

Genome-wide characterization of the rose (*Rosa chinensis*) WRKY family and role of RcWRKY41 in gray mold resistance

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Research article

Keywords: *Rosa* sp., WRKY, transcription factor, *Botrytis cinerea*, grey mold

Posted Date: September 8th, 2019

DOI: <https://doi.org/10.21203/rs.2.14147/v1>

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Version of Record: A version of this preprint was published on November 27th, 2019. See the published version at <https://doi.org/10.1186/s12870-019-2139-6>.

Abstract

Background The WRKYs are a major family of plant transcription factors that play roles in the responses to biotic and abiotic stresses; however, a comprehensive study of the WRKY family in roses (*Rosa* sp.) has not previously been performed.

Results In the present study, we performed a genome-wide analysis of the WRKY genes in the rose (*Rosa chinensis*), including their phylogenetic relationships, gene structure, chromosomal locations, and collinearity. Using a phylogenetic analysis, we divided the 56 RcWRKY genes into three subgroups. The RcWRKYs were unevenly distributed across all seven rose chromosomes, and a study of their collinearity suggested that genome duplication may have played a major role in RcWRKY gene duplication. A Ka/Ks analysis indicated that they mainly underwent purifying selection. Botrytis cinerea infection induced the expression of 19 RcWRKYs, most of which had undergone gene duplication during evolution. These RcWRKYs may regulate rose resistance against *B. cinerea*. Based on our phylogenetic and expression analyses, RcWRKY41 was identified as a candidate regulatory gene in the response to *B. cinerea* infection, which was confirmed using virus-induced gene silencing.

Conclusions This study provides useful information to facilitate the further study of the function of the rose WRKY gene family.

Introduction

Transcription factors play crucial roles in plant growth, development, metabolism, and stress responses. Transcription factors usually possess a DNA-binding domain, a transactivation domain, an oligomerization site, and a nuclear localization signal, among other domains. The WRKYs are one of the most important transcription factor families in plants. These proteins all possess at least one WRKY domain [1], a DNA-binding domain which binds the W-box (TTGACC/T) sequence of the promoter region in their target genes to regulate their expression. In addition, the C-terminal of the WRKY transcription factors usually contains a zinc finger structure. In *Arabidopsis thaliana*, the WRKY protein family can be divided into three different groups: Group I proteins contain two WRKY domains and Group II WRKYs contain only one WRKY domain. Group III proteins also possess a single WRKY domain, but their zinc finger structure is unique from those of the other two groups [2].

WRKY transcription factors participate in the regulation of various plant processes, including growth and development, the response to abiotic stresses, and disease resistance; for example, AtWRKY45 is involved in the regulation of plant leaf senescence through the gibberellin signaling pathway [3]. In rice (*Oryza sativa*), OsWRKY53 positively regulates brassinosteroid signals to influence the plant architecture [4]. The expression of AtWRKY22 in Arabidopsis is strongly induced by submergence during flooding, and its protein product binds to the promoter of *TREHALASE1*, which involved in stomatal function, to inhibit its expression [5]. AtWRKY8 is highly expressed in plant roots and is significantly upregulated under salt stress, with *atwrky8* knockout mutants showing a greater sensitivity to salt [6]. The WRKYs are also key

players in plant resistance responses against pathogens; for example, AtWRKY33 is activated by a MAPK signaling pathway and regulates the biosynthesis of phytoalexin to enhance pathogen resistance in Arabidopsis [7]. By contrast, *AtWRKY38* and *AtWRKY62* encode two structurally similar WRKYs that negatively regulate the defense against *Pseudomonas syringae*; the overexpression of these two genes decreased plant resistance to this pathogen, and the *atwrky38*, *atwrky62*, and *atwrky38 atwrky62* loss-of-function mutants displayed an enhanced disease resistance [8]. These results indicate that the WRKYs play both positive and negative regulatory roles in plant basal disease resistance.

Roses (*Rosa* sp.) are one of the most important commercial flower crops worldwide [9]. The major rose-producing areas include the tropical plateau regions of Africa and South America (including Kenya, Ethiopia, Ecuador, and Colombia), which have suitable climatic conditions and low labor costs, while rose purchasing is largely concentrated in developed countries in Europe and North America [10]. The long-distance logistics and transportation of roses pose a challenge for their post-harvest preservation, with flowers often being affected by post-harvest diseases such as gray mold caused by the necrotrophic fungal pathogen *Botrytis cinerea* [11].

Some WRKYs enhance the resistance of crops and model plants such as Arabidopsis against various diseases, including *B. cinerea*; however, the *WRKY* genes involved in gray mold resistance in roses have not yet been identified. We previously explored the molecular basis of rose resistance against *B. cinerea* using a de novo RNA-Seq analysis, revealing that large numbers of genes, including *WRKY* family genes, were significantly upregulated in roses upon *B. cinerea* infection [10]. In the present study, we performed a genome-wide analysis of the *WRKY* family in roses, and used virus-induced gene silencing (VIGS) to confirm that *RcWRKY41* plays an important role in rose resistance against gray mold.

Methods

Identification and Characteristics of the WRKY Genes in the Rose Genome

The complete rose (*Rosa chinensis* 'Old Blush') genome sequence was obtained from <https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/>. To identify the non-redundant *WRKY* genes in the rose genome, the consensus protein sequence of the *WRKY* Hidden Markov Model (HMM) was downloaded from Pfam (PF03106; <http://pfam.xfam.org>). This HMM profile was then used as a query to search the rose genome, resulting in the identification of all rose sequences containing a *WRKY* domain with an E-value < $1e^{-3}$. Finally, all candidate *RcWRKY*s were validated using the Pfam and the Conserved Domains Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to determine that they contained the core domains.

Phylogenetic Analyses

A total of 66 Arabidopsis *WRKY* protein sequences were collected from The Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org/>). Based on the results of previous studies, additional sequences of *WRKY* genes involved in plant disease resistance were collected from GenBank, including

those from cotton (*Gossypium hirsutum*), rice (*Oryza sativa*), oilseed rape (*Brassica napus*), grape (*Vitis vinifera*), tobacco (*Nicotiana benthamiana*), barley (*Hordeum vulgare*), and pepper (*Capsicum annuum*). A phylogenetic analysis was used to determine whether orthologs of these genes are present in the rose genome. Phylogenetic dendrograms were constructed using the neighbor-joining (NJ) method in MEGA 5.2 software, with default parameter values.

