

# Genome-wide characterization of the WRKY gene family in radish (*Raphanus sativus* L.) reveals its critical functions under different abiotic stresses

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## Abstract

**Key message** The radish WRKY gene family was genome-widely identified and played critical roles in response to multiple abiotic stresses.

**Abstract** The WRKY is among the largest transcription factors (TFs) associated with multiple biological activities for plant survival, including control response mechanisms against abiotic stresses such as heat, salinity, and heavy metals. Radish is an important root vegetable crop and therefore characterization and expression pattern investigation of WRKY transcription factors in radish is imperative. In the present study, 126 putative WRKY genes were retrieved from radish genome database. Protein sequence and annotation scrutiny confirmed that RsWRKY proteins possessed highly conserved domains and zinc finger motif. Based on phylogenetic analysis results, RsWRKYs candidate genes were divided into three groups (Group I, II and

III) with the number 31, 74, and 20, respectively. Additionally, gene structure analysis revealed that intron–exon patterns of the WRKY genes are highly conserved in radish. Linkage map analysis indicated that RsWRKY genes were distributed with varying densities over nine linkage groups. Further, RT-qPCR analysis illustrated the significant variation of 36 RsWRKY genes under one or more abiotic stress treatments, implicating that they might be stress-responsive genes. In total, 126 WRKY TFs were identified from the *R. sativus* genome wherein, 35 of them showed abiotic stress-induced expression patterns. These results provide a genome-wide characterization of RsWRKY TFs and baseline for further functional dissection and molecular evolution investigation, specifically for improving abiotic stress resistances with an ultimate goal of increasing yield and quality of radish.

**Keywords** *Raphanus sativus* · WRKY transcription factor · Abiotic stress · RT-qPCR

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## Abbreviations

aa	Amino acids
BLAST	Basic local alignment search tool
bp	Base pair
Cd	Cadmium
CDS	Coding sequence
GO	Gene ontology
HM	Heavy metal
LG	Linkage group
MW	Molecular weight
Pb	Lead
pI	Isoelectric point
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
TF	Transcription factor

## Background

Sessile organisms such as plants usually encounter several biotic and abiotic environmental challenges, such as drought, salinity, and heat conditions (Chen et al. 2012). Due to the recent global climatic changes, the optimal productivity of crops has continued to decline due to the increase of abiotic and biotic stresses in plants. Furthermore, the high solubility of toxic metals in water results in uptake by plants, which has deleterious effects on the food chain and human health. To overcome these stresses, higher plants continuously develop more complex strategies for their survival under these conditions (Kulhari et al. 2013). Heavy metals (HMs) could negatively influence the morphology, physiology, and biochemistry of plants (Gautam et al. 2016). HMs have also been shown to significantly decline biomass accumulation (Zhao et al. 2012) in addition to detrimental effects on critical metabolic mechanisms including photosynthesis, inorganic nutrition, and water uptake (Mukhopadhyay and Mondal 2015; Rodriguez et al. 2012; Vernay et al. 2007). Transcription factors (TFs) including WRKY are crucial to the adjustment of gene expression in response to environmental stresses (Zhao et al. 2015). More than 20% of the genes in *Arabidopsis thaliana* genome seem to translate into proteins involved in gene transcription (Rushton et al. 2010).

Transcription factors (TFs) have sequence-specific DNA-binding domains and perform integral roles in plant development by temporarily and spatially regulating their target genes (Zhang et al. 2011). WRKY family members have been found to play key roles in biotic and abiotic stress responses in plants (Zhao et al. 2015). WRKY protein can interact with the W-box [TGACC (A/T)] in the promoter of their target genes to induce or repress the expression of downstream genes to activate their stress response (Zentgraf et al. 2010). In *Arabidopsis*, *AtWRKY22* initiates submergence tolerance by interacting with the ACS7 promoter and activating downstream ethylene response (Hsu et al. 2013). *BhWRKY1* interacts with *BhGolS1* promoter involved in promoting dehydration and cold tolerance (Wang et al. 2009). Some WRKY TFs are implicated in the coordination of several biological activities such as salt (Chen et al. 2012), heat (Tang et al. 2013), HM (Wang et al. 2013), and other biotic and abiotic stresses (Tang et al. 2013). Additionally, double mutants, *wrky26–wrky25* and *wrky33–wrky25*; and triple mutants, *wrky33wrky26wrky25* in *A. thaliana* exhibit susceptibility to thermal stress (Li et al. 2011). In *Tamarix hispida*, *ThWRKY7* under CdCl<sub>2</sub> treatment improved Cd tolerance by acting as an upstream regulator of *ThVHAc1* gene (Yang et al. 2016). Noteworthily, single WRKY gene can regulate multiple abiotic responses by interacting with several VQ proteins (having VQ-linked motif including

SIB1 and SIB2 (Sigma Factor-Interacting Protein) to modulate multiple abiotic stresses (Lai et al. 2011). In *O. sativa*, *OsWRKY74* positively regulates phosphate (*Pi*) homeostasis and signal transduction, Fe deficiency, and cold stress in rice (Dai et al. 2015). Overexpression of *GhWRKY41* in tobacco promotes salinity and desiccation tolerance by modulating stomatal conductance and ROS levels (Chu et al. 2016). *PsWRKY* from *Papaver somniferum* is implicated to be induced by multiple treatments, including salinity, cold, dehydration, wounding, phytohormones (ABA, MeJA) as well as modulating benzyloquinoline pathway (Mishra et al. 2013).

Radish (*Raphanus sativus* L.) production continues to be affected by biotic and abiotic factors. Thus, it is critical to explore the molecular mechanisms regulating multiple biological processes in radish. Recently, drafts of the *R. sativus* databases were published (Kitashiba et al. 2014; Mitsui et al. 2015), providing a valuable gateway for genome-wide and comparative analysis of putative TFs. Previous transcriptome studies on radish have mentioned the expression of TFs including, NAC, MYB, ERF, and WRKY under stress and normal growth conditions, Pb (Wang et al. 2013), salt (Sun et al. 2016), heat (Wang et al. 2015b), and taproot thickening (Yu et al. 2015). In this study, genome-wide exploration and classification of WRKY TFs were performed. The conserved motif and exon–introns distribution, linkage group localization, and in silico transcriptome expression analysis were conducted. Additionally, the expression of the *RsWRKY* gene validation was subsequently investigated under different abiotic stresses.

