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Genome Wide Transcriptomic Analysis of *WRKY* Gene Family Response to Biotic Stresses in *Malus* × *domestica*

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Abstract

Apple (*Malus* × *domestica* Borkh.) is a perennial woody plant that often suffers from various biological stresses. Many harmful pathogens can infect apple trees and lead to reduced production. We comprehensively identified the *WRKY* genes in the apple genome and analyzed their expression in response to several biological stressors, including *Alternaria alternata*, *Pythium ultimum*, *Botryosphaeria dothidea*, *Erwinia amylovora*, *Penicillium expansum*, *Gymnosporangium yamadae*, and Apple replant disease. There were 113 *MdWRKYs* identified in the apple genome. Twenty-two *MdWRKYs* were differentially expressed in response to at least five pathogens. Promoter sequence analysis showed that these genes carried many defense- and stress-responsive elements, such as MeJA-response elements, salicylic acid-response elements, and W-box elements, in their promoters. Transient expression assays showed that *MdWRKY*40*a* and *MdWRKY*54*h* played negative roles in defense against *B. dothidea* infection. *WRKY*40 and *WRKY*60 and the *MdWKRY*33*s* might play important roles in responding to pathogens and are conserved in some plants. These differentially expressed *MdWRKYs* might play key roles in the apple response to multiple pathogens.

Keywords

Apple, *WRKY*, Transcription Factor, Pathogens, Big Data

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1. Introduction

Apple (*Malus* × *domestica* Borkh.) is an important fruit crop cultivated on a great deal of land around the world. Many harmful pathogens infect apple trees and lead to a reduction in yields [1] [2]. Fungicides are commonly used in orchards to control and prevent fungal diseases. But fungicides have adverse effects on the environment and can result in pathogen resistance. Screening disease-resistant genetic resources and breeding disease-resistant cultivars combine to form one of the effective strategies to resist pathogens. Therefore, it is important to understand the molecular mechanism of pathogen infection in apple and to identify disease resistance genes.

WRKY transcription factors are known to participate in the defense responses of higher plants [3]. A growing number of *WRKY* transcription factors have been proved to play roles in host-pathogen interactions between different plants and pathogens. The *WRKY* transcription factors are characterized by the conserved 7-amino acid sequence *WRKY*GQK at the N-terminal and the zinc finger motif at the C-terminal. The *WRKY* family was divided into 3 groups based on the number of *WRKY* sequences and the zinc finger sequence. Group I WKRY proteins contain two *WRKY* domains and a C2H2 zinc-finger motif, while the group II and group III have only one *WRKY* domain and either a C2H2 or C2HC zinc-finger motif, respectively. The *WRKY* domain can bind to a W-box (TTGACC/T) cis-element in a promoter to stimulate or repress target gene expression. The W-box appears in the promoters of many plant genes that are associated with defense [4].

In *Arabidopsis*, several *WRKY* genes have been proved to associate with responses to pathogen infections. The Group IIa members At*WRKY*18, At*WRKY*40 and At*WRKY*60 interact with each other to regulate defense pathways [5]. *WRKY*18, *WRKY*40, and *WRKY*33 each bind to the promoters of more than 1000 genes involved in signal perception and transduction not only during microbial-associated molecular pattern-triggered immunity (MTI) but also upon damage-associated molecular pattern-triggered immunity [6]. *WRKY*22 and *WRKY*29 are induced by the MAPK pathway involved in plant responses to both bacterial and fungal pathogens, and transient expression of *WRKY*29 in leaves leads to reduced disease symptoms [7]. *WRKY*53 and *WRKY*70 both positively modulate systemic acquired resistance (SAR) [8]. The Group I members *WRKY*3 and *WRKY*4 play positive roles in plant resistance to necrotrophic pathogens. *WRKY*4 has a negative effect on plant resistance to biotrophic pathogens [9]. The group IId members *WRKY*11 and *WRKY*17 are negative regulators of basal resistance in *Arabidopsis* [10].

Some of the apple *WRKY* genes have been demonstrated to be involved in plant defense. *MdWRKYN*1 and *MdWRKY*26 are targeted by miRNAs and are involved in apple resistance to leaf spot disease caused by *Colletotrichum* spp. [11]. Md*WRKY*100 positively regulates apple resistance to *Colletotrichum gloeosporioides* infection [12]. Ectopic expression of *MdWRKY*1 (homolog of *At*WRKY15) in tobacco plants enhances resistance to *Phytophthora parasitica*

and activates the expression of PR genes [13]. Md *WRKY*15 improves apple resistance to *Botryosphaeria dothidea* via the salicylic acid-mediated pathway by directly binding the *MdICS*1 promoter [14] (Zhao *et al.*, 2020). Md *WRKY*46 enhances apple resistance to *B. dothidea* by activating the expression of *MdPBS*3.1 in the salicylic acid signaling pathway [15]. Md *WRKY*31 regulates plant resistance to *B. dothidea* through the SA signaling pathway by interacting with MdHIR4 [16].

Apple is a commercially cultivated fruit that is important economically and is favored by consumers, and thus is extensively studied. There are large amounts of publicly available data on apples, including genomic sequences, transciptomic and metabolic datasets. Although the *WRKY* gene family has been analysed genome-wide in several species, including *Arabidopsis*, wheat, grapes, poplar, and strawberry [4] [16] [17] [18] [19] [20]. The responses of *WRKY* genes in apple to drought, flooding, and plant hormone have also been studied [21] [22]. Numerous *WRKY* genes were identified that play roles during infection by multiple pathogens. Considering the important roles of the *WRKY* family in plant disease responses, this study aimed to analyze the responses of *WRKY* transcription factors in apple to biotic stress through analysis of several published transcriptomic datasets.

2. Material and Methods

2.1. Identification of *MdWRKY* Genes in the Apple Genome

Arabidopsis AtWRKY protein sequences retrieved from TAIR (The Arabidopsis Information Resource: http://www.arabidopsis.org/) were used as BLASTP queries against the apple genome GDDH13_1-1 [23] (https://iris.angers.inra.fr/gddh13/the-apple-genome-downloads.html) using a stand-alone version of BLAST (Basic Local Alignment Search Tool: http://blast.ncbi.nlm.nih.gov) [24]. Similar sequences with e-values < 0.0001 were further inspected for conservation of the *WRKY* domain (*WRKY*GQK signature amino sequence) using the domain analysis programs Pfam (Protein family: http://xfam.org/) [25] and Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) [26] with default cutoff parameters. ExPASY (http://www.expasy.org/tools/) was used to predict the isoelectric point (pI) and molecular weight (MW) of each Md *WRKY*. The position of each gene on the apple chromosomes and their exon/intron structure were depicted with TBtools [27] based on the genome annotation information of the apple genome GDDH13_1-1. Conserved motifs of the Md *WRKY*s were searched using the Multiple Expectation maximization for Motif Elicitation tool (MEME version 4.9.1, http://meme.nbcr.net/meme/cgi-bin/meme.cgi).

2.2. Phylogenetic Analysis and Classification of Apple *MdWRKY* Genes

The *Arabidopsis* and apple *WRKY* amino sequences were used for phylogenetic tree construction. The phylogenetic tree was constructed using the MEGA 7

program with the neighbor-joining (NJ) method, 1000 bootstrap replicates, and partial deletion parameters. The apple *WRKY*s were divided into different groups according to the conserved *WRKY* and zinc finger domains.

2.3. Expression Analysis of the *MdWRKY* Genes in Apple

The expression of the *MdWRKYs* members in different tissues was determined by published transcriptomics data (Supplemental Table S1). qRT-PCR was also used to measure the expression of several *MdWRKYs* in the leaf, shoot, root, flower, and fruit from the 4 years old apple rootstock M9-T337. Primers for qRT-PCR were designed to amplify 100 - 200 bp target fragments using NCBI Primer Blast (Supplemental Table S3). Quantitative real-time PCR was performed using the Bio-Rad CFX Connect Real-Time PCR Detection System. The reaction volume was 20 μL with 100 ng of template cDNA. PCR amplification conditions were as follows: 95˚C for 5 min for initial denaturation, then 45 cycles of 94˚C for 20 s, 60˚C for 20 s (determined by the primer), and 72˚C for 10 s. Fluorescence was measured at the end of each cycle. The apple *Actin* gene was used as an internal standard in the analysis. The relative expression level of each gene was calculated according to the $2^{-\Delta\Delta CT}$ method. Values for mean expression and standard error (SE) were calculated from the results of three independent replicates.

The expression responses of the *MdWRKYs* to apple replant disease (ARD), *Alternaria alternata*, *Pythium ultimum*, *Botryosphaeria dothidea*, *Erwinia amylovora*, *Penicillium expansum*, and *Gymnosporangium yamadae* were determined by the transcriptome data downloaded from the NCBI SRA (Supplemental Table S2).

After filtering low quality reads and contaminant sequences, the clean reads were aligned to the *Malus* × *domestica* genome GDDH13_1-1 using the HISAT2 software. The Stringtie software was used to assemble the transcripts [28]. Gene expression was calculated using the Fragments Per Kilobase of transcript per Million fragments mapped reads method (FPKM). DESeq2 software was used to estimate differentially expressed genes [29]. Genes with an FDR < 0.1 and $|\log_2(\text{fold change})| \geq 1$ between two samples were identified as differentially expressed genes.

2.4. Promoter Analysis for Cis-Acting Regulatory Elements

For each Md *WRKY* gene, a 2000-bp sequence upstream of the start codon was retrieved from the GDDH13_1-1 genome and was submitted to the PlantCARE website to search the cis-acting regulatory elements [30].

2.5. *Botryosphaeria dothidea* Infection Assays

Botryosphaeria dothidea was isolated from the apple orchard and maintained on Potato Dextrose Agar medium in the dark at 28˚C.

Full coding sequences of *MdWRKY*40*a* and *MdWRKY*54*h* were ligated into

the overexpression vector SAK-227 to generate the vectors *MdWRKY*40*a-OE* and *MdWRKY*54*h-OE*. About 300-bp fragments specific to either *MdWRKY*40*a* or *MdWRKY*54*h* were ligated into the virus induced gene silence (VIGS) vector TRV2 to generate *TRV-MdWRKY*40*a* or *TRV-MdWRKY*54*h*. *Agrobacterium tumefaciens* transformed with the VIGS or OE recombinant vectors was injected into mature 'Pink Lady' apple fruits as described previously [31]. The empty vectors were the controls. After *A. tumefaciens* infiltration, the injection holes were inoculated with freshly grown *B. dothidea* mycelia. The apples inoculated with *Agrobacterium tumefaciens* and *B. dothidea* were stored in darkness at 28˚C, and the symptoms were recorded on 4 days post inoculation (dpi). Fifteen apples were inoculated with each treatment combination. Each apple was inoculated with two holes, one as control, and the other as silence or overexpression treatment, on the opposite side of the apple fruit peels. The area of each spot was measured and compared to control.

3. Results

3.1. Identification and Classification of Apple *MdWRKY* Genes

A total of 113 members homologous to the *WRKY* transcription factor family were identified from the apple genome. All members were systematically numbered, as shown in **Table 1**, based on their similarity to genes in *Arabidopsis thaliana*. Among the 113 apple *WRKY* transcription factors, the peptide length ranged from 80 amino acid (aa) residues (*MdWRKY*44*b*) to 924 aa (*MdWRKY*44*e*). The molecular weight of the predicted proteins ranged from 9.28 (*MdWRKY*44*b*) to 102.78 kDA (*MdWRKY*44*e*). The isoelectric point ranged from 4.81 (*MdWRKY*69*b*) to 9.99 (*MdWRKY*11*c*).

