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ARTICLE

Identification and Analysis of the *WRKY* Transcription Factor Gene Family in *Verbena bonariensis*

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ABSTRACT

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 seed germination, etc. However, little information is available about *WRKY* transcription factors in *Verbena bonariensis*. In this study, 70 *VbWRKY* genes were identified from the whole genome. The phylogenetic analysis of the *WRKY* gene family in *V. bonariensis* and *Arabidopsis* shows that the *WRKY* genes in *V. bonariensis* can be divided into three groups: I, II, and III, which contain 13, 47, and 10 members, respectively. 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 the *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 *VbWRKYs*, 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 about low-temperature, and 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 and different tissues transcriptome, 70 *VbWRKY* genes played their respective roles in various tissues and stages to regulate plant growth, Also, some of them participated in the process of cold stress tolerance, 52 *VbWRKYs* showed significant differences in expression under cold stress, and *37 VbWRKY* genes were up-regulated under cold stress. 9 *VbWRKY* genes were selected for quantitative real-time PCR (qRT-PCR) analysis under low-temperature stress, and the results showed that all 9 genes were upregulated under low-temperature stress. Ultimately, the present study provides a comprehensive analysis of the predicted *V. bonariensis* WRKY genes family, which provided a theoretical basis for the study of low-temperature resistance and growth and development of *V. bonariensis*.

KEYWORDS

Verbena bonariensis; *WRKY* gene family; cold resistance

1 Introduction

The *WRKY* transcription factors are one of the largest families of transcriptional regulators in plants [1], named for the highly conserved WRKY domain. Each WRKY protein contains one or two conserved domains with approximately 60 amino acid residues, including a highly conserved WRKYGQK heptapeptide at the N-terminus and a C_2H_2 or C_2HC zinc finger motif at the C-terminus [2,3]. According to the number of WRKY domains and the type of zinc finger motif, the *WRKY* family can be divided into three main groups (I, II, and III). Group I contains two WRKY heptapeptide domains and one C_2H_2 zinc finger motif. Group II contains one WRKY heptapeptide domain and one C_2H_2 zinc finger motif. Group III contains a WRKY heptapeptide domain and a C_2HC-type zinc finger motif. Group II can be divided into five subfamilies (IIa, IIb, IIc, IId, and IIe) based on evolutionary relationships [4,5]. 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 [6].

The first *WRKY* transcription factor was identified in sweet potatoes in 1994 [7], followed by those identified in potatoes [8], tobacco [9], wheat and barley [10], *Arabidopsis* [11–13], rice [14], poplar [15], rapeseed [16], cucumber [17], cotton [18], etc. Many 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 in response to various biotic and abiotic stresses [19,20]. At the same time, abiotic stress can induce a large number of *WRKY* transcription factors to regulate plant tolerance to stress and acquire corresponding resistance [21]. In addition, it is involved in the regulation of plant physiological development, including hormone signal transduction, biosynthesis regulation, leaf senescence, embryo formation, and seed germination [22,23]. In *Arabidopsis*, *AtWRKY33*, *AtWRKY46*, and *AtWRKY57* can enhance the tolerance of *Arabidopsis* to drought and salt stress by regulating the ABA signaling network [24–26]. 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 [27]. Wang et al. found in their study on *Gossypium hirsutum* that the *GhWRKY22* gene can participate in pollen development through transcriptional regulation [28]. Wheat (*Triticum aestivum*) *WRKY7* is an important regulatory factor for leaf senescence, with its expression continuously increasing during the process of natural leaf senescence [29]. *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 [30]. Compared with the wild type, overexpression of *CsWRKY46* enhances cold resistance in cucumber [26]. *OsWRKY71* plays a positive role in cold resistance by regulating downstream target genes in rice [31]. Under cold stress, the induction of *VbWRKY32* in *V. bonariensis* leaves was greater than that in the stems and roots. and overexpression (OE) in *V. bonariensis* increased cold resistance compared with wild type (WT) [32].

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 growing temperature for *V. bonariensis* is 20°C–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 *V. 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 *V. bonariensis*, the *V. bonariensis* is not tolerant to the cold and grows slowly when the temperature is below 10°C. The low-temperature environment in winter seriously affects the yield and ornamental value of *V. bonariensis* [32]. Many studies have suggested that when plants are subjected to cold stress, they will improve their tolerance by regulating the expression of a series of genes. Many transcription factors, including WRKY,

ERF, and MYB, are important regulatory factors related to cold stress [33,34]. The transcriptomic data of *V. bonariensis* under low-temperature stress indicate that WRKY-TFs play a crucial role in helping plants cope with low-temperature stress.

In this study, genome-wide identification of members of *WRKY* gene family in *V. bonariensis* was performed using genomic data measured by our research group, and analyzed their phylogeny, classification, chromosome distribution, conserved motifs, gene structure, *cis*-acting elements. Moreover, we further explored the expression patterns of *VbWRKY* genes in response to cold stresses. Furthermore, 9 *VbWRKY*s under cold stress were analyzed by qRT-PCR. The results provide a theoretical foundation for the study of *V. bonariensis* growth, development, and low-temperature resistance.

2 Materials and Methods

2.1 Plant Materials and Stress Treatment

V. bonariensis seedlings were placed in a chamber with a mean temperature of 25.0 ± 1.0°C, relative humidity of 60% ± 10%, and a day/light cycle of 16/8 h. For the cold treatment, *V. bonariensis* seedlings were placed in low-temperature refrigerator at 4°C and samples were gathered at 0, 3, 6, 9, 12 and 24 h with 0 h as control. The samples were snap frozen in liquid nitrogen and then stored at −80°C freezer to extract total RNA.

2.2 Identification of WRKY Gene Family Members in V. bonariensis

The genome data of *V. bonariensis* were obtained by our research group (unpublished), and the protein sequences of the *Arabidopsis WRKY* gene family were downloaded from the *Arabidopsis* database TAIR (http://www.arabidopsis.org/, accessed on 27 July 2023) [35]. The hidden Markov model (HMM) file of the WRKY domain (PF03106) was downloaded from the Pfam database (http://Pfam.xfam.org/, accessed on 25 July 2023) [36], and used for a search in hmmer (3.0) [37] 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 *V. bonariensis* genome database. Merge and remove duplicates to obtain the candidate *WRKY* transcription factor protein sequence of *V. 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, accessed on 28 July 2023) and Pfam (http://Pfam.xfam.org/, accessed on 28 July 2023) to analyze and screen candidate genes, and ultimately obtain all *WRKY* transcription factor family members of *V. bonariensis*.

2.3 Physicochemical Properties of WRKY Gene Family Players in V. bonariensis

The online tool ProtParam (https://web.expasy.org/protparam/, accessed on 27 July 2023) [38] was used to predict the molecular weight and theoretical isoelectric point (pI) of the protein of the *WRKY* gene family in *V. bonariensis*. Online tools WoLF PSORT (https://wolfpsort.hgc.jp/, accessed on 19 August 2023) [39] were used to predict the subcellular localization of the *VbWRKYs*.

2.4 Phylogenetic Analysis and Multiple Sequence Alignment of WRKY Gene Family Members in V. 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) [40], 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/, accessed on 21 August 2023) [41].

Software DNAMAN and online software Weblogo3 (http://weblogo.berkeley.edu/logo.cgi, accessed on 19 September 2023) were used to perform multiple sequence alignment of the WRKY domain of *V. bonariensis* WRKY protein.

2.5 Visualization of Gene Structure and Conserved Motif of WRKY Gene Family Members in V. bonariensis

The MEME (https://meme-suite.org/meme/tools/meme, accessed on 19 September 2023) [42] tool was used to perform conservative motif analysis on the *WRKY* family protein sequence of *V. bonariensis*, and the number of motif was set to 10. Based on the *V. bonariensis* genome GFF3 annotation file, the gene structure of *VbWRKY* gene was analyzed and visualized by Tb tools software version 2.096 [43].

2.6 Cis-Acting Elements of WRKY Gene Family Members in V. bonariensis

The promoter of the start codon 2000 bp upstream was separated from the genomefile of the *V. bonariensis* using TBtools software version 2.096 [43], and then the *cis*-acting elements of the *VbWRKYs* were found using the online tool PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 5 August 2023) [44] and then visualized using TBtools software version 2.096 [43].