Collinearity Analyses

In order to identify collinearity, a Multiple Collinearity Scan toolkit [12] was used to detect the microsyntenic relationships between the *WRKY* genes. The resulting microsynteny chains were then evaluated using ColinearScan (E-value < $1e^{-10}$).

Calculation of the Non-Synonymous (Ka) to Synonymous (Ks) Nucleotide Substitution Ratio

An analysis of the Ka/Ks ratios was used to determine the selection modes driving the evolution of the *RcWRKYs*. These ratios were calculated using EasyCodeML software [13].

Expression of the RcWRKYs in Response to B. cinerea

RNA-Seq data from rose petals infected with *B. cinerea* were obtained from the National Center for Biotechnology Information (NCBI) database (accession number PRJNA414570). Clean sequencing reads were mapped to the rose reference genome, and the number of reads per kb per million reads (RPKM) were used to determine the gene expression levels.

VIGS

To obtain the *TRV-RcWRKY41* construct, a fragment from the coding region of *RcWRKY41* was amplified using the primer pairs RcWRKY41-F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTACCAAGCCACAATACCAA-3') and RcWRKY41-R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAACACAGCAATGATTCAAAA-3') and cloned into the *Tobacco rattle virus* vector *TRV2* [14]. To establish VIGS in rose petals, petals were detached from the outermost whorls of rose flowers (*R. hybrida* 'Samantha') during stage 2 of flower opening. A 15-mm disk was then punched from the center of each petal. *Agrobacterium tumefaciens* cultures containing constructs expressing *TRV1* [14] and recombinant *TRV2* were mixed in a 1:1 ratio and vacuum-infiltrated into the petal disks. At six days after the TRV infection, the petal disks were inoculated with *B. cinerea*. The VIGS was repeated at least three times using at least 48 disks. After the *B. cinerea* inoculation, the lesion sizes were recorded, and a Student's *t*-test was conducted to identify any significant differences.

Results

Identification of the RcWRKY Genes in Rose

To identify the rose *WRKY* family gene, the *WRKY* HMM profile (Pfam: 03106) was used as a query to search the rose genome database (*Rosa chinensis* Homozygous Genome v2.0; available at <https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/>) [15]. The HMM search led to the identification of 56 candidate *RcWRKY* genes in the rose genome. We examined the sequences of all candidate proteins using the Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), verifying that all 56 *RcWRKY* proteins contained the *WRKY* DNA-binding domain. A total of 25 of the candidate *RcWRKY* proteins contained two *WRKY* domains, while the other 31 contained one *WRKY* domain. All 56 *RcWRKY* genes could be mapped onto the rose chromosomes, and were named *RcWRKY1* to *RcWRKY56* according to their order on the chromosomes.

The sizes of the *RcWRKY* proteins varied dramatically. *RcWRKY19* was the longest, containing 729 amino acids, while the shortest was *RcWRKY10*, comprising just 120 amino acids. The average length of the *RcWRKY* proteins was 359 amino acids. Details of the *RcWRKY* genes, including their accession numbers, chromosomal locations, number of introns and exons, protein sizes, and gene classifications, are provided in Table 1.

Phylogenetic Analysis of the Rose WRKY Genes

A phylogenetic analysis of the *RcWRKY* genes was performed using the neighbor-joining method (Fig. 1). Our subsequent analysis of the exon-intron structures of the *RcWRKYs* was consistent with the results of the phylogenetic analysis. The *RcWRKYs* contain one to six introns, and most of the *RcWRKY* genes in the same clade exhibited similar exon-intron structures, such as *RcWRKY8*, *RcWRKY51*, *RcWRKY4*, *RcWRKY5*, *RcWRKY21*, *RcWRKY6*, and *RcWRKY22* (Table 1; Fig. 1). Some exceptions were also observed; for example, *RcWRKY39* and *RcWRKY40* were grouped into the same clade, but *RcWRKY40* has two introns and *RcWRKY39* has five. Moreover, the lengths of the *RcWRKY* introns are highly variable, ranging from tens to thousands of nucleotides. *RcWRKY11* contains the longest intron, comprising 2,369 bp, while the shortest intron (51 bp) was found in *RcWRKY16*. In addition, we analyzed the conserved *WRKY* motif sequence in the rose *WRKY* proteins (Fig. 2).

A total of 66 *AtWRKY* genes were previously identified in Arabidopsis [16]. There is also increasing evidence that the *WRKY* transcription factors play a key role in disease resistance in various plant species (Table 2). To evaluate the evolutionary relationships among the *RcWRKYs*, *AtWRKYs*, and the plant *WRKYs* known to be involved in the regulation of disease resistance, we generated a composite phylogenetic tree using the neighbor joining method (Fig. 3). The *AtWRKYs* were previously divided into three groups, according to their evolutionary relationships, with those in Group I containing two *WRKY* domains and those in Groups II and III containing just one *WRKY* domain [16]. In the present study, we found that the evolutionary relationships of the *RcWRKYs* were consistent with the Arabidopsis *WRKY* Groups; the *RcWRKY* genes clustered with the Group I *AtWRKY* genes contained two *WRKY* domains, while the other *RcWRKY* genes contained just one *WRKY* domain. We found that the *WRKYs* reported to take part in the regulation of the disease response were distributed across all three Groups.

Chromosomal Locations, Gene Duplication, and Microsynteny

The *RcWRKY* genes are unevenly distributed across all seven rose chromosomes (Table 1; Fig. 4; Supplemental Fig. S1). We observed a high density of *RcWRKY*s in several regions, including the short arm of chromosome 3 and the long arm of chromosomes 1 and 6. In contrast, *RcWRKY* genes were not found on the short arm of chromosomes 1 and 6. Chromosomes 2 and 5 contain the largest numbers of *RcWRKY* genes (10), followed by chromosome 3 (9), while the lowest numbers of *RcWRKY* genes (6) were found on chromosomes 4 and 6. Chromosomes 1, 3, and 7 contain *RcWRKY* genes from all three Groups, whereas the other chromosomes only included *RcWRKY* genes from Groups I and II. The imbalance of the *RcWRKY* locations across the rose chromosomes implied that genetic variation occurred during evolution.

