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 biotic stressors, including Alternaria alternata, Pythium ultimum, Botryosphaeria dothidea, Erwinia amylovora, Penicillium expansum, Gymnosporangium yamadai, 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 MdWRKY40a and MdWRKY54h played negative roles in defense against B. dothidea infection. WRKY40 and WRKY60 and the MdWKRY33s 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 WRKYGQK 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 WRKY 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 AtWRKY18, AtWRKY40 and AtWRKY60 interact with each other to regulate defense pathways [5]. WRKY18, WRKY40, and WRKY33 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]. WRKY22 and WRKY29 are induced by the MAPK pathway involved in plant responses to both bacterial and fungal pathogens, and transient expression of WRKY29 in leaves leads to reduced disease symptoms [7]. WRKY53 and WRKY70 both positively modulate systemic acquired resistance (SAR) [8]. The Group I members WRKY3 and WRKY4 play positive roles in plant resistance to necrotrophic pathogens. WRKY14 has a negative effect on plant resistance to biotrophic pathogens [9]. The group IIId members WRKY11 and WRKY17 are negative regulators of basal resistance in Arabidopsis [10].

Some of the apple WRKY genes have been demonstrated to be involved in plant defense. MdWRKYN1 and MdWRKY26 are targeted by miRNAs and are involved in apple resistance to leaf spot disease caused by Colletotrichum spp. [11]. MdWRKY100 positively regulates apple resistance to Colletotrichum gloeosporioides infection [12]. Ectopic expression of MdWRKY1 (homolog of AtWRKY15) in tobacco plants enhances resistance to Phytophthora parasitica.
and activates the expression of PR genes [13]. MdWRKY15 improves apple resistance to *Botryosphaeria dothidea* via the salicylic acid-mediated pathway by directly binding the *MdICS1* promoter [14] (Zhao et al., 2020). MdWRKY46 enhances apple resistance to *B. dothidea* by activating the expression of *MdPBS3.1* in the salicylic acid signaling pathway [15]. MdWRKY31 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, transcriptomic 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 (WRKYGQK 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 MdWRKY. 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 MdWRKYs 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 WRKYs 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^−ΔΔCT 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 |log₂(fold change)| ≥ 1 between two samples were identified as differentially expressed genes.

2.4. Promoter Analysis for Cis-Acting Regulatory Elements

For each MdWRKY 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 MdWRKY40a and MdWRKY54h were ligated into
the overexpression vector SAK-227 to generate the vectors *MdWRKY40a-OE* and *MdWRKY54h-OE*. About 300-bp fragments specific to either *MdWRKY40a* or *MdWRKY54h* were ligated into the virus induced gene silence (VIGS) vector TRV2 to generate *TRV-MdWRKY40a* or *TRV-MdWRKY54h*. *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 (*MdWRKY44b*) to 924 aa (*MdWRKY44e*). The molecular weight of the predicted proteins ranged from 9.28 (*MdWRKY44b*) to 102.78 kDA (*MdWRKY44e*). The isoelectric point ranged from 4.81 (*MdWRKY69b*) to 9.99 (*MdWRKY11c*).

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

Because the apple genome underwent chromosomal doubling events during evolution, most of the apple *MdWRKY*s that are orthologous to *Arabidopsis* have two homologous genes, such as *MdWRKY1*, *MdWRKY6*, *MdWRKY7*, *MdWRKY9*, *MdWRKY13b*, *MdWRKY14*, *MdWRKY15*, *MdWRKY20*, *MdWRKY22*,
Table 1. Informations of *MdWRKY* genes.