2.7 Collinearity and Chromosome Mapping of WRKY Gene Family Members in V. bonariensis

To identify the pattern of gene duplication, One Step MCScanX from TBtools version 2.096 [43] with default parameters (E-value cut-off < 1 × 10−10 and num of BlastHits with 5) was used to analyze *WRKY* genes in *V. bonariensis*. The results were visualized using TBtools version 2.096. To assess the selection pressure of genes encoding WRKY proteins, the ratio of nonsyn-onymous (*Ka*)/synonymous (*Ks*) (*Ka*/*Ks* is an indicator of selective pressure) was used to evaluate its evolutionary pressure. The values of *Ka*, *Ks*, and *Ka*/*Ks* were calculated by simple *Ka*/*Ks* calculator in TBtools version 2.096 [43].

2.8 Differences in Expression of WRKY Gene Family Members in V. bonariensis

The expression level of *V. bonariensis* in different tissues and under cold stress was measured by our research group. Take materials from different tissues (flowers, leaves, old stems, and tender stems) of *V. bonariensis* for transcriptome sequencing. *V. bonariensis* was treated at low temperature of 4°C, and after 0 and 12 h, take a mixture of uncooled and 4°C cold treated young and old leaves for transcriptome sequencing. The expression levels (FPKM) were calculated with log2 (FPKM+1) [45] and visualized with TBtools software version 2.096 [43]. Analyze the expression changes of *WRKY* gene in the leaves of *V. bonariensis* in different tissues and under cold stress.

2.9 qRT-PCR Analyses of Expression in Response to Cold Stress

Total RNA was extracted using the E.Z.N.A.®Plant RNA Kit (Omega, Xibao Biotech, Shanghai, China) following the manufacture's instruction. The concentration of the isolated RNA samples were examined in a biophotometer (D30, Eppendorf, Germany). The gene-specific primers used in this study were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 24 April 2024), and *VbActin* was applied as reference gene. The first-strand cDNA was synthesized using the StarScript III RT Mix with gDNA Remover StarScript III (GenStar, Beijing, China). 2 × RealStar Fast SYBR qPCR Mix (GenStar, Beijing, China) reagents were used to detect the target sequence. Each PCR mixture (10 μL) contained 1 μL of cDNA, 5 μL of SYBR qPCR Mix, 0.5 μL of each primer (10 μM), and 3 μL of ddH_2O. The qRT-PCR were performed using the following program: 95°C for 2 min and 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. Each processing is repeated 3 times, relative gene expression was calculated with the $2^{-\triangle\triangle Ct}$ method, and we used 0 h as an untreated control to calculate the fold change in the expression level of the relevant genes.

3 Results

3.1 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 *V. 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 *V. 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 results showed that 70 *WRKY* members of *V. bonariensis* are located in the nucleus.

Table 1: Basic *V. 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	Ⅱa	WRKYGQK	C_2H_2	210	23564.67	9.17	Nucleus (12)
VbWRKY2	evm.model.Chr01.196	Ⅱc	WRKYGKK	C_2H_2	191	21714.95	6.91	Nucleus (14)
VbWRKY3	evm.model.Chr01.352	Ⅲ	WRKYGQK	C_2HC	343	38671.55	5.49	Nucleus (12)
VbWRKY4	evm.model.Chr01.1236	Ⅱc	WRKYGQK	C_2H_2	370	41334.48	5.31	Nucleus (14)
VbWRKY5	evm.model.Chr01.1680	Ⅱa	WRKYGQK	C_2H_2	267	29952.61	6.45	Nucleus (12.5)
VbWRKY6	evm.model.Chr01.2901	Ⅱb	WRKYGQK	C_2H_2	560	60480.41	5.82	Nucleus (11)
VbWRKY7	evm.model.Chr01.3012	Ⅰ	WRKYGQK	C_2H_2	566	63963.55	6.62	Nucleus (12)
VbWRKY8	evm.model.Chr01.3242	Ⅲ	WRKYGQK	C_2HC	345	37765.82	5.46	Nucleus (13)
VbWRKY9	evm.model.Chr01.3292	Ⅱe	WRKYGQK	C_2H_2	376	41173.74	4.54	Nucleus (14)
VbWRKY10	evm.model.Chr01.3573	Ⅲ	WRKYGQK	C_2HC	376	41662.45	6.19	Nucleus (13)
VbWRKY11	evm.model.Chr01.3574	Ⅲ	WRKYGQK	C_2HC	283	31642.27	6.11	Nucleus (13)
VbWRKY12	evm.model.Chr01.4171	Ⅰ	WRKYGQK	C_2H_2	683	74096.16	6.12	Nucleus (14)
VbWRKY13	evm.model.Chr01.4361	Ⅱb	WRKYGQK	C_2H_2	562	60561.52	8.42	Nucleus (13)
VbWRKY14	evm.model.Chr01.4582	Ⅱe	WRKYGQK	C_2H_2	391	42891.24	5.37	Nucleus (14)
VbWRKY15	evm.model.Chr05.888	Ⅱc	WRKYGQK	C_2H_2	334	37774.58	5.42	Nucleus (12)
VbWRKY16	evm.model.Chr05.1400	Ⅰ	WRKYGQK	C_2H_2	508	55442.03	8.64	Nucleus (14)
VbWRKY17	evm.model.Chr05.1615	Ⅱb	WRKYGQK	C_2H_2	476	52551.49	6.22	Nucleus (12)
VbWRKY18	evm.model.Chr05.2053	Ⅱc	WRKYGKK	C_2H_2	97	11299.6	9.52	Nucleus (12.5)
VbWRKY19	evm.model.Chr05.2061	Ⅱc	WRKYGQK	C_2H_2	240	27956.36	6.92	Nucleus (13)
VbWRKY20	evm.model.Chr05.2645	Ⅱb	WRKYGQK	C_2H_2	562	60993.92	7.66	Nucleus (14)
VbWRKY21	evm.model.Chr05.2938	Ⅱe	WRKYGQK	C_2H_2	297	33655.17	5.45	Nucleus (11)
VbWRKY22	evm.model.Chr05.3002	Ⅱc	WRKYGQK	C_2H_2	339	37730.16	6.38	Nucleus (13)
VbWRKY23	evm.model.Chr05.3162	Ⅱe	WRKYGQK	C_2H_2	457	49183.06	5.22	Nucleus (14)
VbWRKY24	evm.model.Chr05.3545	Ⅱa	WRKYGQK	C_2H_2	314	34895.12	8.78	Nucleus (10.5)
VbWRKY25	evm.model.Chr05.4588	Ⅱc	WRKYGQK	C_2H_2	320	35779.9	5.52	Nucleus (11)
VbWRKY26	evm.model.Chr05.4752	Ⅱd	WRKYGQK	C_2H_2	340	37931.89	9.8	Nucleus (13)
VbWRKY27	evm.model.Chr09.767	Ⅱd	WRKYGQK	C_2H_2	331	36544.25	9.65	Nucleus (14)
VbWRKY28	evm.model.Chr09.1872	Ⅰ	WRKYGQK	C_2H_2	499	55138	6.13	Nucleus (14)
VbWRKY29	evm.model.Chr09.2632	Ⅰ	WRKYGQK	C_2H_2	388	43309.09	6.72	Nucleus (13)
VbWRKY30	evm.model.Chr09.3590	Ⅱe	WRKYGQK	C_2H_2	332	37282.15	5.14	Nucleus (14)
VbWRKY31	evm.model.Chr09.3592	Ⅱc	WRKYGQK	C_2H_2	199	22704.51	9.26	Nucleus (12)

(Continued)

Table 1 (continued)