We further investigated the gene duplication events of the *RcWRKY*s. A total of 17 gene pairs were found in the rose genome (Table 3). Where two repeated genes are located on the same chromosome (*RcWRKY2/RcWRKY7* and *RcWRKY34/RhRKYY38*), they are likely to be tandem repeats. Other *RcWRKY* gene pairs are located on different chromosomes, suggesting that segmental duplications occurred within these regions, which may have arisen during full genome duplication in the roses [15]. The collinear relationship of the *RcWRKY* genes across the chromosomes is shown in Figure 4.

In order to study the selective constraints among the duplicated *RcWRKY* genes, the Ka/Ks nucleotide substitution ratios were calculated for the 17 gene pairs (Table 3). Generally, a Ka/Ks ratio > 1 is consistent with positive selection, while Ka/Ks < 1 indicates a purifying selection. The Ka/Ks ratios of all 17 duplicated gene pairs were < 1 (Table 3), indicating that the duplicated *RcWRKY*s had undergone a purifying selection with limited functional divergence during their evolutionary history.

Expression Patterns of the RcWRKY Genes in Response to B. cinerea

There is increasing evidence to suggest that members of the *WRKY* family play key roles in plant defense responses against various pathogens. This involves the upregulation of *WRKY* expression upon pathogen infection. To study the *RcWRKY* responses to *B. cinerea*, we obtained RNA-seq transcriptomic data from rose petals exposed to this pathogen at 30 hours post inoculation (hpi) and 48 hpi [10]. In rose petals, *B. cinerea* conidia germinate at 24 hpi, and the early response to infection is considered to occur at 30 dpi, as no visible disease lesions form by this point. The 48 hpi timepoint corresponds to the later response, when the lesions were starting to expand from the inoculation points [10].

The expression of 19 *RcWRKY* genes (*RcWRKY2*, *RcWRKY4*, *RcWRKY7*, *RcWRKY8*, *RcWRKY13*, *RcWRKY18*, *RcWRKY21*, *RcWRKY23*, *RcWRKY28*, *RcWRKY29*, *RcWRKY30*, *RcWRKY33*, *RcWRKY34*, *RcWRKY35*, *RcWRKY38*, *RcWRKY41*, *RcWRKY46*, *RcWRKY51*, and *RcWRKY54*) was significantly increased at 48 hpi with *B. cinerea*, suggesting they might be involved in rose resistance against this pathogen. Among these *B. cinerea*-induced *RcWRKY*s, the expression of seven *RcWRKY* genes was also significantly increased at 30 hpi. These results suggest these *WRKY*s might be specific regulators of the early stages of the defense response to *B. cinerea* (Table 4).

RcWRKY41 is Required for Rose Resistance Against B. cinerea

Using RNA-seq data taken from rose petals infected with *B. cinerea*, we identified 19 *B. cinerea*-inducible *WRKY* genes. To further illustrate the potential roles of these genes in the rose resistance against *B. cinerea*, we knocked down the expression of *RcWRKY41* using VIGS. *RcWRKY41* was selected for this VIGS study because 1) its expression is induced in both the early (30 hpi) and late (48 hpi) stages of *B. cinerea* infection, and it is therefore considered an important candidate regulator of resistance against this pathogen; and 2) *RcWRKY41* belongs to Group I of the *RcWRKYs*, and is closely related to many *WRKYs* shown to play roles in disease resistance in various plant species, such as *NbWRKY7*, *NbWRKY8*, *NbWRKY9*, *VvWRKY33*, *OsWRKY53*, and *AtWRKY33* (Fig. 3; Table 4)

To test whether *RcWRKY41* is involved in providing resistance against *B. cinerea*, we knocked down the expression of *RcWRKY41* in rose petals. To this end, we cloned a fragment of the *RcWRKY41* coding sequence into pTRV2 vector [14] to generate *TRV-RcWRKY41*. *Agrobacterium* cells carrying *TRV-RcWRKY41* and *TRV1* [14] constructs were mixed in a 1:1 ratio, then vacuum-infiltrated into the rose petal disks to generate *RcWRKY41*-silenced rose petals. The silenced petals were subsequently inoculated with *B. cinerea*. Compared with the control petals inoculated with the empty TRV vectors (*TRV-00*), plants inoculated with *TRV-RcWRKY41* showed more severe disease symptoms and their lesion sizes increased significantly (Fig. 5). These results indicate that *RcWRKY41* plays an important role in the resistance of roses against *B. cinerea*.

Discussion

Transcription factors often control a cluster of functionally related genes, and are therefore appropriate targets for the genetic engineering of (broad-spectrum) resistant crops. The *WRKY* genes are a major family of plant transcription factors with many important functions, including in the response to pathogens. Systematic and comprehensive genome-wide analyses of the *WRKY* family have previously been performed in *Arabidopsis* [16], rice [17], tomato [18], cotton (*Gossypium raimondii* and *G. hirsutum*) [19], cucumber (*Cucumis sativus*) [20], poplar (*Populus trichocarpa*) [21], and other species; however, a comprehensive analysis of the *RcWRKY* gene family has not previously been reported, leaving the functions of the rose *WRKYs* largely unclear. The rose (*R. chinensis*) genome sequencing project was recently completed, providing useful tools for the genome-wide analysis of the *RcWRKY* gene family. In this study, we comprehensively analyzed the *WRKY* family in rose, including their phylogeny, gene structures, chromosomal locations, gene duplication events, and expression profiles under *B. cinerea* infection.

The rose *RcWRKY* family contains more genes (56) than were reported in cucumber (55), but fewer than the number reported in *Arabidopsis* (66), rice (98), tomato (81), cotton (116 in *G. raimondii* and 102 in *G. hirsutum*), and poplar (104), indicating that the *WRKY* family expanded to varying degrees in different plant species following various gene duplication events during their evolution. Gene duplication was found to play a very important role in the expansion of this gene family in rose; a total of 17 duplication events were identified in the 56 *RcWRKYs*, the majority of which (15) involved segmental duplications, while two involved tandem duplications. The Ka/Ks ratios of all 17 *RcWRKY* pairs were < 1, indicating

that this gene family has undergone purifying selection rather than positive selection, and suggested that the RcWRKYs were highly conserved. In plants, the *Resistance* (*R*) genes encoding the immune receptors that recognize a specific pathogen are often under positive selection pressure [22]. The purifying selection detected for all *RcWRKYs* therefore suggests they may be involved in the basal defense of plants, rather than in race-specific resistance.