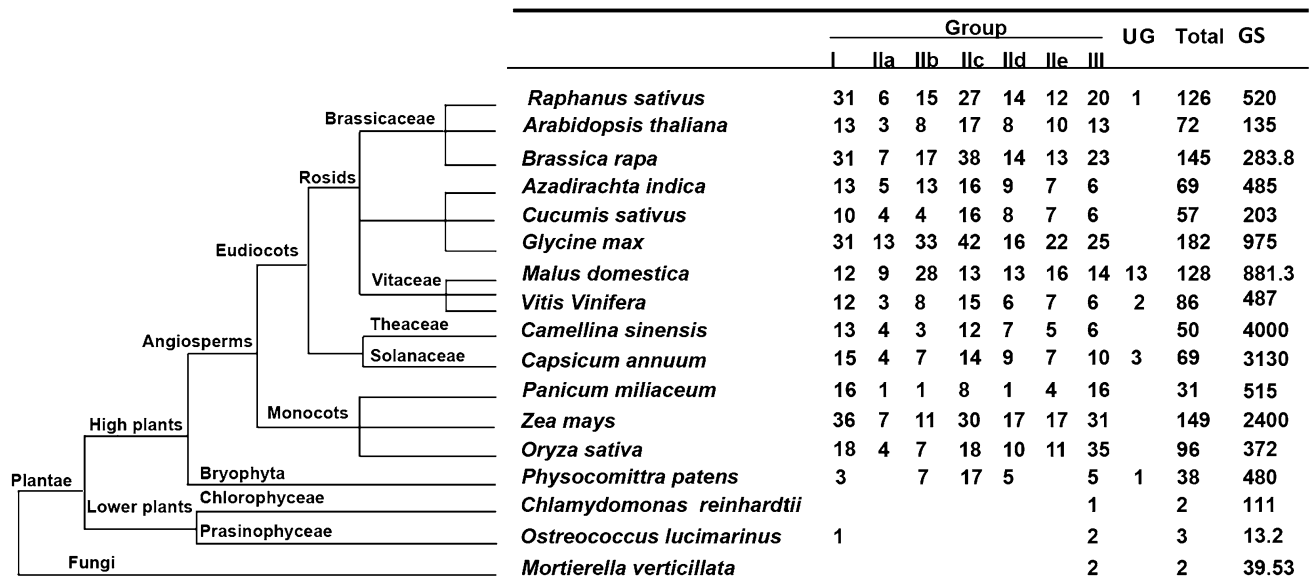
## Materials and methods

### Sequence data retrieval

The sequences of WRKY genes were retrieved from radish genome database using Hidden Markov Model profile (PF03106). Furthermore, CDD (<http://www.ncbi.nlm.nih.gov/Structure>) and InterProScan (Letunic et al. 2006) online programs were utilized to ascertain the presence of WRKY domain in the non-redundant sequences. The data for the sequences of WRKY TFs from other plants were obtained from (<http://plantfdb.cbi.pku>). The proportional study was performed using additional 16 previously studied species including plant and fungi (He et al. 2012; Huh et al. 2012; Bencke-Malato et al. 2014) (Fig. 1).

### Protein properties and phylogenetic analysis

The WRKY domains were obtained and utilized for plotting phylogenetic tree using MEGA6 (Hall 2013). The



**Fig. 1** Evolutionary relationships of radish with other species and their number detail of the WRKY family of each species. The left of this figure indicates the categories of the species; the right of this

figure shows the number detail of the WRKY family of each species; UG (ungrouped) indicates the subfamily members from a distinct group in a combined phylogenetic tree; GS (Genome size, M)

following parameters were adopted: Poisson correction, pairwise deletion, and 1000 bootstrap replicate. To obtain a reliable classification of the different groups, eight highly conserved representative WRKY domains from *Arabidopsis* were included during tree construction. Furthermore, the online ExPASy proteomics server (<http://web.expasy.org/protparam>) was used to analyze protein properties of the putative WRKY proteins.

### Motif and gene structure analysis of *RsWRKY* genes

To predict conserved motifs of WRKY proteins were performed using the Multiple EM for motif elicitation software (<http://meme-suite.org/tools/meme>). Diverse gene structural organizations of *RsWRKY* genes were identified using GSDS tool (<http://gsds.cbi.pku.edu.cn>).

### GO annotation and prediction of miRNA targeting the *RsWRKY* genes

Gene ontology annotation of *RsWRKY* protein sequences was analyzed using an online program (<http://www.blast2go.com>) as previously described (Conesa et al. 2005). Furthermore, all candidate *RsWRKY* genes were sought against available radish reference miRNA sequences from our transcriptome sequences (Xu et al. 2013a; Nie et al. 2015; Yu et al. 2015; Sun et al. 2015a; Zhang et al. 2016) using psRNATarget Server (<http://plantgrn.noble.org/psRNATarget/>). Cytoscape software (Shannon et al. 2003) was applied to visualize the interactions between predicted miRNA and corresponding target *RsWRKY* genes.

### Linkage group localization and distribution of *RsWRKY* genes

The *RsWRKY* sequence scaffolds were anchored to the integrated high-density linkage map published by Kitashiba et al. (2014). Gene sequences with similarity  $\geq 98\%$  and length difference  $\leq 5$  bp were treated as similar genes between two genomes (Wang et al. 2015c) and subsequently localized to their linkage groups according to their corresponding location parameters using MapInspect software (Liu and Meng 2003). Physical interactions were constructed using *Arabidopsis* association model in the STRING Protein–Protein Interaction Networks software (Franceschini et al. 2013).

### In silico patterns survey of *RsWRKY* genes

To predict the *RsWRKY* gene expression profiling, RNA-seq library from NODAI radish genome database was used to retrieve FPKM values of five tissues (cortical, cambium, xylem, root tip and leaf) and six leaf stages (7, 14, 20, 40, 60 and 90 days). Transcriptomic analysis of WRKY-related transcript expression levels in taproot thickening and stresses (heat, salt, Cd, Pb, and Cr) of radish were conducted based on our radish transcriptome data (Wang et al. 2013, 2015b; Xie et al. 2015; Yu et al. 2015; Sun et al. 2016). The MeV version 4.2 software (Saeed et al. 2003) was utilized to plot heat maps for the annotated WRKY genes against their corresponding expression values.

## Plant treatments

Seeds of ‘NAU-YH’ radish genotype were grown in the greenhouse for four weeks before further treatments. For heat treatments, seedlings were placed at 42 °C, with 25 °C for the control. For salt treatment, seedlings were treated with 200 mM NaCl and 0 mM NaCl for control. HM treatment involved Cd and Pb, where seedlings were treated with 0 (CK) and 20 mg L<sup>-1</sup> CdCl<sub>2</sub>·2.5H<sub>2</sub>O and 100 mg L<sup>-1</sup> Pb (NO<sub>3</sub>)<sub>2</sub> and 0 (CK), respectively. The samples were harvested at 24 h after each treatment and frozen immediately in liquid nitrogen and stored at -80 °C until further analysis.

## Reverse transcription-quantitative polymerase chain reaction

The expression pattern was validated using RT-qPCR, as previously described (Xu et al. 2013b). Beacon Designer 7.7 software was used to design gene-specific primers as listed in Table S1. Total RNAs from triplicate samples were isolated using Trizol (Invitrogen) according to manufacturer’s protocols. PCR was carried out using Start Universal SYBR Green Master mix and performed using iCycler iQ machine (BIO-RAD). The reaction was performed using the following program, 95 °C for 30 s and 45 cycles of 95 °C for 5 s, and 58 °C for 15 s and 72 °C for 20 s. The 2<sup>-ΔΔC<sub>t</sub></sup> method was used for data quantification (Livak and Schmittgen 2001).

## Results

### Identification of WRKY genes in radish

In the current study, a sum of 126 potential *RsWRKY* genes was detectable through genome-wide analysis and designated according to their generic order (Table S2). Additionally, 125 *RsWRKY* genes confirmed to have complete WRKY domain, and only one (*RsWRKY96*) had a missing zinc finger motif. Characteristic analysis indicated that the number of aa (amino acids) for these 126 varied from 144 (*RsWRKY44*) to 1086 (*RsWRKY15*) aa, with an average protein length of 387 aa. The coding sequence (CDS) length ranged from 435 to 3261 bp with an average length of ~1184 bp (Table S2). The MW (molecular mass) ranged from 1.09 kDa (Kilodalton) (*RsWRKY23*) to 12.9 kDa (*RsWRKY15*), and the theoretical isoelectric points varied from 4.96 (*RsWRKY103*) to 9.89 (*RsWRKY54*) with 58 members having more than 7 kDa (Table S2).

Relative analysis using pIs (Isoelectric points) and MWs among the three major groups showed that higher proportion of Group II (WRKY GII, 58%) and Group III (WRKY

GII, 53%) were acidic, while Group I (WRKY GI) had most of the members (58%) being basic with a wide fluctuation in MWs (Fig. S1). The majority of Group I proteins indicated broader variation in MW’s size and higher pIs compared to Groups II and III members. The detailed information on *RsWRKY* genes is listed in Table S2.

