

Article

Genome-Wide Identification and Expression Analysis of WRKY Transcription Factors in *Siraitia siamensis*

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Abstract: WRKY transcription factors, as the largest gene family in higher plants, play an important role in various biological processes including growth and development, regulation of secondary metabolites, and stress response. In this study, we performed genome-wide identification and analysis of WRKY transcription factors in *S. siamensis*. A total of 59 *SsWRKY* genes were identified that were distributed on all 14 chromosomes, and these were classified into three major groups based on phylogenetic relationships. Each of these groups had similar conserved motifs and gene structures. We compared all the *S. siamensis SsWRKY* genes with *WRKY* genes identified from three diverse plant species, and the results implied that segmental duplication and tandem duplication play an important roles in the evolution processes of the *WRKY* gene family. Promoter region analysis revealed that *SsWRKY* genes included many *cis*-acting elements related to plant growth and development, phytohormone response, and both abiotic and biotic stress. Expression profiles originating from the transcriptome database showed expression patterns of these *SsWRKY* genes in four different tissues and revealed that most genes are expressed in plant roots. Fifteen *SsWRKY* genes with low-temperature response motifs were surveyed for their gene expression under cold stress, showing that most genes displayed continuous up-regulation during cold treatment. Our study provides a foundation for further study on the function and regulatory mechanism of the *SsWRKY* gene family.

Keywords: *Siraitia siamensis*; WRKY transcription factor; bioinformatics analysis; expression pattern; cold stress



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1. Introduction

WRKY transcription factors are one of the largest families of transcriptional regulators in plants and are integral to signaling webs that modulate many important plant processes [1]. WRKY refers to a highly conserved protein domain, the WRKY domain, with nearly 60 amino acids [2]. The most significant feature of these proteins is that they all contain a WRKYGQK sequence in N-terminal and a C₂H₂ or C₂HC (C-X₄₋₅-C-X₂₂₋₂₃-H-X₁-H or C-X₇-C-X₂₃-H-X₁-H) zinc finger motif at the C-terminal [3,4]. Previous studies have shown that several variants of the WRKYGQK motif exist, including WRKYGEK and WRKYGKK. A typical WRKY motif has therefore been suggested, embracing the consensus sequence W(R/K)(K/R)Y [5]. According to the number of WRKY domains and the type of

zinc-finger motif, WRKY proteins can be divided into three major groups, Group I, Group II, or Group III [6]. Those in Group I contain two WRKY domains and one C₂H₂ (C-X₄₋₅-C-X₂₂₋₂₃-H-X₁-H) zinc finger motif; Group II includes proteins with one WRKY domain and a C₂H₂ zinc finger motif, and can be further divided in five subgroups (IIa, IIb, IIc, IId and IIe) according to the primary amino acid sequence [7]. Group III proteins have one WRKY domain and one C₂HC zinc finger motif. WRKY proteins are capable of binding to the W-box cis-elements [(T)TGAC(C/T)] in the promoter region of the target genes, and can activate, repress or depress transcription, alone or in combination with other regulators [8–10]. Owing to their structure, WRKYs have many important biological functions.

Many studies have shown that WRKY proteins play critical roles in plant growth and development, biotic and abiotic stress response, senescence, secondary metabolism, and phytohormone signaling [11–15]. For example, three *Arabidopsis thaliana* Group III WRKY transcription factors, AtWRKY46, AtWRKY54, AtWRKY70 are positively involved in brassinosteroid-regulated plant growth [16]. In *Solanum lycopersicum*, SlWRKY8 promotes resistance to pathogen infection, and mediates tolerance of drought and salt stress [17]. In *Polygonatum odoratum*, expression of PoWRKY1 is induced by cold and drought stress, and overexpression in *A. thaliana* improved seed germination vigor and root growth of transgenic plants under cold stress and drought [18]. At least 15 WRKY genes in *Acer truncatum* are expressed in cold stress [19]. Furthermore, WRKY40-D and WRKY42-B from *Taraxacum antungense* have been demonstrated to promote initiation of leaf senescence by promoting the jasmonic acid biosynthesis pathway [20,21]. Overexpression of SmWRKY1 in *Salvia miltiorrhiza* and of CrWRKY1 in *Catharanthus roseus* were each shown to positively promote biosynthesis of secondary metabolites, elevating transcripts from genes coding for enzymes in biosynthetic pathways and resulting in their increased accumulation [22,23]. In *Hylocereus polyrhizus*, HPWRKY3 activates sucrose metabolic genes, thus increasing sugar accumulation in pitaya fruit [24]. Many WRKYs have been identified in various plants, including 72 in *A. thaliana* [25], 46 in *Ophiorrhiza pumila* [5], 61 in *Cucumis sativus* [26], and 63 in *Citrullus lanatus* [27]. However, to date there has been no information on WRKY transcription factors in *Siraitia siamensis*.

S. siamensis has been used in traditional Chinese medicine for many years as treatment for lung congestion, colds, and sore throats [28,29]. Its main bioactive ingredients are mogrosides, a kind of triterpenoid sweetener. Among these, mogroside V is a herbal sweetener used in the management of diabetes, and its content is higher in *S. siamensis* than in *Siraitia grosvenorii*. When Siamenoside I is separated from the fruit of *S. siamensis*, it is approximately 560 times sweeter than sucrose [30], ~1.4-fold sweeter than aspartame, and is used as a sugar substitute for diabetic patients [31]. *Siraitia* plants (*S. grosvenorii* and *S. siamensis*) are important perennial vines of the Cucurbitaceae family, with high economic value [32]. The natural distribution range of *S. siamensis* is mainly within Guangxi, Nanning province in China, Thailand, and Vietnam. Compared with *S. grosvenorii*, *S. siamensis* offers many advantages including disease resistance and improved fruit-setting percentage [33]. Therefore, *S. siamensis* may in future become an important source of mogroside V. In recent years, there have been many studies on *S. grosvenorii* [34–36], but research on the molecular biology of *S. siamensis* has to date been focused only on molecular markers [31]. Genome analysis of *S. siamensis* is lacking, especially for gene families that may play an important role in economically important aspects of this species.

In this study, based on the genome data of *S. siamensis* from NCBI (<https://dataview.ncbi.nlm.nih.gov/object/SRR22947134?reviewer=qmfs7mc075pohiv011jqjceh94>, accessed on 2 January 2023), we comprehensively identified and characterized the WRKY gene family with respect to chromosomal location, classification, collinearity, cis-acting elements, expression patterns, and potential protein interactions. Furthermore, 15 SsWRKYs under cold stress were analyzed by qRT-PCR. The results lay a foundation for deeper research on WRKY genes and provide a new perspective on the function of WRKY genes under abiotic and biotic stress.

2. Results

2.1. Identification and Characterization of SsWRKY in *S. siamensis*

To identify potential WRKY genes, the *S. siamensis* genome database (<https://dataview.ncbi.nlm.nih.gov/object/SRR22947134?reviewer=qmfs7mc075pohiv011jqjceh94>, accessed on 2 January 2023) was screened using HMM3.0, and a total of 61 candidates were identified. Of these, two candidate SsWRKY with incomplete WRKY domains were not considered to be SsWRKY. These were classified as SsWRKY-like genes, and were removed from our further analysis. The remaining 59 SsWRKY genes were named SsWRKY1–SsWRKY59 according to their position on chromosome of *S. siamensis*. The detailed characterization of 59 SsWRKY genes is listed in Table 1. The protein length ranged from 128 (SsWRKY14) to 745 (SsWRKY39) amino acids, and the corresponding predicted molecular weights varied from 14.59 kDa to 81.91 kDa, with an average of 40.46 kDa. The theoretical pI ranged from 4.83 (SsWRKY51) to 9.92 (SsWRKY23). Subcellular localization prediction assigned all 59 proteins to the nucleus. The great majority of proteins were considered unstable, except SsWRKY3, SsWRKY10, SsWRKY13, SsWRKY22, SsWRKY23, and SsWRKY50 proteins.

Table 1. Information for the WRKY transcription factor family in *S. siamensis*.