A phylogenetic tree was constructed with the *WRKY* protein sequences of apple and *Arabidopsis* (**Figure 1**). According to the results of the phylogenetic tree and conserved domain analysis, the apple *WRKY* family could also be divided into three subgroups: Group I, Group II and Group III. There were 31 *MdWRKYs* in Group I, 65 in Group II, and 17 in Group III. Group II was further divided into five subgroups, with Group IIa containing 6 members, Group IIb containing 14 members, Group IIc containing 18 members, Group IId containing 14 members, and Group IIe containing 13 members.

The 113 *MdWRKYs* were distributed across the 17 apple chromosomes (**Figure 2**). Chromosomes 12 and 15 each carried the most *MdWRKYs*, 10. Chromosomes 01, 07, and 09 each carry 9 *MdWRKYs*. Chromosomes 04 and 17 have 8 *MdWRKYs*. The other chromosomes carried between 2 and 6 *MdWRKYs*. Three genes were mapped to the unassembled scaffolds.

Because the apple genome underwent chromosomal doubling events during evolution, most of the apple *MdWRKYs* that are orthologous to *Arabidopsis* have two homologous genes, such as *MdWRKY*1, *MdWRKY*6, *MdWRKY*7, *MdWRKY*9, *MdWRKY*13*b*, *MdWRKY*14, *MdWRKY*15, *MdWRKY*20, *MdWRKY*22,

Table 1. Informations of *MdWRKY* genes.

Gene name	Gene ID	Group	Location	Strand	mRNA length	aa	MW	pI
Md *WRKY*1a	MD09G1121600	I	Chr09:9379281-9382725	+	2100	484	53.15	5.94
Md *WRKY*1b	MD17G1112600	I	Chr17:9643152-9646837	+	1988	471	51.66	6.67
Md *WRKY*2a	MD03G1044400	I	Chr03:3511777-3516305	−	2595	732	79.51	5.96
Md *WRKY*2b	MD04G1244700	I	Chr04:32067852-32071212	−	2154	717	78.55	5.99
Md *WRKY*2c	MD12G1260600	I	Chr12:32647306-32651367	−	2587	718	78.96	6.41
Md *WRKY*3a	MD13G1067600	I	Chr13:4637048-4640408	+	2230	526	57.34	7.37
Md *WRKY*3b	MD16G1066500	I	Chr16:4642014-4644789	+	2125	528	57.20	8.39
Md *WRKY*9a	MD09G1048300	IIb	Chr09:3166930-3174316	+	1413	470	51.18	7.70
Md *WRKY*9b	MD17G1048400	IIb	Chr17:3528939-3531555	+	1729	455	49.96	7.70
Md *WRKY*9c	MD08G1227200	IIb	Chr08:29356105-29358646	+	1858	570	62.31	5.35
Md *WRKY*9d	MD15G1419600	IIb	Chr15:52086817-52089361	+	1767	582	64.17	5.10
Md *WRKY*11a	MD08G1127200	IId	Chr08:11928202-11930014	+	1335	341	36.96	9.33
Md *WRKY*11b	MD15G1106600	IId	Chr15:7467131-7469028	+	1376	338	36.70	9.46
Md *WRKY*11c	MD13G1239100	IId	Chr13:24320203-24321656	−	1233	281	30.73	9.99
Md *WRKY*11d	MD16G1244300	IId	Chr16:26579453-26581081	−	1370	284	30.92	9.87
Md *WRKY*11e	MD10G1096000	IId	Chr10:15057933-15058421	+	489	162	17.96	9.30
Md *WRKY*12	MD07G1110400	IIc	Chr07:12683567-12688395	+	1092	236	26.78	8.21
Md *WRKY*13a	MD01G1013500	IIc	Chr01:6515683-6519808	+	1393	270	30.43	8.93
Md *WRKY*13b	MD15G1337100	IIc	Chr15:37891029-37895909	−	1173	271	30.24	8.93
Md *WRKY*14a	MD05G1265200	IIe	Chr05:40011060-40015621	+	1479	492	53.78	5.85
Md *WRKY*14b	MD10G1243000	IIe	Chr10:33776504-33781157	+	2075	493	53.24	6.05
Md *WRKY*15a	MD02G1177500	IId	Chr02:15663260-15665127	−	1629	330	35.97	9.65
Md *WRKY*15b	MD15G1287300	IId	Chr15:26365198-26366819	−	1369	331	36.24	9.54
Md *WRKY*15c	MD08G1094900	IId	Chr08:7968389-7970451	+	1455	356	38.64	9.41
Md *WRKY*15d	MD15G1078200	IId	Chr15:5334685-5336858	−	1691	342	37.21	9.26
Md *WRKY*20a	MD03G1188900	I	Chr03:25924164-25929514	−	2258	584	63.85	5.97
Md *WRKY*20b	MD11G1205000	I	Chr11:29914448-29918933	−	2118	588	63.96	5.87
Md *WRKY*21a	MD04G1226400	IId	Chr04:30603205-30604718	+	1222	325	36.59	9.77
Md *WRKY*21b	MD12G1243400	IId	Chr12:31414877-31416020	+	942	313	35.30	9.96
Md *WRKY*21c	MD06G1062800	IId	Chr06:10770799-10772691	−	1419	318	35.88	9.58
Md *WRKY*22a	MD01G1071300	IIe	Chr01:17602924-17604669	+	1512	349	37.65	8.20
Md *WRKY*22b	MD07G1131400	IIe	Chr07:18815423-18816949	−	1301	348	37.66	6.82
Md *WRKY*23a	MD09G1285400	IIc	Chr09:36364455-36366937	+	2092	350	38.39	5.86
Md *WRKY*23b	MD17G1278100	IIc	Chr17:33812003-33814316	+	1656	346	38.17	6.29
Md *WRKY*27a	MD01G1210200	IIe	Chr01:30413680-30415409	+	1413	470	51.76	5.08
Md *WRKY*27b	MD07G1280300	IIe	Chr07:34424687-34425782	+	837	278	31.07	7.25

Continued

Md *WRKY*29a	MD06G1091200	IIe	Chr06:21994255-21995556	+	888	295	33.55	5.49
Md *WRKY*29b	MD14G1112200	IIe	Chr14:18037604-18039324	+	1218	315	35.65	5.05
Md *WRKY*30a	MD06G1104100	III	Chr06:24218568-24220572	−	1489	351	39.42	5.73
Md *WRKY*30b	MD14G1123000	III	Chr14:19736610-19738943	−	1594	355	39.87	5.66
Md *WRKY*32a	MD02G1007900	I	Chr02:496443-499890	−	1844	473	52.03	8.82
Md *WRKY*32b	MD15G1152100	I	Chr15:11267047-11270466	−	1479	434	48.12	7.37
Md *WRKY*33a	MD03G1057400	I	Chr03:4579079-4582074	−	2168	571	62.55	7.08
Md *WRKY*33b	MD11G1059400	I	Chr11:5068503-5071526	−	2174	572	62.90	6.81
Md *WRKY*33c	MD12G1181000	I	Chr12:26084482-26086791	−	1870	512	56.72	6.80
Md *WRKY*33d	MD04G1167700	I	Chr04:25792146-25794577	−	2022	520	57.71	7.23
Md *WRKY*40a	MD09G1224500	IIa	Chr09:27404228-27406362	−	1397	320	35.31	7.72
Md *WRKY*40b	MD17G1223100	IIa	Chr17:27209201-27211741	+	1743	321	35.67	8.23
Md *WRKY*41a	MD01G1215300	III	Chr01:30851473-30853003	−	1044	347	38.71	5.94
Md *WRKY*41b	MD07G1285200	III	Chr07:34745168-34746713	−	1029	342	38.05	5.50
Md *WRKY*41c	MD07G1285400	III	Chr07:34761880-34763425	−	1029	342	38.05	5.50
Md *WRKY*42a	MD05G1349800	IIb	Chr05:46759691-46762704	−	2242	606	65.35	7.78
Md *WRKY*42b	MD10G1324500	IIb	Chr10:40512430-40515449	−	2354	611	65.65	6.63
Md *WRKY*42c	MD09G1111200	IIb	Chr09:8267211-8270166	−	2013	625	68.20	6.34
Md *WRKY*42d	MD17G1099000	IIb	Chr17:8402127-8405223	−	2341	645	69.96	6.44
Md *WRKY*43a	MD01G1071600	I	Chr01:17669166-17671888	+	657	218	24.56	9.36
Md *WRKY*43b	MD07G1131000	I	Chr07:18748505-18750267	−	935	222	24.93	9.36
Md *WRKY*43c	MD01G1123900	I	Chr01:23690319-23691083	−	653	208	23.62	8.92
Md *WRKY*44a	MD04G1112800	I	Chr04:19827951-19828792	−	441	146	16.66	9.60
Md *WRKY*44b	MD12G1129000	I	Chr12:20418703-20419358	+	243	80	9.28	9.85
Md *WRKY*44c	MD12G1128800	I	Chr12:20397217-20403016	+	2791	470	51.46	9.00
Md *WRKY*44d	MD04G1113100	I	Chr04:19846116-19851149	−	2824	470	51.52	8.93
Md *WRKY*44e	MD06G1115200	I	Chr06:25425907-25429273	+	3026	924	102.78	5.33
Md *WRKY*45a	MD06G1138500	I	Chr06:28356819-28357724	+	581	150	17.15	9.56
Md *WRKY*45b	MD14G1154500	I	Chr14:24914608-24915904	+	955	148	17.09	9.59
Md *WRKY*46a	MD01G1078000	III	Chr01:18445739-18447924	−	1224	353	39.17	5.30
Md *WRKY*46b	MD07G1146900	III	Chr07:21450190-21453542	−	2205	356	39.73	5.48
Md *WRKY*47a	MD03G1197600	IIb	Chr03:27003520-27006281	+	1781	538	58.93	6.44
Md *WRKY*47b	MD11G1213500	IIb	Chr11:31232215-31235905	+	1626	541	58.97	6.46
Md *WRKY*48a	MD13G1150700	IIc	Chr13:11807823-11809934	−	1539	385	42.70	6.11
Md *WRKY*48b	MD16G1151000	IIc	Chr16:11906129-11908067	−	1368	371	41.14	5.60
Md *WRKY*49a	MD04G1131000	IIc	Chr04:21800375-21802338	−	967	297	33.41	5.73
Md *WRKY*49b	MD12G1144100	IIc	Chr12:22324583-22326472	−	894	297	33.08	5.94
Md *WRKY*50a	MD08G1067700	IIc	Chr08:5387244-5388373	−	741	161	18.24	8.56