### Phylogenetic classification of *RsWRKY* genes

Twenty-five *RsWRKY* proteins from the 126 *RsWRKY* identified proteins in the current study, were found to have two WRKY domains, while the rest had only one. When *RsWRKY* protein had two domains, the domain was designated *RsWRKY* together with either letter N for N-terminal or C for the C-terminal domain (Fig. 2). To predict the degree to which the WRKY and zinc finger motif were conserved in each group, the sequence logos were produced using weblogo online software (Crooks et al. 2004) (Fig. 2; Fig. S2). The phylogenetic relationship of the *RsWRKY* proteins was constructed by multiple sequence alignment of their WRKY domains (Fig. 3) which clustered into three main groups (Group I, Group II and Group III). For each group, at least one representative was randomly selected from *Arabidopsis AtWRKY* genes (*AtWRKY1*, 58, 61, 14, 40, 56, 11 and 64).

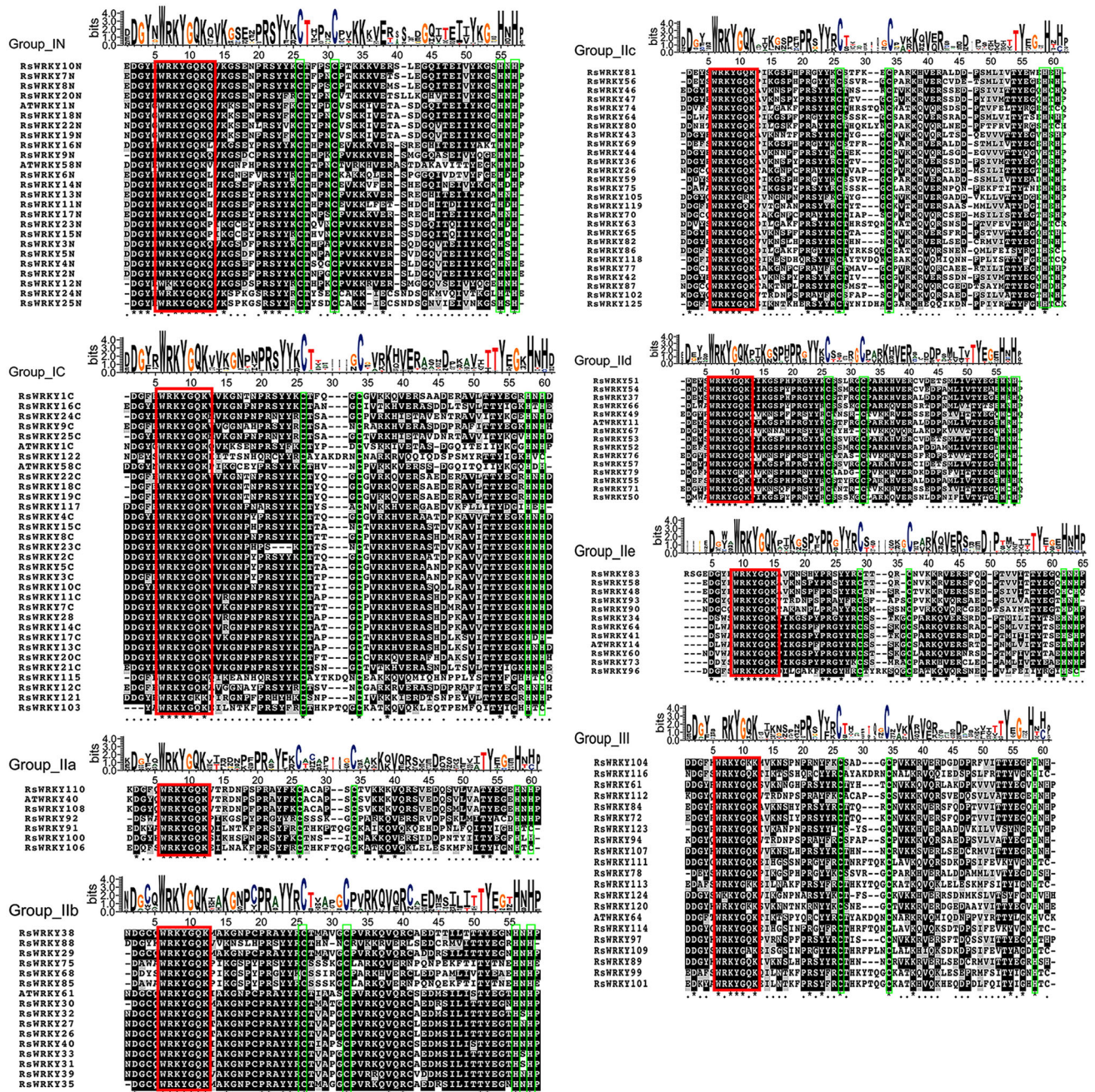
Although *RsWRKY98* had one WRKY domain similar to Group II and III, it was considered as an orphan gene termed as ‘ungrouped’ due to its low homology and divergence in WRKY domain and therefore not clustering in any group. It was also noted that group one had 31 WRKY proteins, out of which 25 had two WRKY domains. Nonetheless, some others (*RsWRKY28*, 117, 122, 115, 103, and 121) contained only one WRKY domain and clustered with C-terminal WRKY domains, indicating that they might have either undergone domain loss or acquisition events during evolution (Ross et al. 2007).

Despite high conservation of the WRKYGQK in WRKY TFs, four variants in the signature WRKY domain were identified in six *RsWRKY*s (Table 1): WRKYGQR (*RsWRKY105*); WRKYGKK (*RsWRKY79*, *RsWRKY121*); WRKYGYA (*RsWRKY109*), and WRKYGYK (*RsWRKY113*, *RsWRKY120*). In addition, one zinc finger form variation, C<sub>2</sub>H was identified in *RsWRKY96* gene (Table 1).

### Linkage group localization and distribution of *RsWRKY* genes

Sixty-seven *RsWRKY* genes (53%) of total *RsWRKY* genes were anchored onto the approximate locations of the nine linkage groups (LGs) R1–R9 of the radish genome (Fig. 4; Table S3). Among the nine LGs, LG R2, R3, and R4





