinc finger structural motifs. Motif 1 and motif 2 make up the C-terminal WRKY box, and motif 5 and otimf 8 make up the N-terminal WRKY box. Class I has two WRKY boxes, with four motifs: motif 1, motif 2, motif 5, and motif 8, while the other classes only have N-terminal WRKY boxes, with only motif 1 and motif 2, and no motif 5 and motif 8. Motif1, motif2, and motif3 are landmark conserved motifs of the VbWRKYs protein in Verbena bonariensis, with most (64/70) having motif 1, 2, and 3. Generally speaking, different subclasses contain different motifs, and the motif elements in the same subclass are roughly the same. Genes with the same motif elements have similar biological functions. The conservative elements in subclasses IIa and IIb, IId and IIe are roughly similar, which also proves that in higher plants, IIa and IIb can be classified into the same subclass, while IId and IIe can be classified into the same subclass. Motif 6 and motif 7 are specifically present in group IIa+b; Motif 9 is a unique conservative element in group IIb; Motif 10 is a unique conservative element of group I. The number and types of various motifs are relatively fixed, consistent with the phylogenetic tree results (Figure 2) and identification classification results (Table 1).

Gene structure analysis showed that most Verbena bonariensis *WRKY* genes had 2-6 exons, except that *VbWRKY38* and *VbWRKY47* had a large number of exons and introns (*VbWRKY38* had 10 exons and *VbWRKY47* had 11 exons) . Among them, the number of genes with 3 exons (2 introns) was the most, accounting for 54% (38/70) of all *VbWRKY* genes, and the number of genes with 2 and 6 exons was the least, with 5 each. In addition, 11 *VbWRKY* genes contained 4 exons and 9 *VbWRKY* genes contained 5 exons. All members of group III, IId, and IIe contain 3 exons. group I contains 2-6 exons, and subclasses IIa and IIb contain 3-6 exons. Subclass IIc contains 2-4 exons. The UTR distribution pattern showed that there were 25 *VbWRKY* genes with UTR region, 7 genes with 5 'UTR region and 5 genes with 3 'UTR region, a total of 13 genes contained both 5' UTR and 3' UTR regions.

cis-Acting Elements analysis of *WRKY* Gene Family Members in Verbena bonariensis

In order to further understand the transcriptional regulation and potential function of *VbWRKY*, PlantCARE was used to predict the cis acting elements of the *VbWRKY* promoter. The results showed that in addition to promoter related elements and WRKY binding site elements, three types of cis regulatory elements were found to be highly concentrated in the promoter region of *VbWRKYs*, including light-responsive elements, plant hormone response elements, and environmental stress response related elements (Figure 5). Among them, The cis acting elements related to environmental stress response include low-temperature (LTR), drought (MBS),

trauma (WUN motif), defense and stress (Tc rich repeats), and anaerobic induction (ARE) response elements. The number of light-responsive elements is the highest, including Box4, G-box, etc, and 70 *VbWRKY* genes all contain light-responsive elements. Plant hormone responsive elements include methyl jasmonate responsive elements (CGTCA-Motif and TGACG-Motif), abscisic acid responsive element (ABRE), auxin responsive elements (TGA-element and AuxRR-core), salicylic acid responsive element (MBS), gibberellin responsive element (p-box and TATC-box) and ethylene responsive element (ERE). Specifically, ABRE elements are commonly present in 61 *VbWRKY* promoters, and 30 *VbWRKY* genes contain low-temperature responsive elements. These results indicate that most of the cis acting elements of *VbWRKY* are related to stress, and *VbWRKY* may play an important role in participating in stress responses.

Collinearity Analysis of *WRKY* Gene Family Members in Verbena bonariensis

To explore the conservatism of *VbWRKY* gene family members during evolution, collinearity analysis of *VbWRKY* gene family players was carried out. It was found that 44 pairs of highlighted homologous genes existed among the *VbWRKY* gene family members (Figure 6). Furthermore, the values of Ka and Ks were calculated for these 44 pairs of highlighted homologous genes (Table 2). With the exception of *VbWRKY3/ VbWRKY8 VbWRKY8/ VbWRKY32 VbWRKY9/ VbWRKY30 VbWRKY10/ VbWRKY37 VbWRKY22/ VbWRKY67 VbWRKY30/ VbWRKY62 VbWRKY8/ VbWRKY53 VbWRKY9/ VbWRKY54* and *VbWRKY7/ VbWRKY69*, the value of Ka/Ks of the other 35 pairs of homologous genes was less than 1, which in-dicates that the 35 pairs of homologous genes evolved under great purifying selection or negative selection pressure[35]. The value of Ka/Ks of the other 9 pairs of highlighted genes was NaN, which may be due to synonymous mutations in most sites where synonymous mutations can occur[36].

Table 2. Ka and Ks values of highlighted homologous gene pairs in *VbWRKYs*.

Differences in expression of Verbena bonariensis *WRKY* gene family members under low temperature stress

To further explore the regulation of *VbWRKYs* on low-temperature stress, the transcriptome data (FPKM) of cold-stressed plants obtained from the Verbena bonariensis database were calculated and visualized (Figures 7). It was found that the members of the old *WRKY* gene family in Verbena bonariensis could be separated into three categories under cold stress: The expression of 37 members increased under cold stress, 17 of which were significantly up-regulated; the expression quantity of 15 members decreased under cold stress; and the remaining 18 members did not respond to cold stress. 17 genes with upregulated expression under cold stress all possessed cis-acting elements (LTR, W box and ABRE) related to cold stress, indicating that some *VbWRKY* members may participate in the process of resistance to cold stress.

Discussion

The *WRKY* transcription factor family is a unique class of transcription factors in plants that participate in various signaling pathways by regulating gene expression in response to biotic and abiotic stresses faced by plants[37]. With the continuous completion of genome sequencing for different species, the *WRKY* gene family has been identified in multiple species. Including 72 in Arabidopsis[21], 102 in rice[38], 104 in tomato[39], 139 in Apple[40], 61 in cucumber[41], 89 in Camellia oleifera[42], 59 in grape[43] and 116 in cotton[44]. This study identified 70 *VbWRKY* members from the genome of Verbena bonariensis.

In this study, 70 members of the *VbWRKY* family were divided into three groups based on phylogenetic evolution: I, II, and III. Among them, Group II has 5 subfamilies. *VbWRKY38* and *VbWRKY69* in Group I lost one WRKY domain during evolution, and the loss of WRKY domains in Group I members is also present in the Arabidopsis genome, for example, Arabidopsis Group I member *AtWRKY10* contains only one WRKY domain[16]. There is a phenomenon of loss and variation of structural domains and zinc finger motifs in Groups II and III, such as *VbWRKY47* in class IIc and *VbWRKY57* and in Group III. Related studies have shown that members of Group II and III evolved from the loss or variation of C-terminus and N-terminus domains and zinc finger motifs in Group I during plant evolution[45]. Thus, loss of the *VbWRKY47*, *VbWRKY57*, and *VbWRKY58* domains may have contributed to the expansion of the Verbena bonariensis *VbWRKY* gene family. *GmWRKY6* and *GmWRKY21*, which contain WRKYGKK variants in soybeans, fail to bind W-box normally[46]. The *NtWRKY12* of tobacco with the WRKYGKK variant recognizes another binding sequence TTTTCCAC instead of the normal Wbox[47]. In the Verbena bonariensis, the conserved heptapeptide sites WRKYGQK of

the *VbWRKY2*, *VbWRKY18*, and *VbWRKY35* coding products in IIc were transformed into WRKYGKK. It is speculated that the WRKY heptapeptide domain variants of Verbena bonariensis may cause the WRKY protein to lose its ability to recognize and bind to DNA, or to recognize other new motifs and generate new functions. The function of zinc finger motifs is equivalent to chelating agents, and the lack of zinc finger motifs can reduce W-box binding ability or generate new biological functions[48]. The zinc finger motifs of *VbWRKY18*, *VbWRKY57*, and *VbWRKY58* in Verbena bonariensis have been lost or mutated, possibly resulting in the loss of their original binding domain function and the emergence of new biological functions.