Gene	Gene ID	Type	WRKY area	Zinc finger	Number of amino acids /aa	Molecular Weight/Da	Theoretical/ PI	Subcellular location
VbWRKY32	evm.model.Chr09.3639	Ⅲ	WRKYGQK	C_2HC	335	37618.83	5.71	Nucleus (13.5)
VbWRKY33	evm.model.Chr09.3841	Ⅱd	WRKYGQK	C_2H_2	353	37949.73	9.65	Nucleus (14)
VbWRKY34	evm.model.Chr09.3921	Ⅱa	WRKYGQK	C_2H_2	329	36884.94	5.94	Nucleus (8.5)
VbWRKY35	evm.model.Chr09.4152	Ⅱc	WRKYGKK	C_2H_2	182	20696.69	5.55	Nucleus (8)
VbWRKY36	evm.model.Chr13.546	Ⅱc	WRKYGQK	C_2H_2	235	26932.46	9.17	Nucleus (12)
VbWRKY37	evm.model.Chr13.854	Ⅲ	WRKYGQK	C_2HC	318	35560.8	6.21	Nucleus (8)
VbWRKY38	evm.model.Chr13.1453	Ⅰ	WRKYGQK	C_2H_2	803	88405.53	6.32	Nucleus (12)
VbWRKY39	evm.model.Chr13.2013	Ⅱd	WRKYGQK	C_2H_2	331	36035.83	9.53	Nucleus (14)
VbWRKY40	evm.model.Chr13.2667	Ⅰ	WRKYGQK	C_2H_2	564	61499.51	6.52	Nucleus (13)
VbWRKY41	evm.model.Chr13.3038	Ⅰ	WRKYGQK	C_2H_2	587	65107.59	5.72	Nucleus (14)
VbWRKY42	evm.model.Chr13.3777	Ⅱc	WRKYGQK	C_2H_2	329	35835.24	5.95	Nucleus (14)
VbWRKY43	evm.model.Chr13.3790	Ⅰ	WRKYGQK	C_2H_2	487	53315.63	6.93	Nucleus (13)
VbWRKY44	evm.model.Chr17.292	Ⅱc	WRKYGQK	C_2H_2	295	32602.86	7.68	Nucleus (14)
VbWRKY45	evm.model.Chr17.314	Ⅰ	WRKYGQK	C_2H_2	467	51934.35	6.48	Nucleus (14)
VbWRKY46	evm.model.Chr17.406	Ⅱb	WRKYGQK	C_2H_2	491	53888.67	8.05	Nucleus (14)
VbWRKY47	evm.model.Chr17.1018	Ⅱc			1011	112752.07	5.93	Nucleus (4)
VbWRKY48	evm.model.Chr17.1133	Ⅱc	WRKYGQK	C_2H_2	175	20370.81	9.21	Nucleus (9)
VbWRKY49	evm.model.Chr17.1141	Ⅱc	WRKYGQK	C_2H_2	175	20370.81	9.21	Nucleus (9)
VbWRKY50	evm.model.Chr17.1578	Ⅱd	WRKYGQK	C_2H_2	202	21680.58	9.53	Nucleus (9)
VbWRKY51	evm.model.Chr17.1588	Ⅱd	WRKYGQK	C_2H_2	334	36182.8	9.51	Nucleus (6)
VbWRKY52	evm.model.Chr17.2328	Ⅰ	WRKYGQK	C_2H_2	725	80519.63	6.48	Nucleus (13)
VbWRKY53	evm.model.Chr17.2472	Ⅲ	WRKYGQK	C_2HC	290	33037.09	5.24	Nucleus (9)
VbWRKY54	evm.model.Chr17.2549	Ⅱe	WRKYGQK	C_2H_2	337	36986.11	5.65	Nucleus (14)
VbWRKY55	evm.model.Chr17.3711	Ⅱb	WRKYGQK	C_2H_2	457	51091.26	5.94	Nucleus (13)
VbWRKY56	evm.model.Chr21.189	Ⅱb	WRKYGQK	C_2H_2	634	68722.31	6.01	Nucleus (13)
VbWRKY57	evm.model.Chr21.575	Ⅲ			192	21939.14	8.5	Nucleus (11)
VbWRKY58	evm.model.Chr21.578	Ⅲ			114	12632.38	9.84	Nucleus (5)
VbWRKY59	evm.model.Chr21.1488	Ⅰ	WRKYGQK	C_2H_2	462	52266.82	6.46	Nucleus (14)
VbWRKY60	evm.model.Chr21.2647	Ⅱa	WRKYGQK	C_2H_2	329	36508.7	8.45	Nucleus (13)
VbWRKY61	evm.model.Chr25.84	Ⅲ	WRKYGQK	C_2HC	301	34157.05	5.2	Nucleus (9)
VbWRKY62	evm.model.Chr25.121	Ⅱe	WRKYGQK	C_2H_2	315	34602.29	5.27	Nucleus (10)
VbWRKY63	evm.model.Chr25.425	Ⅱc	WRKYGQK	C_2H_2	227	25629.08	8.2	Nucleus (11)
VbWRKY64	evm.model.Chr25.816	Ⅱc	WRKYGQK	C_2H_2	400	43910.46	6.46	Nucleus (14)
VbWRKY65	evm.model.Chr25.1964	Ⅱb	WRKYGQK	C_2H_2	489	54001.83	5.81	Nucleus (12)
VbWRKY66	evm.model.Chr25.2524	Ⅱe	WRKYGQK	C_2H_2	262	28629.52	5.63	Nucleus (14)
VbWRKY67	evm.model.Chr25.2599	Ⅱc	WRKYGQK	C_2H_2	348	39220.93	6.4	Nucleus (12)
VbWRKY68	evm.model.Chr25.2950	Ⅱb	WRKYGQK	C_2H_2	573	61739.46	6.31	Nucleus (14)
VbWRKY69	evm.model.Chr25.3048	Ⅰ	WRKYGQK	C_2H_2	423	46429.14	5.92	Nucleus (13)
VbWRKY70	evm.model.Chr25.3548	Ⅱb	WRKYGQK	C_2H_2	542	59514.72	6.02	Nucleus (14)

3.2 Chromosome Mapping of VbWRKYs

Using TBtools software version 2.096, the *VbWRKY* gene was mapped to *V. bonariensis* chromosomes. The results showed that the 70 *V. 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, Chr7 has 10 *WRKY* genes, 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 [46], chromosomal regions containing two or more genes within 200 KB can be defined as gene clusters. In *V. bonariensis*, a total of 16 *VbWRKY* genes are clustered into 8 gene clusters, marked in blue in the figure (Fig. 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.

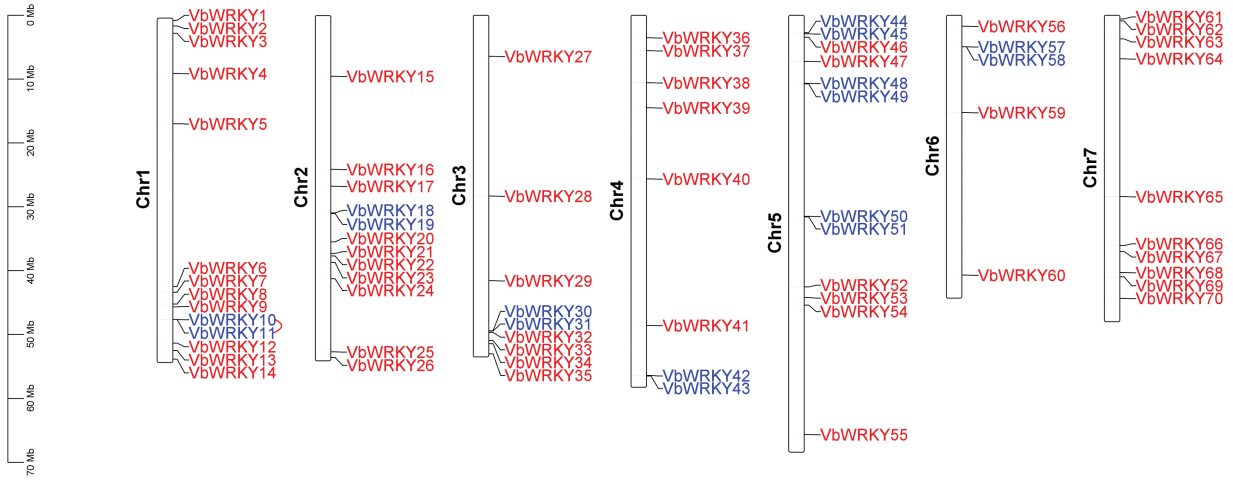

Figure 1: Chromosomal location of *WRKY* gene family members in *V. bonariensis*, gene clusters are indicated in blue

3.3 Phylogenetic Analysis and Multiple Sequence Alignment of VbWRKY Gene Family

Perform phylogenetic analysis on the identified 70 WRKY protein sequences of *V. bonariensis* and 72 known WRKY protein sequences in *Arabidopsis*. The results showed that the identified 70 *V. bonariensis WRKY* transcription factors could be classified into three major groups (Fig. 2), which are in line with the definitions of group I, group II, and group III in *Arabidopsis* by Mangelsen 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. 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 *V. bonariensis WRKY* gene was analyzed by DNAMAN sequence analysis software (Fig. 3, Table 1), The results showed that group I contained two WRKY domains, located at the N and C ends of the sequence, including a WRKYGQK sequence and a C_2H_2-like zinc finger motif, with *VbWRKY38* and *VbWRKY69* having only one WRKY domain. Group II contained one WRKY domain and a C_2H_2-like zinc finger motif. Among them, *VbWRKY47* of IIc has no conserved WRKY domain and zinc finger motif, *VbWRKY18* has a missing zinc finger motif, and WRKYGQK at the conserved sites of *VbWRKY2*, *VbWRKY18*, and *VbWRKY35* has become

WRKYGKK. Group III had a WRKYGQK sequence and the C_2HC zinc finger motif. Among them, *VbWRKY57* and *VbWRKY58* have no conserved WRKY domain and zinc finger motif.