Most of the clades identified in the phylogenetic analysis contained WRKYs from both *Arabidopsis* and rose, implying that these two species underwent fairly conservative evolution. There are some exceptions however; for example, AtWRKY38, AtWRKY62, AtWRKY63, AtWRKY64, AtWRKY66, and AtWRKY67 belong to an evolutionary clade that does not contain any RcWRKYs. This indicated that, after diverging from their common ancestor, these WRKY genes were either lost in rose or acquired (through duplication and divergence) in *Arabidopsis*.

Many *WRKY* genes have been shown to be involved in disease resistance in plants, prompting us to search for candidate *WRKY* genes involved in the rose response to *B. cinerea* infection. The elucidation of gene expression patterns often provides clues about their functions; therefore, we examined the expression changes in the *RcWRKYs* when exposed to *B. cinerea* infection. A total of 19 *RcWRKY* genes were found to be significantly upregulated upon *B. cinerea* infection in rose petals, most of which (14 of 19) had undergone gene duplication events. We further identified the *RcWRKYs* that might participate in *B. cinerea* resistance by adding them to a phylogenetic tree of the plant *WRKYs* known to be involved in the disease responses. Among the 19 *B. cinerea*-induced *RcWRKYs*, *RcWRKY41* was shown to be evolutionally close to a number of disease resistance *WRKYs* from various plant species, and its expression was found to increase from the early to late stages of the *B. cinerea* infection. *RcWRKY41* was therefore considered to be a candidate gene participating in *B. cinerea* resistance, which was confirmed when VIGS was used to silence its expression in rose petals, resulting in their reduced resistance to *B. cinerea*. This indicates *RcWRKY41* plays an important positive regulatory function in the resistance of rose petals against grey mold.

Conclusions

We performed a genome-wide analysis of the *RcWRKYs*, exploring their phylogenetic relationships, collinearity, and expression profiles. A total of 56 non-redundant rose *RcWRKY* family members were identified, which could be divided into three groups based on our analyses of their phylogeny and conserved domains; 22 of them belonged to Group I, 26 belonged to Group II, and nine belonged to Group III. Our expression analysis indicated that 19 *RcWRKY* family genes were induced in rose petals subjected to a *B. cinerea* infection. By comparing these sequences with other plant *WRKYs* known to be involved in disease resistance, we revealed that *RcWRKY41* is involved in the regulation of gray mold resistance in rose petals, which was confirmed using VIGS. These results provide new information that may facilitate the further functional analysis of the *RcWRKYs* in roses.

Abbreviations

hpi, hours post inoculation;

NJ, neighbor-joining;

RPKM, number of reads per kb per million reads;

HMM, Hidden Markov Model;

CDD, Conserved Domains Database;

VIGS, virus-induced gene silencing

Declarations

Ethics approval and consent to participate

Not applicable. Our research did not involve any human or animal subjects, material, or data. The plant materials used in this study were provided by the China Agricultural University and are freely available for research purposes following institutional, national and international guidelines.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study has been included within supplemental data. The plant materials are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This study was supported by the National Natural Science Foundation of China (grant number 31772344) to Zhao Zhang. The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Z. Z. and X. L. conceived and designed the experiments. X. L., D. L., and Y. X. performed the experiments. X. L. and S. Z. analyzed the data. Z. Z. and X. L. wrote the paper. All the authors have read and approved the final version of the manuscript.

Acknowledgements

None.

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Tables

Table 1 Members of the *RcWRKY* gene family, as predicted in *R. chinensis* genome sequence