| Gene name | Gene ID   | Group | Location       | Strand | mRNA length | aa    | MW   | pI |
|-----------|-----------|-------|----------------|--------|-------------|-------|------|----|
| MdWRKY1a  | MD09G1121600 | I     | Chr09:9379281-9382725 | +      | 2100        | 484   | 53.15| 5.94|
| MdWRKY1b  | MD17G1112600 | I     | Chr17:9643152-9646837  | +      | 1988        | 471   | 51.66| 6.67|
| MdWRKY1c  | MD03G1044400 | I     | Chr03:3511777-3516305  | –      | 2595        | 732   | 79.51| 5.96|
| MdWRKY2a  | MD04G1244700 | I     | Chr04:32067852-32071212 | –      | 2154        | 717   | 78.55| 5.99|
| MdWRKY2b  | MD12G1260600 | I     | Chr12:32647306-32651367 | –      | 2587        | 718   | 78.96| 6.41|
| MdWRKY2c  | MD13G1067600 | I     | Chr13:4637048-4640408   | +      | 2230        | 526   | 57.34| 7.37|
| MdWRKY3a  | MD16G1066500 | I     | Chr16:4642014-4644789   | +      | 2125        | 528   | 57.20| 8.39|
| MdWRKY3b  | MD09G1048300 | IIb   | Chr09:3166930-3174316   | +      | 1413        | 470   | 51.18| 7.70|
| MdWRKY3c  | MD17G1048400 | IIb   | Chr17:3528939-3531555   | +      | 1729        | 455   | 49.96| 7.70|
| MdWRKY3d  | MD08G1227200 | IIb   | Chr08:29356105-29358646 | +      | 1858        | 570   | 62.31| 5.35|
| MdWRKY4a  | MD15G1419600 | IIb   | Chr15:52086817-52089361 | +      | 1767        | 582   | 64.17| 5.10|
| MdWRKY4b  | MD08G1127200 | IId   | Chr08:11928202-11930014 | +      | 1335        | 341   | 36.96| 9.33|
| MdWRKY4c  | MD15G1106600 | IId   | Chr15:7467131-7469028   | +      | 1376        | 338   | 36.70| 9.46|
| MdWRKY4d  | MD13G2391000 | IId   | Chr13:24320203-24321656 | –      | 1233        | 281   | 30.73| 9.99|
| MdWRKY4e  | MD16G1244300 | IId   | Chr16:26579453-26581081 | –      | 1370        | 284   | 30.92| 9.87|
| MdWRKY5a  | MD10G1096000 | IId   | Chr10:15057933-15058421 | +      | 489         | 162   | 17.96| 9.30|
| MdWRKY5b  | MD07G1110400 | Iic   | Chr07:12683567-12688395 | +      | 1092        | 236   | 26.78| 8.21|
| MdWRKY5c  | MD01G1013500 | Iic   | Chr01:6515683-6519808   | +      | 1393        | 270   | 30.43| 8.93|
| MdWRKY5d  | MD15G1337100 | Iic   | Chr15:37891029-37895909 | –      | 1173        | 271   | 30.24| 8.93|
| MdWRKY5e  | MD05G1265200 | Ile   | Chr05:40011060-40015621 | +      | 1479        | 492   | 53.78| 5.85|
| MdWRKY5f  | MD10G1243000 | Ile   | Chr10:33776504-33781157 | +      | 2075        | 493   | 53.24| 6.05|
| MdWRKY5g  | MD12G1177500 | IId   | Chr12:15663260-15665127 | –      | 1629        | 330   | 35.97| 6.65|
| MdWRKY5h  | MD15G1287300 | IId   | Chr15:26365198-26366819 | –      | 1369        | 331   | 36.24| 9.54|
| MdWRKY5i  | MD08G1094900 | IId   | Chr08:7968389-7970451   | +      | 1455        | 356   | 38.64| 9.41|
| MdWRKY5j  | MD15G1078200 | IId   | Chr15:5334685-5336858   | –      | 1691        | 342   | 37.21| 9.26|
| MdWRKY6a  | MD03G1188900 | I     | Chr03:25924164-25929514 | –      | 2258        | 584   | 63.85| 5.97|
| MdWRKY6b  | MD11G1205000 | I     | Chr11:29914448-29918933 | –      | 2118        | 588   | 63.96| 5.87|
| MdWRKY6c  | MD04G1226400 | IId   | Chr04:30603205-30604718 | +      | 1222        | 325   | 36.59| 9.77|
| MdWRKY6d  | MD12G1243400 | IId   | Chr12:31414877-31416020 | +      | 942         | 313   | 35.30| 9.96|
| MdWRKY6e  | MD06G1062800 | IId   | Chr06:10770799-10772691 | –      | 1419        | 318   | 35.88| 9.58|
| MdWRKY6f  | MD01G1071300 | Ile   | Chr01:17602924-17604669 | +      | 1512        | 349   | 37.65| 8.20|
| MdWRKY6g  | MD07G1311400 | Ile   | Chr07:18815423-18816949 | –      | 1301        | 348   | 37.66| 6.82|
| MdWRKY6h  | MD09G1285400 | Ile   | Chr09:36364455-36366937 | +      | 2092        | 350   | 38.39| 5.86|
| MdWRKY6i  | MD17G1278100 | Ile   | Chr17:33812003-33814316 | +      | 1656        | 346   | 38.17| 6.29|
| MdWRKY6j  | MD01G1210200 | Ile   | Chr01:30413680-30415409 | +      | 1413        | 470   | 51.76| 5.08|
| MdWRKY7a  | MD07G1280300 | Ile   | Chr07:34424687-34425782 | +      | 837         | 278   | 31.07| 7.25|