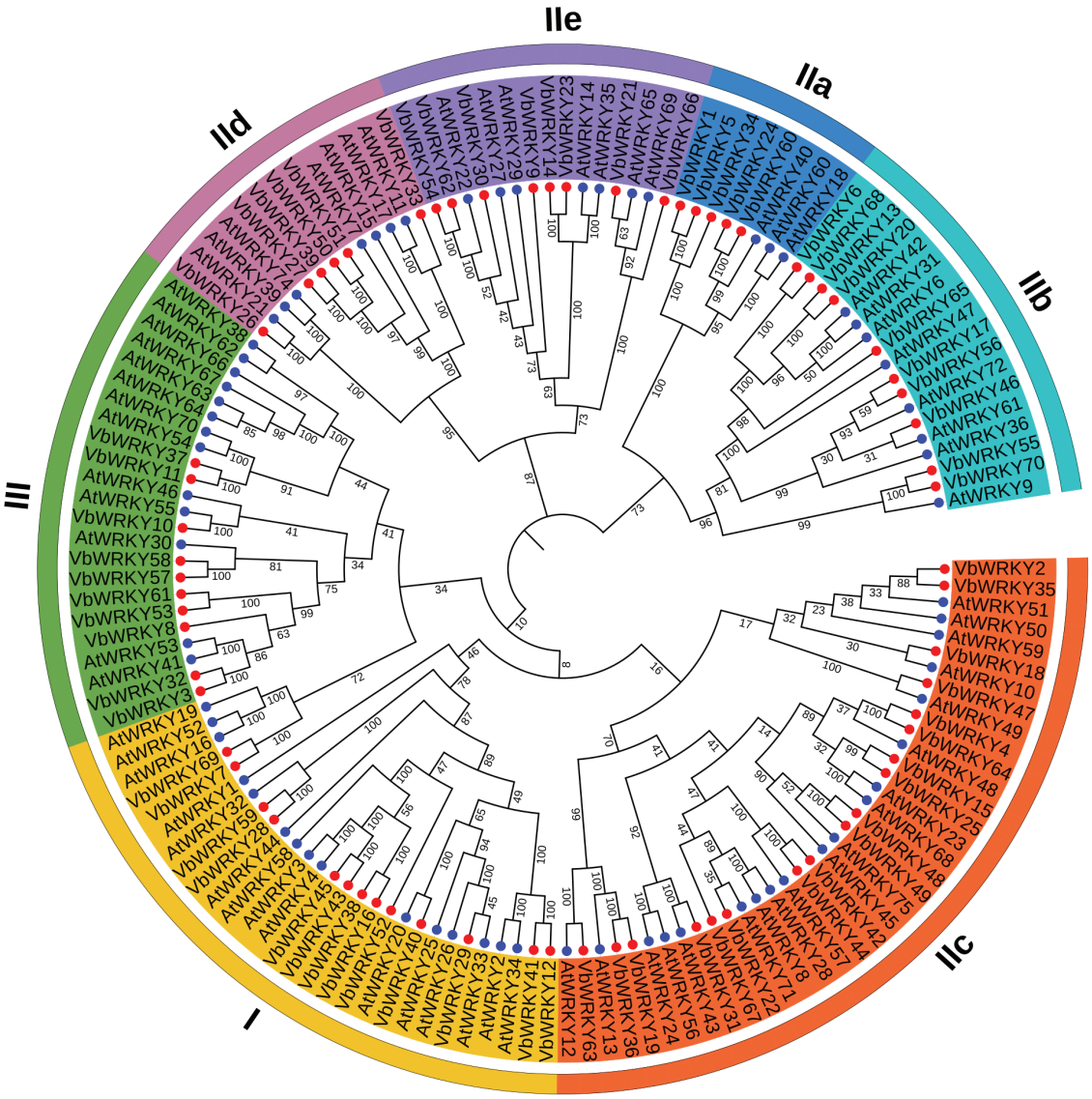

Figure 2: Phylogenetic tree of *WRKY* gene family members in *V. bonariensis* and *Arabidopsis*

3.4 Gene Structure and Conserved Motif Analysis of WRKY Family in V. bonariensis

Using MEME online software to analyze 70 VbWRKY protein sequences, 10 conserved motifs were found (Fig. 4). The frequency of motif occurrence 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 constitute the C-terminal WRKY box, motif 5 and otimf 8 form the N-terminal WRKY box (Fig. 5). Group I has two WRKY boxes, with motif 1 and motif 2, motif 5 and motif 8, while the other groups only have N-terminal WRKY boxes, which consists of only motif 1 and motif 2, without motif 5 and motif 8. Motif 1, motif 2, and motif 3 are landmark conserved motifs of the VbWRKYs protein in *V. bonariensis*, with

most (64/70) having motif 1, 2, and 3. In general, 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 (Fig. 2) and identification classification results (Table 1).

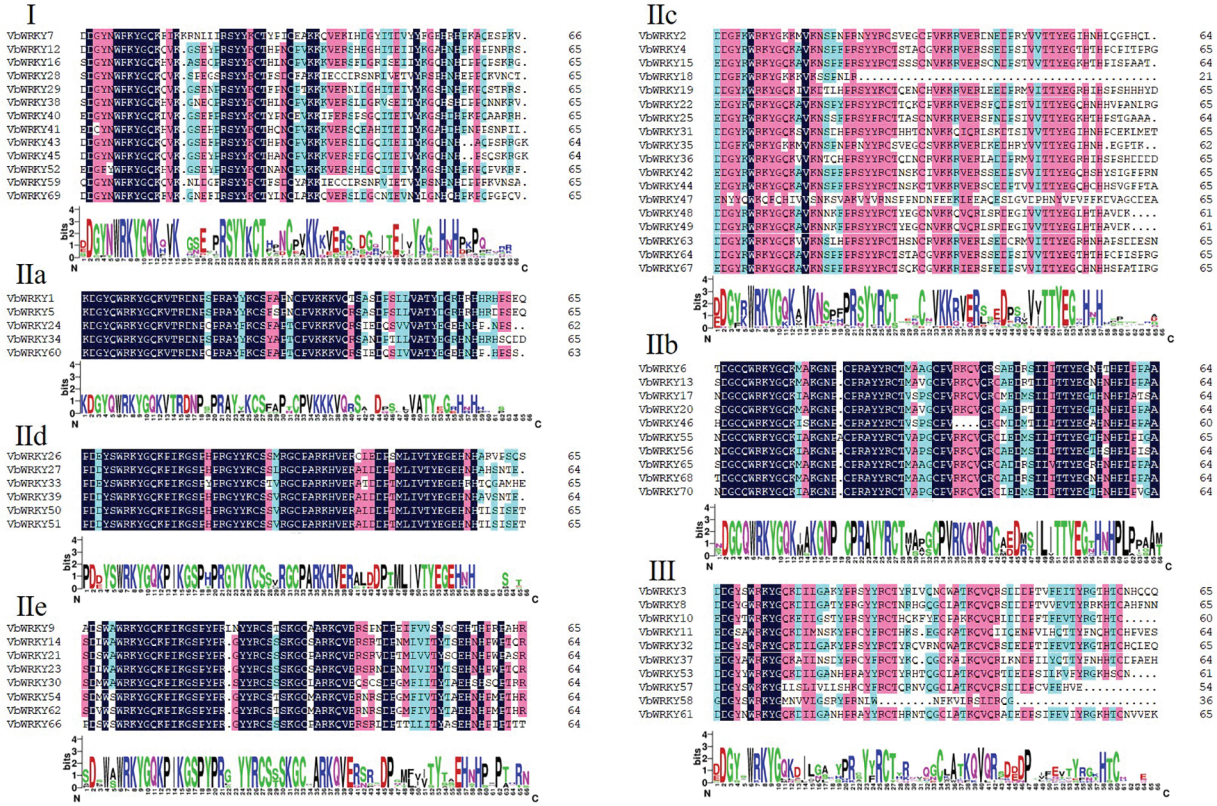

Figure 3: Multiple sequence alignment of *VbWRKY* family members

Gene structure analysis showed that most *V. 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) (Fig. 4). 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 group IIa and IIb contain 3–6 exons, group IIc contains 2–4 exons. The UTR distribution pattern showed that there were 25 *VbWRKY* genes with UTR region, 7 genes only with 5′ UTR region and 5 genes only with 3′ UTR region, a total of 13 genes contained both 5′ UTR and 3′ UTR regions.