Gene	Accession number ¹	Chr. ²	Position ³	Intron	Exon	CDS (bp)	Amino Acids	Clade
RcWRKY1	RchiOBHm_Chrlg0348121	1	40.91	4	5	894	297	□
RcWRKY2	RchiOBHm_Chrlg0357671	1	50.01	2	3	990	329	□
RcWRKY3	RchiOBHm_Chrlg0357751	1	50.05	1	2	681	226	□
RcWRKY4	RchiOBHm_Chrlg0359091	1	51.09	2	3	1053	350	□
RcWRKY5	RchiOBHm_Chrlg0372431	1	61.12	2	3	1047	348	□
RcWRKY6	RchiOBHm_Chrlg0372521	1	61.17	2	3	501	166	□
RcWRKY7	RchiOBHm_Chrlg0378621	1	65.04	2	3	1281	426	□
RcWRKY8	RchiOBHm_Chrlg0380121	1	65.88	2	3	1101	366	□
RcWRKY9	RchiOBHm_Chrg0106361	2	17.77	2	3	945	314	□
rcWRKY10	RchiOBHm_Chrg0117181	2	29.47	1	2	363	120	□
rcWRKY11	RchiOBHm_Chrg0117781	2	30.1	2	3	741	246	□
rcWRKY12	RchiOBHm_Chrg0130891	2	46.96	2	3	741	246	□
rcWRKY13	RchiOBHm_Chrg0133001	2	49.62	4	5	975	324	□
rcWRKY14	RchiOBHm_Chrg0151681	2	69.24	2	3	1113	370	□
rcWRKY15	RchiOBHm_Chrg0156771	2	73.35	4	5	1431	476	□
rcWRKY16	RchiOBHm_Chrg0166991	2	81.67	3	4	1605	534	□
rcWRKY17	RchiOBHm_Chrg0169011	2	83.29	3	4	1473	490	□
rcWRKY18	RchiOBHm_Chrg0175911	2	87.91	1	2	606	201	□
rcWRKY19	RchiOBHm_Chrg0447881	3	0.39	4	5	2190	729	□
rcWRKY20	RchiOBHm_Chrg0450591	3	2.07	2	3	945	314	□
rcWRKY21	RchiOBHm_Chrg0460351	3	8.49	2	3	1122	373	□
rcWRKY22	RchiOBHm_Chrg0460361	3	8.51	2	3	987	328	□
rcWRKY23	RchiOBHm_Chrg0461481	3	9.53	4	5	1521	506	□
rcWRKY24	RchiOBHm_Chrg0466341	3	12.8	2	3	879	292	□
rcWRKY25	RchiOBHm_Chrg0468221	3	14.29	6	7	1437	478	□
rcWRKY26	RchiOBHm_Chrg0485711	3	32.49	4	5	1344	447	□
rcWRKY27	RchiOBHm_Chrg0487201	3	34.38	2	3	963	320	□
rcWRKY28	RchiOBHm_Chrg0398741	4	15.39	2	3	918	305	□
rcWRKY29	RchiOBHm_Chrg0425801	4	51.41	2	3	1167	388	□
rcWRKY30	RchiOBHm_Chrg0429851	4	54.56	1	2	576	191	□
rcWRKY31	RchiOBHm_Chrg0438661	4	61.29	3	4	969	322	□
rcWRKY32	RchiOBHm_Chrg0439041	4	61.49	3	4	1551	516	□
rcWRKY33	RchiOBHm_Chrg0440391	4	62.52	2	3	1668	555	□
rcWRKY34	RchiOBHm_Chrg0002561	5	1.49	5	6	1752	583	□
rcWRKY35	RchiOBHm_Chrg0011581	5	7.68	2	3	834	277	□
rcWRKY36	RchiOBHm_Chrg0013131	5	8.9	2	3	1062	353	□
rcWRKY37	RchiOBHm_Chrg0018041	5	12.58	2	3	1491	496	□
rcWRKY38	RchiOBHm_Chrg0040801	5	35.42	5	6	1629	542	□
rcWRKY39	RchiOBHm_Chrg0042581	5	37.45	5	6	1746	581	□
rcWRKY40	RchiOBHm_Chrg0042601	5	37.46	2	3	381	126	□
rcWRKY41	RchiOBHm_Chrg0071811	5	77.65	5	6	1872	623	□
rcWRKY42	RchiOBHm_Chrg0074411	5	80.32	6	7	2151	716	□
rcWRKY43	RchiOBHm_Chrg0083891	5	89.56	2	3	423	140	□
rcWRKY44	RchiOBHm_Chrg0289301	6	52.4	3	4	1053	350	□
rcWRKY45	RchiOBHm_Chrg0299481	6	60.54	1	2	918	305	□
rcWRKY46	RchiOBHm_Chrg0299501	6	60.55	4	5	1176	391	□
rcWRKY47	RchiOBHm_Chrg0305101	6	64.18	2	3	1020	339	□
rcWRKY48	RchiOBHm_Chrg0308491	6	66.57	2	3	969	322	□
rcWRKY49	RchiOBHm_Chrg0311421	6	68.36	2	3	471	156	□
rcWRKY50	RchiOBHm_Chrg0189781	7	9.05	1	2	501	166	□

RcWRKY51	RchiOBHm_Chr7g0195191	7	13.12	2	3	1080	359	0
RcWRKY52	RchiOBHm_Chr7g0196571	7	14.58	1	2	672	223	0
RcWRKY53	RchiOBHm_Chr7g0202671	7	20.25	2	3	879	292	0
RcWRKY54	RchiOBHm_Chr7g0223361	7	45.05	3	4	1374	457	0
RcWRKY55	RchiOBHm_Chr7g0241021	7	67	2	3	831	276	0
RcWRKY56	RchiOBHm_Chr7g0241041	7	67.01	4	5	429	142	0

¹Available at <https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/>.

²Chromosome

³Starting position (Mb)

Table 2 Plant *WRKY* family genes involved in disease resistance

Gene name	Gene ID	Species	Pathogens	References
AtWRKY3	At2g03340	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[23]
AtWRKY4	At1g13960	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[23]
AtWRKY7	At4g24240	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i> <i>Pseudomonas syringae</i>	[24]
AtWRKY8	At5g46350	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[25]
			Tobacco mosaic virus	
AtWRKY11	At4g31550	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[26]
AtWRKY17	At2g24570	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[27]
AtWRKY18	At4g31800	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[28]
			<i>Pseudomonas syringae</i>	
AtWRKY25	At2g30250	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[29]
AtWRKY27	At5g52380	<i>Arabidopsis thaliana</i>	<i>Ralstonia solanacearum</i>	[30]
AtWRKY28	At4g18170	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[31]
			<i>Sclerotinia sclerotiorum</i>	
AtWRKY33	At2g38470	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[32]
			<i>Alternaria brassicicola</i>	
			<i>Pseudomonas syringae</i>	
AtWRKY38	At5g22570	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[33]
AtWRKY40	At1g80840	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[28]
AtWRKY46	At2g46400	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[34]
AtWRKY48	At5g49520	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[35]
AtWRKY50	At5g26170	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[36]
AtWRKY51	At5g64810	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[36]
AtWRKY53	At4g23810	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[37]
AtWRKY57	At1g69310	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[38]
AtWRKY60	At2g25000	<i>Arabidopsis thaliana</i>	<i>Botrytis cinerea</i>	[28]
AtWRKY61	At1g18860	<i>Arabidopsis thaliana</i>	Turnip crinkle virus	[39]
AtWRKY70	At3g56400	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	[26]
AtWRKY75	At5g13080	<i>Arabidopsis thaliana</i>	<i>Sclerotinia sclerotiorum</i>	[31]
			<i>Pseudomonas syringae</i>	
GhWRKY25	JF899343	<i>Gossypium hirsutum</i>	<i>Botrytis cinerea</i>	[40]
ViWRKY3	XM_002275540.3	<i>Vitis labrusca</i> × <i>V. vinifera</i>	<i>Botrytis cinerea</i>	[41]
VvWRKY33	XP_002264974.1	<i>Vitis vinifera</i>	<i>Plasmopara viticola</i>	[42]