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| Gene   | Chr   | Start    | End      | Genomic Location |
|--------|-------|----------|----------|-----------------|
| MD06G1091200 | IIe    | Chr06:21994255-21995556 | + | 888 | 295 | 33.55 | 5.49 |
| MD14G1112200 | IIe    | Chr14:18037604-18039324 | + | 1218 | 315 | 35.65 | 5.05 |
| MD06G1104100 | III    | Chr06:24218568-24220572 | - | 1489 | 351 | 39.42 | 5.73 |
| MD14G1123000 | III    | Chr14:19736610-19738943 | - | 1594 | 355 | 39.87 | 5.66 |
| MD02G1007900 | I      | Chr02:496443-499890 | - | 1844 | 473 | 52.03 | 8.82 |
| MD15G1152100 | I      | Chr15:11267047-11270466 | - | 1479 | 434 | 48.12 | 7.37 |
| MD03G1057400 | I      | Chr03:4579079-4582074 | - | 2168 | 571 | 62.55 | 7.08 |
| MD11G1059400 | I      | Chr11:5068503-5071526 | - | 2174 | 572 | 62.90 | 6.81 |
| MD12G1181000 | I      | Chr12:26084482-26086791 | - | 1870 | 512 | 56.72 | 6.80 |
| MD04G1167700 | I      | Chr04:25792146-25794577 | - | 2022 | 520 | 57.71 | 7.23 |
| MD09G1224500 | IIa    | Chr09:27404228-27406362 | - | 1397 | 320 | 35.31 | 7.72 |
| MD17G1223100 | IIa    | Chr17:27209201-27211741 | + | 1743 | 321 | 35.67 | 8.23 |
| MD01G1215300 | III    | Chr01:30851473-30853003 | - | 1044 | 347 | 38.71 | 5.94 |
| MD07G1285200 | III    | Chr07:34745168-34746713 | - | 1029 | 342 | 38.05 | 5.50 |
| MD07G1285400 | III    | Chr07:34761880-34763425 | - | 1029 | 342 | 38.05 | 5.50 |
| MD05G1349800 | IIb    | Chr05:46759691-46762704 | - | 2242 | 606 | 65.35 | 7.78 |
| MD10G1324500 | IIb    | Chr10:40512430-40515449 | - | 2354 | 611 | 65.65 | 6.63 |
| MD09G1111200 | IIb    | Chr09:8267211-8270166 | - | 2013 | 625 | 68.20 | 6.34 |
| MD17G1099000 | IIb    | Chr17:8402127-8405223 | - | 2341 | 645 | 69.96 | 6.44 |
| MD01G1071600 | I      | Chr01:17669166-17671888 | + | 657 | 218 | 24.56 | 9.36 |
| MD07G1131000 | I      | Chr07:18748505-18750267 | - | 935 | 222 | 24.93 | 9.36 |
| MD01G1123900 | I      | Chr01:23690319-23691083 | - | 653 | 208 | 23.62 | 8.92 |
| MD04G1112800 | I      | Chr04:19827951-19828792 | - | 441 | 146 | 16.66 | 9.60 |
| MD12G1129000 | I      | Chr12:20418703-20419358 | + | 243 | 80 | 9.28 | 9.85 |
| MD12G1128800 | I      | Chr12:20397217-20403016 | + | 2791 | 470 | 51.46 | 9.00 |
| MD04G1113100 | I      | Chr04:19846116-19851149 | - | 2824 | 470 | 51.52 | 8.93 |
| MD06G1115200 | I      | Chr06:25425907-25429273 | + | 3026 | 924 | 102.78 | 5.33 |
| MD06G1138500 | I      | Chr06:28356819-28357724 | + | 581 | 150 | 17.15 | 9.56 |
| MD14G1154500 | I      | Chr14:24914608-24915904 | + | 955 | 148 | 17.09 | 9.59 |
| MD01G1078000 | III    | Chr01:18445739-18447924 | - | 1224 | 353 | 39.17 | 5.30 |
| MD07G1146900 | III    | Chr07:21450190-21453542 | - | 2205 | 356 | 39.73 | 5.48 |
| MD03G1197600 | IIb    | Chr03:27003520-27006281 | + | 1781 | 538 | 58.93 | 6.44 |
| MD11G1213500 | IIb    | Chr11:31232215-31235905 | + | 1626 | 541 | 58.97 | 6.46 |
| MD13G1150700 | IIc    | Chr13:11807823-11809934 | - | 1539 | 385 | 42.70 | 6.11 |
| MD16G1151000 | IIc    | Chr16:11906129-11908067 | - | 1368 | 371 | 41.14 | 5.60 |
| MD04G1131000 | IIc    | Chr04:21800375-21802338 | - | 967 | 297 | 33.41 | 5.73 |
| MD12G1144100 | IIc    | Chr12:22324583-22326472 | - | 894 | 297 | 33.08 | 5.94 |
| MD08G1067700 | IIc    | Chr08:5387244-5388373 | - | 741 | 161 | 18.24 | 8.56 |
| Genes |Chr15:3683177-3684465| + | 915 | 161 | 18.18 | 5.65 |
|-------|----------------------|---|-----|------|-------|------|