3.5 Cis-Acting Elements Analysis of WRKY Gene Family Members in V. 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 (Fig. 6). 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, The analysis of *cis*-acting elements in the *VbWRKY* gene family may help to understand the stress response of *V. bonariensis*, especially under low temperature stress.

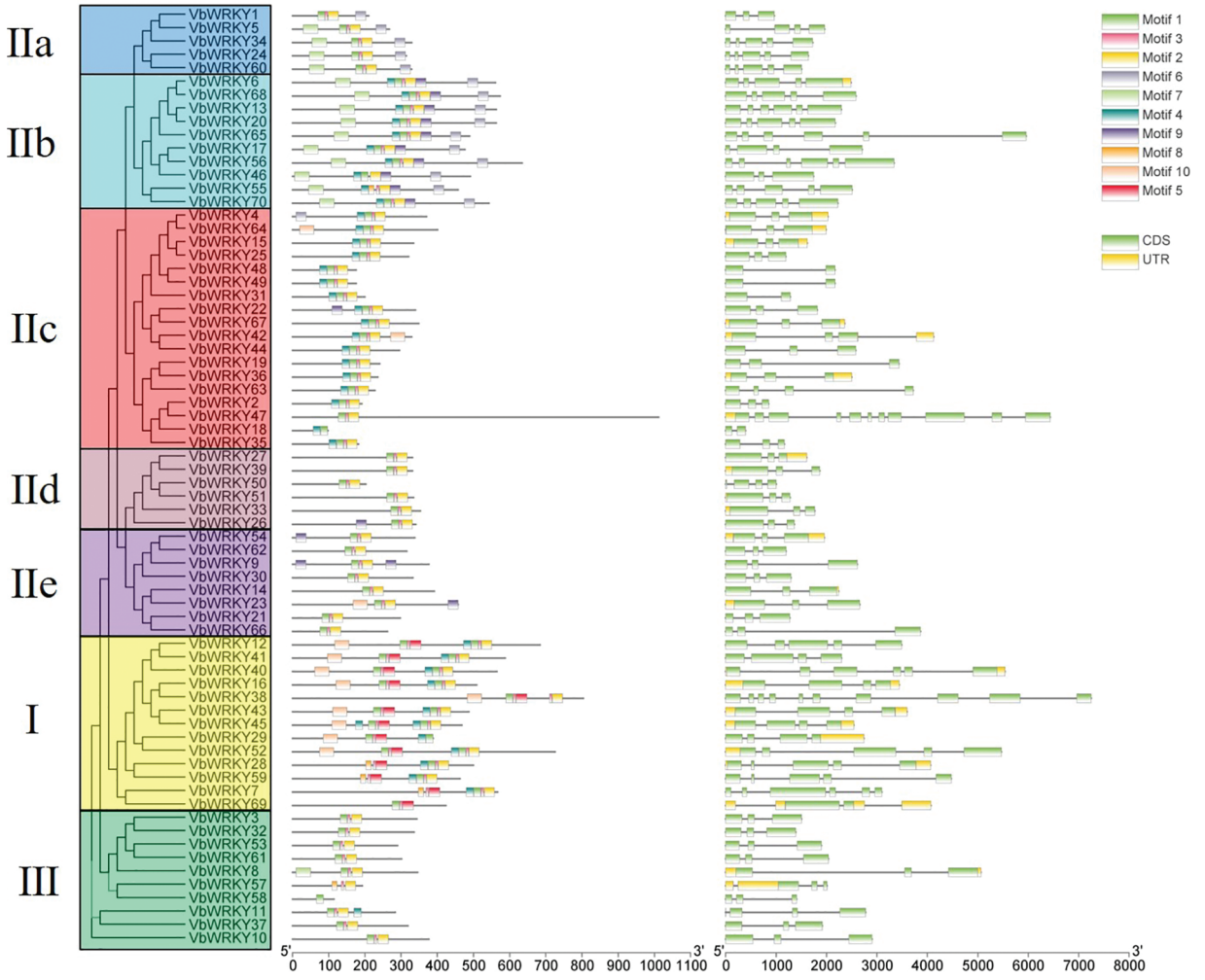

Figure 4: Evolutionary relationship, gene structure, and distribution of conservative motifs of the *WRKY* gene family in *V. bonariensis*

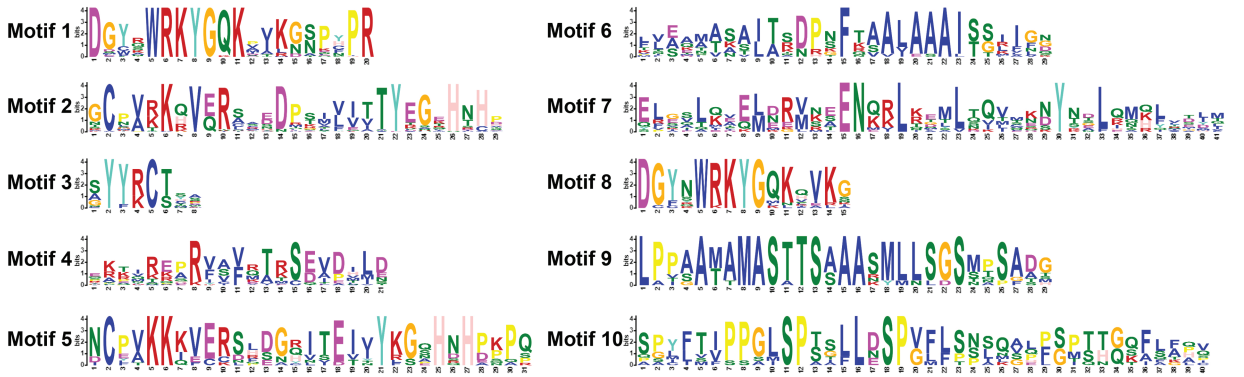

Figure 5: Conservative motif of *WRKY* gene family members in *V. bonariensis*

3.6 Collinearity Analysis of WRKY Gene Family Members in V. 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 (Fig. 7). In addition, *Ka*/*Ks* values were calculated for all members in each group (Table S1) and found that the vast majority of gene pairs with *Ka*/*Ks* values were NaN, this may be due to synonymous mutations in most sites where synonymous mutations can occur [47]. There were no significant differences in *Ka*/*Ks* values between the subgroups, all of which were less than 1, indicating strong purification selection of these *VbWRKY* gene pairs. Among 44 pairs of homologous genes, the *Ka*/*Ks* values of *VbWRKY3*/*VbWRKY8*, *VbWRKY8*/*VbWRKY32*, *VbWRKY9*/*VbWRKY30*, *VbWRKY10*/*VbWRKY37*, *VbWRKY22*/*VbWRKY67*, *VbWRKY30*/*VbWRKY62*, *VbWRKY8*/*VbWRKY53*, *VbWRKY9*/*VbWRKY54* and *VbWRKY7*/*VbWRKY69*, were NaN, the value of *Ka*/*Ks* of the other 35 pairs of homologous genes was less than 1, which indicates that the 35 pairs of homologous genes evolved under great purifying selection or negative selection pressure [48].