Continued

Md *WRKY*50b	MD15G1054000	IIc	Chr15:3683177-3684465	+	915	161	18.18	5.65
Md *WRKY*51	MD15G1331300	IIc	Chr15:36715532-36718649	+	802	199	22.27	5.87
Md *WRKY*54a	MD12G1189200	III	Chr12:27086797-27089009	−	1196	339	37.73	6.42
Md *WRKY*54b	MD12G1189700	III	Chr12:27213135-27215427	−	1370	332	37.18	5.77
Md *WRKY*54c	MD12G1189600	III	Chr12:27190989-27194447	−	1110	369	40.60	5.69
Md *WRKY*54d	MD12G1189900	III	Chr12:27224756-27226237	−	714	237	27.26	8.29
Md *WRKY*54e	MD04G1175500	III	Chr04:26653551-26655300	−	1002	333	37.19	5.38
Md *WRKY*54f	MD04G1175600	III	Chr04:26669452-26671247	−	1035	344	38.25	6.01
Md *WRKY*54g	MD01G1168600	III	Chr01:27287662-27291066	−	1498	303	34.23	5.71
Md *WRKY*54h	MD07G1234700	III	Chr07:30816225-30818950	−	1407	302	33.97	6.40
Md *WRKY*55a	MD01G1168500	III	Chr01:27285229-27286785	+	687	228	25.66	7.86
Md *WRKY*55b	MD07G1234600	III	Chr07:30813251-30815403	+	1029	342	37.44	5.48
Md *WRKY*57a	MD13G1064700	IIc	Chr13:4465565-4471604	−	1653	346	37.73	6.63
Md *WRKY*57b	MD16G1063200	IIc	Chr16:4485285-4490649	−	1905	324	35.54	6.64
Md *WRKY*58a	MD09G1056600	I	Chr09:3741354-3745516	−	2210	528	57.62	8.62
Md *WRKY*58b	MD17G1054100	I	Chr17:4198534-4202033	−	2178	530	57.38	8.41
Md *WRKY*60a	MD00G1143500	IIa	Chr00:31227045-31229422	+	1538	334	36.98	7.14
Md *WRKY*60b	MD15G1039500	IIa	Chr15:2783151-2784898	+	1208	302	33.60	7.22
Md *WRKY*60c	MD00G1143600	IIa	Chr00:31240990-31242443	+	959	278	31.28	8.86
Md *WRKY*60d	MD15G1039600	IIa	Chr15:2798151-2799702	+	905	286	32.09	8.18
Md *WRKY*61a	MD14G1196100	IIb	Chr14:28655099-28658370	+	1755	584	63.31	6.51
Md *WRKY*61b	MD06G1189100	IIb	Chr06:32608598-32612425	+	2298	683	73.47	7.32
Md *WRKY*61c	MD13G1077900	IIb	Chr13:5478407-5482438	−	2008	571	61.85	5.96
Md *WRKY*61d	MD16G1077700	IIb	Chr16:5438696-5444408	−	2259	587	64.14	6.44
Md *WRKY*65a	MD05G1295700	IIe	Chr05:43023949-43025466	−	1133	273	30.20	5.81
Md *WRKY*65b	MD10G1275800	IIe	Chr10:36698263-36699996	−	1310	266	29.50	5.10
Md *WRKY*69a	MD09G1235100	IIe	Chr09:29539556-29542546	−	1140	250	27.40	5.47
Md *WRKY*69b	MD00G1140800	IIe	Chr00:30743431-30745181	−	1196	268	30.83	4.81
Md *WRKY*69c	MD03G1292900	IIe	Chr03:36975267-36977012	−	992	260	30.34	5.17
Md *WRKY*71a	MD10G1266400	IIc	Chr10:35935449-35937762	−	1615	327	35.94	6.49
Md *WRKY*71b	MD05G1290300	IIc	Chr05:42212192-42214208	−	1288	319	35.12	6.64
Md *WRKY*71c	MD09G1150700	IIc	Chr09:11892767-11895678	−	1683	369	41.36	7.84
Md *WRKY*71d	MD17G1138100	IIc	Chr17:12392128-12394598	−	1211	365	41.25	6.79
Md *WRKY*74a	MD05G1204400	IId	Chr05:33402565-33405458	−	2071	354	39.96	9.70
Md *WRKY*74b	MD10G1191400	IId	Chr10:28819078-28822211	−	1821	355	39.95	9.68
Md *WRKY*75a	MD16G1122400	I	Chr16:8821613-8823564	−	486	161	18.04	9.08
Md *WRKY*75b	MD13G1122100	I	Chr13:8993050-8995368	−	1221	190	21.71	9.74
Md *WRKY*75c	MD09G1008800	I	Chr09:616602-619344	+	694	216	24.76	9.14
Md *WRKY*75d	MD17G1001500	I	Chr17:91553-93431	−	672	223	25.60	9.14

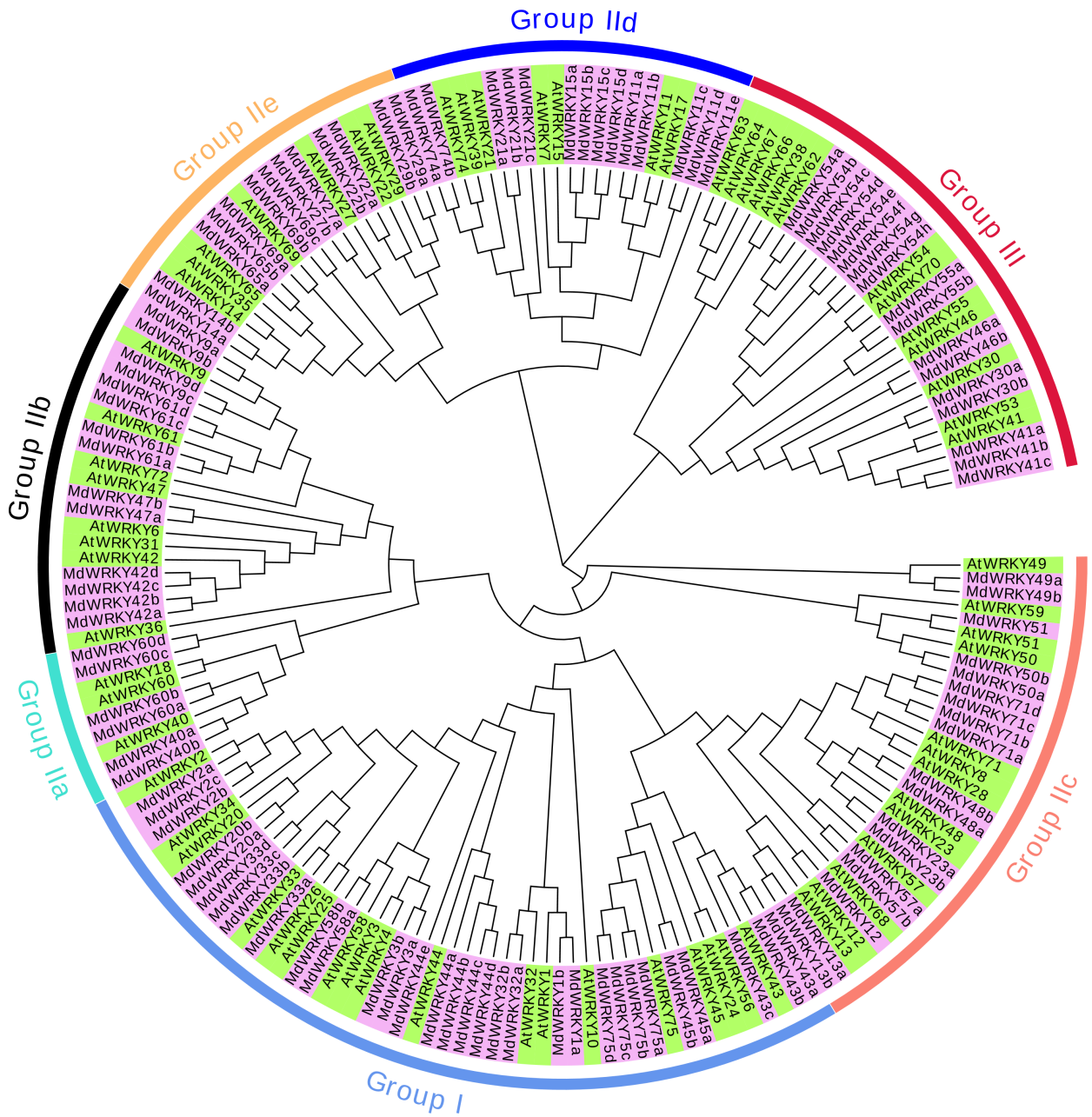

Figure 1. Phylogenetic tree of apple and *Arabidopsis WRKY* transcription factors. Apple (Md) protein sequences are colored in pink, *Arabidopsis* (At) in green. Groups are based on *Arabidopsis*. Genes were first named according to homology with *Arabidopsis*, then within apple, due to genome duplication events.

*MdWRKY*23, *MdWRKY*26, *MdWRKY*29, *MdWRKY*32, *MdWRKY*40, *MdWRKY*45, *MdWRKY*49, *MdWRKY*50, *MdWRKY*55, *MdWRKY*57, *MdWRKY*65, *MdWRKY*72, and *MdWRKY*74. Due to chromosomal fragment duplication, some genes have multiple homologous genes (equal to or greater than 3), such as the 8 homologous *MdWRKY*54 genes.

3.2. Gene Structure and Motif Analysis of *MdWRKYs*

The predicted structures of the *MdWRKY* genes are shown in **Figure 3(b)**. The

number of predicted exons ranged from 1 to 7. *MdWRKY*44*c* and *MdWRKY*44*d* each contained 7 exons. *MdWRKY*11*e* contained 1 exon. MEME detected 10 distinct conserved motifs in the Md *WRKY*s (Figure 3(c)). In general, the homologs had similar motifs. Motifs 1, 2, and 3 are *WRKY* domains and appeared