At the same time, the analysis of introns, exons and conserved motifs can provide important evidence for further understanding of gene evolution. Gene structure analysis showed that most of the Verbena bonariensis *VbWRKY* genes (38/70) contained two introns, similar to the reported in cassava (42/85) , cucumber (29/61) and maize (78/140)[49, 50]. The conserved Motif distribution pattern is the main basis for the classification of gene family members. Motif analysis found that the *VbWRKY* genes in the same subgroup had similar conserved Motif distribution patterns, while there were differences in the conservative motif distribution patterns of *VbWRKY* genes in different subgroups. it is inferred that *VbWRKY* gene has similar or different biological functions due to the difference of conserved motif distribution patterns.

The analysis of cis acting elements shows that the promoter region of the *VbWRKY* gene in Verbena bonariensis is rich in various cis acting elements, such as light responsive elements, hormone responsive elements and stress responsive elements. It is speculated that the *VbWRKY* gene may be activated and expressed under light signals, hormones, and biotic or abiotic stress induction, thereby directly or indirectly regulating various biological processes in plants. The collinearity analysis of the *WRKY* gene family in Verbena bonariensis showed that there were 44 prominent homologous genes in the *WRKY* family, providing impetus for the evolution of Verbena bonariensis.

Numerous studies have confirmed that multiple *WRKY* genes in plants can be rapidly induced to express simultaneously when subjected to extreme stress, thus participating in the regulation of stress response. For example, experiments by Qiu Yuping et al. [51] showed that 10 Oswrkys in rice were rapidly induced to express under abiotic stresses such as NaCl, drought, low temperature (4 °C) and high temperature (42 °C) . Wu et al.[35] found that eight *TaWRKYs* genes in wheat (Triticum aestivum) had rapid responses to low temperature, high salinity, drought and high temperature. This study found that 37 *VbWRKY* genes were involved in the response to low temperature stress, and 17 of them showed increased expression during cold stress. These results suggest that *VbWRKYs* are induced by stress and participate in the regulatory network of stress genes. Moreover, most of the genes were upregulated under abiotic stress, suggesting that these *VbWRKYs* may play a positive regulatory role in abiotic stress of Verbena bonariensis.

This study utilized the genome data of Verbena bonariensis previously measured by the research team to identify the members of the *WRKY* gene family of Verbena bonariensis, and analyzed their phylogeny, subgroup types, chromosome distribution, conserved motifs, gene structure, cis acting elements, and transcriptome data under low temperature stress, it provides a basis for further study on the roles of *WRKY* transcription factors in plant growth and development and in response to low temperature stress, and provides a basis for further study on the functions of Verbena bonariensis *WRKY* transcription factor family.

Conclusion

This study identified a total of 70 members of the *WRKY* gene family in Verbena bonariensis. By analyzing various bioinformatics information such as the WRKY domain, evolutionary relationships, gene structure, conserved motifs, chromosome distribution, and cis acting element distribution of the *WRKY* gene family in Verbena bonariensis, a comprehensive and systematic identification of the *WRKY* gene family was conducted. Its structure is highly conservative and participates in many aspects of Verbena bonariensis growth and development. Analysis of transcriptome data from Verbena bonariensis under low temperature stress revealed that some *VbWRKY* genes can respond to low temperature stress and exhibit a positive regulatory effect. The results of this study provide a theoretical basis for the functional study of *WRKY* gene in cold stress and growth and development of Verbena bonariensis, and may also contribute to the screening of cold resistance.

Materials and Methods

Identification of *WRKY* gene family members in Verbena bonariensis

The genome data of Verbena bonariensis were obtained by our research team, and the protein sequences of the Arabidopsis *WRKY* gene family were downloaded from the Arabidopsis database TAIR (http://www.arabidopsis.org/)[52]. The hidden Markov model (HMM) file of the WRKY domain (PF03106) was downloaded from the Pfam database (http://Pfam.xfam.org/)[53], and used for a search in hmmer (3.0)[54] to obtain the target sequence. at the same time, the Arabidopsis WRKY protein sequence was used as the query sequence, and BLAST program was used to compare the sequences in the Verbena bonariensis genome database. Merge and remove duplicates to obtain the candidate WRKY transcription factor protein sequence of Verbena bonariensis. The *VbWRKY* gene family members were further identified by using the protein conserved domain analysis tools NCBICD-search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and Pfam (http://Pfam.xfam.org/) to analyze and screen candidate genes, and ultimately obtain all WRKY transcription factor family members of Verbena bonariensis.

Physicochemical Properties of *WRKY* Gene Family Players in Verbena bonariensis

The online tool ProtParam ((https://web.expasy.org/protparam/)[55] was used to predict the molecular weight and theoretical isoelectric point (pI) of the protein of the *WRKY* gene family in Verbena bonariensis. Online tools such as WoLF PSORT (https://wolfpsort.hgc.jp/)[56] were used to predict the subcellular localization of the *VbWRKYs*.

phylogenetic analysis and multiple sequence alignment of *WRKY* gene family members in Verbena bonariensis

Using the *WRKY* gene family of Arabidopsis thaliana as the reference sequence, the phylogenetic tree was constructed by using the neighbor-joining (NJ) method of Mega7.0(set the Bootstrap value to 1000 and other parameters as the default value)[57], cluster analysis of *VbWRKY* family members was carried out according to the existing grouping of Arabidopsis thaliana, and the evolutionary tree was beautified by using the online website iTOL (http://iTOL.embl.de/)[58].

Referring to the definition of WRKY domain by Rushton et al, the 10-65 amino acid residues in the core sequence of WRKY domain were used as target sequences, and sequence Analysis Software DNAMAN and online software Weblogo3(http://weblogo.berkeley.edu/logo.cgi) were used to perform multiple sequence alignment of the WRKY domain of Verbena bonariensis WRKY protein.

Visualization of Gene Structure and Conserved Motif of *WRKY* gene family members in Verbena bonariensis

the MEME (https://meme-suite.org/meme/tools/meme)[59] tool was used to perform conservative motif analysis on the *WRKY* family protein sequence of Verbena bonariensis, and parameters were set to search for 10 motif elements. Based on the Verbena bonariensis genome GFF3 annotation file, the gene structure of *VbWRKY* gene was analyzed by Tbtools software[60], and its conserved motifs and gene structure patterns were plotted.

cis-Acting elements of *WRKY* gene family members in Verbena bonariensis

The promoter of the start codon 2000 bp upstream was separated from the genomefile of the Verbena bonariensis using TBtools software[60], and then the cis-acting elements of the *VbWRKYs* were found using the online tool PlantCare ((http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[61] and then visualized using TBtools software[60].

Collinearity and Chromosome Mapping of *WRKY* gene family members in Verbena bonariensis

the collinearity among members of Verbena bonariensis was calculated using the MCScanX method. Based on the annotation information of the Verbena bonariensis genome, obtain the positional information of *VbWRKY* gene family members in the genome. Mapping was carried out using TBtools software[60].

Differences in expression of *WRKY* gene family members under low temperature stress in Verbena bonariensis

The expression level of Verbena bonariensis under cold stress (FPKM) was measured by our research group. Expression levels (FPKM) were calculated with log2(FPKM + 1)[62] and visualized with TBtools software[60].