| Md WRKY50b | MD15G1054000 | Ilc | Chr15:36715332-36718649 | + | 802 | 199 | 22.27 | 5.87 |
| Md WRKY51 | MD15G1331300 | Ilc | Chr15:37086797-27089009 | – | 1196 | 339 | 37.73 | 6.42 |
| Md WRKY54a | MD12G1189200 | III | Chr12:27213135-27215427 | – | 1370 | 332 | 37.18 | 5.77 |
| Md WRKY54b | MD12G1189700 | III | Chr12:27219059-27219447 | – | 1110 | 369 | 40.60 | 5.69 |
| Md WRKY54c | MD12G1189600 | III | Chr12:27224756-27226237 | – | 714 | 237 | 27.26 | 8.29 |
| Md WRKY54d | MD04G1175500 | III | Chr04:26653551-26655300 | – | 1002 | 333 | 37.19 | 5.38 |
| Md WRKY54e | MD04G1175600 | III | Chr04:26669452-26671247 | – | 1035 | 344 | 38.25 | 6.01 |
| Md WRKY54f | MD01G1668000 | III | Chr01:27287662-27291066 | – | 1498 | 303 | 34.23 | 5.71 |
| Md WRKY54g | MD07G1234700 | III | Chr07:30816225-30818950 | – | 1407 | 302 | 33.97 | 6.40 |
| Md WRKY54h | MD07G1234600 | III | Chr07:30813251-30815403 | + | 1029 | 342 | 37.44 | 5.48 |
| Md WRKY54i | MD13G1064700 | Iic | Chr13:4465565-4471604 | – | 1653 | 346 | 37.73 | 6.63 |
| Md WRKY55a | MD01G1168500 | III | Chr01:27285229-27286785 | + | 687 | 228 | 25.66 | 7.86 |
| Md WRKY55b | MD07G1234600 | III | Chr07:30813251-30815403 | + | 1029 | 342 | 37.44 | 5.48 |
| Md WRKY55c | MD01G1039500 | IIa | Chr15:2783151-2784898 | + | 1208 | 302 | 33.60 | 7.22 |
| Md WRKY55d | MD00G1143500 | IIa | Chr00:31227045-31229422 | + | 1538 | 334 | 36.98 | 7.14 |
| Md WRKY56a | MD15G1039500 | IIa | Chr15:2783151-2784898 | + | 1208 | 302 | 33.60 | 7.22 |
| Md WRKY56b | MD01G1168500 | III | Chr01:27285229-27286785 | + | 687 | 228 | 25.66 | 7.86 |
| Md WRKY56c | MD01G1039500 | IIa | Chr15:2783151-2784898 | + | 1208 | 302 | 33.60 | 7.22 |
| Md WRKY56d | MD15G1039600 | IIa | Chr15:2798151-2799702 | + | 905 | 286 | 32.09 | 8.18 |
| Md WRKY61a | MD14G1196100 | IIb | Chr14:28655099-28658370 | + | 1755 | 584 | 63.31 | 6.51 |
| Md WRKY61b | MD06G1189100 | IIb | Chr06:32608598-32612425 | + | 2298 | 683 | 73.47 | 7.32 |
| Md WRKY61c | MD13G1077900 | IIb | Chr13:5478407-5482438 | – | 2008 | 571 | 61.85 | 5.96 |
| Md WRKY61d | MD16G1077700 | IIb | Chr16:5438696-5444408 | – | 2259 | 587 | 64.14 | 6.44 |
| Md WRKY65a | MD05G1295700 | IIe | Chr05:43023494-43025466 | – | 1133 | 273 | 30.20 | 5.81 |
| Md WRKY65b | MD10G1275800 | IIe | Chr10:36698263-36699996 | – | 1310 | 266 | 29.50 | 5.10 |
| Md WRKY65c | MD09G1235100 | IIe | Chr09:29539556-29542546 | – | 1140 | 250 | 27.40 | 5.47 |
| Md WRKY65d | MD00G1140800 | IIe | Chr00:30743431-30745181 | – | 1196 | 268 | 30.83 | 4.81 |
| Md WRKY65e | MD03G1292900 | IIe | Chr03:36975267-36977012 | – | 992 | 260 | 30.34 | 5.17 |
| Md WRKY71a | MD10G1266000 | IIc | Chr10:35935449-35937762 | – | 1615 | 327 | 35.94 | 6.49 |
| Md WRKY71b | MD05G1290300 | IIc | Chr05:42212192-42214208 | – | 1288 | 319 | 35.12 | 6.64 |
| Md WRKY71c | MD09G1150700 | IIc | Chr09:11892767-11895678 | – | 1683 | 369 | 41.36 | 7.84 |
| Md WRKY71d | MD17G1138100 | IIc | Chr17:12392128-12394598 | – | 1211 | 365 | 41.25 | 6.79 |
| Md WRKY71e | MD05G1204400 | IIId | Chr05:33402565-33405458 | – | 2071 | 354 | 39.96 | 9.70 |
| Md WRKY71f | MD10G1191400 | IIId | Chr10:28819078-28822211 | – | 1821 | 355 | 39.95 | 9.68 |
| Md WRKY75a | MD16G1122400 | I | Chr16:8821613-8823564 | – | 486 | 161 | 18.04 | 9.08 |
| Md WRKY75b | MD13G1121100 | I | Chr13:8993050-8995368 | – | 1221 | 190 | 21.71 | 9.74 |
| Md WRKY75c | MD09G1008800 | I | Chr09:616602-619344 | + | 694 | 216 | 24.76 | 9.14 |
| Md WRKY75d | 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.