Gene Name	Gene ID	Gene Start	Gene End	Protein Length (aa)	Molecular Weight	Theoretical pI	Instability Index	Subcellular Localization
SsWRKY1	SsiaChr1G00020800.1	751919	757923	190	21,462.72	5.92	45.09	nucleus
SsWRKY2	SsiaChr1G00023090.1	2499410	2504472	546	60,090.33	7.67	58.86	nucleus
SsWRKY3	SsiaChr1G00040860.1	26599342	26603231	438	47,993.39	6.81	38.10	nucleus
SsWRKY4	SsiaChr1G00041580.1	27084596	27087427	461	49,397.04	8.40	45.55	nucleus
SsWRKY5	SsiaChr1G00042810.1	27853616	27855704	295	31,790.99	5.50	59.35	nucleus
SsWRKY6	SsiaChr1G00043600.1	28351114	28353153	305	34,321.93	7.10	71.15	nucleus
SsWRKY7	SsiaChr2G00001280.1	860173	863790	328	36,013.85	6.21	59.23	nucleus
SsWRKY8	SsiaChr2G00004890.1	3652228	3654428	307	33,802.91	8.57	45.82	nucleus
SsWRKY9	SsiaChr2G00004900.1	3661109	3663016	236	26,486.98	9.57	70.01	nucleus
SsWRKY10	SsiaChr2G00005450.1	4137409	4138214	162	18,458.21	5.17	39.23	nucleus
SsWRKY11	SsiaChr3G00044220.1	197881	199214	283	31,834.41	5.65	58.91	nucleus
SsWRKY12	SsiaChr3G00044550.1	429901	432039	466	50,804.16	6.15	61.60	nucleus
SsWRKY13	SsiaChr3G00046440.1	1628752	1633276	363	40,551.22	9.38	36.13	nucleus
SsWRKY14	SsiaChr3G00054430.1	17366784	17369617	128	14,590.29	6.90	57.03	nucleus
SsWRKY15	SsiaChr3G00056180.1	19990778	19992548	304	33,509.51	6.61	66.41	nucleus
SsWRKY16	SsiaChr3G00062240.1	24823802	24825812	349	37,785.78	5.70	58.09	nucleus
SsWRKY17	SsiaChr3G00062690.1	25101188	25103885	349	38,684.93	4.88	54.01	nucleus
SsWRKY18	SsiaChr3G00063680.1	25790770	25794091	220	24,863.42	8.74	45.10	nucleus
SsWRKY19	SsiaChr4G00082080.1	664352	668450	429	46,841.51	5.15	52.61	nucleus
SsWRKY20	SsiaChr4G00083060.1	1571966	1573595	304	33,330.52	5.93	52.89	nucleus
SsWRKY21	SsiaChr4G00085620.1	3371552	3374085	607	65,921.96	6.77	46.81	nucleus
SsWRKY22	SsiaChr4G00090590.1	6827223	6828826	316	34,224.58	9.59	37.56	nucleus
SsWRKY23	SsiaChr4G00091770.1	7817291	7819338	140	16,308.49	9.92	38.24	nucleus
SsWRKY24	SsiaChr4G00095300.1	10761531	10764705	331	36,407.8	9.15	67.77	nucleus
SsWRKY25	SsiaChr5G00105720.1	2172446	2175101	375	40,443.59	9.54	47.46	nucleus
SsWRKY26	SsiaChr5G00107540.1	3423281	3426794	297	34,150.07	9.39	51.45	nucleus
SsWRKY27	SsiaChr5G00108780.1	4344810	4356332	442	47,253.55	9.06	52.87	nucleus
SsWRKY28	SsiaChr6G00152850.1	3845799	3848486	491	54,198.42	9.11	61.97	nucleus
SsWRKY29	SsiaChr6G00153650.1	4474657	4476865	270	30,054.3	6.83	57.17	nucleus
SsWRKY30	SsiaChr6G00159600.1	8993505	8995000	308	33,360.81	9.38	51.73	nucleus
SsWRKY31	SsiaChr6G00161490.1	12439816	12442437	245	27,878.37	9.08	57.29	nucleus
SsWRKY32	SsiaChr7G00067510.1	4245861	4251877	737	79,659.14	5.94	55.04	nucleus
SsWRKY33	SsiaChr7G00068370.1	6214402	6219491	523	57,736.24	6.89	45.11	nucleus
SsWRKY34	SsiaChr7G00073090.1	18164282	18168358	356	40,012.95	5.73	59.24	nucleus
SsWRKY35	SsiaChr7G00073400.1	18453322	18455025	195	21,397.1	7.00	44.92	nucleus

Table 1. Cont.

SsWRKY36	SsiaChr7G00073410.1	18456961	18458359	214	24,010.52	4.95	58.25	nucleus
SsWRKY37	SsiaChr7G00076520.1	21283722	21287776	492	55,112.31	5.61	51.35	nucleus
SsWRKY38	SsiaChr7G00080620.1	24382740	24385570	368	40,365.31	5.82	50.80	nucleus
SsWRKY39	SsiaChr8G00123950.1	11741204	11749949	745	81,911.12	5.51	48.40	nucleus
SsWRKY40	SsiaChr8G00130410.1	18147025	18152970	512	56,211.49	6.28	57.55	nucleus
SsWRKY41	SsiaChr8G00131810.1	19170655	19172307	332	36,140.06	9.55	52.72	nucleus
SsWRKY42	SsiaChr8G00134260.1	20940110	20944180	565	60,663.57	6.89	59.63	nucleus
SsWRKY43	SsiaChr9G00178500.1	390249	392867	603	65,652.99	7.28	62.23	nucleus
SsWRKY44	SsiaChr9G00184810.1	13512314	13516697	258	28,929.78	6.15	44.44	nucleus
SsWRKY45	SsiaChr9G00186390.1	14756723	14760562	296	33,177.89	9.44	64.08	nucleus
SsWRKY46	SsiaChr9G00190810.1	17742607	17745742	582	62,261.62	6.87	41.80	nucleus
SsWRKY47	SsiaChr10G00168450.1	12831606	12834367	294	32,742.6	6.01	52.60	nucleus
SsWRKY48	SsiaChr10G00168460.1	12838628	12843264	342	37,504.25	7.07	51.72	nucleus
SsWRKY49	SsiaChr10G00175160.1	18327226	18331262	509	55,833.51	8.78	55.31	nucleus
SsWRKY50	SsiaChr11G00215290.1	661155	663681	196	21,975.74	9.28	31.80	nucleus
SsWRKY51	SsiaChr11G00221880.1	13766286	13770658	244	27,386.47	4.83	55.79	nucleus
SsWRKY52	SsiaChr11G00221950.1	13823287	13825620	317	34,872.95	6.06	54.27	nucleus
SsWRKY53	SsiaChr11G00223210.1	14943982	14945840	310	34,106.25	8.20	47.54	nucleus
SsWRKY54	SsiaChr12G00195640.1	3637484	3639541	302	33,579.64	5.30	50.52	nucleus
SsWRKY55	SsiaChr12G00203270.1	18044155	18045982	338	37,423.83	6.18	64.65	nucleus
SsWRKY56	SsiaChr13G00140450.1	15745104	15747555	327	36,421.34	9.65	62.67	nucleus
SsWRKY57	SsiaChr13G00142910.1	18011118	18014237	307	34,072.08	7.05	62.00	nucleus
SsWRKY58	SsiaChr14G00204380.1	659304	662023	329	36,799.93	9.69	47.81	nucleus
SsWRKY59	SsiaChr14G00213550.1	16567231	16569495	352	38,011.63	5.78	60.17	nucleus

2.2. Classification and Phylogenetic Relationships of SsWRKYs

Combining 70 AtWRKY protein sequences with 59 SsWRKY protein sequences, MEGA7.0 software was employed to construct the phylogenetic tree and classify the sequences into three groups based on the features of the WRKY gene family in *A. thaliana* (Figure 1). Eleven SsWRKY proteins were classified into Group I, forty SsWRKY proteins were classified into Group II and were further divided into five subgroups, with 4, 5, 16, 7, and 8 members in subgroups IIa–IIe, respectively. Group III consisted of the remaining eight SsWRKY proteins.

Multiple sequence alignments were used to identify the structural characteristics of SsWRKY proteins. As is shown in Figure 2, the majority of the 59 SsWRKY proteins (48/59, 81%) had a single conserved WRKY domain, and the remaining 11 proteins (19%) contained two WRKY domains. The highly conserved WRKY motif represented by 58 WRKYGQK and only one WRKYGKK (SsWRKY10), was identified in 59 SsWRKY proteins, similar to results of studies from other species including *Isatis indigotica* [37], *O. pumila* [5], and tomato [38]. All Group I members contained two complete WRKY domains, including a WRKYGQK sequence and a C₂H₂-like zinc-finger motif. All 40 Group II proteins contained one WRKY domain and a C₂H₂ motif (C-X₄₋₅-C-X₂₂₋₂₃-H-X₁-H). Eight members of Group III had a WRKYGQK sequence and the C₂HC zinc finger motif (C-X₇-C-X₂₃-H-X₁-H).

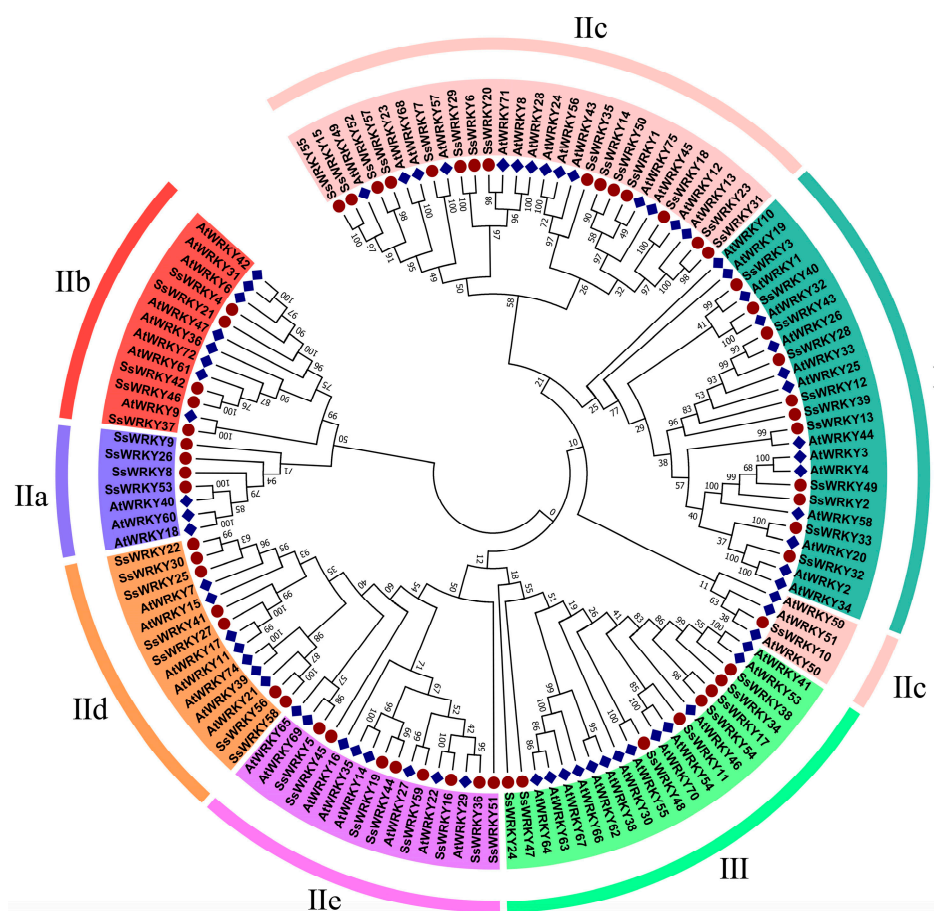


Figure 1. Phylogenetic tree of WRKY proteins from *A. thaliana* and *S. siamensis*. *S. siamensis* and 70 *A. thaliana* WRKY proteins were aligned by ClustW, and the phylogenetic tree was constructed using the neighbor-joining method and MEGA 7.0 with 1000 bootstrap replicates. The WRKY proteins from different groups are indicated with different colors, proteins from *A. thaliana* and *S. siamensis* are denoted by red circles and blue squares.