References

1. Mangelsen E, Kilian J, Berendzen KW, Kolukisaoglu ÜH, Harter K, Jansson C, et al. Phylogenetic and comparative gene expression analysis of barley (Hordeum vulgare) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. BMC genomics. 2008; 9(1):1-17.
2. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. Trends in plant science. 2000; 5(5):199-206.
3. Li H, Xu Y, Xiao Y, Zhu Z, Xie X, Zhao H, et al. Expression and functional analysis of two genes encoding transcription factors, VpWRKY1 and VpWRKY2, isolated from Chinese wild Vitis pseudoreticulata. Planta. 2010; 232:1325-1337.
4. Schluttenhofer C, Yuan L. Regulation of specialized metabolism by WRKY transcription factors. Plant Physiology. 2015; 167(2):295-306.
5. Ishiguro S, Nakamura K. 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 and General Genetics MGG. 1994; 244:563-571.
6. Dellagi A, Birch PR, Heilbronn J, Avrova AO, Montesano M, Palva ET, et al. A potato gene, erg-1, is rapidly induced by Erwinia carotovora ssp. atroseptica, Phytophthora infestans, ethylene and salicylic acid. Journal of plant physiology. 2000; 157(2):201-205.
7. Yoda H, Ogawa M, Yamaguchi Y, Koizumi N, Kusano T, Sano H. Identification of early-responsive genes associated with the hypersensitive response to tobacco mosaic virus and characterization of a WRKY-type transcription factor in tobacco plants. Molecular Genetics and Genomics. 2002; 267:154-161.
8. Sun C, Palmqvist S, Olsson H, Borén M, Ahlandsberg S, Jansson C. A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. The Plant Cell. 2003; 15(9):2076-2092.
9. Chen H, Lai Z, Shi J, Xiao Y, Chen Z, Xu X. Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. BMC plant biology. 2010; 10(1):1-15.
10. Hwang SH, Yie SW, Hwang DJ. Heterologous expression of OsWRKY6 gene in Arabidopsis activates the expression of defense related genes and enhances resistance to pathogens. Plant Science. 2011; 181(3):316-323.
11. Zheng Z, Qamar SA, Chen Z, Mengiste T. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. The Plant Journal. 2006; 48(4):592-605.
12. Ramamoorthy R, Jiang SY, Kumar N, Venkatesh PN, Ramachandran S. A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments. Plant and cell physiology. 2008; 49(6):865-879.
13. Ross CA, Liu Y, Shen QJ. The WRKY gene family in rice (Oryza sativa). Journal of Integrative Plant Biology. 2007; 49(6):827-842.
14. Levée V, Major I, Levasseur C, Tremblay L, MacKay J, Séguin A. Expression profiling and functional analysis of Populus WRKY23 reveals a regulatory role in defense. New Phytologist. 2009; 184(1):48-70.
15. Yang B, Jiang Y, Rahman MH, Deyholos MK, Kav NN. Identification and expression analysis of WRKY transcription factor genes in canola (Brassica napus L.) in response to fungal pathogens and hormone treatments. BMC plant biology. 2009; 9:1-19.
16. Ling J, Jiang W, Zhang Y, Yu H, Mao Z, Gu X, et al. Genome-wide analysis of WRKY gene family in Cucumis sativus. BMC genomics. 2011; 12:1-20.
17. Ding M, Chen J, Jiang Y, Lin L, Cao Y, Wang M, et al. Genome-wide investigation and transcriptome analysis of the WRKY gene family in Gossypium. Molecular Genetics and Genomics. 2015; 290:151-171.
18. Phukan UJ, Jeena GS, Shukla RK. WRKY transcription factors: molecular regulation and stress responses in plants. Frontiers in plant science. 2016; 7:760.
19. Ye J, Wang X, Hu T, Zhang F, Wang B, Li C, et al. An InDel in the promoter of Al-ACTIVATED MALATE TRANSPORTER9 selected during tomato domestication determines fruit malate contents and aluminum tolerance. The Plant Cell. 2017; 29(9):2249-2268.

20. Banerjee A, Roychoudhury A. WRKY proteins: signaling and regulation of expression during abiotic stress responses. The Scientific World Journal. 2015; 2015.
21. Rushton PJ, Somssich IE, Ringler P, Shen QJ. WRKY transcription factors. Trends in plant science. 2010; 15(5):247-258.
22. Ülker B, Somssich IE. WRKY transcription factors: from DNA binding towards biological function. Current opinion in plant biology. 2004; 7(5):491-498.
23. Chu X, Wang C, Chen X, Lu W, Li H, Wang X, et al. The cotton WRKY gene GhWRKY41 positively regulates salt and drought stress tolerance in transgenic Nicotiana benthamiana. PLoS One. 2015; 10(11):e0143022.
24. Duan GF, Li LJ, Liu QL. A WRKY transcription factor from Malus domestica negatively regulates dehydration stress in transgenic Arabidopsis. Acta physiologiae plantarum. 2014; 36:541-548.
25. Zhang Y, Yu H, Yang X, Li Q, Ling J, Wang H, et al. CsWRKY46, a WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by regulating a set of cold-stress responsive genes in an ABA-dependent manner. Plant Physiology and Biochemistry. 2016; 108:478-487.
26. Chen L, Yang Y, Liu C, Zheng Y, Xu M, Wu N, et al. Characterization of WRKY transcription factors in Solanum lycopersicum reveals collinearity and their expression patterns under cold treatment. Biochemical and Biophysical Research Communications. 2015; 464(3):962-968.
27. Wang Y, Li Y, He SP, Gao Y, Wang NN, Lu R, et al. A cotton (Gossypium hirsutum) WRKY transcription factor (GhWRKY22) participates in regulating anther/pollen development. Plant Physiology and Biochemistry. 2019; 141:231-239.
28. Zhang H, Zhao M, Song Q, Zhao L, Wang G, Zhou C. Identification and function analyses of senescence-associated WRKYs in wheat. Biochemical and Biophysical Research Communications. 2016; 474(4):761-767.
29. Niu CF, Wei W, Zhou QY, Tian AG, Hao YJ, Zhang WK, et al. Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. Plant, cell & environment. 2012; 35(6):1156-1170.
30. Kim CY, Vo KTX, Nguyen CD, Jeong DH, Lee SK, Kumar M, et al. Functional analysis of a cold-responsive rice WRKY gene, OsWRKY71. Plant Biotechnology Reports. 2016; 10:13-23.
31. Wang MQ, Huang QX, Lin P, Zeng QH, Li Y, Liu QL, et al. The overexpression of a transcription factor gene VbWRKY32 enhances the cold tolerance in Verbena bonariensis. Frontiers in Plant Science. 2020; 10:1746.
32. Lata C, Prasad M. Role of DREBs in regulation of abiotic stress responses in plants. Journal of experimental botany. 2011; 62(14):4731-4748.
33. Mitsis T, Efthimiadou A, Bacopoulou F, Vlachakis D, Chrousos GP, Eliopoulos E. Transcription factors and evolution: An integral part of gene expression. World Academy of Sciences Journal. 2020; 2(1):3-8.
34. Holub EB. The arms race is ancient history in Arabidopsis, the wildflower. Nature Reviews Genetics. 2001; 2(7):516-527.
35. Wu H, Ni Z, Yao Y, Guo G, Sun Q. Cloning and expression profiles of 15 genes encoding WRKY transcription factor in wheat (Triticum aestivem L.). Progress in Natural Science. 2008; 18(6):697-705.
36. Zhang T, Cai J, Wang S, Lv L, Yuan D, Zeng X, et al. Identification and Expression Analysis of the Ethylene Response Factor Gene Family in Tea Plant (Camellia sinensis). Agronomy. 2023; 13(7):1900.
37. Huang X, Ding F, Peng HX, Pan JC, He XH, Xu JZ, et al. Research progress on family of plant WRKY transcription factors. Biotechnology Bulletin. 2019; 35(12):129.
38. Yamasaki K, Kigawa T, Seki M, Shinozaki K, Yokoyama S. DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. Trends in plant science. 2013; 18(5):267-276.
39. Huang S, Gao Y, Liu J, Peng X, Niu X, Fei Z, et al. Genome-wide analysis of WRKY transcription factors in Solanum lycopersicum. Molecular Genetics and Genomics. 2012,; 287:495-513.
40. Li MY, Xu ZS, Tian C, Huang Y, Wang F, Xiong AS. Genomic identification of WRKY transcription factors in carrot (Daucus carota) and analysis of evolution and homologous groups for plants. Scientific reports. 2016; 6(1):23101.
41. Chen C, Chen X, Han J, Lu W, Ren Z. Genome-wide analysis of the WRKY gene family in the cucumber genome and transcriptome-wide identification of WRKY transcription factors that respond to biotic and abiotic stresses. BMC Plant Biology. 2020; 20:1-19.