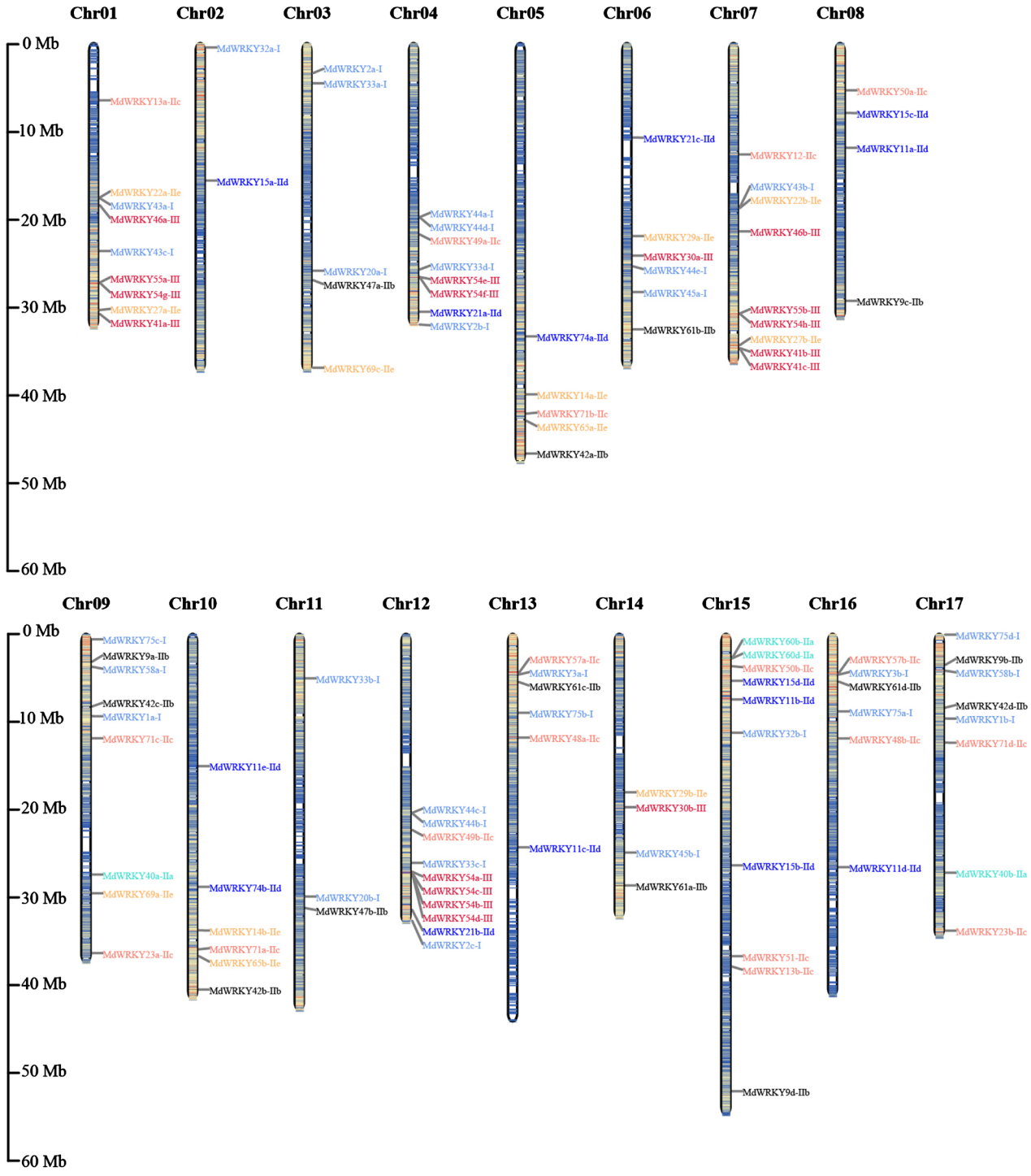

Figure 2. Chromosomal location of the *MdWRKYs* transcription factors. Seventeen apple chromosomes are shown. Three genes did not mapped to any chromosome but mapped to scaffold sequences (not shown).

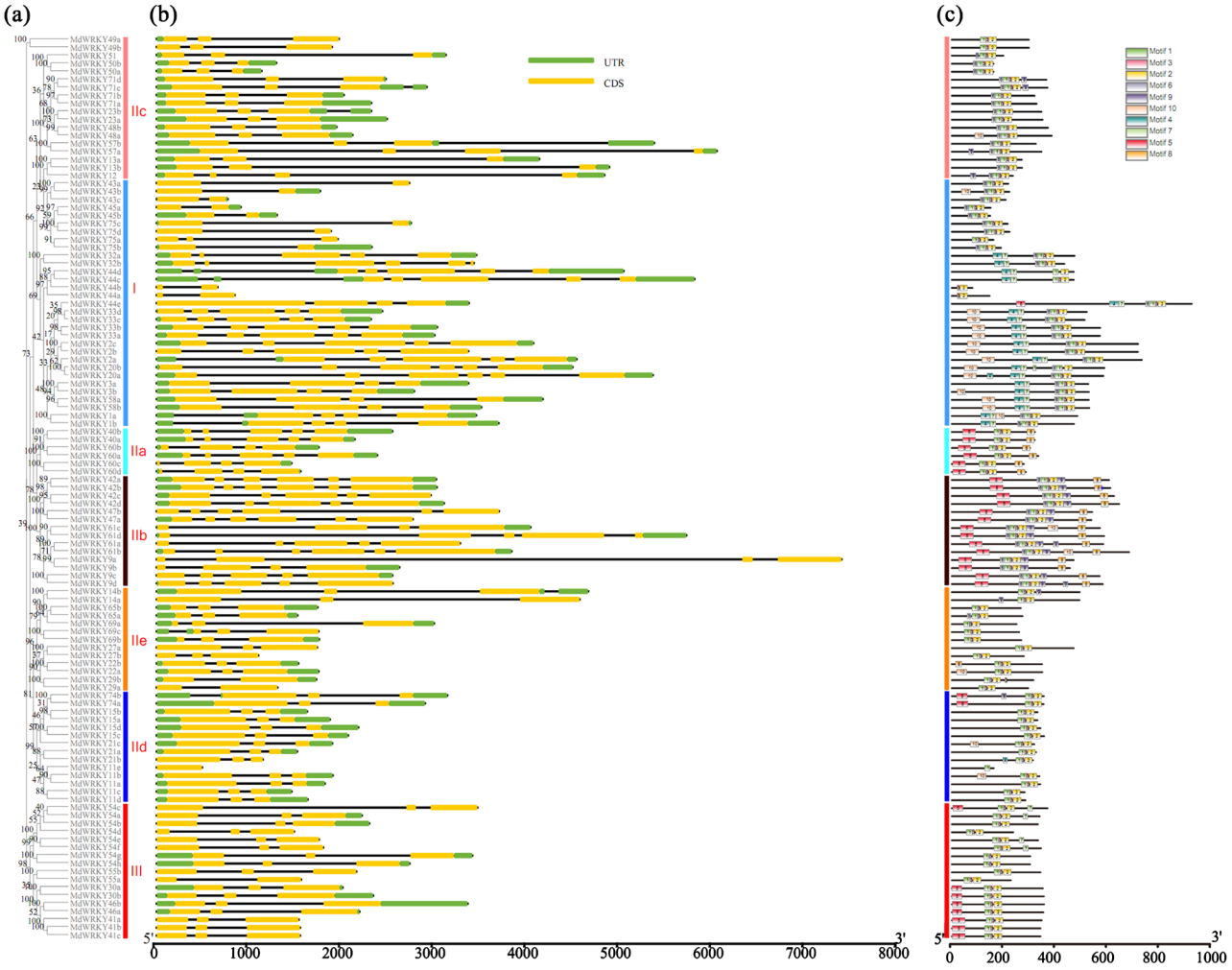

Figure 3. Phylogenetic tree, gene structure and protein motif analysis of *MdWRKYs.* (a) Phylogenetic analysis of Md *WRKY*s, constructed by the neighbor-joining method in MEGA 7.0 software. (b) Gene structure of *MdWRKYs*. Introns, exons, and untranslation region (UTR) are represented by black lines, brown boxes, and light green boxes respectively. The length of each intron and exon is indicated. Each section of bar represents 1 kb. (c) Conserved motif analysis of Md *WRKY*s with MEME. Each colored box represents a conserved motif. Details are included with Supplementary **Figure S1**.

in all Md *WRKY* proteins. In Group IIc, I, IIa, and IIb, motif 6 and motif 9 together with motifs 1, 2, and 3 consist of the *WRKY* domain. Motif 4 and motif 7 compose of another *WRKY* domain that only appeared in some Group I members. Motif 5 was highly similar with leucine zipper (LZ) domains that appear in some At *WRKY*s, which could mediate protein dimerization. Motif 5 appeared in all the Group IIa and IIb members and in some of Group IId and III members. Motif 10 appeared in the N-terminal of some Group I members. Motif 8 appeared in Group IIb.

3.3. Expression Analysis of *MdWRKYs* in Different Tissues

The expression levels of the 113 *MdWRKYs* in different tissues were extracted from published transcriptome data (**Figure 4**). *MdWRKY*11*a*, *MdWRKY*69*b*, *MdWRKY*44*c*, *MdWRKY*58*a*, *MdWRKY*1*a*, *MdWRKY*32*a*, *MdWRKY*15*a*,

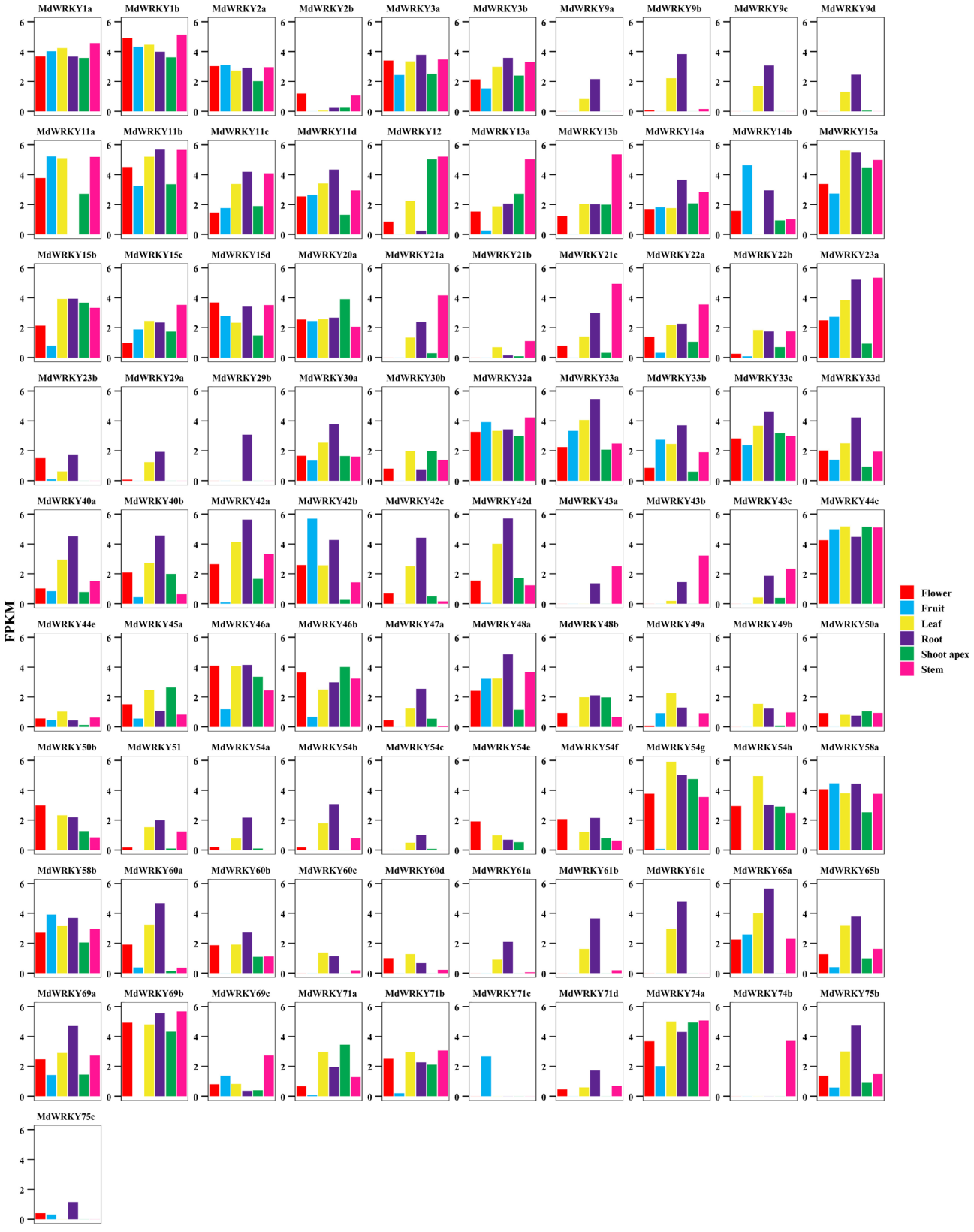

Figure 4. The expression analysis of *MdWRKYs* in different tissues by transcriptome. Gene expression of *MdWRKYs* was quantified in flower (red), fruit (blue), leaf (yellow), root (purple), shoot apex (green), and fruit (pink) and displayed by the $\log_{10}$ (FPKM). Genes that showed low or no expression in these six tissues are not shown in the figure.