*MdWRKY23, MdWRKY26, MdWRKY29, MdWRKY32, MdWRKY40, MdWRKY45, MdWRKY49, MdWRKY50, MdWRKY55, MdWRKY57, MdWRKY65, MdWRKY72,* and *MdWRKY74*. Due to chromosomal fragment duplication, some genes have multiple homologous genes (equal to or greater than 3), such as the 8 homologous *MdWRKY54* 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. *MdWRKY44c* and *MdWRKY44d* each contained 7 exons. *MdWRKY11e* contained 1 exon. MEME detected 10 distinct conserved motifs in the MdWRKYs (*Figure 3(c)*). In general, the homologs had similar motifs. Motifs 1, 2, and 3 are *WRKY* domains and appeared

![Figure 2](image_url)

*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 MdWRKYs, constructed by the neighbor-joining method in MEGA 7.0 software. (b) Gene structure of MdWRKYs. Introns, exons, and untranslated 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 MdWRKYs with MEME. Each colored box represents a conserved motif. Details are included with Supplementary Figure S1.

In all MdWRKY proteins. In Group IIc, I, Ila, and Ilb, 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 AtWRKYs, 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 IId.

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). MdWRKY11a, MdWRKY69b, MdWRKY44c, MdWRKY58a, MdWRKY1a, MdWRKY32a, MdWRKY15a,
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 log₁₀ (FPKM). Genes that showed low or no expression in these six tissues are not shown in the figure.
MdWRKY74a, MdWRKY11b, MdWRKY11b, and MdWRKY54g were highly expressed in the most organs. Among the MdWRKYs, MdWRKY54g showed the highest expression in the leaf, while MdWRKY11a was the highest expressed in the root. MdWRKY69b showed the highest expression in the fruit, stem, and flower. MdWRKY44c was the highest expressed in the shoot apex.

Other genes showed highly tissue-specific expression. MdWRKY29b was only expressed in the root. MdWRKY74b was only expressed in the stem. MdWRKY71c was only expressed in the fruit. About 15 MdWRKYs, MdWRKY47b, MdWRKY55a, MdWRKY27a, MdWRKY45b, MdWRKY44b, MdWRKY141a, MdWRKY75a, MdWRKY27b, MdWRKY27b, MdWRKY11b, MdWRKY41c, MdWRKY75d, and MdWRKY11e showed low abundance in these tissues within this dataset and are not shown in the figure. MdWRKY20b, MdWRKY32b, MdWRKY44d, MdWRKY54d, MdWRKY57a, MdWRKY57b, and MdWRKY61d 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 MdWRKY33a, MdWRKY40a, MdWRKY51, and MdWRKY75b were highly expressed in the root. MdWRKY42a was detected in all examined tissues, with higher expression in the root and flower. MdWRKY54b showed higher expression in the leaf and shoot compared to other tissues. MdWRKY60c showed higher expression in the leaf and fruit. MdWRKY71b showed higher expression in the leaf and root.