42. Su W, Zhou Z, Zeng J, Cao R, Zhang Y, Hu D, et al. Genome-wide identification of the WRKY gene family in Camellia oleifera and expression analysis under phosphorus deficiency. Frontiers in Plant Science. 2023; 14:1082496.
43. Guo C, Guo R, Xu X, Gao M, Li X, Song J, et al. Evolution and expression analysis of the grape (Vitis vinifera L.) WRKY gene family. Journal of experimental botany. 2014; 65(6):1513-1528.
44. Dou L, Zhang X, Pang C, Song M, Wei H, Fan S, et al. Genome-wide analysis of the WRKY gene family in cotton. Molecular genetics and genomics. 2014; 289:1103-1121.
45. Chen M, Tan Q, Sun M, Li D, Fu X, Chen X, et al. Genome-wide identification of WRKY family genes in peach and analysis of WRKY expression during bud dormancy. Molecular Genetics and Genomics. 2016; 291:1319-1332.
46. Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, et al. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant biotechnology journal. 2008; 6(5):486-503.
47. van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJ. A novel WRKY transcription factor is required for induction of PR-1a gene expression by salicylic acid and bacterial elicitors. Plant Physiology. 2008; 146(4):1983-1995.
48. Shen T, Qi H, Luan X, Xu W, Yu F, Zhong Y, et al. The chromosome-level genome sequence of the camphor tree provides insights into Lauraceae evolution and terpene biosynthesis. Plant Biotechnology Journal. 2022; 20(2):244.
49. Hu W, Ren Q, Chen Y, Xu G, Qian Y. Genome-wide identification and analysis of WRKY gene family in maize provide insights into regulatory network in response to abiotic stresses. BMC plant biology. 2021; 21:1-21.
50. Wei Y, Shi H, Xia Z, Tie W, Ding Z, Yan Y, et al. Genome-wide identification and expression analysis of the WRKY gene family in cassava. Frontiers in Plant Science. 2016; 7:25.
51. Qiu Y, Jing S, Fu J, Li L, Yu D. Cloning and analysis of expression profile of 13 WRKY genes in rice. Chinese Science Bulletin. 2004; 49:2159-2168.
52. Heidari P, Ahmadizadeh M, Izanlo F, Nussbaumer T. In silico study of the CESA and CSL gene family in Arabidopsis thaliana and Oryza sativa: Focus on post-translation modifications. Plant Gene. 2019; 19:100189.
53. Miller DR, Leek T, Schwartz RM. A hidden Markov model information retrieval system. In: Proceedings of the 22nd annual international ACM SIGIR conference on Research and development in information retrieval. 1999; 214-221.
54. Finn RD, Clements J, Eddy SR. HMMER web server: interactive sequence similarity searching. Nucleic acids research. 2011; 39(suppl_2):W29-W37.
55. Gasteiger E, Hoogland C, Gattiker A, Duvaud Se, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the ExPASy server: Springer. 2005.
56. Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends in biochemical sciences. 1999; 24(1):34-35.
57. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution. 2016; 33(7):1870-1874.
58. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic acids research. 2019; 47(W1):W256-W259.
59. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, et al. MEME SUITE: tools for motif discovery and searching. Nucleic acids research. 2009; 37(suppl_2):W202-W208.
60. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, et al. TBtools: an integrative toolkit developed for interactive analyses of big biological data. Molecular plant. 2020; 13(8):1194-1202.
61. Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic acids research. 2002; 30(1):325-327.
62. Zhou T, Chen J, Huang Y, Jin Z, Li J, Li Y, et al. Genome-Wide Identification and Expression Analysis of the PIN Auxin Transporter Gene Family in Zanthoxylum armatum DC. Agriculture.2022; 12(9):1318.

Tables

Table 1 Basic Verbena bonariensis of the *WRKY* gene family

Gene	Gene ID	Type	WRKY area	Zinc finger	Number of amino acids /aa	Molecular Weight /Da	Theoretical /PI	Subcellular Location
VbWRKY1	evm.model.Chr01.66	a	WRKYGQK	C2H2	210	23564.67	9.17	Nucleus (12)
VbWRKY2	evm.model.Chr01.196	c	WRKYGKK	C2H2	191	21714.95	6.91	Nucleus (14)
VbWRKY3	evm.model.Chr01.352		WRKYGQK	C2HC	343	38671.55	5.49	Nucleus (12)
VbWRKY4	evm.model.Chr01.1236	c	WRKYGQK	C2H2	370	41334.48	5.31	Nucleus (14)
VbWRKY5	evm.model.Chr01.1680	a	WRKYGQK	C2H2	267	29952.61	6.45	Nucleus(12.5)
VbWRKY6	evm.model.Chr01.2901	b	WRKYGQK	C2H2	560	60480.41	5.82	Nucleus (11)
VbWRKY7	evm.model.Chr01.3012		WRKYGQK	C2H2	566	63963.55	6.62	Nucleus (12)
VbWRKY8	evm.model.Chr01.3242		WRKYGQK	C2HC	345	37765.82	5.46	Nucleus (13)
VbWRKY9	evm.model.Chr01.3292	e	WRKYGQK	C2H2	376	41173.74	4.54	Nucleus (14)
VbWRKY10	evm.model.Chr01.3573		WRKYGQK	C2HC	376	41662.45	6.19	Nucleus (13)
VbWRKY11	evm.model.Chr01.3574		WRKYGQK	C2HC	283	31642.27	6.11	Nucleus (13)
VbWRKY12	evm.model.Chr01.4171		WRKYGQK	C2H2	683	74096.16	6.12	Nucleus (14)
VbWRKY13	evm.model.Chr01.4361	b	WRKYGQK	C2H2	562	60561.52	8.42	Nucleus (13)
VbWRKY14	evm.model.Chr01.4582	e	WRKYGQK	C2H2	391	42891.24	5.37	Nucleus (14)
VbWRKY15	evm.model.Chr05.888	c	WRKYGQK	C2H2	334	37774.58	5.42	Nucleus (12)
VbWRKY16	evm.model.Chr05.1400		WRKYGQK	C2H2	508	55442.03	8.64	Nucleus (14)
VbWRKY17	evm.model.Chr05.1615	b	WRKYGQK	C2H2	476	52551.49	6.22	Nucleus (12)
VbWRKY18	evm.model.Chr05.2053	c	WRKYGKK	C2HC	97	11299.6	9.52	Nucleus(12.5)
VbWRKY19	evm.model.Chr05.2061	c	WRKYGQK	C2H2	240	27956.36	6.92	Nucleus (13)
VbWRKY20	evm.model.Chr05.2645	b	WRKYGQK	C2H2	562	60993.92	7.66	Nucleus (14)
VbWRKY21	evm.model.Chr05.2938	e	WRKYGQK	C2H2	297	33655.17	5.45	Nucleus (11)
VbWRKY22	evm.model.Chr05.3002	c	WRKYGQK	C2H2	339	37730.16	6.38	Nucleus (13)
VbWRKY23	evm.model.Chr05.3162	e	WRKYGQK	C2H2	457	49183.06	5.22	Nucleus (14)
VbWRKY24	evm.model.Chr05.3545	a	WRKYGQK	C2H2	314	34895.12	8.78	Nucleus(10.5)
VbWRKY25	evm.model.Chr05.4588	c	WRKYGQK	C2H2	320	35779.9	5.52	Nucleus (11)
VbWRKY26	evm.model.Chr05.4752	d	WRKYGQK	C2H2	340	37931.89	9.8	Nucleus (13)
VbWRKY27	evm.model.Chr09.767	d	WRKYGQK	C2H2	331	36544.25	9.65	Nucleus (14)
VbWRKY28	evm.model.Chr09.1872		WRKYGQK	C2H2	499	55138	6.13	Nucleus (14)
VbWRKY29	evm.model.Chr09.2632		WRKYGQK	C2H2	388	43309.09	6.72	Nucleus (13)
VbWRKY30	evm.model.Chr09.3590	e	WRKYGQK	C2H2	332	37282.15	5.14	Nucleus (14)
VbWRKY31	evm.model.Chr09.3592	c	WRKYGQK	C2H2	199	22704.51	9.26	Nucleus (12)
VbWRKY32	evm.model.Chr09.3639		WRKYGQK	C2HC	335	37618.83	5.71	Nucleus(13.5)
VbWRKY33	evm.model.Chr09.3841	d	WRKYGQK	C2H2	353	37949.73	9.65	Nucleus (14)