NbWRKY7	AB445391.1	<i>Nicotiana benthamiana</i>	<i>Botrytis cinerea</i>	[43]
NbWRKY8	AB445392.1	<i>Nicotiana benthamiana</i>	<i>Botrytis cinerea</i>	[43]
NbWRKY9	AB711130	<i>Nicotiana benthamiana</i>	<i>Botrytis cinerea</i>	[43]
NbWRKY11	AB711132	<i>Nicotiana benthamiana</i>	<i>Botrytis cinerea</i>	[43]
OsWRKY30	Os08g0499300	<i>Oryza sativa</i>	<i>Rhizoctonia solani</i>	[44, 45]
OsWRKY53	Os05g0343400	<i>Oryza sativa</i>	<i>Magnaporthe oryzae</i>	[46]
OsWRKY12	Os01g0624700	<i>Oryza sativa</i>	<i>Xanthomonas oryzae pv. oryzae</i>	[47]
OsWRKY4	Os03g0758900	<i>Oryza sativa</i>	<i>Rhizoctonia solani</i>	[48]
OsWRKY22	Os01g0820400	<i>Oryza sativa</i>	<i>Magnaporthe oryzae</i>	[49]
OsWRKY71	Os02g0181300	<i>Oryza sativa</i>	<i>Xanthomonas oryzae pv. oryzae</i>	[50]
OsWRKY31	Os03g0321700	<i>Oryza sativa</i>	<i>Magnaporthe oryzae</i>	[51]
HvWRKY2	Aj858838	<i>Hordeum vulgare</i>	<i>Blumeria graminis hordei</i>	[52]
			<i>Melampsora lini</i>	
BnWRKY1	EU912389	<i>Brassica napus</i>	<i>Sclerotinia sclerotiorum</i>	[53]
BnWRKY32	EU912402	<i>Brassica napus</i>	<i>Sclerotinia sclerotiorum</i>	[53]
BnWRKY70	EU912415	<i>Brassica napus</i>	<i>Sclerotinia sclerotiorum</i>	[53]
CaWRKY1a	DQ335599	<i>Capsicum annuum</i>	<i>Xanthomonas axonopodis pv. glycines</i>	[54]

Sequence1	Sequence2	Ka	Ks	Ka/Ks	Effective Len	Average S-sites	Average N-sites
RcWRKY2	RcWRKY7	0.5781	2.173169	0.266017	939	221.0833	717.9167
RcWRKY27	RcWRKY29	0.517668	NaN	NaN	948	217.4167	730.5833
RcWRKY18	RcWRKY30	0.360378	2.11817	0.170136	564	123.0833	440.9167
RcWRKY16	RcWRKY32	0.397724	1.996123	0.199248	1458	339.25	1118.75
RcWRKY17	RcWRKY33	0.47967	1.902171	0.25217	1359	316.5	1042.5
RcWRKY12	RcWRKY35	0.481472	NaN	NaN	699	164.25	534.75
RcWRKY14	RcWRKY36	0.459658	3.074976	0.149484	987	222.9167	764.0833
RcWRKY34	RcWRKY38	0.383131	2.014811	0.190157	1494	342.9167	1151.083
RcWRKY15	RcWRKY39	0.633703	1.385551	0.457365	1395	329.5	1065.5
RcWRKY23	RcWRKY41	0.356736	1.316333	0.271008	1500	339.3333	1160.667
RcWRKY20	RcWRKY44	0.309884	1.32433	0.233993	927	205.75	721.25
RcWRKY13	RcWRKY45	0.620285	NaN	NaN	741	168.9167	572.0833
RcWRKY9	RcWRKY48	0.331141	NaN	NaN	891	218	673
RcWRKY18	RcWRKY50	0.436328	2.474255	0.176347	492	105.3333	386.6667
RcWRKY30	RcWRKY50	0.369102	1.186943	0.310968	480	102.0833	377.9167
RcWRKY4	RcWRKY51	0.512715	2.346934	0.218462	1008	221.5833	786.4167
RcWRKY2	RcWRKY52	0.489159	1.471413	0.332442	654	150.75	503.25

Table 3 Duplication analysis of the *RcWRKY* gene family

Table 4 Expression of the *RcWRKY* genes under *B. cinerea* infection¹

Gene ²	Accession number	Group	log ₂ Ratio	log ₂ Ratio
			30hpi	48hpi
RcWRKY2	RchiOBHm_Chr1g0357671	II	-	2.908
RcWRKY4	RchiOBHm_Chr1g0359091	III	2.104	1.966
RcWRKY7	RchiOBHm_Chr1g0378621	II	-	2.269
RcWRKY8	RchiOBHm_Chr1g0380121	III	2.864	3.92
RcWRKY13	RchiOBHm_Chr2g0133001	II	-	5.947
RcWRKY18	RchiOBHm_Chr2g0175911	I	3.162	5.883
RcWRKY21	RchiOBHm_Chr3g0460351	III	-	6.323
RcWRKY23	RchiOBHm_Chr3g0461481	I	1.22	3.598
RcWRKY28	RchiOBHm_Chr4g0398741	II	-	2.188
RcWRKY29	RchiOBHm_Chr4g0425801	I	-	1.386
RcWRKY30	RchiOBHm_Chr4g0429851	I	2.319	4.448
RcWRKY33	RchiOBHm_Chr4g0440391	II	-	5.654
RcWRKY34	RchiOBHm_Chr5g0002561	II	-	3.618
RcWRKY35	RchiOBHm_Chr5g0011581	II	1.107	2.229
RcWRKY38	RchiOBHm_Chr5g0040801	II	-	4.919
RcWRKY41	RchiOBHm_Chr5g0071811	I	1.673	3.79
RcWRKY46	RchiOBHm_Chr6g0299501	II	1.092	2.166
RcWRKY51	RchiOBHm_Chr7g0195191	III	1.85	1.94
RcWRKY54	RchiOBHm_Chr7g0223361	II	-	1.962

¹The log₂ transformed expression profiles were obtained from the RNA-seq dataset [10].

²The *RcWRKY*s undergo duplicate events are marked in bold.