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)). MdWRKY61c, MdWRKY32b,
Figure 6. Heatmap of differentially expressed MdWRKYs in response to biotic stress. Transcriptome datasets from published studies were mined for data on the 113 MdWRKYs identified in the apple genome. Datasets were from apples infected with (a) Alternaria alternata; (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 log10(FPKM). The color scales of panels (b), (c), (e), (f), and (g) were used to indicate the gene expression level corresponding to the log10(FPKM_Treatment/FPKM_Control).
and MdWRKY61a were downregulated after *Alternaria* inoculation. MdWRKY33b reached its highest value at 12 hpi, MdWRKY15a, MdWRKY50a, MdWRKY75c, MdWRKY42a, MdWRKY15d, MdWRKY71b, MdWRKY54e, MdWRKY50b, MdWRKY40a, and MdWRKY48a reached their highest values at 18 hpi. The expression of MdWRKY14b, MdWRKY47b, MdWRKY2c, and MdWRKY60b peaked at 36 hpi. MdWRKY169a, MdWRKY23b, MdWRKY30b, MdWRKY69b, MdWRKY41a, MdWRKY48b, MdWRKY44d, MdWRKY27a, MdWRKY65a, MdWRKY71a, MdWRKY3b, MdWRKY33d, MdWRKY33c, MdWRKY42b, MdWRKY60a, MdWRKY61d, MdWRKY15b, MdWRKY45a, MdWRKY61b, MdWRKY29b, MdWRKY75b, MdWRKY69c, MdWRKY11c, MdWRKY54g, and MdWRKY15c reached their highest expression levels at 72 hpi. MdWRKY19c, MdWRKY60d, MdWRKY71d, MdWRKY51, MdWRKY42d, MdWRKY30a, MdWRKY54h, MdWRKY29a, MdWRKY40b, MdWRKY32a, MdWRKY47a, MdWRKY161a, MdWRKY160c, MdWRKY64a, MdWRKY33a, and MdWRKY11d 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 MdWRKY75b and MdWRKY51 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 *MdWRKY*s after *Py. ultimum* infection (Figure 6(c)). *MdWRKY*48a, *MdWRKY*2a, *MdWRKY*15c, *MdWRKY*27b, *MdWRKY*41c, *MdWRKY*41b, 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*40b, *MdWRKY*33b, *MdWRKY*71a, *MdWRKY*74a, *MdWRKY*33a, *MdWRKY*93c, *MdWRKY*75c, *MdWRKY*29a, *MdWRKY*45a, *MdWRKY*47a, *MdWRKY*69c, *MdWRKY*44c, *MdWRKY*33a, *MdWRKY*33d, *MdWRKY*33c, *MdWRKY*29b, *MdWRKY*15a, and *MdWRKY*60d were downregulated at 24 hpi, but upregulated at 72 hpi in B9. *MdWRKY*43c, *MdWRKY*15b, *MdWRKY*54c, *MdWRKY*69b, *MdWRKY*65b, *MdWRKY*40a, and *MdWRKY*40b were upregulated at 24 hpi, but downregulated at 48 hpi and 72 hpi in G935. *MdWRKY*46a, *MdWRKY*54b, *MdWRKY*61d, *MdWRKY*60a, *MdWRKY*14b, *MdWRKY*54h, and *MdWRKY*93a were upregulated at 24 hpi in G935. *MdWRKY*22b, *MdWRKY*42b, and *MdWRKY*60c were upregulated at 48 hpi in G935. Also in G935, *MdWRKY*44c, *MdWRKY*33a, *MdWRKY*33d, and *MdWRKY*29b 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)). *MdWRKY71b, MdWRKY42a, MdWRKY47a,* and *MdWRKY71a* were upregulated in the resistant trees, but downregulated in the susceptible trees. *MdWRKY61c* was downregulated at 72 hai but upregulated at 96 hai in the susceptible trees. *MdWRKY2a* was downregulated at 48 hai in the susceptible trees. *MdWRKY71c, MdWRKY75c, MdWRKY71d,* and *MdWRKY46a* were downregulated in the resistant trees, but upregulated in the susceptible trees. *MdWRKY57a* was upregulated at 72 hai in the resistant trees. *MdWRKY50a, MdWRKY51, MdWRKY60c, MdWRKY54g, MdWRKY54h,* and *MdWRKY50b* were upregulated at 48 hai in the susceptible trees. *MdWRKY33d* was upregulated at 72 hai and 96 hai in the susceptible trees. *MdWRKY175d* 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 *MdWRKY65a* was downregulated at 4 dpi in HRA. *MdWRKY42a, MdWRKY33a, MdWRKY30a, MdWRKY11b,* and *MdWRKY20a* were upregulated at 4 dpi in the LRA. *MdWRKY30b* was upregulated at 4 dpi and 8 dpi in the LRA. *MdWRKY48b, MdWRKY71a, MdWRKY60b, MdWRKY71b, MdWRKY48a, MdWRKY50a, MdWRKY75a,* and *MdWRKY51* were downregulated at 8 dpi in the LRA. *MdWRKY175c* and *MdWRKY175b* 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*-Pl613981 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)). *MdWRKY65a* expression peaked at 6 hpi. *MdWRKY42a, MdWRKY33d, MdWRKY75c, MdWRKY75b,* and *MdWRKY40b* were significantly upregulated at 24 hpi. *MdWRKY65a* was significantly upregulated at 6 hpi. In ‘Gala’, there were only 5 differentially expressed *MdWRKYs*. *MdWRKY33d* and *MdWRKY40b* were significantly upregulated at 24 hpi. *MdWRKY58a* was significantly downregulated at 24 hpi. *MdWRKY75c* was significantly downregulated at 24 hpi and 48 hpi. *MdWRKY42a* 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)). *MdWRKY71a, MdWRKY75a, MdWRKY27b, MdWRKY47b, MdWRKY21b, MdWRKY29b, MdWRKY60d, MdWRKY71b, MdWRKY2b,* and *MdWRKY49a* were upregulated at 10 dpi. *MdWRKY57b, MdWRKY74a, MdWRKY45b, MdWRKY24e, MdWRKY69a,* and *MdWRKY22c* were downregulated at 10 dpi. *MdWRKY39c, MdWRKY21a, MdWRKY13b, MdWRKY58a, MdWRKY21c, MdWRKY44c, MdWRKY42c, MdWRKY13a,* and *MdWRKY15d* were upregulated at 30 dpi. *MdWRKY23a, MdWRKY61d,* and *MdWRKY14b* were upregulated at 30 dpi. *MdWRKY30b, MdWRKY50b, MdWRKY46b, MdWRKY61a, MdWRKY40b, MdWRKY30a, MdWRKY55b, MdWRKY54b, MdWRKY54e,* and *MdWRKY50a,* were upregulated at 30 dpi. *MdWRKY30b, MdWRKY50b, MdWRKY46b, MdWRKY61a, MdWRKY40b, MdWRKY30a, MdWRKY55b, MdWRKY54b, MdWRKY54e,* and *MdWRKY50a,* were upregulated at 30 dpi. *MdWRKY9c, MdWRKY21a, MdWRKY13b, MdWRKY58a, MdWRKY71b, MdWRKY2a,* and *MdWRKY49a* were upregulated at 10 dpi and 30 dpi. *MdWRKY75c, MdWRKY47a, MdWRKY45b,* and *MdWRKY60c* were upregulated at 10 dpi and 30 dpi. *MdWRKY69b* and *MdWRKY29b* were downregulated at 10 dpi and 30 dpi. *MdWRKY71c* and *MdWRKY60c* were upregulated at 10 dpi and 30 dpi. *MdWRKY89c* and *MdWRKY29b* were downregulated at 10 dpi and 30 dpi. *MdWRKY33d, MdWRKY41b, MdWRKY41c,* and *MdWRKY33b, MdWRKY60a,* were upregulated at 10 dpi and 30 dpi. *MdWRKY42a, MdWRKY48a, MdWRKY41a,* and *MdWRKY60a, MdWRKY55a,* were upregulated at 10 dpi, but downregulated at 30 dpi. *MdWRKY74b, MdWRKY42d,* and *MdWRKY54f, MdWRKY32a,* and *MdWRKY69b* 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). *MdWRKY33d* and *MdWRKY75c* were differentially expressed after infection with 6 pathogens, including *A. alternata, B. dothidea, E. amylovora, G. yamadae, Pe. expansum,* and *Py. ultimum*. *MdWRKY51* was differentially expressed after infection with 5 diseases, including ARD, *A. alternata, B. dothidea, E. amylovora,* and *Py. ultimum*. *MdWRKY75b* was differentially expressed after infection with 5 diseases, including ARD, *A. alternata,* *E. amylovora,* and *Pe. expansum*. *MdWRKY33a, MdWRKY33b, MdWRKY33c,* and *MdWRKY30a,* were differentially expressed after infection with 5 pathogens, namely *A. alternata, E. amylovora, G. yamadae,* and *Pe. expansum*. *MdWRKY42a* and *MdWRKY71b* were differentially expressed after infection with 5 pathogens, including *A. alternata, B. dothidea, E. amylovora,* *G. yamadae,* and *Pe. expansum*. *MdWRKY71a, MdWRKY54h,* and *MdWRKY60c* 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-MdWRKY40a and TRV-MdWRKY54h constructs significantly decreased the lesion size compared with the control (Figure 9(a) and Figure 9(b)). On the contrary, overexpression of *MdWRKY40a-OE* and *MdWRKY54h-OE* reduced resistance to *B. dothidea* (Figure 9(c) and Figure 9(d)). The disease spot size of apple fruits transiently expressing *MdWRKY40a-OE* and *MdWRKY54h-OE* were significantly larger than the control. These results indicated that *MdWRKY40a* and *MdWRKY54h* 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 WRKYs in group IIa, *MdWRKY40* and *MdWRKY60* mainly responded to infection *A. alternata*, *E. amylovora*, *G. yamadae*, *Pe. expansum*, and *Py. ultimum*. These genes are homologous to *AtWRKY18*, *WRKY40*, and *WRKY60*, 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 *WRKY18*, *WRKY40*, and *WRKY33*, were strongly (>4-fold) induced 30 min after flg22 treatment in *Arabidopsis* seedlings [41]. *WRKY18*, *WRKY40*, and *WRKY33* were identified as hub genes within a proposed WRKY regulatory network [6] [42]. *PtrWRKY40*
Figure 9. The role of *MdWRKY40a* and *MdWRKY54h* in *Botryosphaeria dothidea* infection. (a) The phenotypes of apples inoculated with *B. dothidea* after silencing *MdWRKY40a* and *MdWRKY54h* 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 *MdWRKY40a* and *MdWRKY54h* 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 *MdWRKY40a* and *MdWRKY54h* 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 *MdWRKY40a* and *MdWRKY54h* 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]. *GmWRKY40*, from *Glycine max* L., enhances the resistance to *Phytophthora sojae* [44]. In *Malus hupehensis*, *MhWRKY40b* were induced by the powdery mildew (*Podosphaera leucotricha*) [37]. In *Malus × domestica*, 4 *MdWRKY33s* were induced by *A. alternata*, *Pe. expansum*, *Py. ultimum*, *G. yamadae*, and *E. amylovora*. *MdWRKY33a* and *MdWRKY33d* were also induced by ARD and *B. dothidea*, respectively. *Arabidopsis WRKY33* is a key positive resistance regulator against the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* [45] [46]. Hence, the group IIA members WRKY40 and WRKY60 and group I member WRKY33 may play important roles in responding to pathogens and are conserved in plants.