VbWRKY34	evm.model.Chr09.3921	a	WRKYGQK	C2H2	329	36884.94	5.94	Nucleus (8.5)
VbWRKY35	evm.model.Chr09.4152	c	WRKYGKK	C2H2	182	20696.69	5.55	Nucleus (8)

Table 1(continued)

Gene	Gene ID	Type	WRKY area	Zinc finger	Number of amino acids /aa	Molecular Weight /Da	Theoretical /PI	Subcellular Location
VbWRKY36	evm.model.Chr13.546	c	WRKYGQK	C2H2	235	26932.46	9.17	Nucleus (12)
VbWRKY37	evm.model.Chr13.854		WRKYGQK	C2HC	318	35560.8	6.21	Nucleus (8)
VbWRKY38	evm.model.Chr13.1453		WRKYGQK	C2H2	803	88405.53	6.32	Nucleus (12)
VbWRKY39	evm.model.Chr13.2013	d	WRKYGQK	C2H2	331	36035.83	9.53	Nucleus (14)
VbWRKY40	evm.model.Chr13.2667		WRKYGQK	C2H2	564	61499.51	6.52	Nucleus (13)
VbWRKY41	evm.model.Chr13.3038		WRKYGQK	C2H2	587	65107.59	5.72	Nucleus (14)
VbWRKY42	evm.model.Chr13.3777	c	WRKYGQK	C2H2	329	35835.24	5.95	Nucleus (14)
VbWRKY43	evm.model.Chr13.3790		WRKYGQK	C2H2	487	53315.63	6.93	Nucleus (13)
VbWRKY44	evm.model.Chr17.292	c	WRKYGQK	C2H2	295	32602.86	7.68	Nucleus (14)
VbWRKY45	evm.model.Chr17.314		WRKYGQK	C2H2	467	51934.35	6.48	Nucleus (14)
VbWRKY46	evm.model.Chr17.406	b	WRKYGQK	C2H2	491	53888.67	8.05	Nucleus (14)
VbWRKY47	evm.model.Chr17.1018	c			1011	112752.07	5.93	Nucleus (4)
VbWRKY48	evm.model.Chr17.1133	c	WRKYGQK	C2H2	175	20370.81	9.21	Nucleus (9)
VbWRKY49	evm.model.Chr17.1141	c	WRKYGQK	C2H2	175	20370.81	9.21	Nucleus (9)
VbWRKY50	evm.model.Chr17.1578	d	WRKYGQK	C2H2	202	21680.58	9.53	Nucleus (9)
VbWRKY51	evm.model.Chr17.1588	d	WRKYGQK	C2H2	334	36182.8	9.51	Nucleus (6)
VbWRKY52	evm.model.Chr17.2328		WRKYGQK	C2H2	725	80519.63	6.48	Nucleus (13)
VbWRKY53	evm.model.Chr17.2472		WRKYGQK	C2HC	290	33037.09	5.24	Nucleus (9)
VbWRKY54	evm.model.Chr17.2549	e	WRKYGQK	C2H2	337	36986.11	5.65	Nucleus (14)
VbWRKY55	evm.model.Chr17.3711	b	WRKYGQK	C2H2	457	51091.26	5.94	Nucleus (13)
VbWRKY56	evm.model.Chr21.189	b	WRKYGQK	C2H2	634	68722.31	6.01	Nucleus (13)
VbWRKY57	evm.model.Chr21.575				192	21939.14	8.5	Nucleus (11)
VbWRKY58	evm.model.Chr21.578				114	12632.38	9.84	Nucleus (5)
VbWRKY59	evm.model.Chr21.1488		WRKYGQK	C2H2	462	52266.82	6.46	Nucleus (14)
VbWRKY60	evm.model.Chr21.2647	a	WRKYGQK	C2H2	329	36508.7	8.45	Nucleus (13)
VbWRKY61	evm.model.Chr25.84		WRKYGQK	C2HC	301	34157.05	5.2	Nucleus (9)
VbWRKY62	evm.model.Chr25.121	e	WRKYGQK	C2H2	315	34602.29	5.27	Nucleus (10)
VbWRKY63	evm.model.Chr25.425	c	WRKYGQK	C2H2	227	25629.08	8.2	Nucleus (11)
VbWRKY64	evm.model.Chr25.816	c	WRKYGQK	C2H2	400	43910.46	6.46	Nucleus (14)
VbWRKY65	evm.model.Chr25.1964	b	WRKYGQK	C2H2	489	54001.83	5.81	Nucleus (12)
VbWRKY66	evm.model.Chr25.2524	e	WRKYGQK	C2H2	262	28629.52	5.63	Nucleus (14)

VbWRKY67	evm.model.Chr25.2599	c	WRKYGQK	C2H2	348	39220.93	6.4	Nucleus (12)
VbWRKY68	evm.model.Chr25.2950	b	WRKYGQK	C2H2	573	61739.46	6.31	Nucleus (14)
VbWRKY69	evm.model.Chr25.3048		WRKYGQK	C2H2	423	46429.14	5.92	Nucleus (13)
VbWRKY70	evm.model.Chr25.3548	b	WRKYGQK	C2H2	542	59514.72	6.02	Nucleus (14)

Table 2. Ka and Ks values of highlighted homologous gene pairs in *VbWRKYs*.

Gene ID		Ka	Ks	Ka/Ks
VbWRKY3	*VbWRKY8*	0.430942	NaN	NaN
VbWRKY13	*VbWRKY20*	0.162854	1.159392	0.140465
VbWRKY4	*VbWRKY15*	0.387014	2.504243	0.154543
VbWRKY14	*VbWRKY23*	0.241943	1.041421	0.23232
VbWRKY1	*VbWRKY34*	0.235269	0.955791	0.246151
VbWRKY2	*VbWRKY35*	0.321504	1.274818	0.252196
VbWRKY3	*VbWRKY32*	0.198634	0.844565	0.235191
VbWRKY8	*VbWRKY32*	0.495579	NaN	NaN
VbWRKY9	*VbWRKY30*	0.580768	NaN	NaN
VbWRKY10	*VbWRKY37*	0.780393	NaN	NaN
VbWRKY12	*VbWRKY40*	0.553363	2.330171	0.237478
VbWRKY16	*VbWRKY45*	0.390815	1.741709	0.224386
VbWRKY17	*VbWRKY46*	0.518724	1.534833	0.337968
VbWRKY24	*VbWRKY60*	0.129945	0.884903	0.146847
VbWRKY17	*VbWRKY56*	0.366801	2.033576	0.180372
VbWRKY21	*VbWRKY66*	0.431953	2.720782	0.158761
VbWRKY22	*VbWRKY67*	0.440555	NaN	NaN
VbWRKY15	*VbWRKY64*	0.437978	2.189333	0.200051
VbWRKY27	*VbWRKY39*	0.10397	0.912797	0.113903
VbWRKY30	*VbWRKY54*	0.55281	3.684271	0.150046
VbWRKY32	*VbWRKY53*	0.556961	2.049515	0.271753
VbWRKY28	*VbWRKY59*	0.208147	0.719959	0.28911
VbWRKY32	*VbWRKY61*	0.501291	3.004425	0.166851
VbWRKY38	*VbWRKY43*	0.374072	2.254012	0.165958
VbWRKY42	*VbWRKY44*	0.241859	1.017276	0.237751
VbWRKY43	*VbWRKY45*	0.179849	0.843489	0.21322
VbWRKY38	*VbWRKY45*	0.445031	2.121683	0.209754
VbWRKY46	*VbWRKY56*	0.495144	1.790712	0.276507
VbWRKY53	*VbWRKY61*	0.264801	0.952139	0.278111
VbWRKY54	*VbWRKY62*	0.200399	0.986631	0.203114
VbWRKY55	*VbWRKY70*	0.313535	0.860365	0.364421
VbWRKY39	*VbWRKY51*	0.27303	4.145718	0.065858
VbWRKY30	*VbWRKY62*	0.515135	NaN	NaN
VbWRKY8	*VbWRKY53*	0.616043	NaN	NaN
VbWRKY9	*VbWRKY54*	0.503155	NaN	NaN