Figures

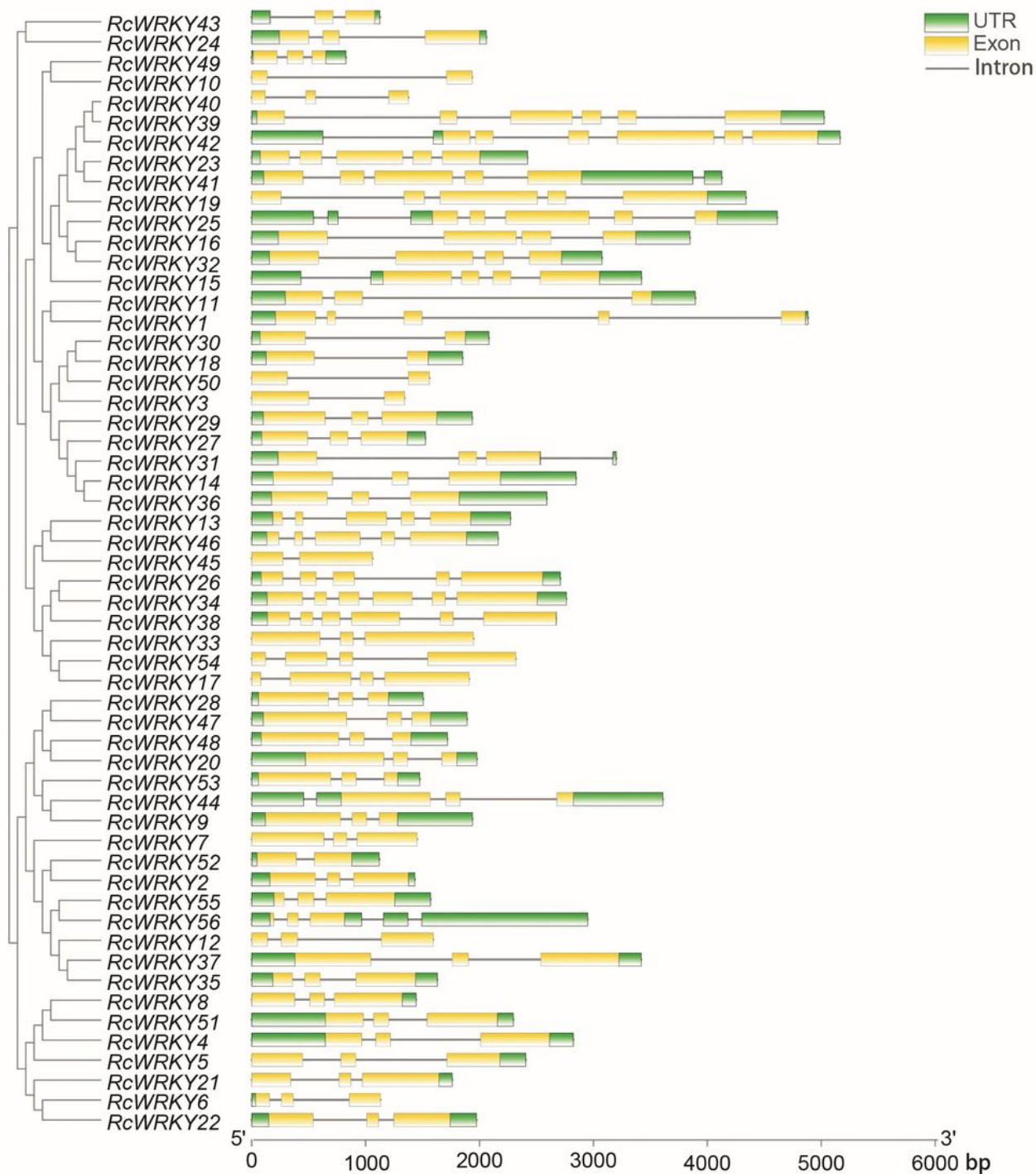


Figure 1

Phylogenetic analysis of the rose WRKY transcription factors. A complete alignment of the rose WRKYs was used to construct the phylogenetic tree.



Figure 2

The sequence of the WRKY motif in rose WRKY proteins. These sequences were determined from the multiple alignment analysis of 56 RcWRKY transcription factors. The bit score indicates the informational content for each position in the sequence.

tree using the Neighbor-Joining method. The bootstrap values are indicated on the nodes of the branches. The WRKYs reported to be involved in plant disease resistance are marked in bold.