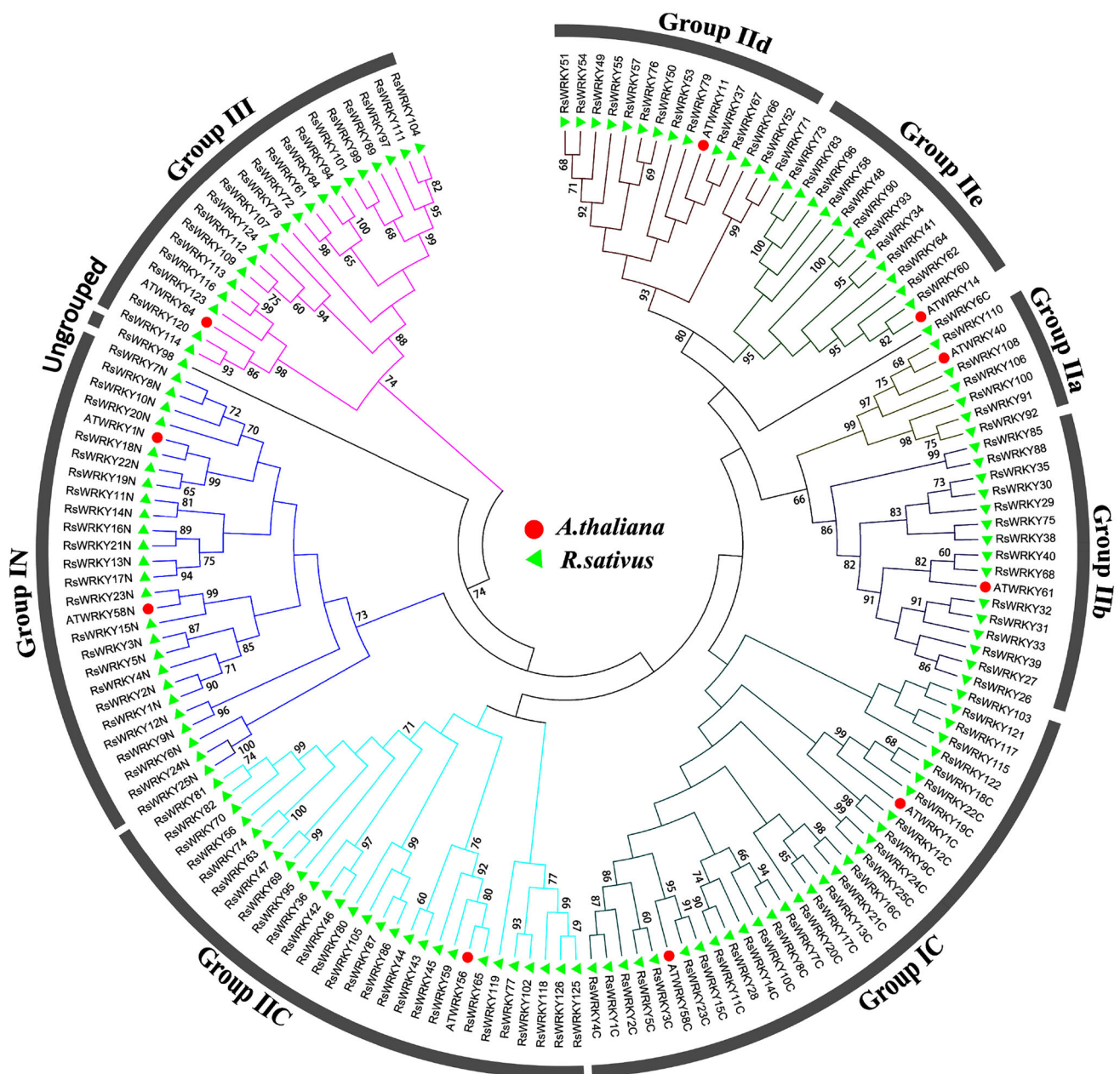
**Fig. 2** Multiple sequence alignment of the WRKY domain among *R. sativus* WRKY genes. Red indicates conserved WRKY amino acid domains; green indicates zinc finger motifs; dashes indicate gaps. ‘N’ and ‘C’ indicate the N-terminal and C-terminal WRKY domain of a specific in Group I WRKY gene. The conserved domains were

constitute the highest number of WRKY TFs, with 9, 8, and 22 genes, respectively, whereas the least number of TFs was located on LG R1 (one gene). The highest WRKY gene number in LG R4 was ascribed to the increased number of Group I, Group Iic and Group III with 6, 6, and 4 genes, respectively. Interestingly, Group II was responsible for 56,

identified using the Pfam program. The overall height of each stack on the logo illustrates the sequence conservation at each position. Each residue letter height symbolizes the relative frequency of the corresponding residue (color figure online)

55, and 38% of the total TF family in LG R2, R4, and R3, respectively. Furthermore, tandem duplications of *RsWRKY* genes were explored along the nine LGs. Tandem duplication was considered when a pair or more homologous WRKY genes confined within the same range distance. Nine *RsWRKY* gene clusters containing 22 tandemly





**Fig. 3** Unrooted phylogenetic tree representing relationships among WRKY domains of radish and selected *Arabidopsis* domains. The domain sequences of all RsWRKY and *Arabidopsis* AtWRKY representatives were aligned using ClustalW and the phylogenetic tree constructed with

the neighbour joining method in MEGA 6.0. Group I proteins with the suffix 'N' or 'C' indicates the N-terminal WRKY domains or the C-terminal WRKY domains. The different colors highlight different groups or subgroups of WRKY domains (color figure online)

duplicated genes were located on LGs R2, R3, R4, R5, R6, and R8 (Fig. 4). Most of the highest tandem duplication was found on LG R4, which had four gene clusters containing nine genes.

### Motif composition and gene structure of RsWRKY proteins

MEME motif analysis revealed that different RsWRKY proteins had distinct conserved motifs (Table 2). Motif 1

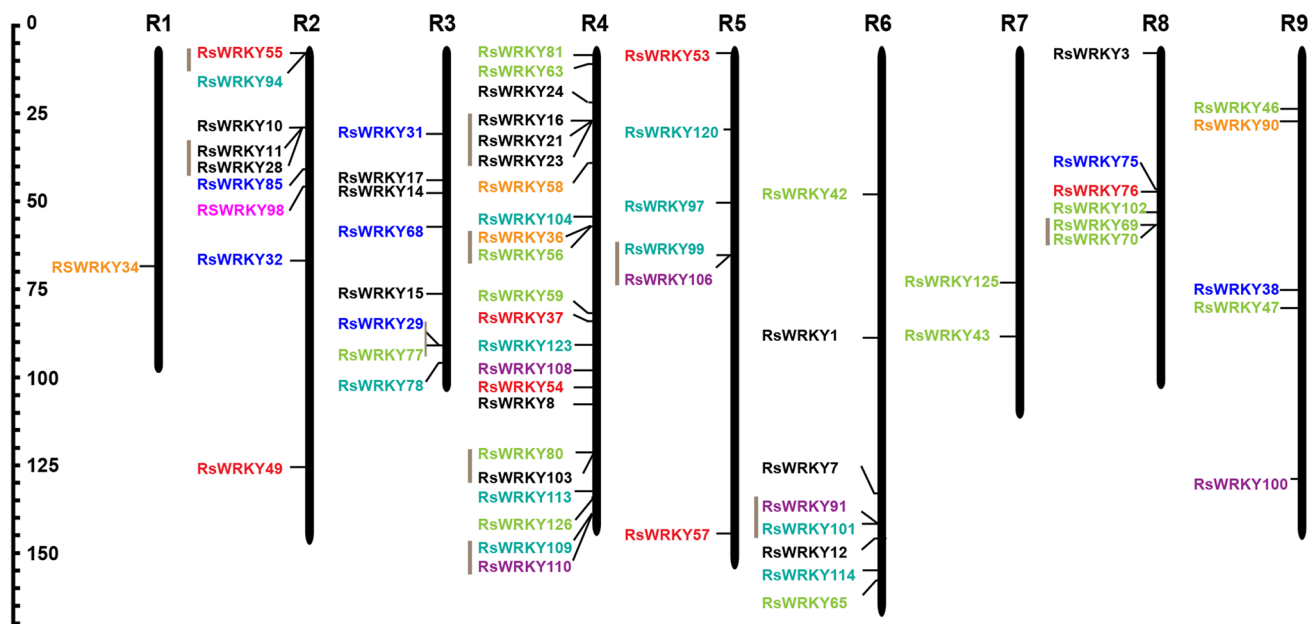
and 2 are closely related to WRKY domain and are found in all radish WRKY amino acid sequences other than RsWRKY49 without motif 2 (Fig. 5). Motif 1, 2, and 9 jointly contain the C-terminal WRKY domain, while, motif 4, 8, and 10 made up the N-terminal WRKY domain in radish, these six motifs were highly conserved in all RsWRKY proteins. Motif 4, 3, and 10 were highly conserved in Group I. Motif 7 was prominently conserved in GIIB. Group IId has four unique motifs 1, 2, 6, and 9. Notably, RsWRKY96 was not clustered in any group due

**Table 1** Variants of the heptapeptide WRKYGQK and zinc finger structure in WRKY domains in radish

Variants	<i>RsWRKY</i> gene	Group
WRKYGQR	<i>RsWRKY105</i>	II(c)
WRKYGKK	<i>RsWRKY79</i>	II(d)
WRKYGYA	<i>RsWRKY109</i>	III
WRKYGYK	<i>RsWRKY113</i>	III
WRKYGYK	<i>RsWRKY120</i>	III
WRKYGKK	<i>RsWRKY121</i>	UG
Variant of zinc finger form		
C <sub>2</sub> H	<i>RsWRKY96</i>	II(e)

to the lack of the complete WRKY domain, but contained two motifs (motif 1 and 2), which are prominent in the C-terminal of Group II-b. Therefore, it was speculated that *RsWRKY96* is a member of Group II-d. The width of the motifs was diverse with motif 8 being the longest while motif 6 had the shortest motif length.