2.3. Gene Structure and Conserved Motif Analysis

To better illustrate the similarity and diversity of motif composition among SsWRKYs, the MEME program was employed to analyze their conserved motifs. A total of ten conserved motifs were identified in the WRKY proteins of *S. siamensis*, ranging from 15 to 38 amino acids (Figure 3d), as shown in Figure 3 and Table S1. Motifs 1 and 2 were extensively distributed in all SsWRKY proteins, corresponding to the conserved WRKY domain. Motifs 3, 5, and 10 were unique to Group I. Motif 6 was found mainly in Groups IIa and IIb; Motifs 7 and 8 were mainly distributed in Group IIe, Group IId, and Group III; Motif 9 was found only in Groups IIa and IIb (Figure 3b). In general, almost all the SsWRKYs that belonged to the same subgroups displayed similar motif composition, implying that these SsWRKY proteins have analogous functions.

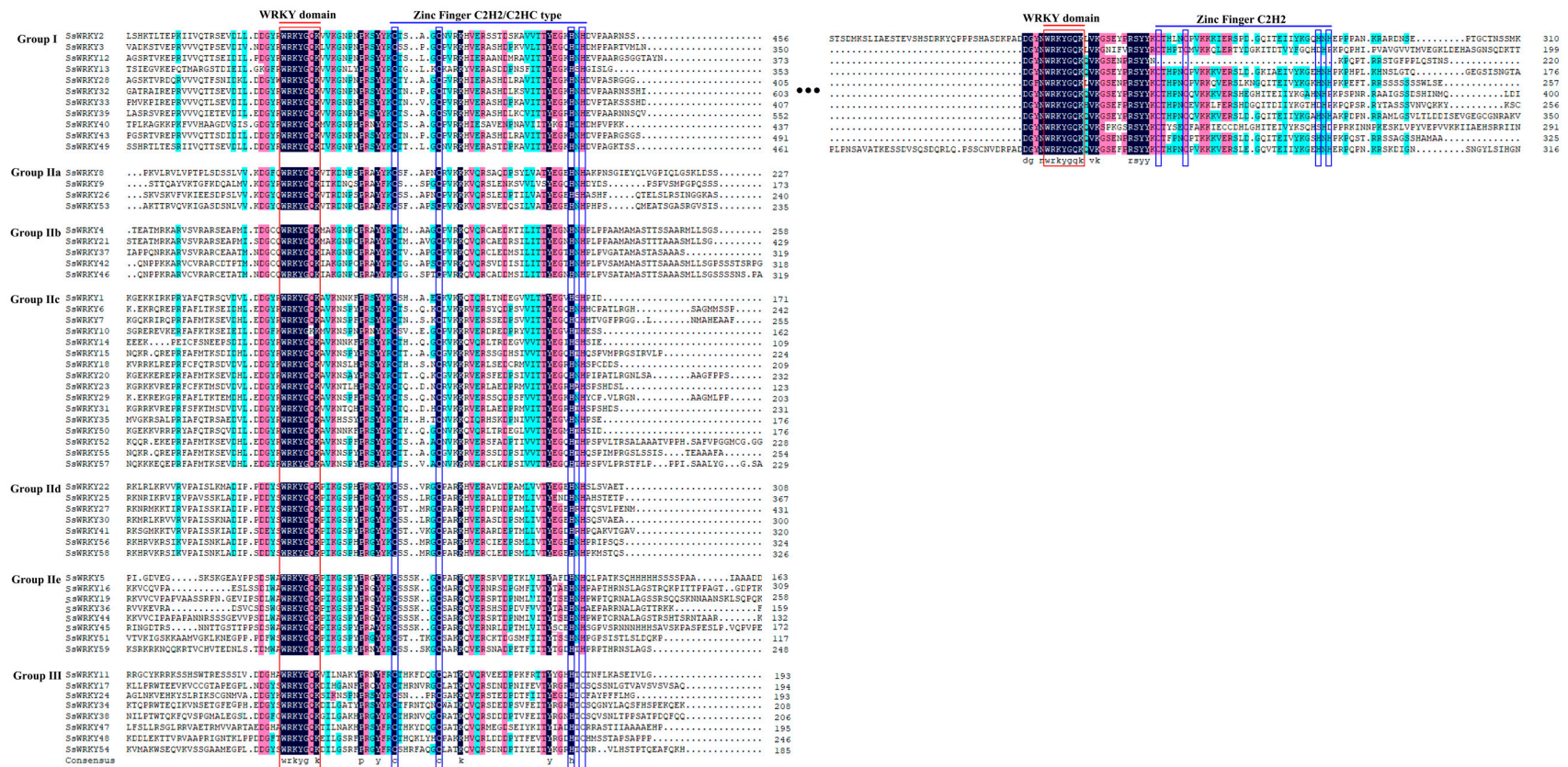


Figure 2. Multiple sequence alignments of the WRKY domain amino acid sequences of 59 SsWRKY proteins, visualized using the DNAMAN7.0 program. The highly conserved regions in each groups are shown in dark blue. The red lines represent the WRKY domains, and the blue lines indicate the conserved zinc finger motifs.

To help clarify the evolution of *SsWRKY* family genes and their genetic structural diversity, the exon–intron organization of the coding sequences of *SsWRKY* genes was examined. As is shown in Figure 3c, the number of the exons in *SsWRKY*s varied from two to seven exons (7 with two exons, 29 with three exons, 8 with four exons, 10 with five exons, 4 with six exons), with *SsWRKY21* containing the highest number of predicted exons ($n = 7$). These results show that the genetic structural diversity among *SsWRKY* genes may be the result of exon-loss and -gain events during the evolution of the *SsWRKY* gene family. *SsWRKY* genes from the same group showed similar exon/intron structures, as in Group IIc. In addition, *SsWRKY* genes with similar structures clustered together, as in Group III and Group IIc which contained three exons and two introns (or three introns) except for *SsWRKY24*, *SsWRKY11*, and *SsWRKY36*.

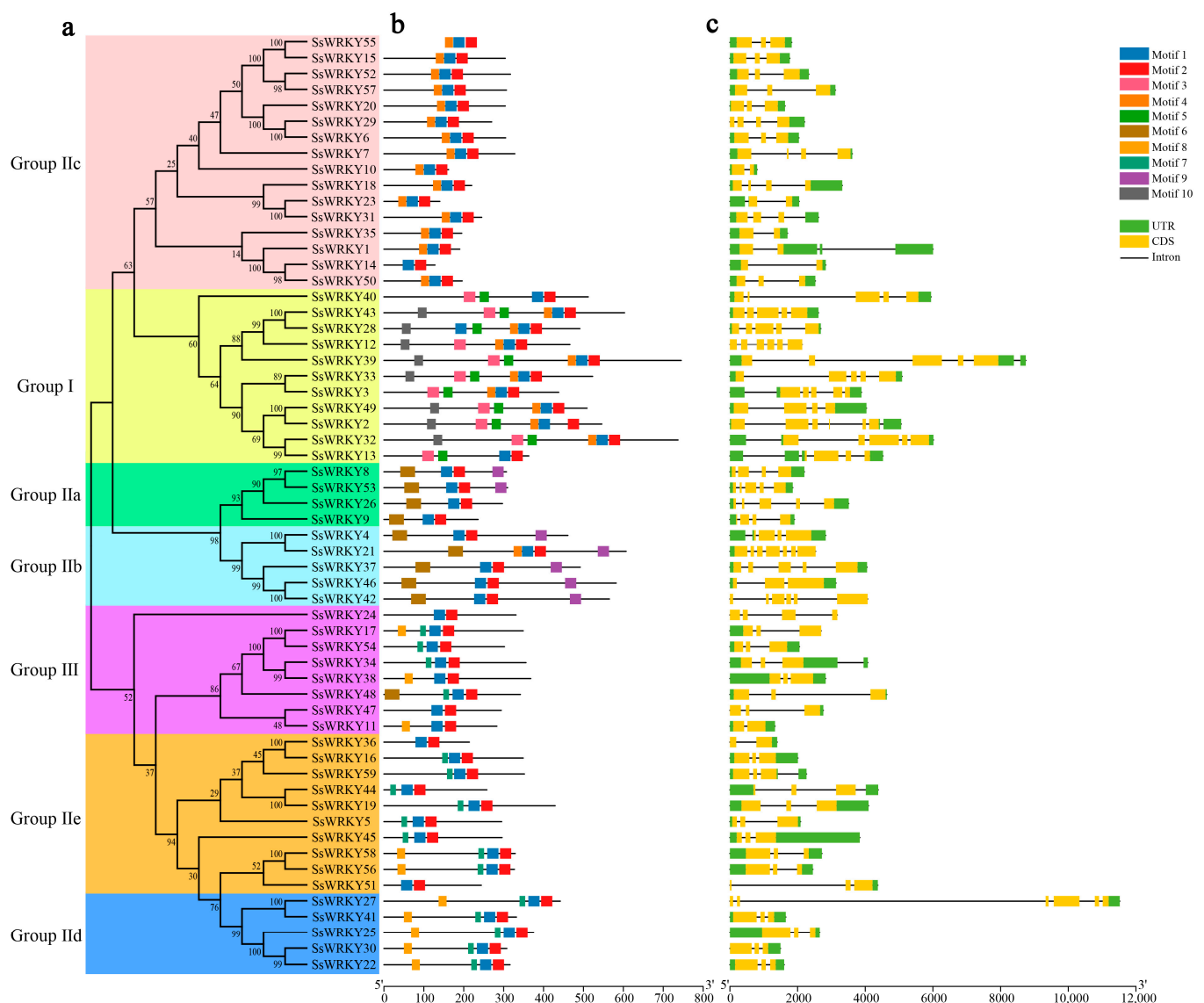


Figure 3. Cont.