VbWRKY3	*VbWRKY53*	0.5133	2.158511	0.237803
VbWRKY6	*VbWRKY68*	0.181267	1.175121	0.154254
VbWRKY8	*VbWRKY61*	0.455098	2.312834	0.196771
VbWRKY4	*VbWRKY64*	0.191156	0.645218	0.296266
VbWRKY15	*VbWRKY25*	0.378693	1.297006	0.291975
VbWRKY19	*VbWRKY36*	0.192127	0.75541	0.254334
VbWRKY16	*VbWRKY45*	0.390815	1.741709	0.224386
VbWRKY3	*VbWRKY61*	0.435519	2.86014	0.152272
VbWRKY7	*VbWRKY69*	0.453792	NaN	NaN

Figures

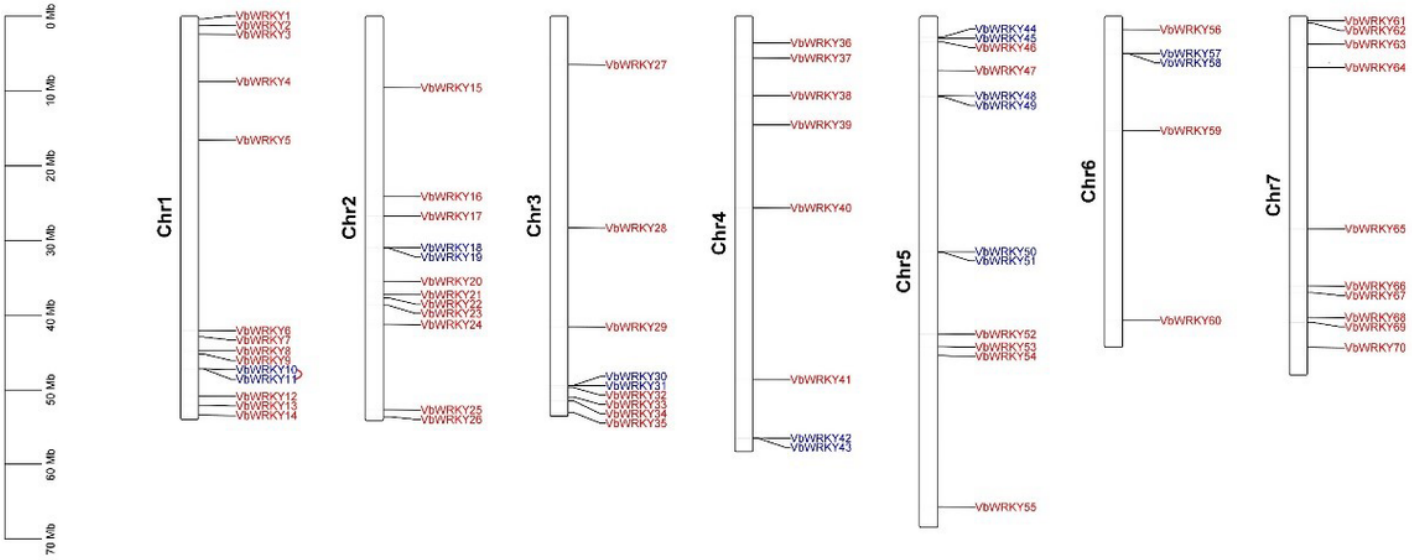

Figure 1

Chromosomal location of *WRKY* gene family members in Verbena bonariensis, gene clusters are indicated in blue

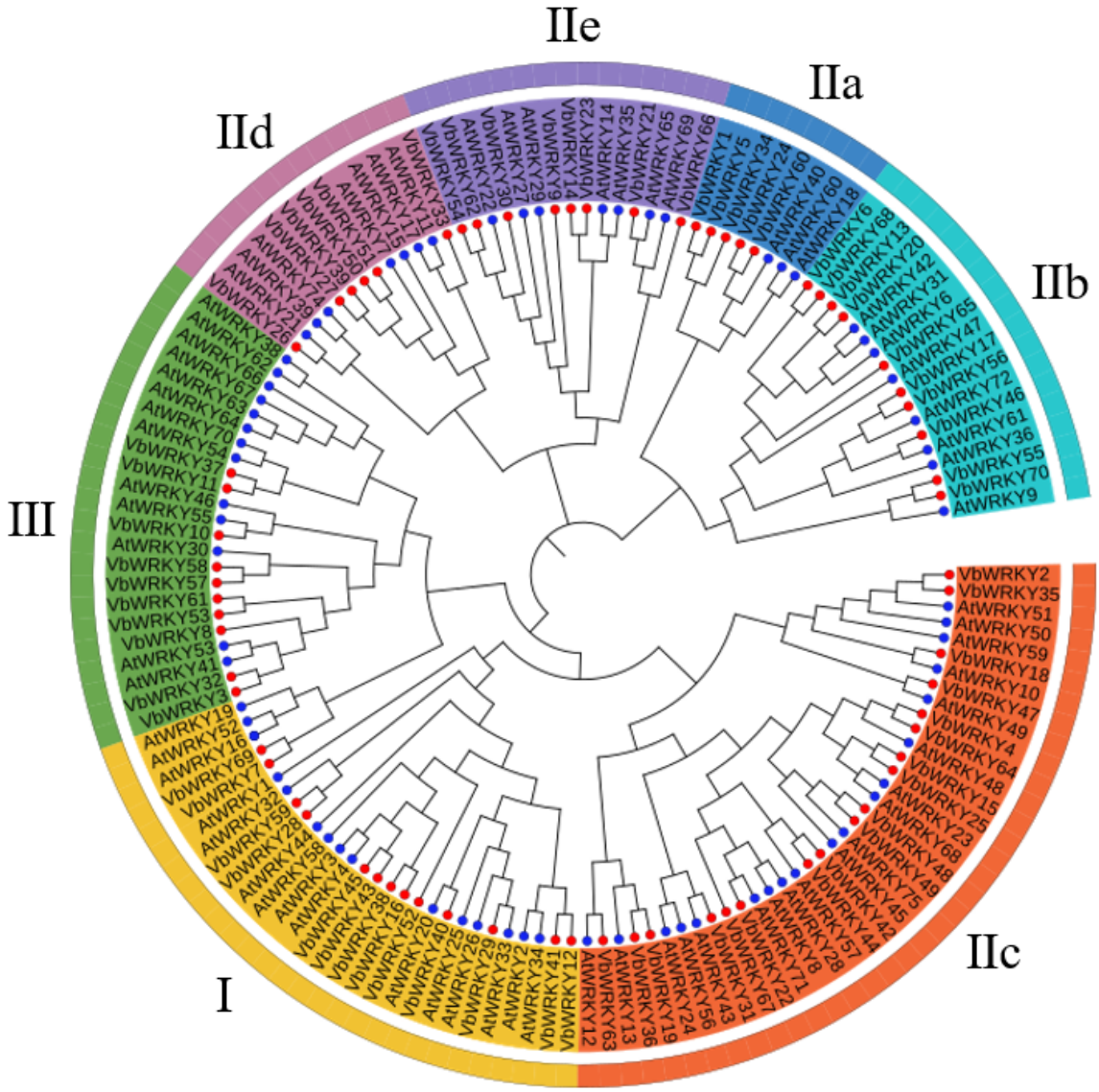

Figure 2

Phylogenetic tree of *WRKY* gene family members in Verbena bonariensis and Arabidopsis.