WRKY15a and WRKY15b 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 *BnWRKY15* si-
multaneously increases the susceptibility of B. napus to S. sclerotiorum and down-regulates BnWRKY33 [47]. Although AtWRKY71 is involved in controlling shoot branching and accelerates flowering in Arabidopsis [48] [49], MdwRKY71a and MdwRKY71b showed differential expression after infecting B. dothidea, E. amylovora, G. yamadae, and Py. Ultimum in apple.

MdwRKY42a and MdwRKY42b showed differential expression in response to 5 pathogen infection. MdwRKY42a (named MdwRKY31 in [16]) regulates plant resistance to B. dothidea through the SA signaling pathway by interacting with MdhIR4. In rice, WRKY42 negatively regulates the rice response to Magnaporthe oryzae by suppressing JA signaling-related genes [50].

MdwRKY54h and MdwRKY40a 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, MdwRKY54h was upregulated in the sensitive genotype. Transient expression assays showed that MdwRKY40a and MdwRKY54h 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 WRKYs 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. MdwRKY40a and MdwRKY54h 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, P161983—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 WRKY33a | GAGGCAGCCAAACATCAGAAG | ATGCATCCCTGCCCTGCTCT | qRT-PCR |
| Md WRKY40a | CTTGTG7CCAGACCGAAGCA | AGGGAAGGACATCTATTGCCA | |
| Md WRKY42a | CTTCCTG7TGTCGTGACACA | CCGGGAAGCTGTAATGTTC | |
| Md WRKY51 | ACAAAATGGGAAGCTGAGGTT | ATAGCTCGACATCATCTCGGT | |
| Md WRKY54h | TCGTCCATTCCCATCGTCA | CGTCCCACTGCAGTTTTGA | |
| Md WRKY60c | TCTCGATCTGTGCAGGATCCA | AGGTCTTGCAATGGAAACGTG | |
| Md WRKY71b | GTATGAAAGGCAAGCAAAC | TGGGGCATTTGAAACAAAGT | |
| Md WRKY75b | TTCTCCCGTGCCTGCGAAC | TCTCAGATGGCTGTCACCAC | |

**Vector construction**

| Gene name | Forward primer | Reverse primer | Purpose |
|-----------|----------------|----------------|---------|
| TRV-Md WRKY40a | ATTCTGTGAGTAAGGTACGGAGATTC | CCGGACATGCGCCCGCTGCAGCTACCTCA | |
| TRV-Md WRKY54h | ATTCTGTGAGTAAGGTACGGAGATTC | TCCCGGCGATGCGCGGCGCTGAGCCTC | |
| WRKY40a-OE | GTGGAATCCCGAAGCAATCCAGTTACGATACATGCAAT | TCCCTTTACCAGTGAATGATGGTTAATGATATATATTGTTTTGA | |
| WRKY54h-OE | GTGGACCAGGCTACGAGCAGAACACACCCAGGAAGAAGA | TCCCTTTACCAGTGAATGATGGTTAATGATATATATTGTTTTGA | |

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