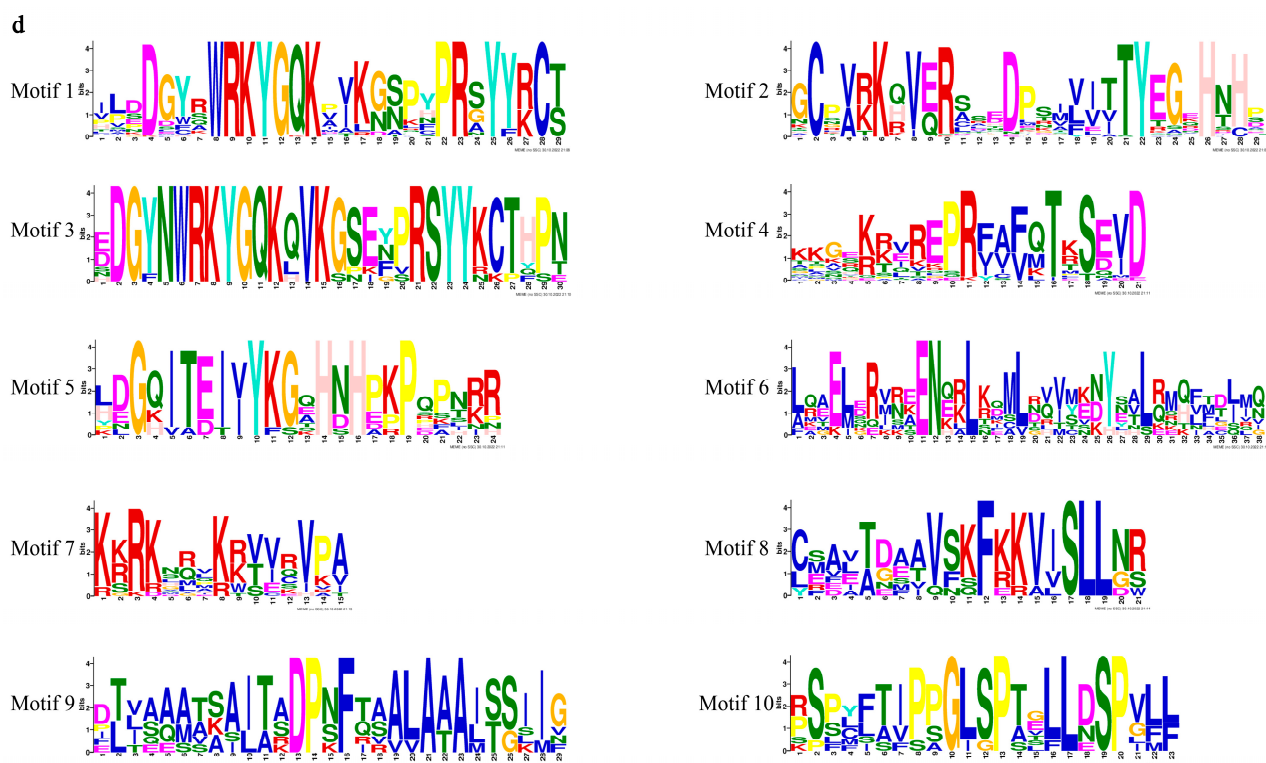


Figure 3. Gene structure and conserved motif analysis of SsWRKY gene family. (a) Phylogenetic tree of SsWRKYs constructed using MEGA7.0 software; (b) Motif compositions of SsWRKY proteins. Ten motifs are represented by different colored boxes numbered 1–10; (c) Exon–intron structural analysis of SsWRKY genes. Green boxes, yellow boxes, and black lines represent UTR, CDS, and introns, respectively; (d) Sequence logos for motif 1–10.

2.4. Chromosomal Location and Collinearity Analysis of SsWRKY Genes

As displayed in Figure 4, all 59 SsWRKY genes were mapped to 14 chromosomes of *S. siamensis*. Remarkably, although WRKY genes were not distributed evenly on every chromosome, they were present on each chromosome. There was no apparent relationship between the number of SsWRKY genes and chromosome length (Figure 4). SsWRKY genes were most abundant on Chr 3, with eight genes, and least abundant on Chr 12, 13, and 14, each with two genes. Collinear blocks within the *S. siamensis* genome were investigated to identify the relationship between SsWRKY genes and gene duplication events (Figure 5a). Twenty-four gene pairs were detected in the *S. siamensis* genome and were located on different chromosomes, suggesting that segmental duplications in these locations potentially contributed to expansion of the SsWRKY family. In addition, only one tandem duplication gene pair was identified, SsWRKY8 and SsWRKY9 located on Chr 2 (Figure 4). Some reports have indicated that tandem gene duplication is an important reason for gene clustering [39]. To enquire into the phylogenetic mechanisms of the SsWRKY gene family, three collinear maps of *S. siamensis* were constructed using three representative species, one monocotyledon (*O. sativa*) and two dicotyledons (*A. thaliana* and *C. sativus*) (Figure 5b–e). A total of 56 SsWRKY genes showed a collinear relationship with *C. sativus* ($n = 56$), followed in terms of frequency by *A. thaliana* ($n = 50$), and *O. sativa* ($n = 20$) (Table S2). There were 85, 72, and 29 orthologous pairs between the other three species, respectively (*C. sativus*, *A. thaliana*, and *O. sativa*). Syntenic pairs were identified with three other species (with 13 SsWRKY genes, SsWRKY7, 8, 11, 12, 16, 26, 36, 38, 40, 45, 47, 49), implying that these gene pairs may have been present before ancestral divergence and played a key role in the evolution of the SsWRKY gene family.

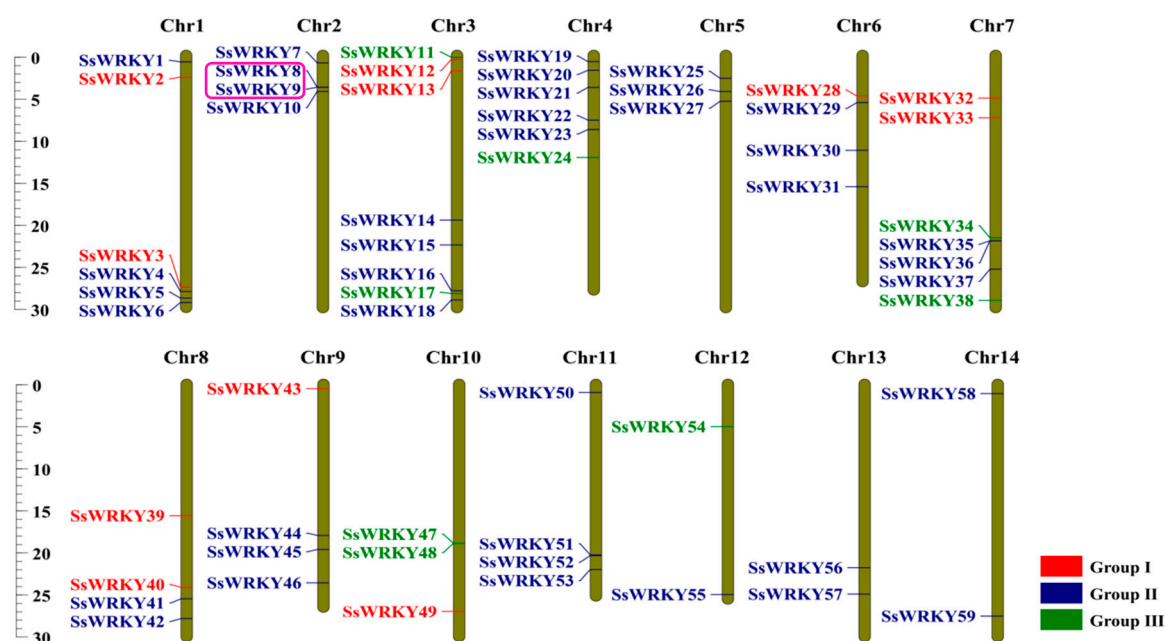


Figure 4. *SsWRKY* gene distribution across 14 chromosomes of *S. siamensis*. Tandem duplicated genes are marked with a pink box. Vertical bars represent chromosomes. The different groups of *SsWRKY* genes are colored red, blue, or green. Base-pair positions are indicated on the left.

2.5. Analysis of Cis-Acting Elements of *SsWRKY* Gene Family

To further our understanding of the potential function and regulation of WRKY transcription factors in *S. siamensis*, nearly 2.0 kb of sequence regions upstream of the translation initiation site was extracted to identify the *cis*-acting elements, using PlantCARE software. Almost all *SsWRKY* genes had at least one *cis*-acting element in the promoter region. These *cis*-acting elements were further classified into three categories, plant growth and development, phytohormone-responsive, and abiotic and biotic stress (Figure 6). The first category, plant growth and development, included light-responsive and development-related elements. Various light-responsive elements were presented in the promoter region of *SsWRKY* genes, containing Box4, G-box, GT1-motif, GATA-motif, Sp1, etc. The Box 4 element (203) was the most abundant *cis*-acting element found in *SsWRKY* promoters. There were also many elements related to development, including CAT-box, MSA-like, and O₂-site. The second category contained phytohormone-responsive elements, composed of the TGACG-motif, TCA-element, ABRE, GARE-motif, etc. For example, the promoter region of *SsWRKY1* had ABRE, AuxRR-core, CGTCA-motif, GARE-motif, and TGACG-motif, indicating that these *cis*-acting elements are related with ABA, auxin, methyl jasmonate (MeJA), gibberellin, and salicylic acid responsiveness. It is speculated that *SsWRKY1* may therefore be involved in multiple hormone responses. The third category included *cis*-acting elements related to abiotic and biotic stress, including antioxidant response element (ARE), low temperature response (LTR) element, drought inducibility elements (MBS), wound responsive elements (Wun-motif), and TC-rich repeats. In *S. siamensis*, 139 ARE elements were present in 53 promoters (90%) of *SsWRKY* genes. In contrast, Wun-motif was the least abundant element in *S. siamensis*. LTR elements were found in 15 promoters of *SsWRKY*. Based on the strong induction in response to cold-stress treatment, *SsWRKY44* and *SsWRKY56* had four copies of the LTR element. Remarkably, the *SsWRKY37* gene did not contain any elements related to abiotic or biotic stress. In general, these results demonstrate that the *SsWRKY* genes might be associated with growth and development, hormones, and stress responses. The analysis of the putative *cis*-acting elements of the *SsWRKY* gene family might improve understanding of stress response, especially low temperature stress in *S. siamensis*.