Intron–exon structure analysis revealed the presence of intron–exon in all *RsWRKY* gene coding sequences. Remarkably, closely related genes in the phylogenetic tree shared similar structure compositions, indicating functional similarities within a subfamily. Moreover, the number of exons ranged from 3 to 12 (*RsWRKY3*). Normally, most *RsWRKY* genes in a common pair or triplets indicated



**Group(I)** **Group(IIa)** **Group(IIb)** **Group(IIc)** **Group(IId)** **Group(IIe)** **Group(III)** **Ungrouped(UG)**

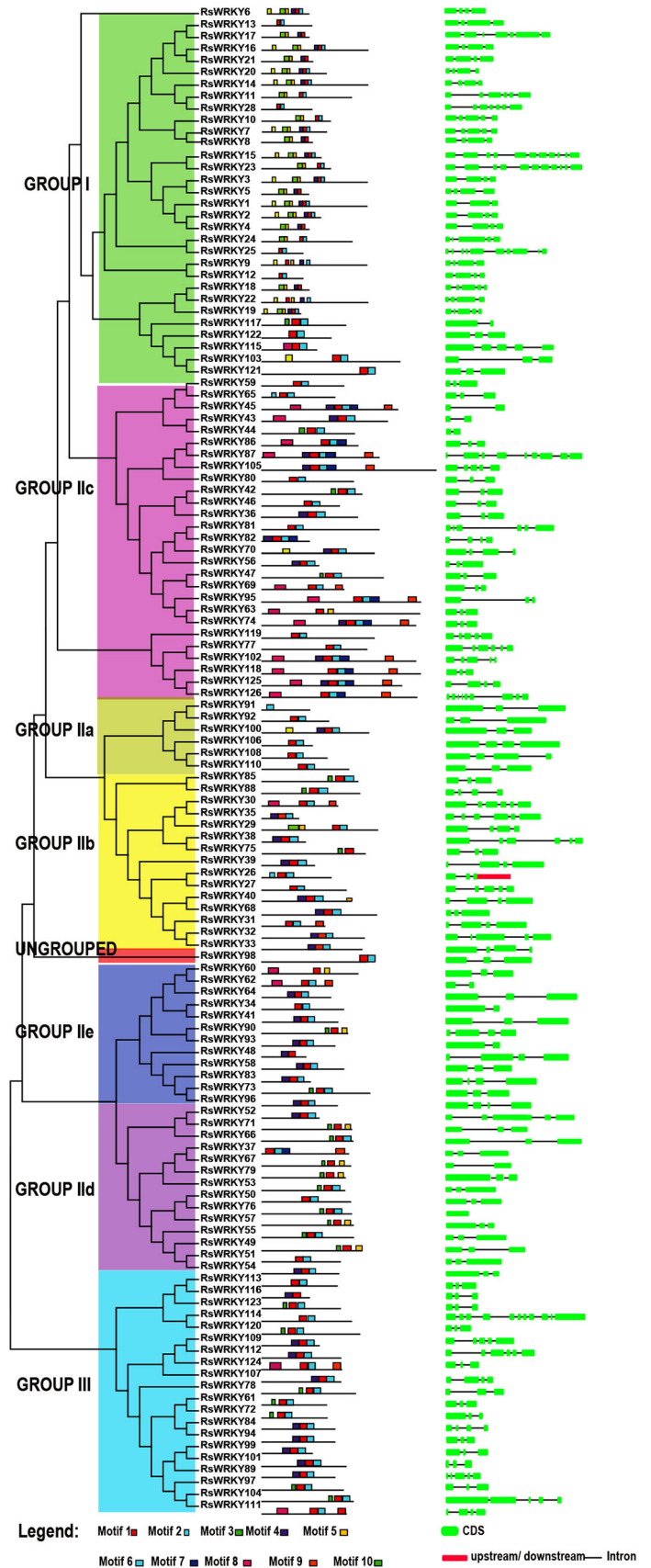
**Fig. 4** (I) Linkage map of group localization and distribution of *WRKY* genes in radish, Tandem-duplicated genes are indicated with vertical gray lines. The size of a linkage group is indicated by its

relative length. (II) The percentages of *RsWRKY* genes on each linkage group are demonstrated by the pie accordingly

**Table 2** Regular expressions of conserved motifs from *RsWRKY* proteins

Motif	<i>E</i> value	Width	Site	Best possible match
1	1.1E–2870	29	125	LDDGYRWRKYGQKVIKGNPYPRSSYYRCTY
2	2.0E–1701	27	112	QGCPVRKHVERCADDPKMVITTYEGKH
3	4.3E–749	41	25	DDGYNWRKYGQKQVKGSEFPRSYYKCTHPNCPVKKKVERS
4	9.0E–408	29	56	KEQAKGKKKAQRKVREPRVCFQTRSVDVI
5	1.50E–300	21	35	DGQITEIYKGEHNHPKPQNT
6	2.90E–115	15	27	KKRKHRVVRVIRVPA
7	1.50E–165	29	13	NHPLPPAATAMASTTSAASMLLSGSSSS
8	2.30E–160	41	17	ALREEMKRVKEENKKLKEMLEQICENYNSLQMHVAVMQQ
9	2.60E–147	29	16	LVEQMAKALTKDPNFTAALAAAITGMIGG
10	4.00E–114	26	20	PPCMFTIPPGLSPAMLLDSPVFFSNF

**Fig. 5** Unrooted phylogenetic tree based on the full-length sequences, conserved motifs composition and gene structure of 126 RsWRKY proteins. The phylogenetic tree was constructed by MEGA6.0. Different colors represented various groups, MEME was used to predict motifs and these motifs represented with boxes, while exon–intron structure of radish *WRKY* genes; blue indicates untranslated 5'- and 3'-regions; yellow indicates exons; black lines indicate introns (color figure online)





similar intron–exon distribution, for example, *RsWRKY28* and *RsWRKY98*.

### Comprehensive analysis of microRNA targeting *RsWRKY* genes

A total of 15 known miRNAs plus 9 candidate novel miRNAs belonging to 25 miRNA families were identified as putative miRNAs targeting 32 *RsWRKYs* transcripts in this study (Table S4). The relationship network between candidate miRNAs and their targets was constructed (Fig. 6). *RsWRKY12* and *RsWRKY82* were the most targeted transcripts with each being successfully targeted by five radish miRNAs. They were closely followed by *RsWRKY 7* and *55* which were successfully targeted by 4 putative miRNAs. A microRNA *rsa-miRn36* with nine *RsWRKY* target transcripts was proven to be the most abundant identified miRNAs. It is noteworthy that *rsa-miRn36* was identified as salt-responsive novel miRNA corresponding to *Unigene1698* (Sun et al. 2015b).

### Gene ontology annotation and interactions among specific *RsWRKY* proteins

GO analysis showed that these 126 *RsWRKY* genes were assigned into 35 biological processes, 4 cellular components, and 3 molecular functions (Fig. S3A). In biological process category, a group of 7 metabolic processes had the highest representation (144 sequences, 90.5%) (Fig. S3A), followed by ‘response to stress’ (50 sequences, 39.7%) and ‘response to chemical’ (49 sequences, 38.9%). In cellular classification, there was an equal number of sequence representation among the four designated categories with each having 102 (81%) sequences (Fig. S3B). In molecular components, ‘sequence-specific DNA binding’ had the highest representation of 115 (91.3%) sequences, followed by ‘transcription factor activity’ with (90.5%) sequences. ‘Calmodulin binding’ was the least represented in this group annotation with only 13 (10.32%) members (Fig. S3C).