*MdWRKY*74*a*, *MdWRKY*11*b*, *MdWRKY*1*b*, and *MdWRKY*54*g* were highly expressed in the most organs. Among the *MdWRKYs*, *MdWRKY*54*g* showed the highest expression in the leaf, while *MdWRKY*11*a* was the highest expressed in the root. *MdWRKY*69*b* showed the highest expression in the fruit, stem, and flower. *MdWRKY*44*c* was the highest expressed in the shoot apex.

Other genes showed highly tissue-specific expression. *MdWRKY*29*b* was only expressed in the root. *MdWRKY*74*b* was only expressed in the stem. *MdWRKY*71*c* was only expressed in the fruit. About 15 *MdWRKYs*, *MdWRKY*47*b*, *MdWRKY*55*a*, *MdWRKY*27*a*, *MdWRKY*45*b*, *MdWRKY*44*b*, *MdWRKY*41*a*, *MdWRKY*44*a*, *MdWRKY*75*a*, *MdWRKY*27*b*, *MdWRKY*2*c*, *MdWRKY*55*b*, *MdWRKY*41*b*, *MdWRKY*41*c*, *MdWRKY*75*d*, and *MdWRKY*11*e* showed low abundance in these tissues within this dataset and are not shown in the figure. *MdWRKY*20*b*, *MdWRKY*32*b*, *MdWRKY*44*d*, *MdWRKY*54*d*, *MdWRKY*57*a*, *MdWRKY*57*b*, and *MdWRKY*61*d* were undetected in these tissues and are not shown in the figure.

We further examined expression of eight *MdWRKY* genes in different tissues by qRT-PCR (Figure 5). The results showed that *MdWRKY*33*a*, *MdWRKY*40*a*, *MdWRKY*51, and *MdWRKY*75*b* were highly expressed in the root. *MdWRKY*42*a* was detected in all examined tissues, with higher expression in the root and flower. *MdWRKY*54*h* showed higher expression in the leaf and shoot compared to other tissues. *MdWRKY*60*c* showed higher expression in the leaf and fruit. *MdWRKY*71*b* showed higher expression in the leaf and root.

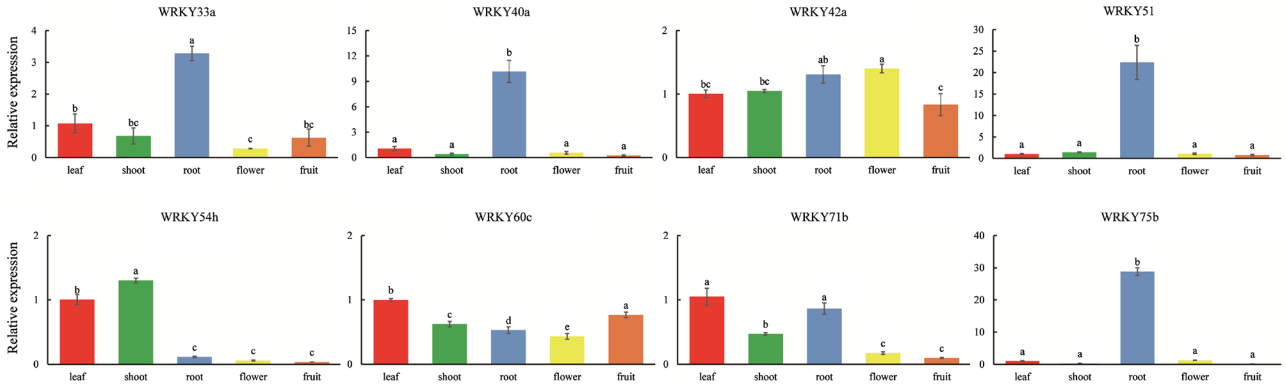

Figure 5. Expression of selected *MdWRKYs* in different tissues by qRT-PCR. Gene expression of eight *MdWRKYs* was measured in leaf, shoot, root, flower, and fruit. The *MdACTIN* gene was used as the internal control to normalize the real-time PCR data. Error bars indicate SEs (standard errors) from 3 biological repetitions.

3.4. Expression Analysis of *MdWRKYs* in Response to Pathogens

The expression of the *MdWRKYs* in response to pathogens was determined (Figure 6). *Alternaria alternata* can cause apple *Alternaria* blotch disease, which often results in defoliation of the tree. Transcriptome analysis was used to determine the response in apple leaves to *A. alternata* infection at 0, 12, 18, 36, and 72 hours post inoculation (hpi) [32]. There were 59 differentially expressed *MdWRKYs* after *Alternaria* infection (Figure 6(a)). *MdWRKY*61*c*, *MdWRKY*32*b*,

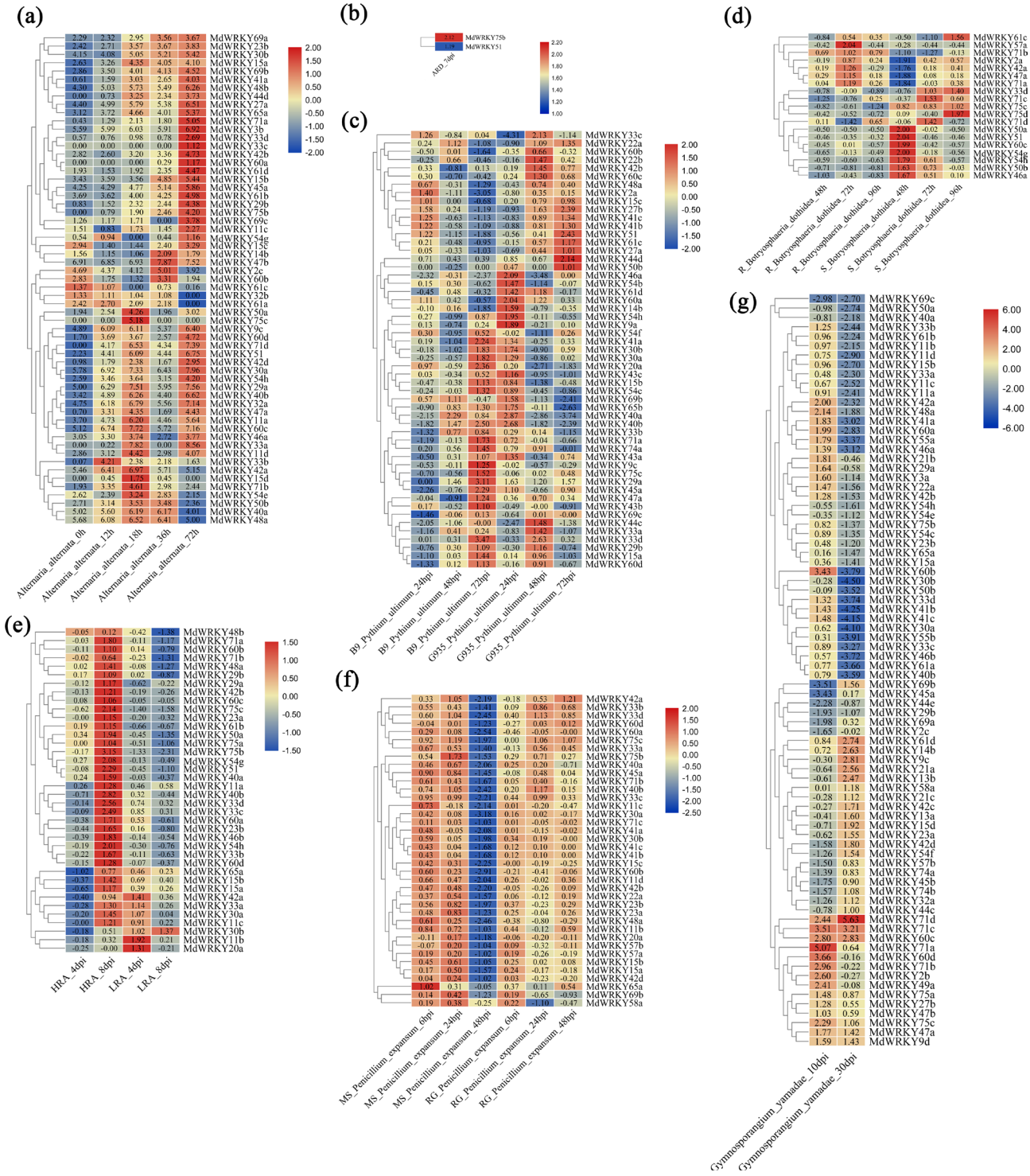

Figure 6. Heatmap of differentially expressed *MdWRKYs* in response to biotic stress. Transcriptome datasets from published studies were mined for data on the 113 Md*WRKY*s identified in the apple genome. Datasets were from apples infected with (a) *Alternaria alternate*; (b) apple replant disease (ARD); (c) *Pythium ultimum*; (d) *Botryosphaeria dothidea*; (e) *Erwinia amylovora*; (f) *Penicillium expansum*; and (g) *Gymnosporangium yamadae.* The color scales of panels (a) and (d) were used to indicate the gene expression level corresponding to the $\log_{10}$(FPKM). The color scales of panels (b), (c), (e), (f), and (g) were used to indicate the gene expression level corresponding to the $\log_{10}(\text{FPKM}_{\text{Treatment}}/\text{FPKM}_{\text{Control}})$.

and *MdWRKY*61*a* were downregulated after *Alternaria* inoculation. *MdWRKY*33*b* reached its highest value at 12 hpi. *MdWRKY*15*a*, *MdWRKY*50*a*, *MdWRKY*75*c*, *MdWRKY*42*a*, *MdWRKY*15*d*, *MdWRKY*71*b*, *MdWRKY*54*e*, *MdWRKY*50*b*, *MdWRKY*40*a*, and *MdWRKY*48*a* reached their highest values at 18 hpi. The expression of *MdWRKY*14*b*, *MdWRKY*47*b*, *MdWRKY*2*c*, and *MdWRKY*60*b* peaked at 36 hpi. *MdWRKY*69*a*, *MdWRKY*23*b*, *MdWRKY*30*b*, *MdWRKY*69*b*, *MdWRKY*41*a*, *MdWRKY*48*b*, *MdWRKY*44*d*, *MdWRKY*27*a*, *MdWRKY*65*a*, *MdWRKY*71*a*, *MdWRKY*3*b*, *MdWRKY*33*d*, *MdWRKY*33*c*, *MdWRKY*42*b*, *MdWRKY*60*a*, *MdWRKY*61*d*, *MdWRKY*15*b*, *MdWRKY*45*a*, *MdWRKY*61*b*, *MdWRKY*29*b*, *MdWRKY*75*b*, *MdWRKY*69*c*, *MdWRKY*11*c*, *MdWRKY*54*g*, and *MdWRKY*15*c* reached their highest expression levels at 72 hpi. *MdWRKY*9*c*, *MdWRKY*60*d*, *MdWRKY*71*d*, *MdWRKY*51, *MdWRKY*42*d*, *MdWRKY*30*a*, *MdWRKY*54*h*, *MdWRKY*29*a*, *MdWRKY*40*b*, *MdWRKY*32*a*, *MdWRKY*47*a*, *MdWRKY*11*a*, *MdWRKY*60*c*, *MdWRKY*46*a*, *MdWRKY*33*a*, and *MdWRKY*11*d* reached their highest expression values at 18 hpi, and showed a second peak at 72 hpi.