3.7 Differences in Expression of WRKY Gene Family Members in V. bonariensis

To further explore the regulation of *VbWRKYs* on growth and low temperature stress, transcriptome data (FPKM) of four tissues (flower, leaf, old stem, tender stem) and cold-stressed plants obtained from the *V. bonariensis* database were calculated and visualized (Figs. 8 and 9). The research found that, except for 7 genes such as *VbWRKY6* and *VbWRKY17*, which are not expressed in different tissues of *V. bonariensis*, there are differences in the expression levels of the remaining 63 *VbWRKY* genes in different tissues of *V. bonariensis* (Fig. 8). *VbWRKY33*, *VbWRKY38*, *VbWRKY39* and other 17 genes had high expressed in almost all tissues. The expression of 30 genes, including *VbWRKY7*, *VbWRKY23*, and *VbWRKY70* was very low in almost all tissues. Sixteen genes, including *VbWRKY2*, *VbWRKY24*, and *VbWRKY52* had highly expressed only in a single tissue. Among them, four genes such as *VbWRKY16*, *VbWRKY25* had highly expressed in flower, and six genes such as *VbWRKY13*, *VbWRK53* had highly expressed in leaf, six genes such as *VbWRKY11* and *VbWRKY14* had highly expressed in stems.

The results showed that 70 *VbWRKYs* genes were expressed in different degrees under low temperature stress, and they were divided into three types according to their expression patterns under low temperature stress (Fig. 9): the first type includes 37 genes, including *VbWRKY2*, *VbWRKY9*, *VbWRKY25*, *VbWRKY40*, *VbWRKY67*, etc., which show increased expression under cold stress, of which 17 are significantly upregulated, with *VbWRKY25* and *VbWRKY45* being the most significantly upregulated. The second type includes 15 genes, including *VbWRKY8*, *VbWRKY23*, *VbWRKY31*, *VbWRKY47*, *VbWRKY51*, etc., which show a decrease in expression under cold stress. The third type includes 18 genes, including *VbWRKY1*, *VbWRKY14*, *VbWRKY21*, *VbWRKY48*, and *VbWRKY63*, which exhibit no response under 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.

Figure 6: *Cis*-acting elements of the *V. bonariensis WRKY* gene family

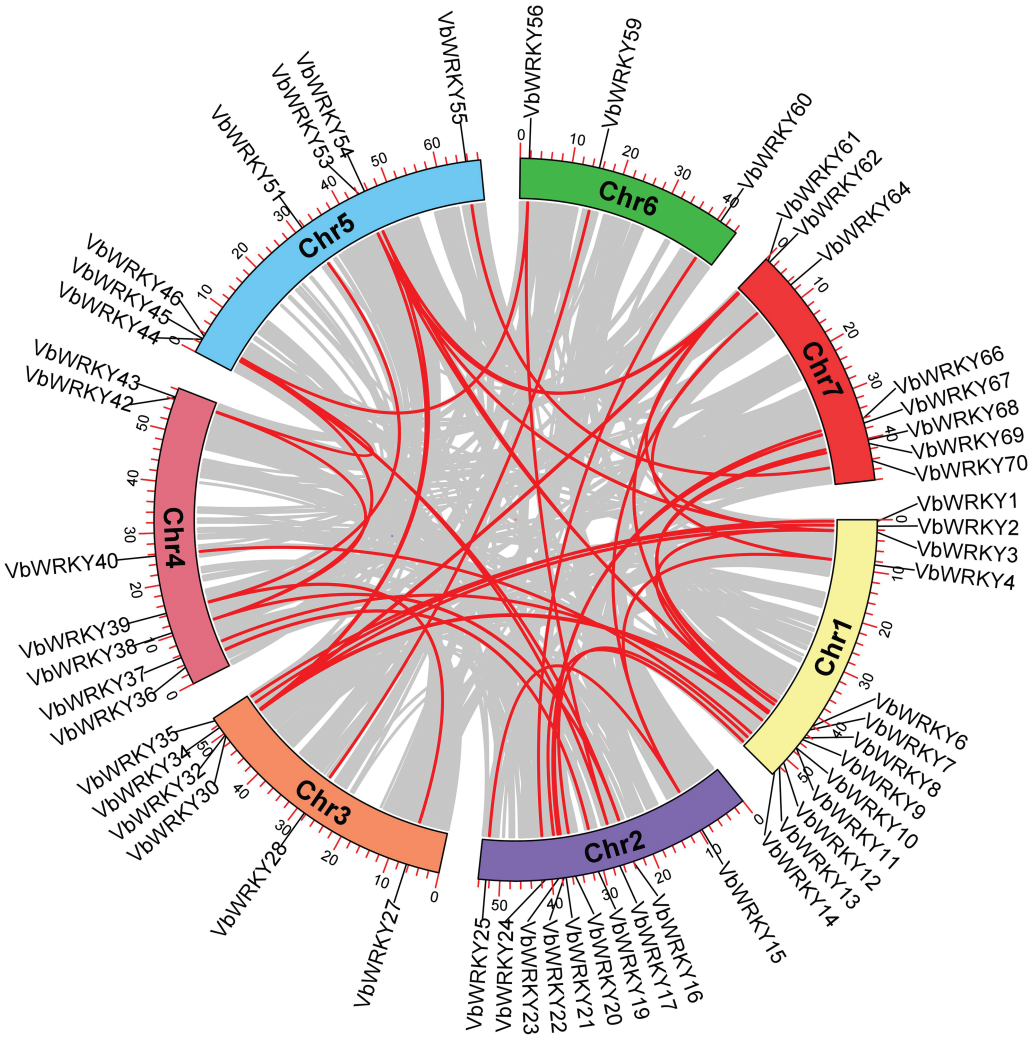

Figure 7: Collinearity relationship of *WRKY* gene family members in *V. bonariensis*. The red lines refer to collinear gene pairs

3.8 Expression Analysis of VbWRKY Genes under Cold Stress

In order to examine the expression patterns of *VbWRKY* genes potentially associated with responses to low temperatures, 9 *VbWRKY* genes from 17 that were significantly upregulated under cold stress were selected and surveyed for their expression levels during different stages of induced low-temperature stress (4°C) (0, 3, 6, 9, 12 and 24 h) (Fig. 10). Under cold treatment, 9 *VbWRKY*s were induced to present the significant up-regulation at different time points. The highest expression levels in the majority of selected *VbWRKY* genes (*VbWRKY*4, *VbWRKY*9, *VbWRKY*22, *VbWRKY*30, *VbWRKY*45, *VbWRKY*70) were found after exposure to low temperature for 24 h. The expression of *VbWRKY13* was the highest levels at 3 h. The expression of *VbWRKY25* was the highest levels at 12 h and the expression of *VbWRKY54* was the highest levels at 9 h.

4 Discussion

The *WRKY* transcription factor family is a class of transcription factors that are specific to plants and regulate gene expression to participate in various signaling pathways in response to biotic and abiotic stresses [49]. With the continuous completion of genome sequencing for different species, the *WRKY*

gene family has been identified in multiple species. Including 72 in *Arabidopsis* [22], 102 in rice [50], 104 in tomato [51], 139 in Apple [52], 61 in cucumber [53], 89 in *Camellia oleifera* [54], 59 in grape [55] and 116 in cotton [56]. In this study, 70 *VbWRKY* members were identified from the genome of *V. bonariensis*.