Subsequently, the 50 *RsWRKY* candidates potentially involved in plant stress responses were utilized for protein–protein interaction using STRING 9.1 software with the confidence parameter set at a threshold of 0.15. The 50 proteins belonged to diverse groups, (12 proteins (Group I), 3 (Group IIa), 6 (Group IIb), 12 (Group IIc), 7 (Group IId), 3 (Group IIe), 6 (Group III) and 1 (Un-grouped) (Table S5). Subsequently, functional and physical interactions were examined using their database to retrieve the protein interactions. Four proteins that showed high sequence similarity with *WRKY33*

(*RsWRKY18*, 19, 22) were involved in the stronger (thicker lines) interaction network (Fig. 7). *MKS1* and *MPK3* genes formed a strong interaction with *WRKY33* and *WRKY25* (*RsWRKY8*, 11, 12), respectively.

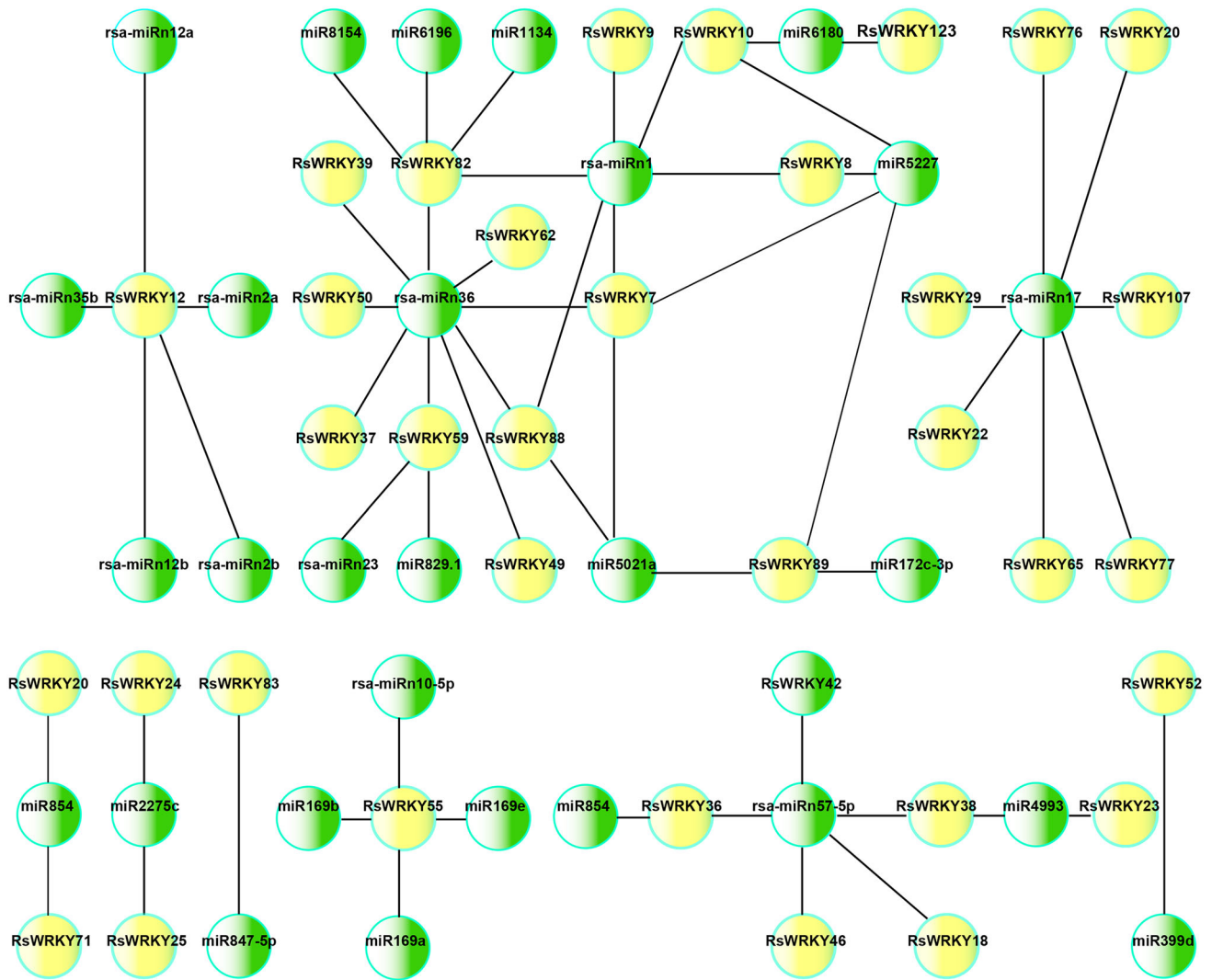
### Reverse transcription-quantitative polymerase chain reaction

Gene profiling indicated that the 36 *RsWRKYs* representative genes exhibited distinct patterns under stress and normal growth conditions (Fig. 8). In detail, 24 and 20 *RsWRKY* transcripts were significantly elevated under Cd and Pb treatments, respectively. Moreover, 30 and 24 genes were found to be potentially responsive to salt and heat stresses, respectively. Co-expression of several *RsWRKYs* plays vital roles in abiotic stress adjustments. For instance, the expression levels of 13 *RsWRKY* genes (*RsWRKY6*, *RsWRKY10*, *RsWRKY11*, *RsWRKY13*, *RsWRKY15*, *RsWRKY17*, *RsWRKY20*, *RsWRKY23*, *RsWRKY24*, *RsWRKY28*, *RsWRKY29*, *RsWRKY30*, and *RsWRKY98*) indicated significant higher expression with a cross-responsive approach to Pb, Cd, salt, and heat. *RsWRKY31* and *RsWRKY114* had the highest expression levels (>22 folds) under all abiotic stresses. Additionally, stress-specific responses were also observed, *RsWRKY28* expression was preferentially expressed under heat stress with no significant response to other stresses. *RsWRKY31* and *RsWRKY114* had 25- and 22-fold changes, respectively, under different stress treatments as compared to normal growth condition. It was noted that the expression level of 29 *RsWRKY* genes was relatively higher under heat treatment, among which *RsWRKY31* was the most upregulated under all stress treatments. The average fold change value was 1.36 and 0.96 in Cd and Pb stresses, respectively. Additionally, the average fold change in heat and salt was 4.23- and 1.96-folds, respectively (Fig. 8; Table S8).