Replanting apple trees in land previously used as apple orchards or nurseries often results in apple replant disease (ARD). ARD weakens apple trees and affects fruit yield and quality [33] [34]. Cultivating the ARD-susceptible apple rootstock M26 on ARD-affected soil significantly upregulated *MdWRKY*75*b* and *MdWRKY*51 expression in leaves (**Figure 6(b)**).

Pythium ultimum is a primary component of the ARD pathogen complex identified in orchard soil [35]. Roots of the replant-tolerant rootstock G935 and the replant-susceptible rootstock B9 were infected by *Py. ultimum* and sampled at 24 hpi, 48 hpi, and 72 hpi for transcriptome analysis [36]. There were 53 differentially expressed *MdWRKYs* after *Py. ultimum* infection (**Figure 6(c)**). *MdWRKY*48*a*, *MdWRKY*2*a*, *MdWRKY*15*c*, *MdWRKY*27*b*, *MdWRKY*41*c*, *MdWRKY*41*b*, and *MdWRKY*51 showed similar expression patterns: upregulated at 24 hpi and downregulated at the 72 hpi in the susceptible B9, but downregulated at 24 hpi and upregulated at 72 hpi in the resistant G935. *MdWRKY*40*b*, *MdWRKY*33*b*, *MdWRKY*71*a*, *MdWRKY*74*a*, *MdWRKY*43*a*, *MdWRKY*9*c*, *MdWRKY*75*c*, *MdWRKY*29*a*, *MdWRKY*45*a*, *MdWRKY*47*a*, *MdWRKY*43*b*, *MdWRKY*69*c*, *MdWRKY*44*c*, *MdWRKY*33*a*, *MdWRKY*33*d*, *MdWRKY*29*b*, *MdWRKY*15*a*, and *MdWRKY*60*d* were downregulated at 24 hpi, but upregulated at 72 hpi in B9. *MdWRKY*43*c*, *MdWRKY*15*b*, *MdWRKY*54*c*, *MdWRKY*69*b*, *MdWRKY*65*b*, *MdWRKY*40*a*, and *MdWRKY*40*b* were upregulated at 24 hpi, but downregulated at 48 hpi and 72 hpi in G935. *MdWRKY*46*a*, *MdWRKY*54*b*, *MdWRKY*61*d*, *MdWRKY*60*a*, *MdWRKY*14*b*, *MdWRKY*54*h*, and *MdWRKY*9*a* were upregulated at 24 hpi in G935. *MdWRKY*22*b*, *MdWRKY*42*b*, and *MdWRKY*60*c* were upregulated at 48 hpi in G935. Also in G935, *MdWRKY*44*c*, *MdWRKY*33*a*, *MdWRKY*33*d*, and *MdWRKY*29*b* showed low levels of expression at 24 hpi, high levels at 48 hpi, and then downregulation at 72 hpi.

Apple fruit ring rot disease caused by *Botryosphaeria dothidea* has severe im-

pacts on China apple production. Transcriptomics was used to analyze gene expression in fruit from resistant and susceptible trees infected with *B. dothidea* at 48 hours after inoculation (hai), 72 hai, and 96 hai [2]. The result showed that there were 19 differentially expressed genes after *B. dothidea* infection (**Figure 6(d)**). *MdWRKY*71*b*, *MdWRKY*42*a*, *MdWRKY*47*a*, and *MdWRKY*71*a* were upregulated in the resistant trees, but downregulated in the susceptible trees. *MdWRKY*61*c* was downregulated at 72 hai but upregulated at 96 hai in the susceptible trees. *MdWRKY*2*a* was downregulated at 48 hai in the susceptible trees. *MdWRKY*71*c*, *MdWRKY*75*c*, *MdWRKY*71*d*, and *MdWRKY*46*a* were downregulated in the resistant trees, but upregulated in the susceptible trees. *MdWRKY*57*a* was upregulated at 72 hai in the resistant trees. *MdWRKY*50*a*, *MdWRKY*51, *MdWRKY*60*c*, *MdWRKY*54*g*, *MdWRKY*54*h*, and *MdWRKY*50*b* were upregulated at 48 hai in the susceptible trees. *MdWRKY*33*d* was upregulated at 72 hai and 96 hai in the susceptible trees. *MdWRKY*75*d* was upregulated at 96 hai in the susceptible trees.

Fire blight disease incited by *Erwinia amylovora* is a serious disease of susceptible apple, pear, quince, and other rosaceous hosts. Transcriptomics was used to analyze Malling 7 rootstock with high root area (HRA) or low root area (LRA) response to *E. amylovora* on 4 days post inoculation (dpi) and 8 dpi [37]. A total of 38 *MdWRKYs* were differentially expressed after *E. amylovora* infection (**Figure 6(e)**). About 31 of the *MdWRKYs* were upregulated 8 dpi in the HRA. Only *MdWRKY*65*a* was downregulated at 4 dpi in HRA. *MdWRKY*42*a*, *MdWRKY*33*a*, *MdWRKY*30*a*, *MdWRKY*11*b*, and *MdWRKY*20*a* were upregulated at 4 dpi in the LRA. *MdWRKY*30*b* was upregulated at 4 dpi and 8 dpi in the LRA. *MdWRKY*48*b*, *MdWRKY*71*a*, *MdWRKY*60*b*, *MdWRKY*71*b*, *MdWRKY*48*a*, *MdWRKY*50*a*, *MdWRKY*75*a*, and *MdWRKY*51 were downregulated at 8 dpi in the LRA. *MdWRKY*75*c* and *MdWRKY*75*b* were downregulated at 4 dpi and 8 dpi in the LRA.

Penicillium expansum can infect apple fruit through wounds, causing blue mold disease that results in fruit rot. Transcriptomics was used to analyze the mature apple fruit of the susceptible 'Royal Gala' and resistant *Malus sieversii*–PI613981 in response to *Pe. expansum* inoculation at 6 hpi, 24 hpi, and 48 hpi [38]. In the *Malus sieversii*, most of the differentially expressed *MdWRKYs* were significantly downregulated at 48 hpi (**Figure 6(f)**). *MdWRKY*65*a* expression peaked at 6 hpi. *MdWRKY*42*a*, *MdWRKY*33*d*, *MdWRKY*75*c*, *MdWRKY*75*b*, and *MdWRKY*40*b* were significantly upregulated at 24 hpi. *MdWRKY*65*a* was significantly upregulated at 6 hpi. In 'Gala', there were only 5 differentially expressed *MdWRKYs*. *MdWRKY*33*d* and *MdWRKY*40*b* were significantly upregulated at 24 hpi. *MdWRKY*58*a* was significantly downregulated at 24 hpi. *MdWRKY*75*c* was significantly downregulated at 24 hpi and 48 hpi. *MdWRKY*42*a* was significantly upregulated at 48 hpi.

Apple rust disease, caused by *Gymnosporangium yamadae*, is one of the major threats to apple orchards. Transcriptomics was used to analyze gene expres-

sion in apple leaves infected by *G. yamadae* at 10 dpi and 30 dpi [39]. There were 80 differentially expressed *MdWRKYs* after infection (**Figure 6(g)**). *MdWRKY*71*a*, *MdWRKY*75*a*, *MdWRKY*27*b*, *MdWRKY*47*b*, *MdWRKY*21*b*, *MdWRKY*29*a*, *MdWRKY*60*d*, *MdWRKY*71*b*, *MdWRKY*2*b*, and *MdWRKY*49*a* were upregulated at 10 dpi. *MdWRKY*57*b*, *MdWRKY*74*a*, *MdWRKY*45*b*, *MdWRKY*45*a*, *MdWRKY*44*e*, *MdWRKY*69*a*, and *MdWRKY*2*c* were downregulated at 10 dpi. *MdWRKY*9*c*, *MdWRKY*21*a*, *MdWRKY*13*b*, *MdWRKY*58*a*, *MdWRKY*21*c*, *MdWRKY*44*c*, *MdWRKY*42*c*, *MdWRKY*13*a*, *MdWRKY*15*d*, *MdWRKY*23*a*, *MdWRKY*61*d*, and *MdWRKY*14*b* were upregulated at 30 dpi. *MdWRKY*30*b*, *MdWRKY*50*b*, *MdWRKY*46*b*, *MdWRKY*61*a*, *MdWRKY*40*b*, *MdWRKY*30*a*, *MdWRKY*55*b*, *MdWRKY*54*h*, *MdWRKY*54*e*, *MdWRKY*50*a*, *MdWRKY*40*a*, *MdWRKY*75*b*, *MdWRKY*54*c*, *MdWRKY*65*a*, *MdWRKY*15*a*, *MdWRKY*23*b*, *MdWRKY*33*c*, *MdWRKY*11*d*, *MdWRKY*15*b*, *MdWRKY*11*c*, *MdWRKY*33*a*, *MdWRKY*11*a*, *MdWRKY*61*b*, and *MdWRKY*11*b* were downregulated at 30 dpi. *MdWRKY*75*c*, *MdWRKY*47*a*, *MdWRKY*9*d*, *MdWRKY*71*d*, *MdWRKY*71*c*, and *MdWRKY*60*c* were upregulated at 10 dpi and 30 dpi. *MdWRKY*69*c* and *MdWRKY*29*b* were downregulated at 10 dpi and 30 dpi. *MdWRKY*33*d*, *MdWRKY*41*b*, *MdWRKY*41*c*, *MdWRKY*33*b*, *MdWRKY*60*b*, *MdWRKY*42*a*, *MdWRKY*48*a*, *MdWRKY*41*a*, *MdWRKY*60*a*, *MdWRKY*55*a*, *MdWRKY*46*a*, *MdWRKY*3*a*, *MdWRKY*22*a*, and *MdWRKY*42*b* were upregulated at 10 dpi, but downregulated at 30 dpi. *MdWRKY*74*b*, *MdWRKY*42*d*, *MdWRKY*54*f*, *MdWRKY*32*a*, and *MdWRKY*69*b* were downregulated at 10 dpi, but upregulated at 30 dpi.

In summary, about 22 *MdWRKYs* showed differential expression in response to at least five pathogens (**Figure 7**). *MdWRKY*33*d* and *MdWRKY*75*c* were differentially expressed after infection with 6 pathogens, including *A. alternata*, *B. dothidea*, *E. amylovora*, *G. yamadae*, *Pe. expansum*, and *Py. ultimum*. *MdWRKY*51 was differentially expressed after infection with 5 diseases, including ARD, *A. alternata*, *B. dothidea*, *E. amylovora*, and *Py. ultimum*. *MdWRKY*75*b* was differentially expressed after infection with 5 diseases, including ARD, *A. alternata*, *E. amylovora*, *G. yamadae*, and *Pe. expansum*. *MdWRKY*33*a*, *MdWRKY*33*b*, *MdWRKY*33*c*, *MdWRKY*30*a*, *MdWRKY*42*b*, *MdWRKY*40*a*, *MdWRKY*30*b*, *MdWRKY*40*b*, *MdWRKY*60*a*, *MdWRKY*60*b*, *MdWRKY*60*d*, *MdWRKY*15*a*, and *MdWRKY*15*b* showed differential expression after infection with 5 pathogens, namely *A. alternata*, *E. amylovora*, *G. yamadae*, *Pe. expansum*, and *Py. ultimum*. *MdWRKY*42*a* and *MdWRKY*71*b* were differentially expressed after infection with 5 pathogens, including *A. alternata*, *B. dothidea*, *E. amylovora*, *G. yamadae*, and *Pe. expansum*. *MdWRKY*71*a*, *MdWRKY*54*h*, and *MdWRKY*60*c* showed differential expression after infection with 5 pathogens, namely *A. alternata*, *B. dothidea*, *E. amylovora*, *G. yamadae*, and *Py. ultimum*.