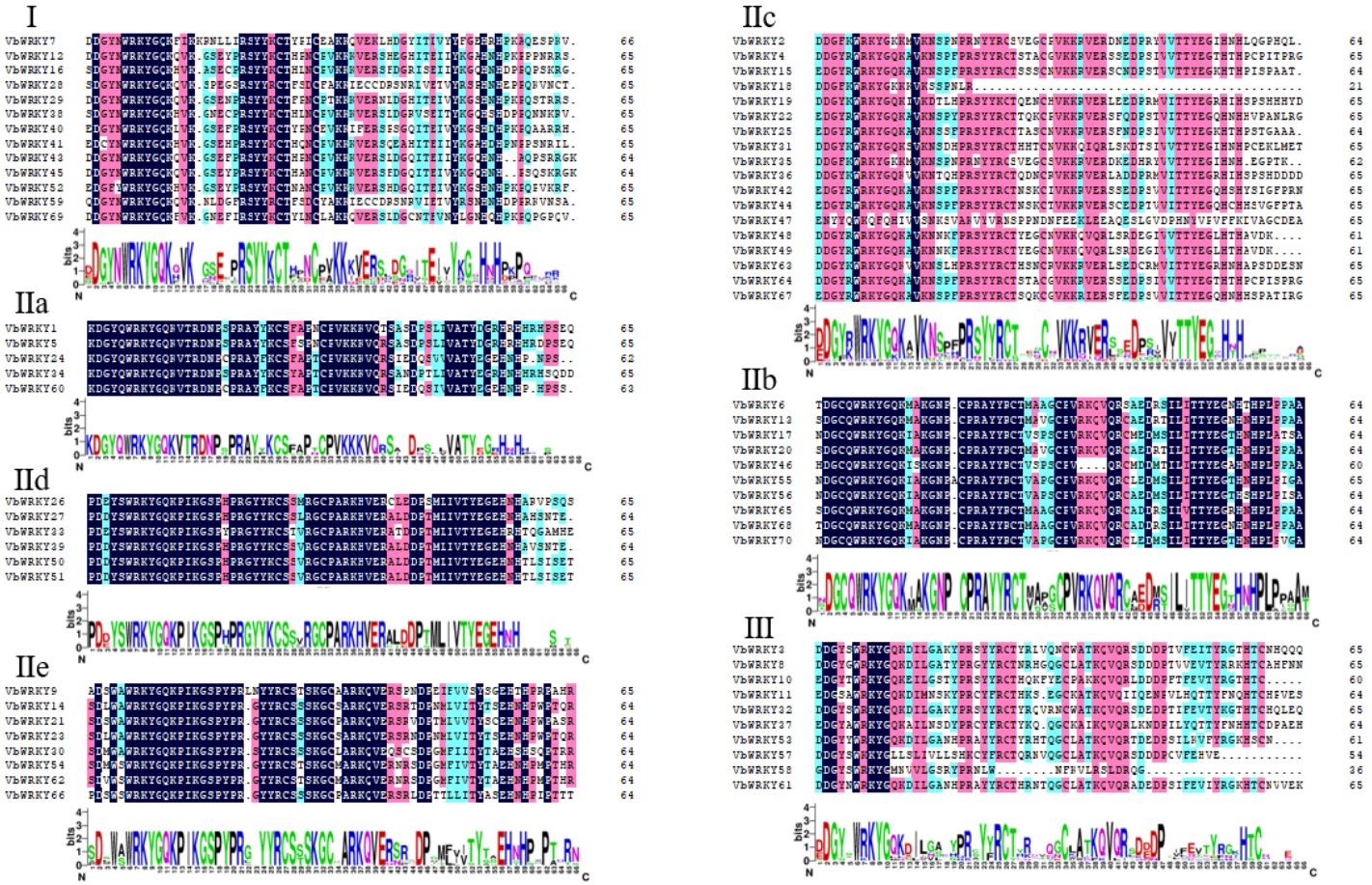

Figure 3

Multiple sequence alignment of *VbWRKY* family members

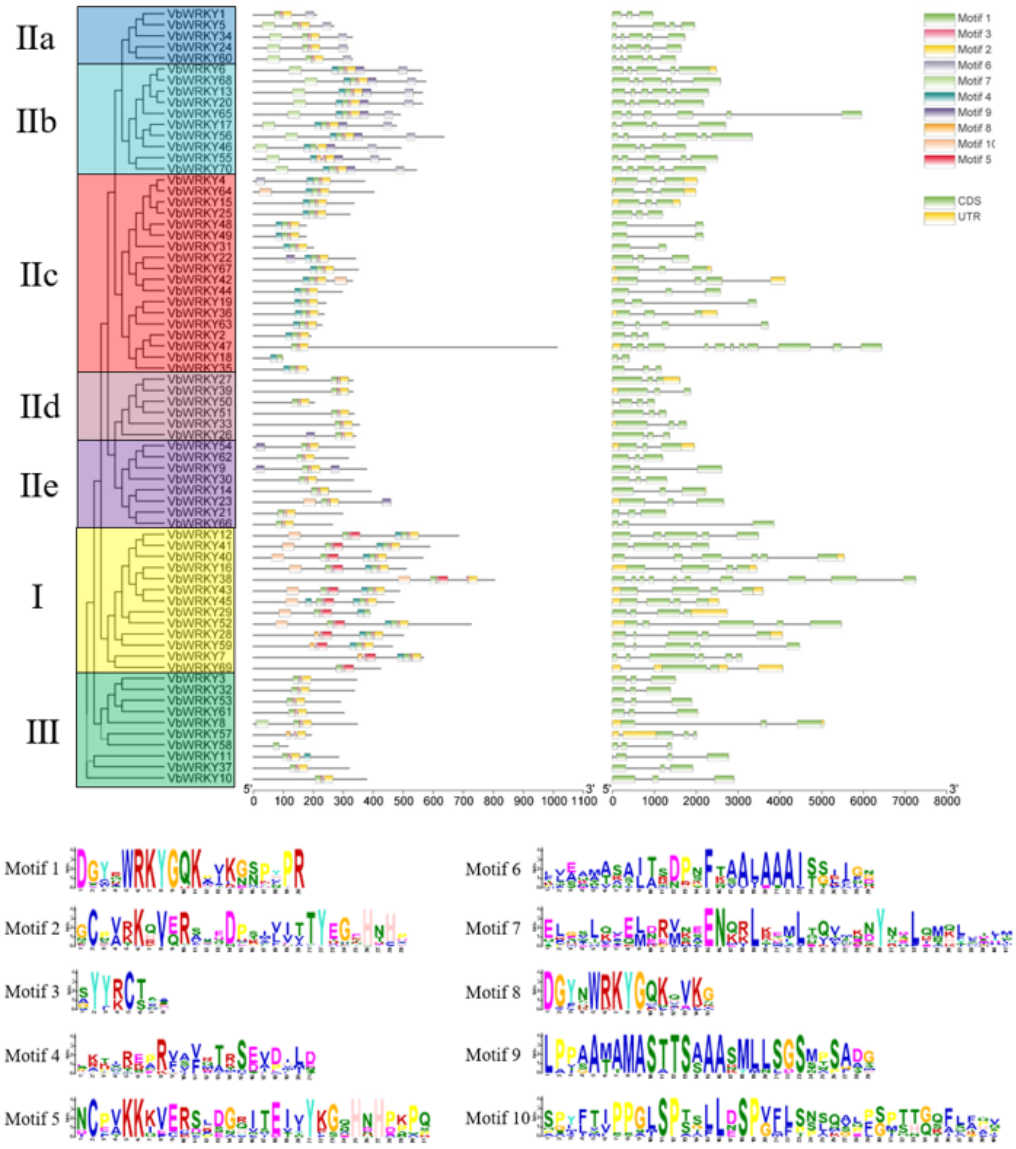

Figure 4

Evolutionary relationship, gene structure, and distribution of conservative motifs of the *WRKY* gene family in Verbena bonariensis