## Discussion

### Identification and characterization of *RsWRKY* genes

In higher plants, *WRKY* proteins regulate multiple biological processes specifically in stress response (Chen et al. 2015), and have been found throughout protozoa, fungi, and other plant species (Wu et al. 2015). In the present study, a total of 126 *WRKY* putative genes were identified from radish genome database; additional 16 species were incorporated for comparative analysis. It was found that *WRKY* genes in



**Fig. 6** A schematic representation of targeted regulatory relations between putative miRNAs and their targets

*O. lucimarinus* were relatively advanced compared to *C. reinhardtii* about their number of WRKY genes (Fig. 1). Surprisingly, *O. lucimarinus* had higher WRKY protein coverage in the genome compared to most of the higher plant species. Such extensive coverage illustrated the basic and complex importance of WRKY proteins through the evolution of this gene superfamily in the green lineage. The coverage of WRKY proteins in *Brassica rapa* and *Arabidopsis thaliana* was the highest in comparison to all other species used in this study, indicating that WRKY proteins play critical and diverse functions in *Brassicaceae* family. With the highly advanced evolutionary level, higher species normally acquire additional genes for their complex biological mechanisms. Conversely, the genome WRKY gene proportion in complex plants do not necessarily tally with their evolutionary stage (Wu et al. 2015). Stress normally curtails optimal biological processes in plants, WRKY genes in conjunction with other stress responsive genes may

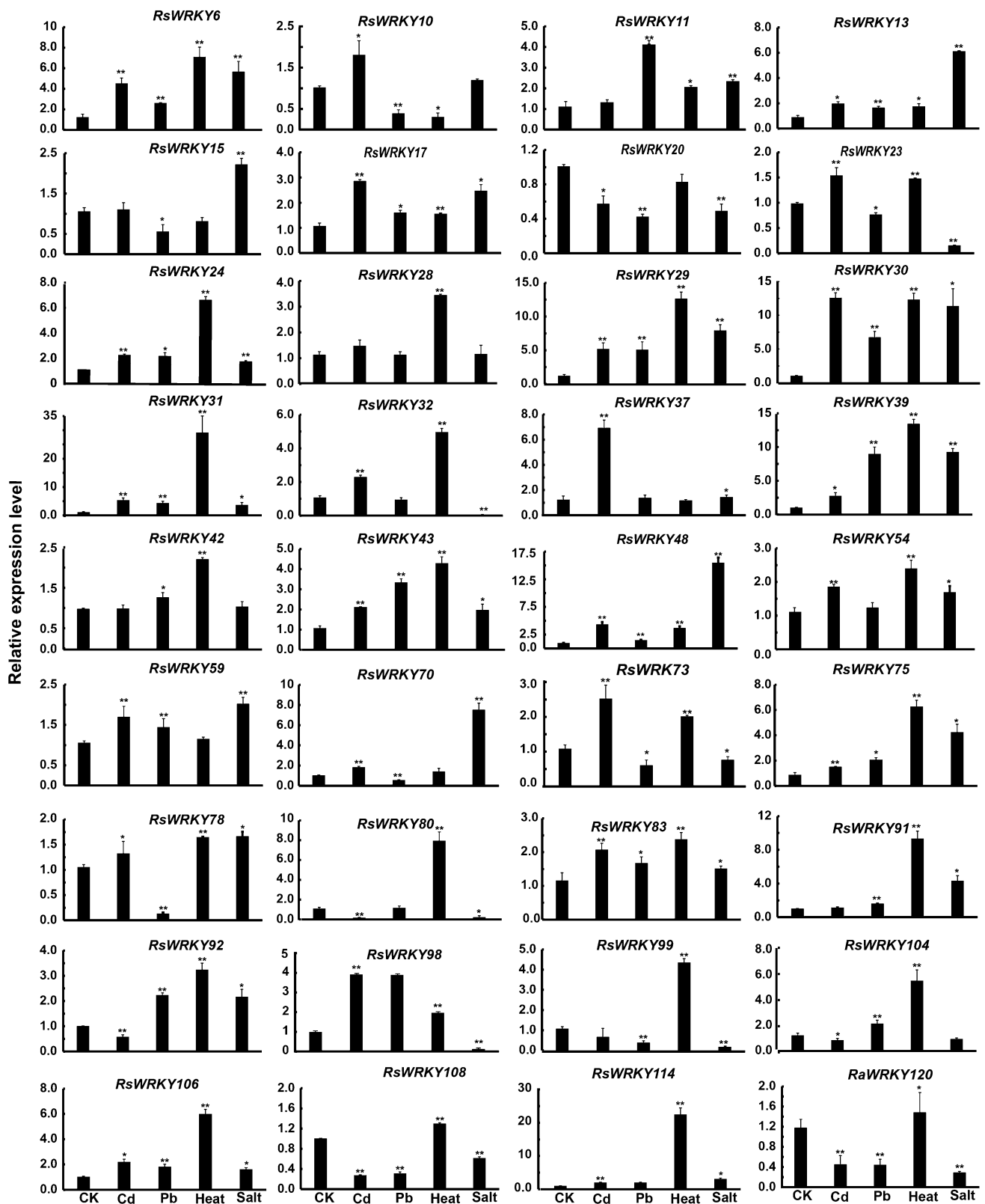
synergistically promote tolerance in plants by adjusting their expression patterns.

### Structural conservation and divergence of radish WRKY gene family

The phylogenetic tree indicated the classification of 126 *RsWRKY* genes into three broad groups (Group I, Group II, and Group III), Group II was expanded further to five subgroups (IIa to IIe) according to *Arabidopsis*. Group I members had a pair of WRKY domains with six members (*RsWRKY*28, 117, 122, 115, 103, and 121) having a single domain. The same phenomenon was consistent with other species such as *Arabidopsis* (Wei et al. 2012). The evolutionary loss of WRKY domain seems lower in dicotyledons than in monocotyledons. Therefore, Group I can be inferred from the origin of other groups and appeared early in the evolution.







**Fig. 8** RT-qPCR was used to analyze the expression profiles of 36 *RsWRKY* genes under cadmium, lead, heat, and salt stress. CK is untreated seedling. The *Actin* gene was used as an internal control for

RT-qPCR. Error bars were obtained from three technical replicates. Asterisks reveal the gene significantly upregulated or downregulated under abiotic stresses by *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ )

as a binding site located beside TTGACY motif in *AtWRKY* TFs (Ciolkowski et al. 2008). If the genes are targeted by a variant WRKY domain, the motif may result to an alternative function from that of the usual WRKYGQK motif (Eulgem et al. 2000), therefore, WRKY TFs with altered WRKY domain may end up recognizing a DNA-binding sequence other than a W-box sequence [(C/T) TGAC(C/T)] (Maeo et al. 2001). It can be deduced that the variations of the WRKY domain are likely to impact on expression patterns of stress-responsive genes targeted by *RsWRKY* TFs.

Gene structure of all the 126 *RsWRKY* indicated variation in exon–intron pattern. Members located on the same subgroup showed closely related conserved patterns; this is an indication of functional similarities among members in the same evolutionary group (Wang et al. 2014). Furthermore, exon–intron reorganization by addition or losing may result from and combinations of various linkage groups (Guo et al. 2014). The present study shows an example of such diversification on *WRKY* gene (*RsWRKY61*) which has a single exon, while other *RsWRKY* members within the same phylogenetic group (Group III) possess two to five exons in their genomic sequences. Moreover, *RsWRKY3* was found to bear 12 exons. Notably, the members with the highest number of exons categorically clustered in Group I followed by Group II. Therefore, it could be deduced that Group I and II constitute the ancestral genes, from which other groups originate.

### The evolutionary relationship and protein interaction networks of *RsWRKY* genes

A large number of gene duplication occurrences in radish facilitate gene function prediction and evolution analysis (Fig. 4). In many species, whole-genome duplication events mostly result in the enlargement of a gene family (Cannon et al. 2004). This phenomenon also occurred in *R. sativus* (Shen et al. 2013). In WRKY TFs, tandem gene duplication has also occurred in many other plant species including rice and cucumber (Ling et al. 2011). In cucumber, duplication events in *CsWRKY* family generated additional Group-III *WRKY* genes (Ling et al. 2011). In this study, 28.5% (20/70) *RsWRKY* genes evolved from tandem gene duplication (Fig. 4). It was observed that 40% (9 out of 22) *RsWRKY* genes on LG R4 were involved in duplication as indicated by the higher number of *RsWRKY* genes on LG R4. Consequently, tandem duplication probably took an integral role in the enlargement of *RsWRKY* gene family in radish.