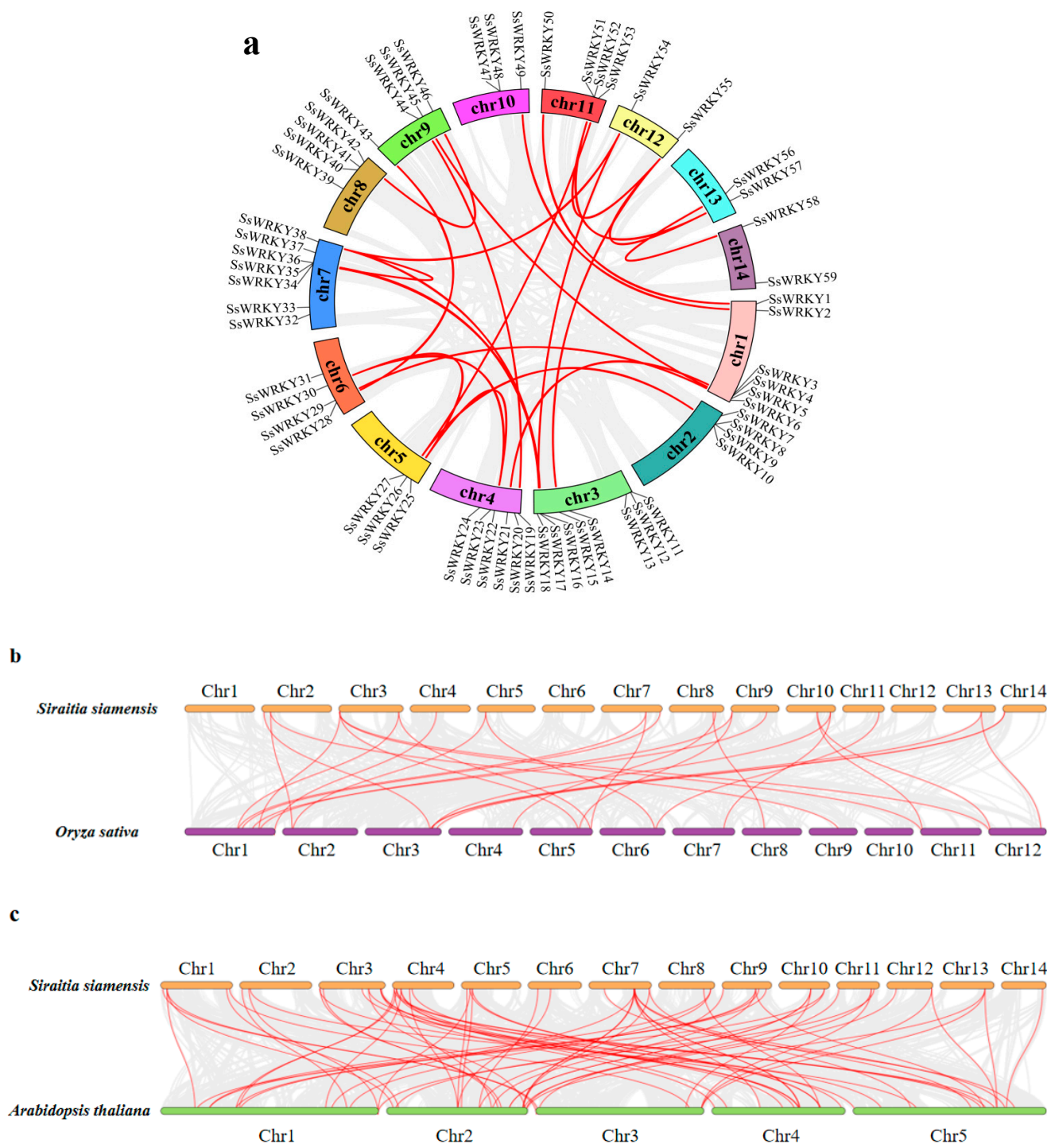


Figure 5. Cont.

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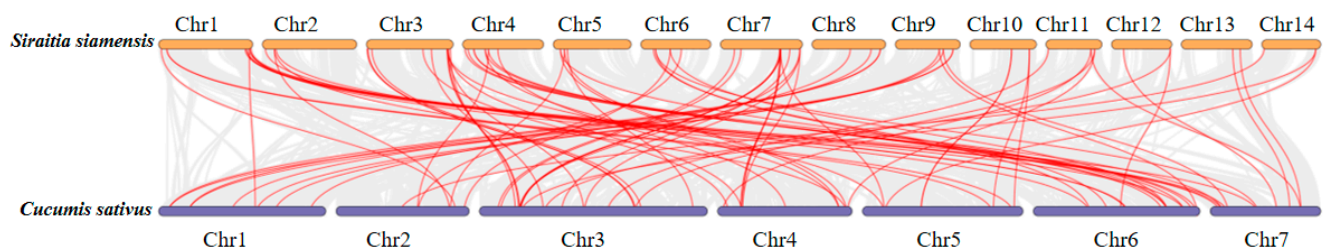


Figure 5. Chromosome-level analysis of the *SsWRKY* gene family in the *S. siamensis* genome assembly. (a) Chromosomal location of *SsWRKY* genes and their collinear relationships are shown in the circle diagram. Gray lines indicate all syntenic blocks in the *S. siamensis* genome, and red lines represent segmentally duplicated gene pairs. Chromosomes 1–9 are denoted by different colors; (b–d) Collinearity analysis of *WRKY* genes between *S. siamensis* and *O. sativa*, *A. thaliana*, and *C. sativus*. The syntenic gene pairs of *SsWRKY* genes are tagged with red lines, and the collinear blocks are presented by gray lines.

2.6. Protein Interaction Network and Gene Co-Expression of *SsWRKYs*

String software was used to construct a protein–protein interaction network of 18 *SsWRKY* proteins based on the *A. thaliana* association model. In the interaction network diagram (Figure 7a and Table S2), connected genes might have close functional relationships. The thicker the connecting line, the stronger is the predicted interaction between the two proteins. Among the 18 *SsWRKY* proteins, there were three proteins from Group I, eleven proteins belonging to Group II, and four proteins in Group III. In addition, *SsWRKY53*, *SsWRKY54*, *SsWRKY11*, *SsWRKY12*, *SsWRKY38*, *SsWRKY47*, *SsWRKY3*, and *SsWRKY39* were shown to have stronger predicted interaction networks with other proteins. These proteins might play a key role in the whole process of *WRKY* regulation. The results of *SsWRKYs* co-expression showed that most genes were co-expressed, with the red color representing higher co-expression levels. As shown in Figure 7b, the cluster members *SsWRKY4*, 5, 27, 28, 41, 42, 44, 45, 46, 47 had high co-expression levels. Among them, the co-expression levels between *SsWRKY44–47* and *SsWRKY4–5*, *SsWRKY27–28*, *SsWRKY41–42* were significantly higher than those among other members.

2.7. Expression Profiles of the *SsWRKY* Genes in Different Tissues

The expression levels of the 59 *SsWRKY* genes were investigated in four different tissues of *S. siamensis* (roots, stems, tubers, and leaves), using the *S. siamensis* transcriptome database (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA917212?reviewer=qmfs7mc075pohiv011jqiceh94>, accessed on 2 January 2023). All 59 *SsWRKY* genes were found to have significant expression in at least one tissue, and changes in expression were found between the different tissues surveyed, except for *SsWRKY12* (Figure 8). The results showed that *SsWRKY* genes had the highest expression levels in the roots; 37% (22/59), 31% (18/59), 29% (17/59), and 2% (1/59) of the *SsWRKY* genes displayed their highest expression levels in roots, tubers, stems, or leaves, respectively. These differences in the expression profiles of the *WRKY* genes indicate potentially different roles in these tissues.