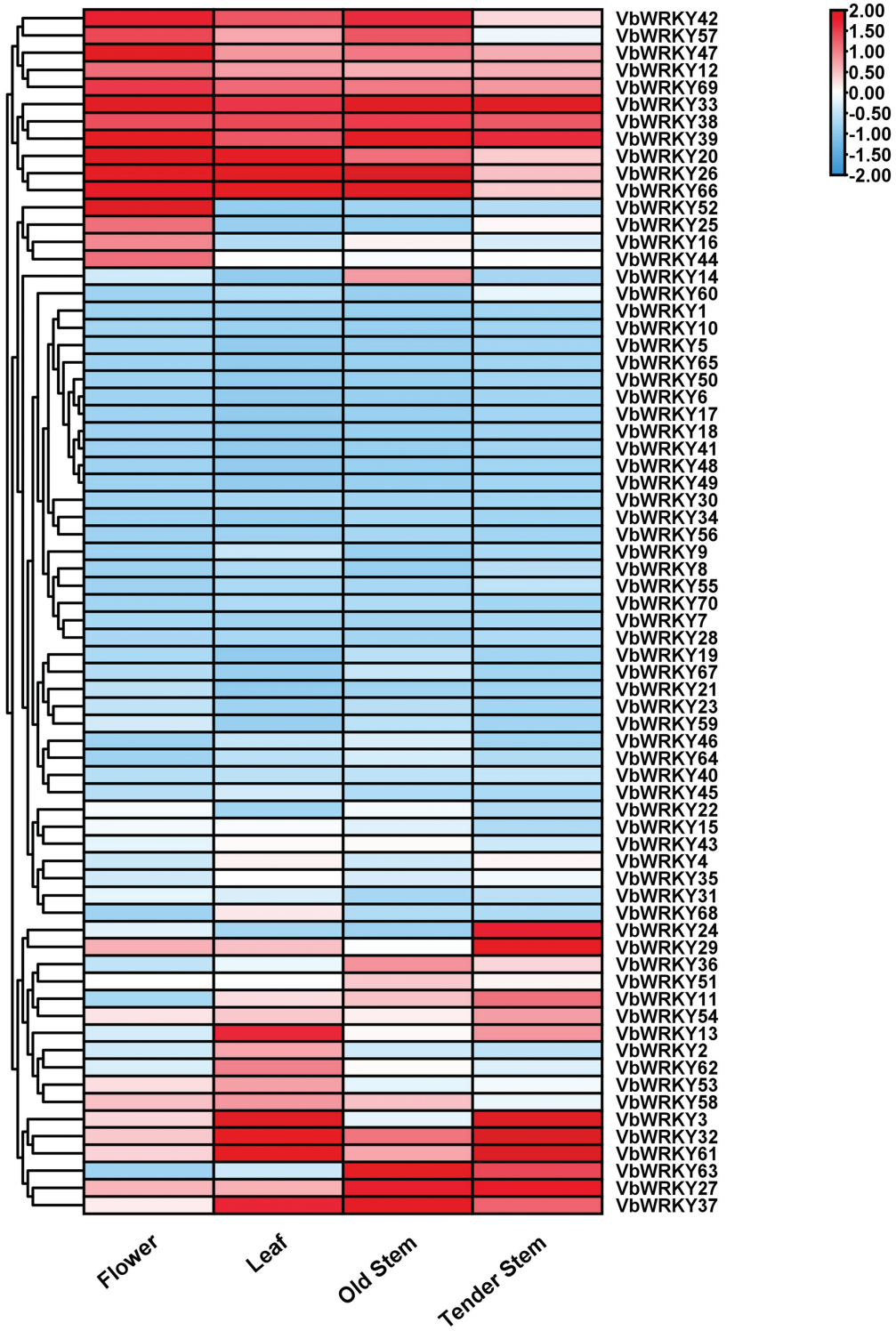

Figure 8: Expression difference of *WRKY* gene family members in different region of *V. bonariensis*

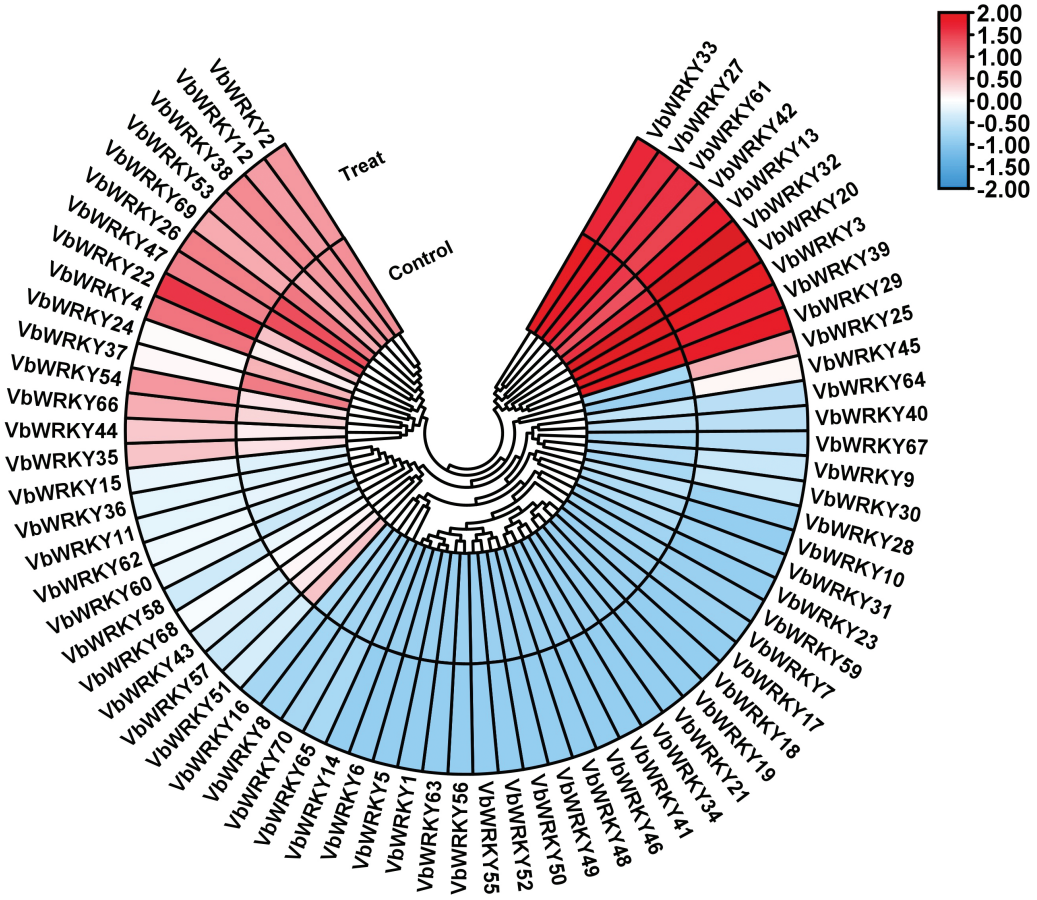

Figure 9: Differences in the expression of *WRKY* gene family members in *V. bonariensis* under cold stress. Control, before cold treatment; Treat, after cold treatment

In this study, 70 members of the *VbWRKY* family were divided into three groups (I, II, and III) based on phylogenetic evolution. Group II was further divided into 5 subfamilies. *VbWRKY38* and *VbWRKY69* in group I lost one WRKY domain during evolution. This phenomenon of WRKY domains loss in group I members is also present in the *Arabidopsis* genome, for example, *AtWRKY10* only contains one WRKY domain [17]. There is a phenomenon of loss and variation of structural domains and zinc finger motifs in groups II and III, such as *VbWRKY47* in group 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 [57]. Thus, loss of the *VbWRKY47*, *VbWRKY57*, and *VbWRKY58* domains may have contributed to the expansion of the *V. bonariensis VbWRKY* gene family. *GmWRKY6* and *GmWRKY21*, which contain WRKYGKK variants in soybeans, fail to bind W-box normally [58]. The *NtWRKY12* of tobacco with the WRKYGKK variant recognizes another binding sequence instead of the normal W-box [59]. In the *V. bonariensis*, the conserved amino acid motif WRKYGQK in the *VbWRKY2*, *VbWRKY18*, and *VbWRKY35* encoded proteins of group IIc was altered to WRKYGKK. It is speculated that the WRKY heptapeptide domain variants of *V. 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 [60]. The zinc finger motifs have been lost or mutated in *VbWRKY18*,

VbWRKY57, and *VbWRKY58* in *V. bonariensis*, possibly resulting in the loss of their original binding domain function and the emergence of new biological functions.