3.5. *MdWRKYs* Promoter Analysis

We further analyzed the promoters of the 22 differentially expressed *MdWRKYs*

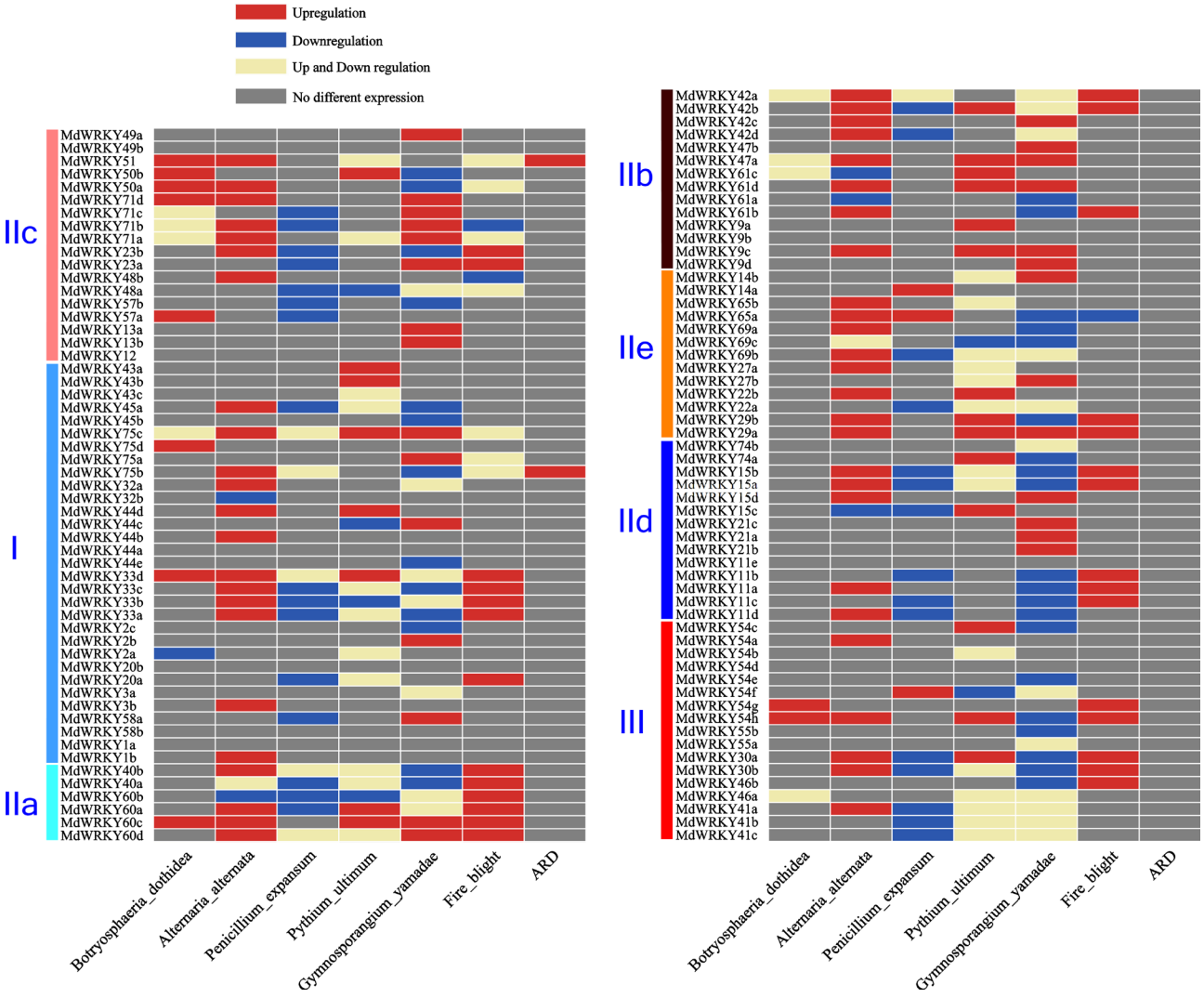

Figure 7. Summary of the differential expression of the *MdWRKYs* in response to biotic stresses. Data is from published transcriptomes.

(**Figure 8**). These genes carried many defense- and stress-responsive elements. The promoters of 17 *MdWRKYs* contained a MeJA-response cis-element. The G-Box, ABRE, CAAT-box, and TATA-box cis-elements appeared in the 15 *MdWRKYs* members promoters. The promoters of 14 *MdWRKYs* contained ARE cis-elements. The promoters of 12 *MdWRKYs* contained salicylic acid response element cis-elements.

3.6. The Role of *MdWRKY40a* and *MdWRKY54h* in *Botryosphaeria dothidea* Infection

Through big data analysis, we have identified 22 differentially expressed *MdWRKYs* in response to at least five pathogens. *MdWRKY40a* and *MdWRKY54h* were further selected to test for their roles during *B. dothidea* infection. Especially for *MdWRKY54h*, there is less reports about its function in pathogens infection. When apple fruits transiently silenced and inoculated with

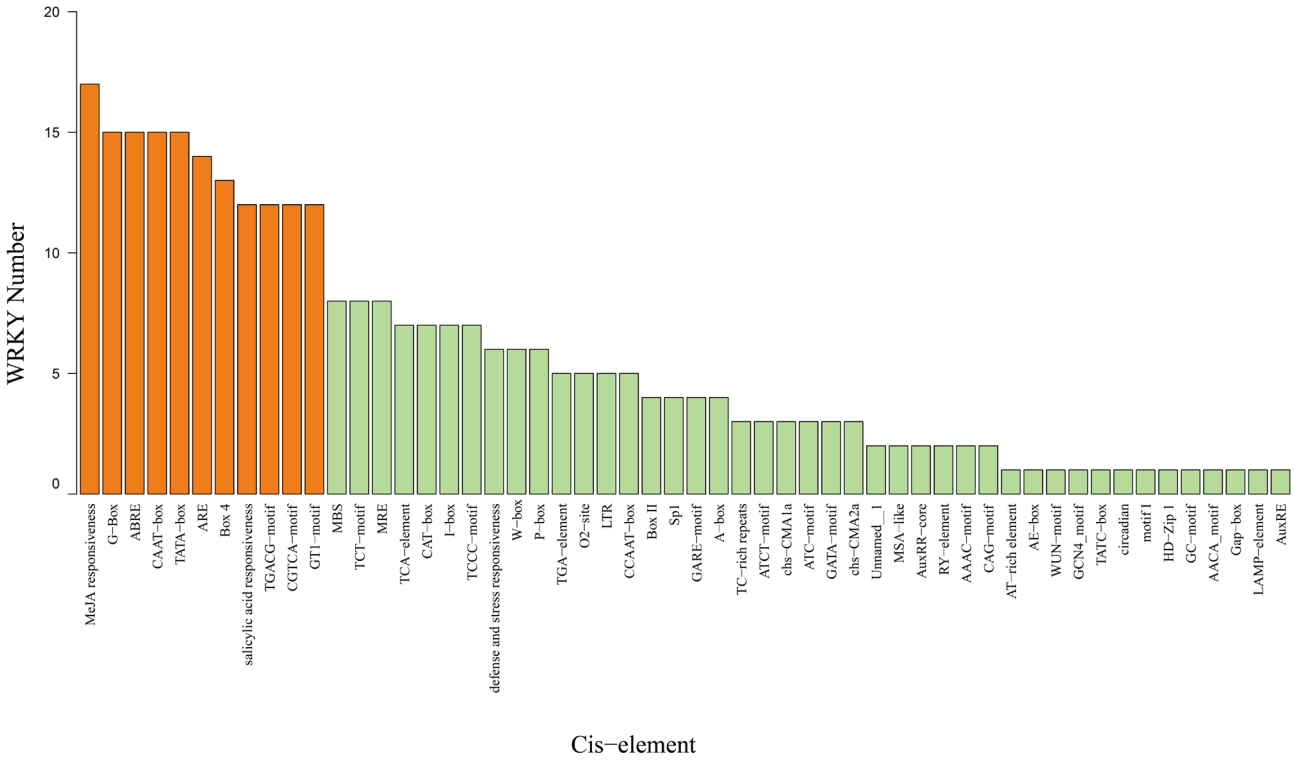

Figure 8. Cis-element analysis of the *MdWRKYs* promoters.

B. dothidea, the *TRV-MdWRKY*40*a* and *TRV-MdWRKY*54*h* constructs significantly decreased the lesion size compared with the control (**Figure 9(a)** and **Figure 9(b)**). On the contrary, overexpression of *MdWRKY*40*a-OE* and *MdWRKY*54*h-OE* reduced resistance to *B. dothidea* (**Figure 9(c)** and **Figure 9(d)**). The disease spot size of apple fruits transiently expressing *MdWRKY*40*a-OE* and *MdWRKY*54*h-OE* were significantly larger than the control. These results indicated that *MdWRKY*40*a* and *MdWRKY*54*h* promote growth of *B. dothidea* or decrease plant resistance.

4. Discussion

In this paper, we systematically identified 113 *MdWRKYs* in the apple genome and analyzed their response to seven pathogens. Among these *MdWRKYs*, 22 *MdWKRYs* showed differential expression in response to at least 5 pathogens. The 22 differentially expressed *MdWKRYs* may play roles during the apple response to pathogens. The two *WRKY*s in group IIa, *MdWRKY*40 and *MdWRKY*60 mainly responded to infection *A. alternata*, *E. amylovora*, *G. yamadae*, *Pe. expansum*, and *Py. ultimum*. These genes are homologous to *AtWRKY*18, *WRKY*40, and *WRKY*60, which have been intensively studied and shown to be induced in response to biotrophic, hemibiotrophic and necrotrophic fungi [5] [6] [40]. Fifteen *WRKY* TF genes, including *WRKY*18, *WRKY*40, and *WRKY*33, were strongly (>4-fold) induced 30 min after flg22 treatment in *Arabidopsis* seedlings [41]. *WRKY*18, *WRKY*40, and *WRKY*33 were identified as hub genes within a proposed *WRKY* regulatory network [6] [42]. *PtrWRKY*40