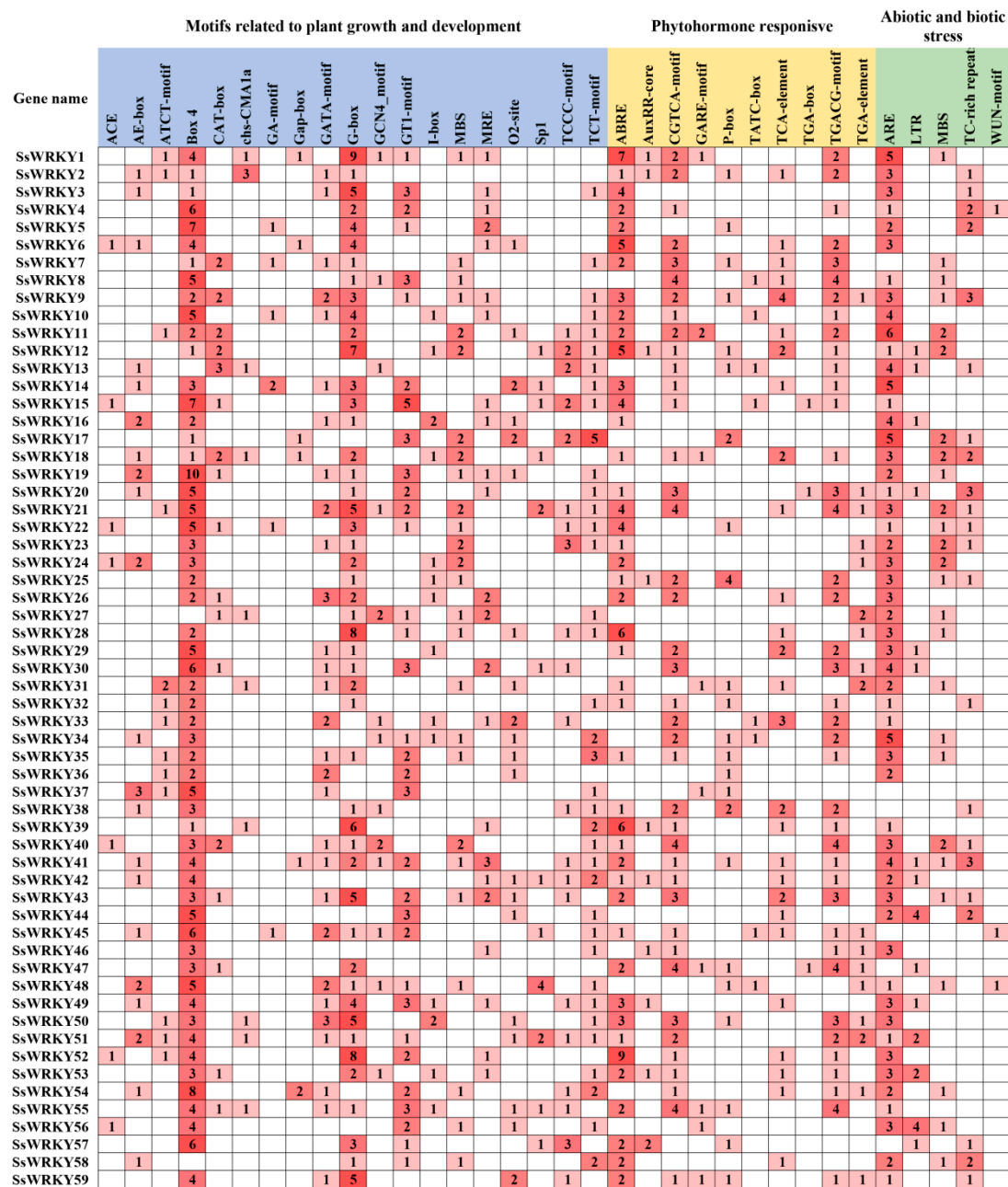


Figure 6. Cis-acting elements analysis in the promoter region of SsWRKY genes. The number of cis-acting elements are shown by the digits. The greater the quantity, the darker the color. Light blue, yellow, and green represent three categories of cis-acting elements related to plant growth and development, phytohormone response, and abiotic and biotic stress.

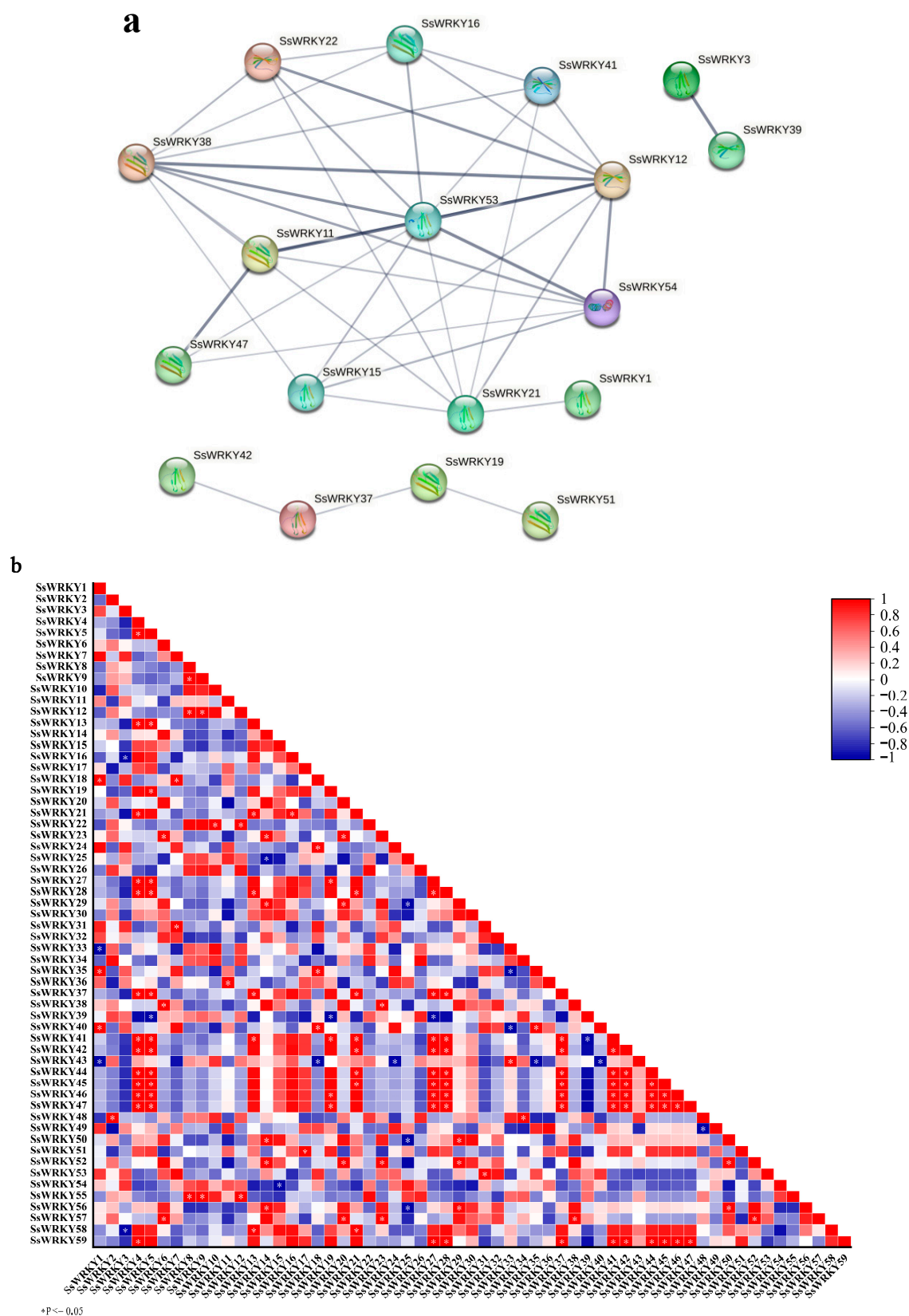


Figure 7. Functional interaction network diagram of SsWRKY protein and co-expression of SsWRKY genes. **(a)** Interaction network diagram of SsWRKY proteins. The thicker the gray connecting line, the stronger is the predicted interaction between the two proteins; **(b)** co-expression analysis of SsWRKY genes. * $p < 0.05$.

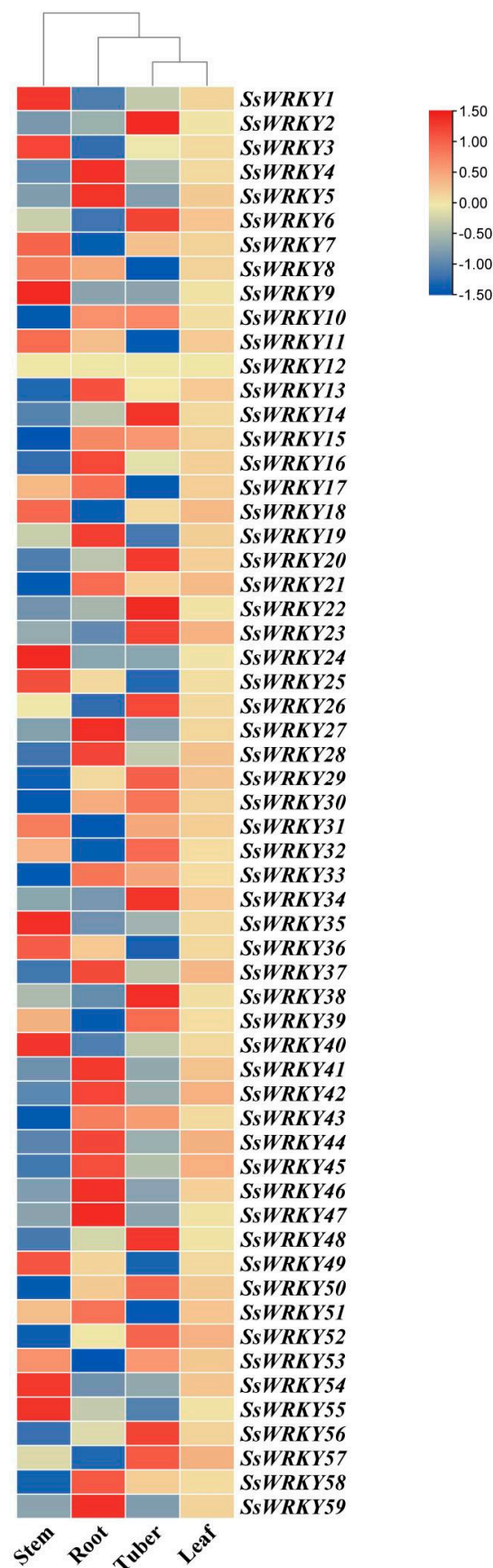


Figure 8. Expression patterns of *SsWRKY* genes in four different tissues of *S. siamensis*. RPKM value was assessed to investigate the expression levels of *SsWRKY* genes in stems, roots, tubers, and leaves. The color variation from blue to red represents low to high expression. Our findings will provide references for the function and regulation mechanism of the *WRKY* gene family in other plants.

2.8. Expression Analysis of *SsWRKY* Genes under Cold Stress

In order to examine the expression patterns of *SsWRKY* genes potentially associated with responses to low temperatures, 15 *SsWRKY* genes containing LTR *cis*-acting elements in their promoter were selected and surveyed for their expression levels during different stages of induced low-temperature stress (4 °C) (0, 6, 12, 24, 36 and 48 h). The expression of the selected *SsWRKY* genes could be classified into three groups according to their expression patterns, as shown in Figure 9. The highest expression levels in the majority of selected *SsWRKY* genes (*SsWRKY12*, *SsWRKY13*, *SsWRKY16*, *SsWRKY20*, *SsWRKY29*, *SsWRKY30*, *SsWRKY47*, *SsWRKY49*, *SsWRKY57*) were found after exposure to low temperature for 48 h. *SsWRKY44*, *SsWRKY51*, and *SsWRKY56* showed their highest levels at 6 h, and then gradually decreased, returning to basal expression levels after 48 h cold treatment, whereas *SsWRKY41*, *SsWRKY42*, *SsWRKY53* were highly expressed at 24 h, falling again to initial expression levels after 48 h cold treatment.