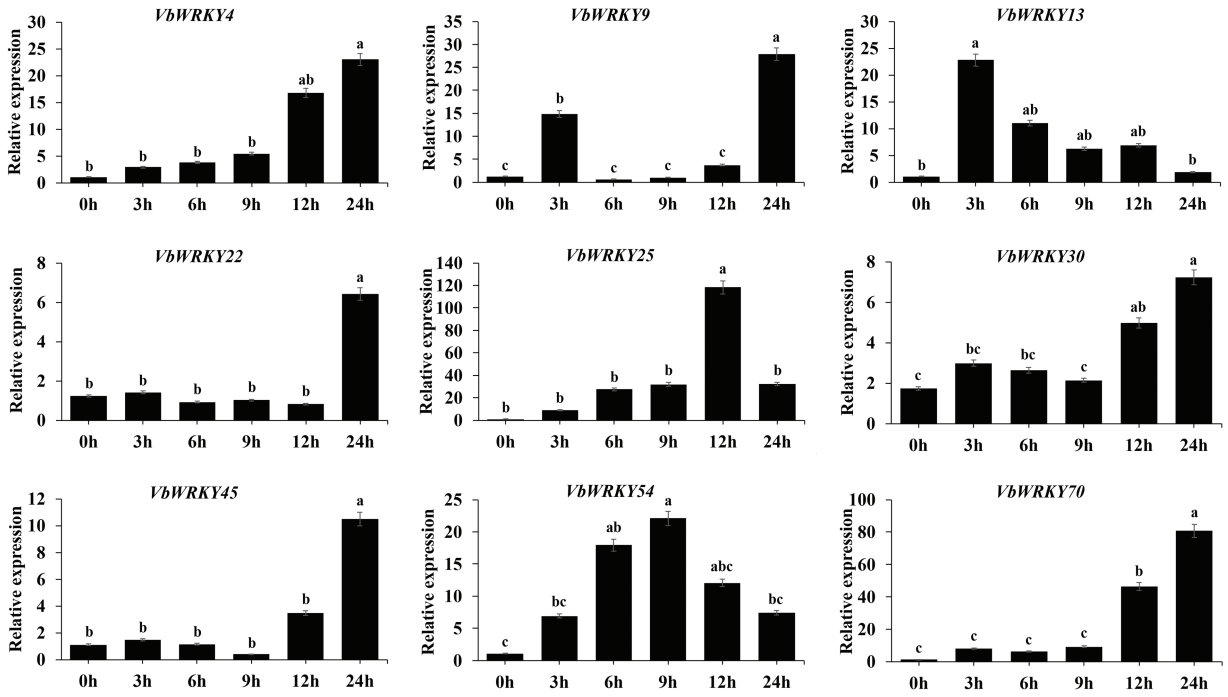

Figure 10: Expression profiles of 9 *VbWRKY* genes under cold treatment. Gene expression of these *VbWRK* genes was analyzed by qRT-PCR. Data represents the mean ± SD of three technical repetition. 0 h was used as an untreated control to calculate the fold change in the expression level of the relevant genes ($p < 0.05$, n = 3)

Gene family members can be distributed in clusters on one chromosome or on different chromosomes. By observing the positions of gene family members on chromosomes, we can judge whether the genes are clustered on the chromosomes. In this study, WRKY members are distributed unevenly on the chromosome, which is consistent with the results of Xu et al. [61] and Mu et al. [62]. 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 *VbWRKY* genes (38/70) contained two introns, similar to the reported in cassava (42/85), cucumber (29/61) and maize (78/140) [63,64]. Previous studies have shown that members of the WRKY family have diverse functions in plant growth and various stress responses, but the functions in WRKY genes within the same group or subgroup usually remain similar [65]. 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. Almost all VbWRKYs contain motif 1 and motif 2, with motif 1 being the conserved seven peptide sequence of WRKYGQK and motif 2 being the zinc finger structural motif, which may have been retained as core elements during evolution. The conserved motifs of the IId and IIe subgroups are similar, which may indicate that they established genetic relationships through evolution. This research result is similar to the conservative motif analysis of SsWRKYs genes by Mu et al. [62] and the conservative motif analysis of *GhWRKYs* genes by Ehsan et al. [66].

The analysis of *cis*-acting elements shows that the promoter region of the *VbWRKY* gene in *V. 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, thereby directly or indirectly regulating various biological processes in plants. Gene duplication events played prominent roles in a succession of genomic rearrangements and expansions, and it is also the main motivation of plants evolution [67]. The gene family expansion occurs via three mechanisms: TDs, SDs and transposition events [68]. Gene duplication was found to play a very important role in the expansion of the *WRKY* gene family. In *V. bonariensis*, a total of 44 segmental duplication events and a pair of tandem repeat genes are identified in *VbWRKYs*. Moreover, for all pairs, the *Ka*/*Ks* ratios are <1, indicating that the *WRKY* gene family in *V. bonariensis* has undergone purifying selection, providing impetus for the evolution of *V. bonariensis*.

It has been found that the *WRKY* gene family is constitutively expressed in many plants. In Salix suchowensis, Bi et al. found that some *WRKY* family genes were expressed in different parts of plants [69]. In tea plant, Pengjie Wang et al. also found that some *WRKY* genes were expressed in different parts of plants [70]. In this study, we analyzed the expression patterns of 70 *VbWRKY* genes in four tissues (flower, leaf, old stem and tender stem). The results showed that 17 *VbWRKY* genes were highly expressed, these 17 genes are presumed to regulate the entire growth and development of *V. bonariensis*. The expression levels of 16 *VbWRKY* genes in only one tissue were high, among which four genes, *VbWRKY25* and *VbWRKY52*, were highly expressed in the flowers of *V. bonariensis*. It is speculated that they are involved in the growth and development process of *V. bonariensis* flowers; 6 genes, including *VbWRKY2* and *VbWRKY62*, have high expression levels in the leaves, suggesting their involvement in the growth and development process of *V. bonariensis* leaves.

The *WRKY* gene family is especially associated with responses to biotic and abiotic stress. Previous studies have shown that *WRKY* genes function in response to cold stress in many plants. For example, experiments by Qiu et al. [71] 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). Holub [46] found that eight *TaWRKYs* genes in wheat (*Triticum aestivum*) had rapid responses to low temperature, high salinity, drought and high temperature. Du et al.'s research results showed that the expression levels of most *KoWRKY* genes, especially *KoWRKY16*, *KoWRKY28*, *KoWRKY32*, *KoWRKY43*, *KoWRKY45*, and *KoWRKY55*, etc., were upregulated after freezing treatment, and their expression increased by more than twice. It was also verified that the expression levels of these 9 genes were upregulated under 4°C low temperature stress [72]. In this study, we investigated the expression patterns of genes in *V. bonariensis* leaves under low temperature stress, which is a common abiotic stress in the production and cultivation of *V. bonariensis*, a total of 52 *VbWRKY* genes were involved in the response to cold stress, 17 (*VbWRKY45*, *VbWRKY25*, *VbWRKY32*, *VbWRKY22*, *VbWRKY4*, etc.) of which were significantly up-regulated. All of these 17 genes had *cis*-acting elements related to cold stress, which further confirmed that these 17 genes were induced by low temperature. 9 *VbWRKY* genes were surveyed for their response to cold-temperature stress in leaf tissue. The results showed that all had altered expression throughout the experiment, and that 9 *VbWRKY*s were induced to present the significant up-regulation at different time points. The highest expression levels in the majority of selected *VbWRKY* genes (*VbWRKY*4, *VbWRKY*9, *VbWRKY*22, *VbWRKY*30, *VbWRKY*45, *VbWRKY*70) were found after exposure to low temperature for 24 h. The expression of *VbWRKY13* was the highest levels at 3 h. The expression of *VbWRKY25* was the highest levels at 12 h and the expression of *VbWRKY54* was the highest levels at 9 h, indicating that these *VbWRKY* transcription factors may function variously at different periods of the stress response. Although WRKYs have been observed to function in many plants in response to low temperature, the mechanism of how WRKYs respond to cold signals and regulate the

expression of downstream genes remains largely unknown. Further research is required to demonstrate the function of these genes in relation to low temperatures and their involvement in cold signal pathways. The function of *VbWRKY* can be verified through experiments such as *cis*-acting elements and transgenic experiments to determine its role.

This study utilized the genome data of *V. bonariensis* previously measured by the research team to identify the members of the *WRKY* gene family of *V. bonariensis*, and analyzed their phylogeny, subgroup types, chromosome distribution, conserved motifs, gene structure, cis acting elements, and transcriptome data in different tissues and under low temperature stress, furthermore, 9 *VbWRKY*s under cold stress were analyzed by qRT-PCR, 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 *V. bonariensis WRKY* transcription factor family.

5 Conclusion

This study identified a total of 70 members of the *WRKY* gene family in *V. 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 *V. bonariensis*, a comprehensive and systematic identification of the *WRKY* gene family was conducted. Its structure is highly conservative and participates in many aspects of *V. bonariensis* growth and development. Analysis of transcriptome data from *V. bonariensis* under low-temperature stress revealed that some *VbWRKY* genes can respond to low-temperature stress and exhibit a positive regulatory effect. Expression profiling revealed that most *VbWRKY* genes were found to have a positive or negative response to cold stresses, and their response changed with the degree of stress. The results of this study provide a theoretical basis for the functional study of the *WRKY* gene in cold stress and the growth and development of *V. bonariensis*, and may also contribute to the screening of cold resistance.

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Author Contributions: Study conception and design: Yan Li; data collection: Tao Zhang, Sisi Wang, Xiuliu Yang; analysis and interpretation of results: Dandan Yuan, Ju Cai; draft manuscript preparation: Dandan Yuan. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

Supplementary Materials: The supplementary material is available online at https://doi.org/10.32604/phyton.2024.052190.

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