Figure 5

cis-acting elements of the Verbena bonariensis *WRKY* gene family

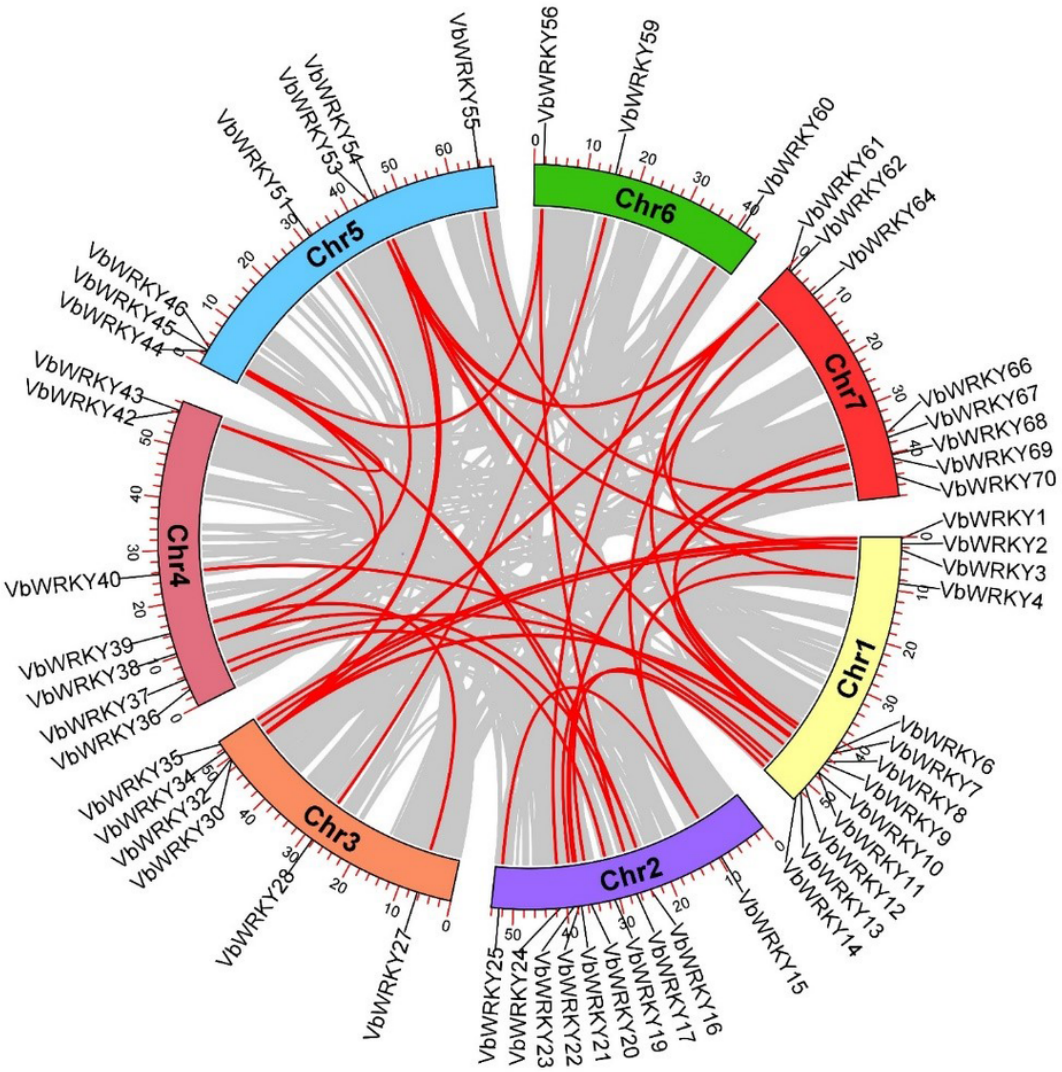

Figure 6

Collinearity relationship of *WRKY* gene family members in Verbena bonariensis

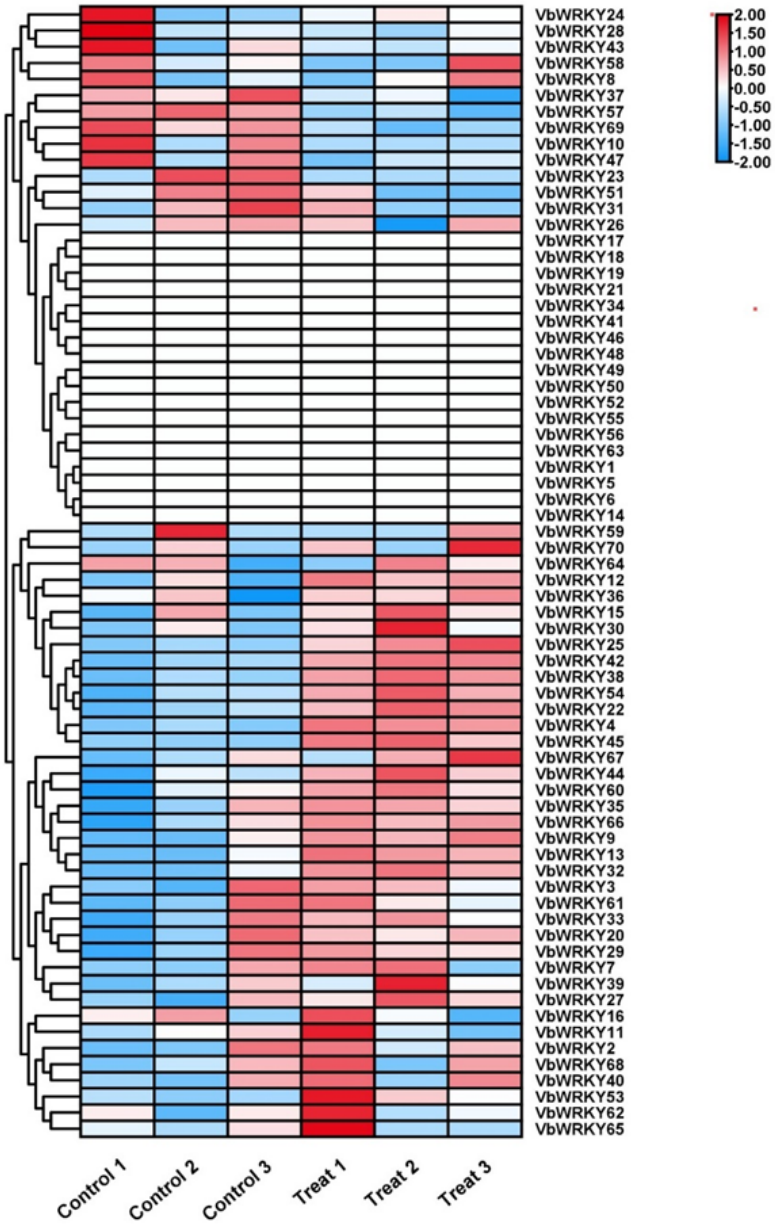

Figure 7

Differences in the expression of *WRKY* gene family members in Verbena bonariensis under cold stress. Control, before cold treatment; Treat, after cold treatment.