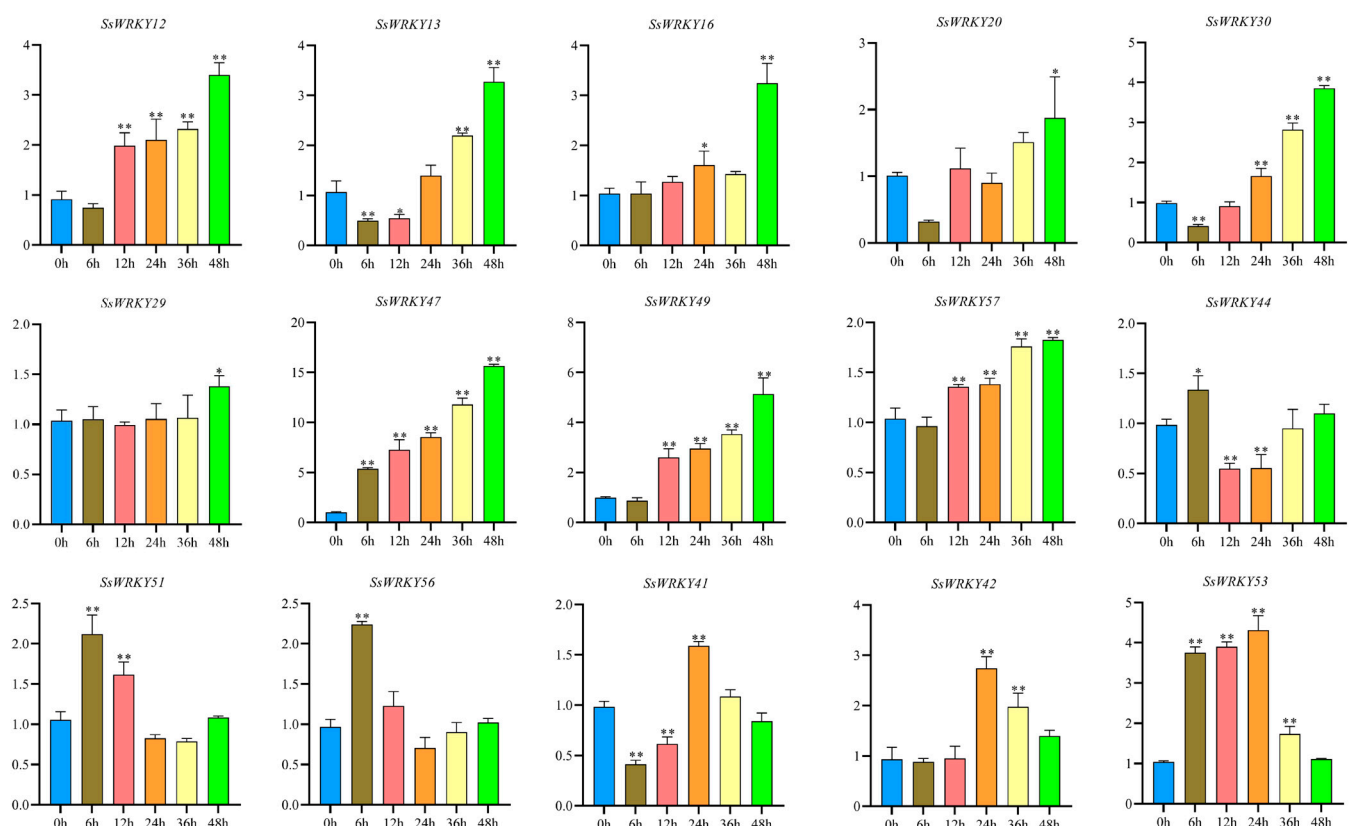


Figure 9. qRT-PCR analysis of *SsWRKY* genes under cold stress. The Y-axes represent the relative expression and X-axes represent the duration of the cold treatment. Data represents the mean ± SD of three technical repetitions. * $p < 0.05$, ** $p < 0.01$.

3. Discussion

S. siamensis is important for its economic and medicinal value [31]. However, little research has been conducted at the molecular level due to a lack of genome and transcriptome data. Studies in many plant species have shown that the *WRKY* gene family is the largest transcription factor family, and members often participate in important plant processes, especially stress response [40]. Based on whole genome sequences, the structure and function of the *WRKY* gene family has been widely investigated in several species including *A. thaliana* [41], *O. sativa* [42], *C. sativus* [26], and *Cucumis melon* [27]. However, there have been no equivalent studies in *S. siamensis*. Genome sequencing of *S. siamensis* has been recently completed (unpublished), providing a means for performing genome-wide

analysis. In this study, we provide identification and analysis of the *SsWRKY* gene family for the first time.

Based on the number of the conserved WRKY domains and the feature of the zinc finger motif, the WRKY gene family was classified into three groups. Following phylogenetic analysis with *AtWRKY* genes, the 59 *SsWRKYs* were divided into three main groups, 11 *SsWRKY* proteins belonging to Group I, 40 *SsWRKY* proteins classified into Group II, accounting for the largest proportion (68%), indicating that this group may have experienced gene duplication events during its evolution, and the remaining eight *SsWRKY* proteins belonged to Group III. Similar results have been reported from *Zea mays* [43], *C. sativus* [26], *Coffea canephora* [44]. Furthermore, variation of the conserved WRKY domain (WRKYGKK) was observed in the *SsWRKY10* protein sequence from the subgroup IIc. A similar situation has also been described in other plants such as *O. pumila* (OpWRKY16, OpWRKY27, OpWRKY28, OpWRKY30, and OpWRKY47), and *C. sativus* (CsWRKY10 and CsWRKY47) [5,26]. Previous research has shown that the WRKYGQK domain can bind to W-box *cis*-elements in the promoter region to interact with target genes [22], and changes of the WRKYGQK domain may influence their binding specificity. Therefore, the function and DNA-binding specificities of the *SsWRKY10* protein should be further analyzed. Based on the analysis of conserved motifs, WRKY members in the same group or subgroup were found to have similar motif composition. Almost all *SsWRKY* contained motif 1 and motif 2, which may be a core element retained during evolution. The conserved motifs of IId and IIe subgroups were similar, which may suggest their genetic relationship through evolution.

Gene duplication events, including tandem, segmental, and whole-genome duplications, are important for acquiring new genes and enabling expansion on gene families in organisms [45]. Among 69 *SsWRKY* genes, only *SsWRKY8* and *SsWRKY9* displayed tandem duplication (Group IIa). Among the *SsWRKY* genes, the number of segmental duplications exceeded the number of tandem duplications. Twenty-four gene pairs were detected in the *S. siamensis* genome and were located on different chromosomes, suggesting that segmental duplications in these locations potentially contributed to expansion of the *SsWRKY* family. Moreover, three collinear maps of *S. siamensis* were constructed with three representative species, and it was shown that 56 *SsWRKY* genes (nearly 95%) were orthologous between *CsWRKY* genes (Table S2), implying that segmental duplication of WRKY genes might have occurred in diploid plants.

A gene's expression profile can often be related to its function. In this study, 59 *SsWRKYs* were analyzed in four different tissues of *S. siamensis*; root, stem, tuber, and leaf. Among *SsWRKYs*, 22 *SsWRKY* genes were expressed in the roots (Figure 7). These results concur with research reported for other species, such as *A. thaliana* [46], *A. truncatum* [19], and *C. sativus* [26]. It may be that roots, as an important organ for absorbing water and nutrients, respond first to stress when a plant is subjected to drought, high salt, or other stresses. For example, *AtWRKY33* is a negative regulator that mediates Pi-deficiency-induced remodeling of root architecture [47]; *AtWRKY6* and *AtWRKY23* were shown to regulate root growth and development [48]. *SsWRKY12*, *SsWRKY21*, and *SsWRKY52* are homologous genes of *AtWRKY6*, *AtWRKY23*, and *AtWRKY33*. These genes may play key roles in root growth and development, and may perform a similar function in *S. siamensis* as they do in *A. thaliana*. *SsWRKY46* is more highly expressed in stems than in other tissues, indicating that some *SsWRKY* genes may be involved in specific organ responses including stress. These results imply that *SsWRKY* genes showing different patterns of expression in different tissues are involved in numerous biological metabolism processes in *S. siamensis*.

The possible regulatory function of *SsWRKY* protein can be better understood using interaction network analysis of WRKY proteins from model plants. Five *SsWRKYs* (*SsWRKY11*, *SsWRKY12*, *SsWRKY38*, *SsWRKY53*, *SsWRKY54*) with high sequence similarity with *AtWRKY62*, *AtWRKY33*, *AtWRKY30*, *AtWRKY40*, and *AtWRKY46* were identified as potential central nodes of the interaction network. Studies have reported that *AtWRKY40* (homolog of *SsWRKY53*) can modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins [49]. Also, *AtWRKY40* can alter resistance

to pathogens. Notably, SsWRKY12 is also a node in the interaction network diagram. AtWRKY33 (homolog of SsWRKY12) has been proposed to interact with AtWRKY30 and AtWRKY15 (homolog of SsWRKY38, 22). Related studies showed that overexpression of AtWRKY33 in *Arabidopsis* could improve plants' salt-tolerance [50]. Furthermore, SsWRKY47 was predicted to interact with SsWRKY11, SsWRKY54, AtWRKY70 (homolog of SsWRKY47), and AtWRKY46 (homolog of SsWRKY54), and to play a key role in the regulation of brassinolide in plant growth, development, and drought tolerance [16]. In addition, a strong interaction between SsWRKY3 and SsWRKY39 was predicted. AtWRKY1 (homolog of SsWRKY3) negatively regulates plants' defense responses to Pst DC3000 through SA signaling pathways [51]. It is known that homologous proteins with similar sequences from different plants may have similar functions. In general, based on previous studies and prediction results of protein-function interaction, it can be inferred that the corresponding SsWRKY gene in *S. siamensis* may also have similar functions, and that closely related SsWRKY genes may have close functional relationships.