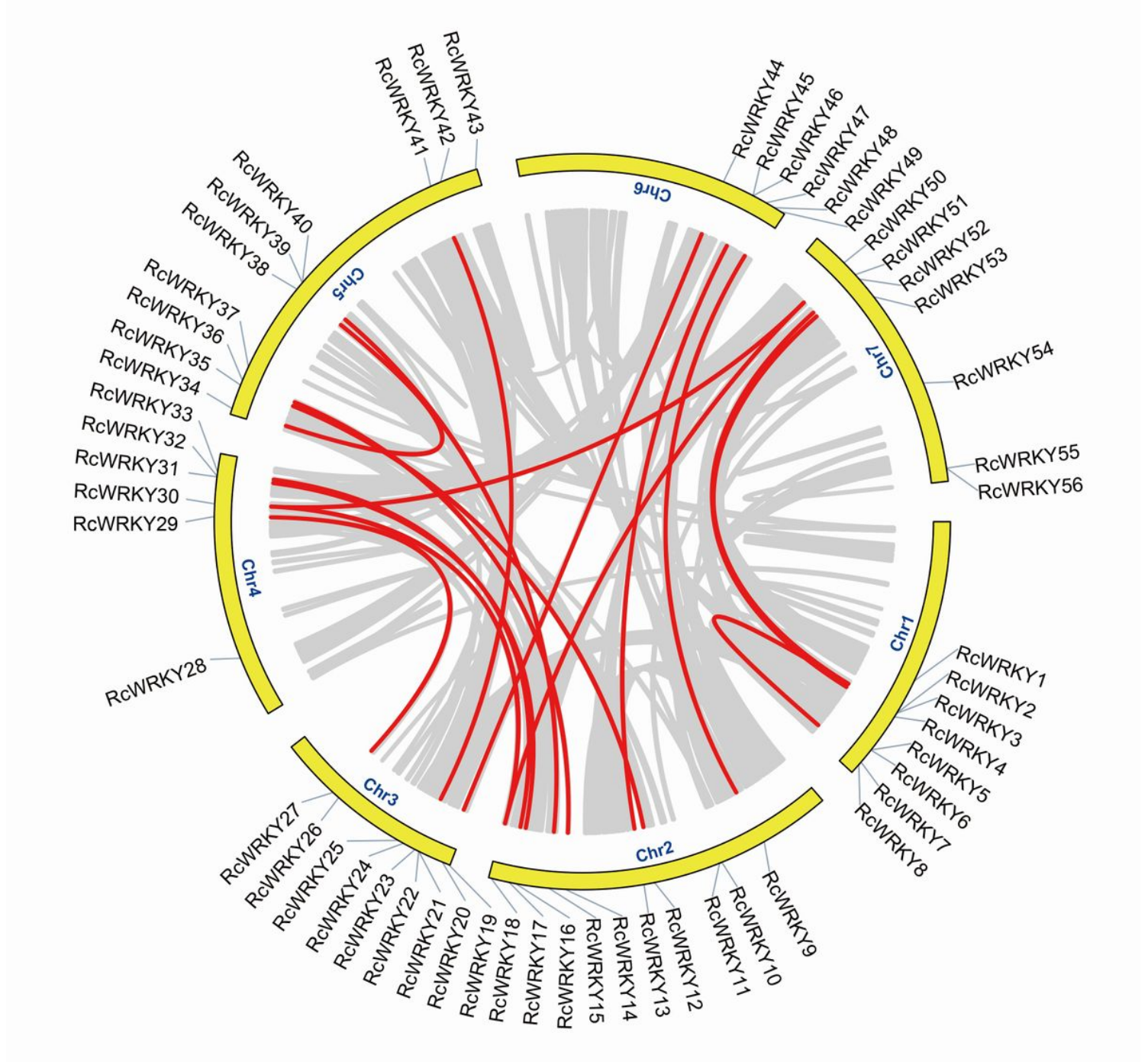


Figure 4

Localization and synteny of the WRKY transcription factors in the rose genome. The RcWRKYs were mapped to the rose chromosomes. Those with a syntenic relationship are joined by red lines. The gray lines indicate all syntenic blocks in the rose genome.

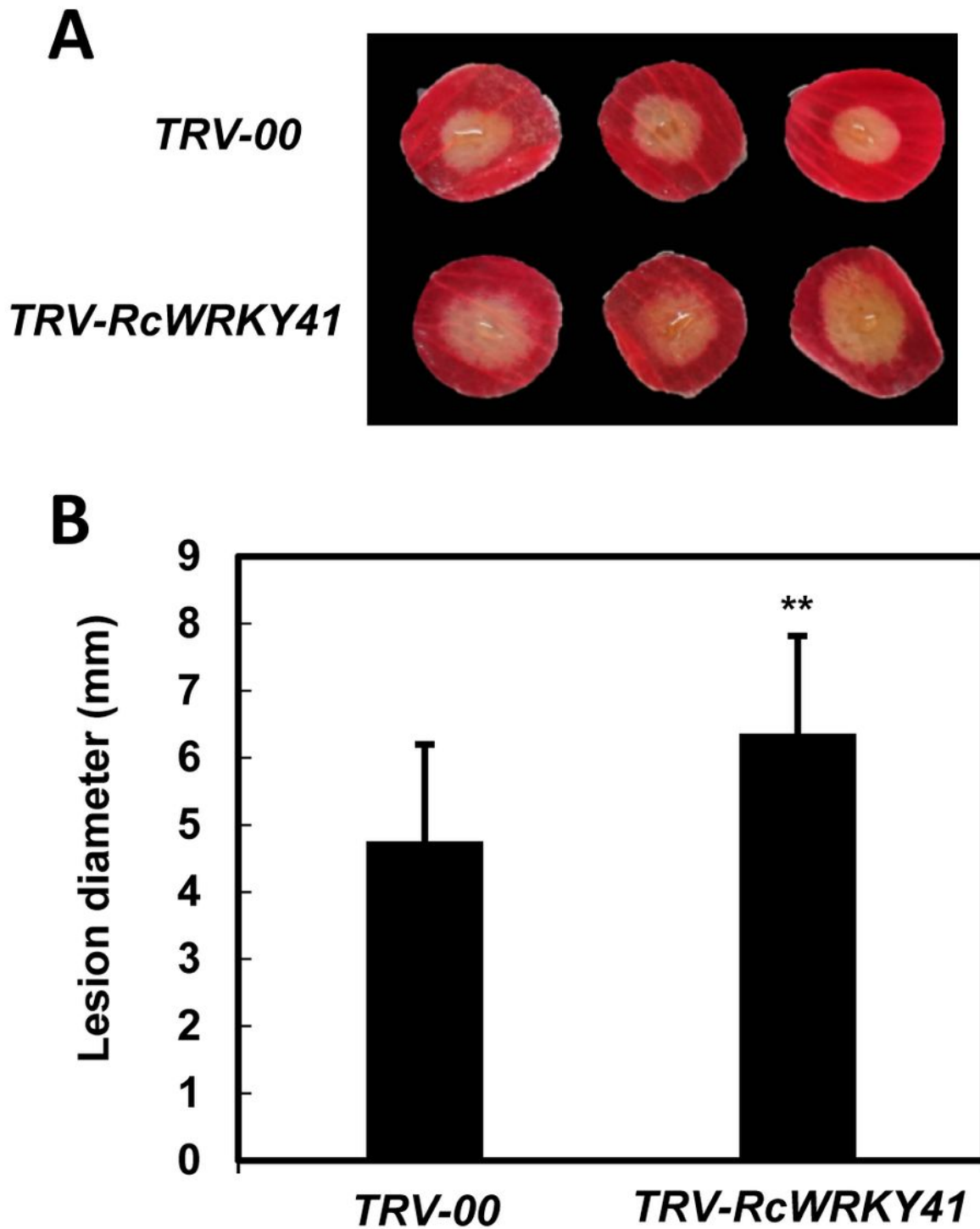


Figure 5

Functional analysis of rose transcription factor gene *RcWRKY41*. (A) Compromised *B. cinerea* resistance symptoms on rose petal disks upon the silencing of *RcWRKY41*, shown at 60 hpi (hours post inoculation). A recombinant tobacco rattle virus (TRV) targeting *RcWRKY41* (*TRV-RcWRKY41*) was used for the gene silencing, and an empty TRV (*TRV-00*) was used as the control. (B) Quantification of the

average diameter of the disease lesions on the control and RcWRKY41-silenced petals at 60 hpi. Error bars = standard deviation. The statistical analysis was performed using a Student's t-test; ** $P < 0.01$.

Supplementary Files

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