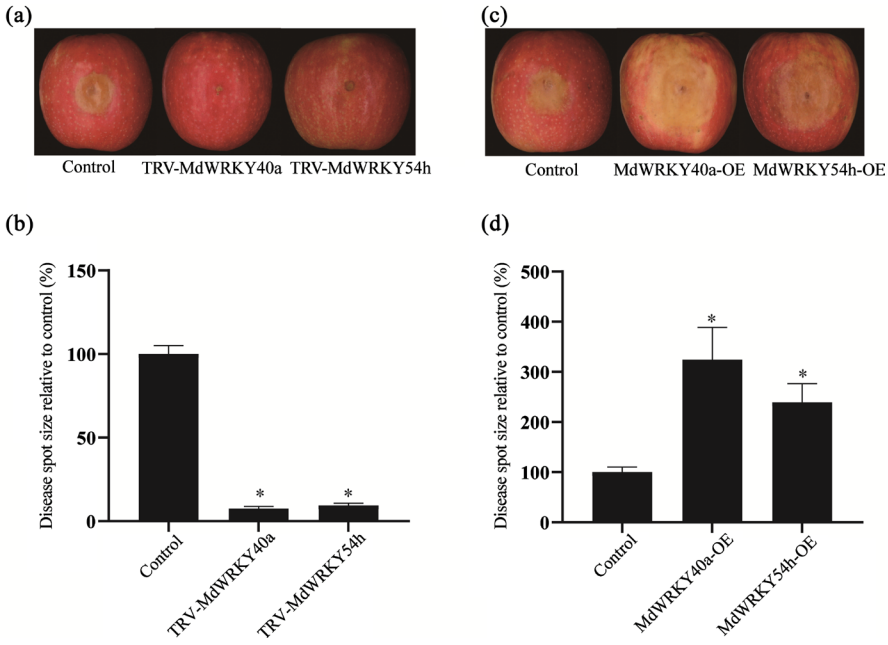

Figure 9. The role of *MdWRKY*40*a* and *MdWRKY*54*h* in *Botryosphaeria dothidea* infection. (a) The phenotypes of apples inoculated with *B. dothidea* after silencing *MdWRKY*40*a* and *MdWRKY*54*h* by VIGS were recorded 4 days post inoculation (dpi). Fruit injected with *Agrobacterium tumefaciens* containing empty vector (pTRV2) and inoculated with *B. dothidea* were the control. (b) Relative spot size on apples after silencing *MdWRKY*40*a* and *MdWRKY*54*h* by VIGS and inoculation with *B. dothidea* at 4 dpi. The area of each spot was measured and compared to control. Fifteen apples were inoculated with each treatment combination. (c) The phenotypes of apple inoculated with *B. dothidea* during transient overexpression of *MdWRKY*40*a* and *MdWRKY*54*h* were recorded at 4 dpi. Fruit injected with *Agrobacterium tumefaciens* containing empty vector (SAK-277) and inoculation with *B. dothidea* were the control. (d) Relative spot size on apples during transient overexpression of *MdWRKY*40*a* and *MdWRKY*54*h* and inoculation with *B. dothidea* 4 dpi. The area of each spot was measured and compared to control. Fifteen apples were inoculated with each treatment combination.

plays a negative role in resistance to hemibiotrophic fungi in poplar but functions as a positive regulator of resistance toward the necrotrophic fungi in *Arabidopsis* [43]. *GmWRKY*40, from *Glycine max* L., enhances the resistance to *Phytophthora sojae* [44]. In *Malus hupehensis*, *MhWRKY*40*b* were induced by the powdery mildew (*Podosphaera leucotricha*) [37]. In *Malus* × *domestica*, 4 *MdWRKY*33*s* were induced by *A. alternata*, *Pe. expansum*, *Py. ultimum*, *G. yamadae*, and *E. amylovora*. *MdWRKY*33*a* and *MdWRKY*33*d* were also induced by ARD and *B. dothidea*, respectively. *Arabidopsis WRKY*33 is a key positive resistance regulator against the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* [45] [46]. Hence, the group IIa members *WRKY*40 and *WRKY*60 and group I member *WKRY*33 may play important roles in responding to pathogens and are conserved in plants.

*WRKY*15*a* and *WKRY*15*b* were also differentially expressed in response to pathogen infection, including *A. alternata*, *E. amylovora*, *G. yamadae*, *Pe. expansum*, and *Py. ultimum*. In oilseed rape, overexpression of *BnWRKY*15 si-

multaneously increases the susceptibility of *B. napus* to *S. sclerotiorum* and down-regulates *BnWRKY*33 [47]. Although *AtWRKY*71 is involved in controlling shoot branching and accelerates flowering in *Arabidopsis* [48] [49], *MdWRKY*71*a* and *MdWRKY*71*b* showed differential expression after infecting *B. dothidea*, *E. amylovora*, *G. yamadae*, and *Py. Ultimum* in apple.

*MdWRKY*42*a* and *MdWRKY*42*b* showed differential expression in response to 5 pathogen infection. Md*WRKY*42a (named Md*WRKY*31 in [16]) regulates plant resistance to *B. dothidea* through the SA signaling pathway by interacting with MdHIR4. In rice, *WRKY*42 negatively regulates the rice response to *Magnaporthe oryzae* by suppressing JA signaling-related genes [50].

*MdWRKY*54*h* and *MdWRKY*40*a* showed differential expression after infection with *B. dothidea*, *A. alternata*, *E. amylovora*, *G. yamadae*, and *Py. ultimum*. In the transcriptome of apple fruit inoculated *B. dothidea*, *MdWRKY*54*h* was upregulated in the sensitive genotype. Transient expression assays showed that *MdWRKY*40*a* and *MdWRKY*54*h* play negative roles in defense against *B. dothidea* infection.

These *MdWRKYs* are conserved in apple and other plants. Some of them had been verified to played roles in the pathogen response in plants. The identified *WRKYs* genes will provide clues for apple and other plants in pathogens infection research.

In *Arabidopsis*, apple and other plants, many *WRKY* genes are responsive to pathogen infection. About 75 *MdWRKYs* were differentially expressed in response to at least 2 pathogens. About one-quarter of the *MdWRKYs* contain a W-box element. The *WRKY*-*WRKY* regulation network complex has been characterized based on the auto- and cross-regulation patterns through the WKRY domain/W-box and physical interaction between *WRKY* members [5] [6] [47] [50]. In addition, plant hormones, like MeJA and SA, are involved in systemic acquired resistance [14] [51] [52] [53] [54]. Many *WRKY* promoters contain MeJA- and SA-responsive elements. Some *WRKY*s also enhance disease resistance by involvement in MeJA and SA synthesis or signal transduction [14] [44] [53]. Therefore, pathogens, WKRY proteins, and hormones come together in a regulatory network that may be the cause of the many different expression patterns seen for the *WRKY* gene family after inoculation with pathogens.

5. Conclusion

In short, we identified 113 *MdWRKY* members in the apple genome and analyzed their expression patterns in response to various biological stressors. Twenty-two *MdWRKYs* showed differential expression in response to at least five pathogens. *MdWRKY*40*a* and *MdWRKY*54*h* played negative roles in resistance to *Botryosphaeria dothidea*. Autoregulation, cross-regulation, and physical interaction between *WRKY* members and cross-regulation between pathogens, *WRKY* proteins, and hormones may work together to create the many *MdWRKY* expression patterns after inoculation with pathogens.

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Author Contribution Statement

XZ and CS conceived and designed research; HZ, LY, RZ, YZ and CS conducted experiments; XZ, ZW, HP, TB, SS, JJ, MW and JF revised manuscript and edited language; HZ and CS analyzed the data and finalized the manuscript. All authors read and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental Data Legends

Table S1. Transcriptome data used for tissues expression analyses.

Tissue	SRR NO.	Cutivar
Leaf	SRR767668	hybrid M49
Root	SRR768132	Galaxy
Fruit	SRR768133	hybrid M67
Shoot apex	SRR768134	Granny Smith
Stem	SRR768135	Granny Smith
Flower	SRR768137	Gala

Table S2. Transcriptome data used for meta-analysis of biotic stress responses.

Pathogens	Pathogen	Cutivar	Tissue	Objective	BioProject	Reference article
Alternaria alternate	Fungi	Starking Delicious	Leaf	Response to Alternaria alternata	PRJNA349086	[32]
apple replant disease (ARD)	-	M26	Leaf	Response to apple replant disease (ARD)	PRJNA362843	[34]
Pythium ultimum	Fungi	B.9, G.935	Root	Response to Pythium ultimum	PRJNA407578	[36]
Botryosphaeria dothidea	Fungi	Royal Gala, PI61983—Malus sieversii	Fruit	Response to Penicillium expansum	PRJNA383305	[38]
Erwinia amylovora	Bacteria	Malling 7	Root	Response to Erwinia amylovora	PRJNA507638	[37]
Penicillium expansum	Fungi	'Jonathan'—'Golden Delicious'	fruit	Apple Fruit Ring Rot Disease Resistance	PRJNA392908	[2]
Gymnosporangium yamadae	Fungi	Fuji	Leaf	Response to Gymnosporangium yamadae	PRJNA549565	[39]

Table S3. Primer sequences for qRT-PCR.

Gene name	Forward primer	Reverse primer	Purpose
Md *WRKY*33a	GAGGCAGCCAACATCAGAAG	ATGCATCATCCCTTGGCTCT	qRT-PCR
Md *WRKY*40a	CTTGTGTCCAGACCGAAGCA	AGGGACGGATCCTATTGCCA	
Md *WRKY*42a	CTTCCTCGTTTGCTGACACA	CCGGGAAGCTGCTAATGTTC	
Md *WRKY*51	ACAAAATCGGAGCTGGAGGT	ATAGCTCGCATCATCTCGGT	
Md *WRKY*54h	TCGTCCATTCCCATCGTCAA	CGTCCCACTGCATGTTTGAA	
Md *WRKY*60c	TCTCAGTCTCTCGGGATCCA	AGGTCTTGCAATCGAACGTG	
Md *WRKY*71b	GTATGAAGGCCAGCACAACC	TGGGGCATTTGAAACAAGAGT	
Md *WRKY*75b	TTCTCCCCTGTCGTTGAACA	TCTCACAGTTGCTTCACCAC	
TRV-Md *WRKY*40a	ATTCTGTGAGTAAGGTTACCGAATTC GTCCCTTGCTCAACCTCCCT	TCTTCGGGACATGCCCGGGCCTCGAGCATCACCTCA CCCTTTCCACT	Vector construction
TRV-Md *WRKY*54h	ATTCTGTGAGTAAGGTTACCGAATTC TCATCGGCCCTGATCTTTGG	TCTTCGGGACATGCCCGGGCCTCGAGGTCGTCCCAC TGCATGTTTG	
*WRKY*40a-OE	GTGGATCCAAAGAATTCATGGACTAC TCAGCTGCAAAT	CTCCTTTACCCATGAATTCGTAAGTATTGTGTTGAA GTAT	
*WRKY*54h-OE	GTGGATCCAAAGAATTCATGGGAACC AACCACAAGAGA	CTCCTTTACCCATGAATTCAACAGCATCAAAACCTT CATC	

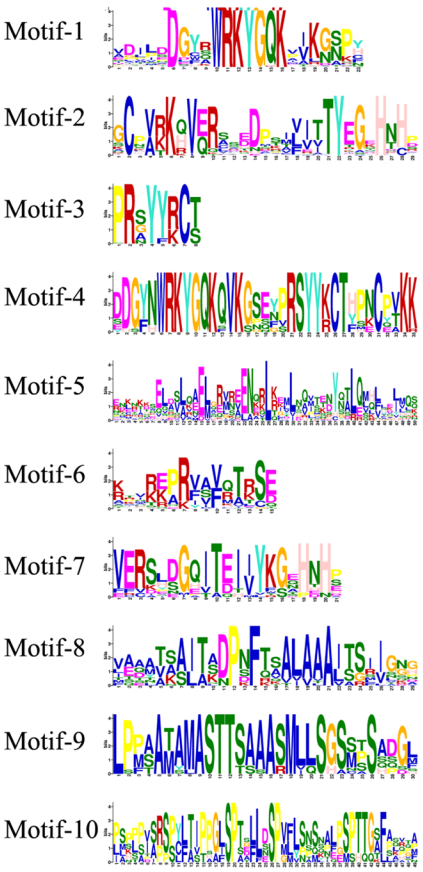

Figure S1. Motif consensus sequences for **Figure 3**.