The WRKY gene family is especially associated with responses to biotic and abiotic stress [52]. Previous studies have shown that WRKY genes function in response to cold stress in many plants. For example, AtWRKY34 negatively effects the CBF-mediated cold response pathway [53]. In *Vitis vinifera*, cold stress induced the most rapid upregulation of VvWRKY genes of all the tested abiotic stress treatments [54]. In *Brassica campestris*, BcWRKY46 is induced by cold stress and ABA, improving the plant's tolerance of low temperature by activating related genes in the ABA signal pathway [55]. VbWRKY32 positively regulates the expression level of genes in response to cold, which increases antioxidant activity and maintains membrane stability, improving survival ability under cold stress [56]. In this study, 15 SsWRKY genes that contained one LTR *cis*-acting element in the promoter region were surveyed for their response to cold-temperature stress in leaf tissue (Figure 8). The results showed that all had altered expression throughout the experiment, and that nine SsWRKY genes were maximally expressed under cold stress after 48 h. Similar expression patterns were also found in *V. vinifera* [57]. In addition, several SsWRKY genes were highly expressed at 24 h, returning to the initial expression level after 48 h cold treatment, indicating that these SsWRKY 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.

4. Materials and Methods

4.1. Plant Materials and Stress Treatment

S. siamensis plants used in this research were collected from Guangxi Academy of Agricultural Sciences (108.24 E; 22.84 N) (Nanning, China). Tissue culture seedlings of *S. siamensis* were placed in a chamber with a mean temperature of 25.0 ± 1.0 °C, relative humidity of $60\% \pm 10\%$, and a day/light cycle of 12/12 h. For the cold treatment, *S. siamensis* seedlings were placed in low-temperature refrigerator at 4 °C and samples were gathered at 6, 12, 24, 36, and 48 h with 0 h as control. The leaves of six tissue culture seedlings were collected and mixed to provide one sample. The samples were snap frozen in liquid nitrogen and then stored at -80 °C freezer to extract total RNA.

4.2. Sequence Retrieval and Candidate SsWRKYs Identification

The *S. siamensis* genome files (*S._siamensis.gff*, *each_chr.stats*, and *S._siamensis_genome.fa*) were prepared in order to identify SsWRKY genes. The WRKY protein sequences from *A. thaliana* were downloaded from the TAIR database (<https://www.arabidopsis.org/index.jsp>, accessed on 20 October 2022) as the query sequences for identification of homologous SsWRKY sequences, using TBtools software. The HMM file of the WRKY domain (PF03106) was downloaded from the Pfam database (<http://pfam.xfam.org/>, accessed on

21 October 2022) and candidate *SsWRKY* genes were identified using HMMER3.0 (<http://hmmer.janelia.org/>, accessed on 21 October 2022) with E-value $< 1.0 \times 10^{-5}$ [36]. Then, two online software programs, PfamScan (<https://www.ebi.ac.uk/Tools/pfa/pfamscan/>, accessed on 21 October 2022) and SMART (<http://smart.embl-heidelberg.de/>, accessed on 21 October 2022) were employed to identify whether all candidate *SsWRKY* proteins had complete WRKY DNA binding domains including the conserved WRKYGQK/WRKYGKK and zinc-finger motif [58]. Furthermore, we made use of the online website ExPASy (<https://web.expasy.org/protparam/>, accessed on 23 October 2022) to calculate the amino acid length, molecular weight (MW), theoretical isoelectric point (pI), and instability index of all candidate *SsWRKY* proteins, and the online website WoLF PSORT (<https://wolfsort.hgc.jp/>, accessed on 23 October 2022) to predict their subcellular localization [59,60].

4.3. Multiple Protein Sequence Alignment and Phylogenetic Tree Construction

Based on the conserved domain of the *SsWRKY* protein, the family was divided into different groups using DNAMAN7.0 software. The ClusterW tool in MEGA7.0 software was applied to perform multiple protein sequence alignment analysis of WRKYs from *S. siamensis* and *A. thaliana*, using default parameter values and the neighbor-joining method to construct a phylogenetic evolution tree, with 1000 bootstrap iterations [61]. Subsequently, the graphic was transformed using Illustrator 2020 (v24.0.0.330). Based on the reported classification of WRKYs from *A. thaliana*, the *SsWRKY*s were classified into relevant groups.

4.4. Gene Structure and WRKY Protein Conserved Motif Analysis

The DNA and CDS sequences of the *SsWRKY* gene family were screened from the whole genome sequence and gene annotation file of *S. siamensis*. Then, the exon–intron structures of *SsWRKY*s were analyzed and visualized using TBtools software. The MEME v5.1.1 online program (<https://meme-suite.org/meme/>, accessed on 23 October 2022) was used to predict the conserved motifs of WRKY protein, with the maximum number of motifs set to 10 [62]. The diagrammatic sketches of gene structures and conserved motifs were re-edited using Photoshop 2022 software.

4.5. Chromosomal Location, Gene Duplication and Collinearity Analysis

All *SsWRKY* genes were mapped to the 14 chromosomes of *S. siamensis* according to their positions on the *S. siamensis* genome sequence, which was displayed using Circos (<http://circos.ca/>, accessed on 23 October 2022). Blastp was used to analyze the gene duplication events with default parameters and MCSanX to detect collinearity relationship between *S. siamensis* and three other species (*Oryza sativa*, *A. thaliana*, *C. sativus*) [63,64].

4.6. Promoter Cis-Acting Element Analysis of *SsWRKY* Gene Family

TBtools software (GTF/GFF3 sequences extractor) was applied to extract the 2000 bp sequence upstream of the CDS sequences of *S. siamensis*, and the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 23 October 2022) was used for prediction of cis-acting elements in the promoter regions [65].

4.7. Protein Interaction Network and Gene Co-Expression of *SsWRKY*s

For the protein interaction network, String (<http://string-db.org/>, accessed on 23 October 2022) was employed to analyze the *SsWRKY* protein interaction with reference to AtWRKY protein, with the parameter threshold set to 0.15 [66]. *A. thaliana* was selected as the model plant to analyze the co-expression of *SsWRKY*. The network was visualized using Cytoscape version 3.7.0 [67].

4.8. Expression Analysis of *SsWRKY* in Different Tissues

In order to analyze the expression patterns of *SsWRKY* genes in different tissues, the RNA-seq data of *SsWRKY* genes were gathered from the transcriptome database (unpublished), which includes gene expression levels in roots, tubers, leaves, and stems. The values for fragments per kilobase of transcript per million fragments mapped (FPKM) were utilized to calculate the genome-wide expression of the *SsWRKY* genes, which was then visualized on a heatmap using TBtools software, with the expression levels shown by different colors from blue to red.

4.9. Total RNA Extraction, cDNA Synthesis, and qRT-PCR Validation

Samples consisting of 50–100 mg of tissue in liquid nitrogen were fully milled to a fine powder, and total RNA was extracted using the Trizol method as previously reported [68]. The purity and concentration of RNA was measured using a Micro Drop system (BIO-DL, Shanghai, China), and the RNA integrity was checked on 1% agarose gels. Total RNA (1000 ng) was used for reverse transcription using an Evo M-MLV RT mix kit for qPCR, with gDNA Clean (Accurate Biology, Changsha, China) to remove genomic DNA contamination, at 20 µL volume according to the manufacturer's instructions. The cDNA samples were diluted 5-fold with RNase-free water to use as templates for qRT-PCR analysis. Specific primer pairs were designed using Beacon Designer 7.0 software and were restricted to primer sequences of 18–25 bp, amplicon length 80–150 bp, melting temperature (T_m) 55–60 °C, and GC content of 45–55%; the primers used for this study are shown in Table S3. The reaction was carried out in 96-well plates with a DiceTM Real Time System III thermal cycler (Takara, Japan) and a 20 µL reaction system: 2 × SYBR Green Pro Taq HS premix 10 µL (Vazyme, China), 2 µL cDNA, 0.4 µL each of forward primer and reverse primer, 7.2 µL RNase free water. The amplification reaction procedure was: 95 °C, 30 s; 95 °C, 5 s; 60 °C, 30 s, for 40 cycles. Each qRT-PCR analysis was performed with three technical replicates and three biological replications, the relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method. The relative expression under cold-stress treatment of *SsWRKYs* containing LTR-motifs was visualized using GraphPad Prism 9 software.

5. Conclusions

We identified 59 *WRKY* genes distributed on 14 chromosomes in *S. siamensis* based on a genome-wide analysis. These 59 *SsWRKY* genes were divided into three major groups based on their phylogenetic relationships. The comparison of *WRKY* genes in *S. siamensis* with three diverse species helped to visualize the expansion and contraction of the *SsWRKY* gene family through its evolution. Expression profiles based on the RNA-seq data from four different tissues showed that these genes have complex expression profiles and may therefore have diverse but important roles in roots, stems, tubers, and leaves. Furthermore, a protein–protein interaction network was predicted for 18 of 59 *SsWRKYs*. Finally, 15 *SsWRKY* genes with LTR *cis*-acting elements revealed altered and complex expression in response to cold stress. Our study describes a functional framework for *WRKY* genes, which can provide the basis for further exploration of the function and regulatory mechanism of *WRKY* genes in *S. siamensis*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants12020288/s1>, Table S1: The details of 10 motifs in the protein sequences of *SsWRKYs*; Table S2: Collinearity analysis of *WRKY* genes between *S. siamensis* and *A. thaliana*, *O. sativa*, and *C. sativus*, respectively; Table S3: The primers used for expression analysis for 15 *SsWRKYs* under cold stress.

Author Contributions: Q.T. and C.M. initiated the project, helped to conceive the study, revised the manuscript and participated in the design and coordination. D.M. and W.C. conceived the study, participated in the experiments and data analysis, and drafted the manuscript. I.W.W. and D.Q. participated in data analysis and manuscript revision. Y.S., H.Z., Z.L. and X.L. participated in the

experiments and data analysis. J.H. and Y.Z. participated in the experiments. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All WRKY protein sequences are provided in additional file 2.

Conflicts of Interest: The authors declare no conflict of interest.

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