The current study deduced that gene expansion mainly happened in Group II which constituted 50% (11/22) of the total genes involved in the duplication. Among the Group

II subgroups Group IIc was leading in the number (5) of *WRKY* sequences involved in duplication event, these findings were contrary to *Arabidopsis* and rice, where most of the tandem duplication occurred in Group III. Additionally, the conserved *WRKY* genes and physical proximity of TFs shared a common group and were located on the same tandem cluster, R1 (*RsWRKY10*, 11 and 28), R4 (*RsWRKY16*, 21 and 23) in Group I, R3 (*RsWRKY29* and 77), and R8 (*RsWRKY69* and 70) in Group II. This phenomenon has also been observed in poplar (Jiang et al. 2014) and grape (Guo et al. 2014) genomes, suggesting paralogous segments resulting from ancestral polyploidization occurrence (Song et al. 2013).

The genomic comparison or protein interaction is a technique for transferring available genomic information from a model plant to those currently studied. Six proteins, which exhibited increased sequence similarity with *Arabidopsis* WRKY18 (*RsWRKY93*, 94 and 102), WRKY33 (*RsWRKY18*, 19), and WRKY53 (*RsWRKY63* and 74) were located at the center of the network node, are involved in stronger interaction network illustrated by thicker lines (Fig. 7). MPK3 and MKS1 are also essential phosphorylation partner genes in the function of *WRKY* genes (Li et al. 2012). According to previous studies, *AtWRKY8* via its C-terminal domain, interacts with target genes to regulate multiple abiotic stresses including salt and heat (Wang et al. 2015a). *AtWRKY 33*, 25, and 26 showing high homology to 7 *RsWRKY* genes (Fig. 6), were found to coordinate induction of plant thermotolerance (Li et al. 2011). Intriguingly, the soloist gene was featured in the protein interaction network which indicated homology with *AtWRKY29*. The results indicated multiple interactions among *WRKY* genes; this was commensurate to the RT-qPCR result indicating co-expression of *RsWRKY* genes under multiple stress responses.

### WRKY proteins play roles in abiotic stress response

Accumulating reports propose that the abiotic stresses deter the optimal physiological and biochemical mechanism in plants (Joshi et al. 2016). In the dynamic growing conditions, a critical factor underlying stress resistance in sessile plants is their ability to conceptualize, integrate, and adjust to biotic and abiotic environmental cues, which could consequently modulate molecular and physiological metabolism for their survival. These deleterious conditions sometimes occur simultaneously and negatively affect plant growth causing significant losses in agricultural production. Such abiotic stresses also activate the WRKY TFs and trigger a network of signal transduction to enhance the stress tolerance in plants (Schlutenhofer and Yuan 2015). Recent advances suggest that WRKY TF family is crucial in numerous plant processes such as plant

development and patterns to abiotic stresses (Zhai et al. 2016). In *Arabidopsis*, rice, and Chinese cabbage, at least 26, 54, and 28 *WRKY* genes were identified in response to abiotic stress, respectively (Jiang and Deyholos 2006; Zhou et al. 2008; Goel et al. 2016). The majority of *RsWRKY* genes showed higher expression under abiotic stress treatments than control. We also found involvement of 13 *RsWRKY* genes in all the abiotic stresses and almost all (35/36) to more than one stressor. Similarly, *AtWRKY25* and *AtWRKY33* has been known to respond to both heat and salt treatments (Li et al. 2011), while *TcWRKY53* is simultaneously induced by cold, salt, and PEG treatments (Wei et al. 2008). These findings support that *WRKY* genes can co-regulate more than one stressor by a synergistic or antagonistic mechanism (Shaik and Ramakrishna 2013). In addition, co-expression of *WRKYs* creates a complex network, which resists stress by multiple expression. In rice, overexpression of two *WRKY* TFs (*OsWRKY11* and *OsWRKY45*) promotes water stress tolerance under drought condition (Qiu and Yu 2009). The result from this study also indicated that 29 *RsWRKY* genes responded significantly to salt stress, and confirms cross regulation among *WRKY* genes in response to abiotic stress. Overexpression of *ZmWRKY33* in *Arabidopsis* improved salt stress tolerance of the transgenic plants (Li et al. 2013a). Consistently, *RsWRKY90* was upregulated in salt stress similar to its orthologue *ATWRKY70*, which highly expresses under osmotic stress (Li et al. 2013b).

Surprisingly, the *RsWRKY98* showed preferential expression in responses to HMs as compared to the control, while in the salt condition it was downregulated. Comparatively, its orthologous gene (*AtWRKY29*) has been implicated to be involved in mitogen-activated protein kinase (MAPK) pathway during stress responses (Göhre et al. 2012), implying that the *RsWRKY98* could be among the few TFs phosphorylated and activated by kinases. In particular, MEKK1–MAPK kinase 2(MKK2)–MPK4/MPK6 cascade has been shown to function as part of cold and salt stress signaling (Teige et al. 2004). It could provide underpin for the unique positioning of *RsWRKY98* gene on the phylogenetic tree. This affirms that the *WRKY* family continuously evolve as the plants adjust to the ever-changing environmental cue from selective pressure, resulting in new *RsWRKY* members by either duplication or polyploidization. In *Sorghum bicolor*, tolerance to desiccation could be associated with the gene duplication events (Paterson et al. 2009). In *Brassica napus*, evolutionary expansion of *BnaWRKY* gene family results in new members with conserved or different roles in multiple stress responses (He et al. 2016).

Notably, *RsWRKY78* and *RsWRKY59* were found to be upregulated under salt treatments, while their orthologous

genes, *AtWRKY7* and *AtWRKY59* were downregulated (Scarpeci et al. 2013). Conversely, the *RsWRKY023*, *RsWRKY32*, and *RsWRKY80* were downregulated under salt conditions, indicating that the complex responses among *WRKY* genes in response to abiotic stresses. Additionally, overexpression of *ZmWRKY33* in *Arabidopsis* plants showed enhanced osmotic stress tolerance compared to wild-type (Liu et al. 2013). Furthermore, current study reported that most *RsWRKY* genes were negatively regulated during HM treatments (Pb and Cd). In *Musa* species, most of the *WRKY* genes were downregulated Pb and Cd, Arsenic, and chromium HM stresses (Goel et al. 2016). In *Arabidopsis*, double and triple mutations of *WRKY18*, *WRKY40*, and *WRKY60* were highly resistant to Cd stress compared to single mutant or wild-type (Chen et al. 2010). Additionally, overexpression of *ThWRKY7* exhibited increased tolerance to Cd stress in *T. hispida* by binding to the W-box in the promoter region of *ThVHAc1* genes (Yang et al. 2016). These results indicate the critical roles of *WRKY* TFs in modulations of stress responsive genes in abiotic stress tolerances and provide the possibility of engineering for multiple stress tolerances in radish and other root vegetable crops.

## Conclusion

In this study, 126 *RsWRKY* genes were identified from the *R. sativus* genome sequence. Classification and expression analyses revealed that some *RsWRKY* genes broadly participate in the regulation of plant response to abiotic stress. Meanwhile, 36 candidate *RsWRKY* genes were identified to be involved in abiotic stress responses and their expression profiles were confirmed by RT-qPCR. In particular, a total of 13 genes were confirmed to have multiple stress responses in Cd, Pb, heat, and salt stress. Thus, it could be concluded that *WRKY* genes may have multifunctional roles under various abiotic stresses, and could contribute in controlling signaling processes linked to transcriptional adjustments in plants under harsh environmental conditions. These *WRKYs* may potentially be utilized for breeding new radish cultivars resistant to multiple stresses. This study facilitates further identification of critical stress-related *WRKYs* for their transgenic application in radish.

**Author contribution statement** BKK designed the experiments and wrote the manuscript. BKK, FL, TM, and ZF performed validation experiments. WY and WR contributed powerful analytical tools. ZX and EMM contributed to proofreading of this manuscript. LL and XL conceived the study and managed the experiments. All authors read and approved the final manuscript.



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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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