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TRANSCRIPTIONAL PROFILES OF *MDWRKY33* IN APPLE ROOT IN RESPONSE TO INFECTION BY *PYTHIUM ULTIMUM*, ABIOTIC STRESSES AND CHEMICAL TREATMENTS

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A B S T R A C T

Plant resistance responses to pathogen infection involve massive transcriptional reprograming and widespread redirection of cellular pathways to adjust the plant from growth to defense. Transcription factors (TFs) function at the major regulating points of gene expression, and specific TFs are known to play crucial roles in plant defense activation. Molecular defense activation in apple root from infection by oomycete necrotrophic pathogen *Pythium ultimum,* a primary component in a pathogen complex inciting apple replant disease, has not been investigated in detail. Based on previous transcriptome analyses, members of apple WRKY gene family have been identified as the primary candidates in regulating defense response in apple root. Among them, *MdWRKY33*, an orthologue of *AtWRKY33* in apple genome, demonstrated as a highly-expressed WRKY with genotype-specific induction patterns during *P. ultimum* infection. The sequence features of *MdWRKY33* and its tissue-specific expression, as well as its responses to abiotic and pharmacological treatments, added to the evidence for its functional roles in defense activation in apple root. In response to *P. ultimum* infection, *MdWRKY33* was consistently upregulated in all eight tested apple rootstock genotypes at all timepoints. Between genotypes, the stronger induction patterns at the earlier stage of infection in resistant genotypes suggest its essential roles of contributing to apple root resistance, although plant resistance to necrotrophic pathogens is polygenetic quantitative resistance in nature. Transgenic manipulation of this gene is underway to provide more definitive functional identity in contributing to apple root resistance to *P. ultimum* infection.

Keywords: Apple rootstock, Replant disease, Defense activation, Resistance responses, Transcriptome profiling, Transcription factor, Gene expression, *Pythium ultimum*, Necrosis patterns.

INTRODUCTION

Coordinated regulation of gene expression is essential for plant growth, development, and response to environmental factors. Plant resistance responses towards pathogen infection involve a complex transcriptional re-programming which includes hundreds of host genes (Van Verk *et al.*, 2009). Proper and swift transcriptional control is a crucial step for a plant to mount effective defense activation to pathogen infection (Birkenbihl *et al.*, 2017). Transcription factors (TFs) are among the key regulators contributing to plant resistance responses and coordinating multiple pathways of plant immune responses, such as pathogen detection, phosphorylation cascades, hormones signaling and secondary metabolism (Alves *et al.*, 2014; Singh *et al.*, 2002). TFs function by binding certain upstream elements of target genes, which temporarily and spatially turn on or off the transcription of a set of genes according to specific cellular context (Jin *et al.*, 2016). It is known that plants dedicate up to 5% of genome sequences to encode as many as 58 TF families (Jin *et al.*, 2014). For example, more than 2100 and 2300 TFs have been identified in the Arabidopsis and rice genomes, respectively (Riechmann *et al.*, 2000). TFs act as one of the master switches of transcriptional regulation of plant defense activation and optimized defense output requires the coordinated action of multiple members of various TF families.

Members from several plant TF families are known to directly participate in transcriptional regulation of plant

defense responses under pathogenic pressure (Buscaill and Rivas, 2014; Birkenbihl *et al.*, 2017). WRKY, one of the largest plant-specific TF families, have received more attention for their central role during defense activation against plant necrotrophic pathogens. TF family is defined by the presence of specific motifs such as bZIP, zinc finger, and/or helix turn helix for their ability to bind the specific cis-regulatory elements presenting in the promoter sequences of target genes (Zhang *et al.*, 2011). WRKY family contains one or two conserved WRKYGQK sequences and a novel zinc finger motif. WRKY TFs bind to the specific *cis*-element of W-box, or (T)(T)TGAC(C/T) sequence, in the promoter of target genes (Eulgem *et al.*, 2000). The first WRKY gene was reported from sweet potato in 1994 (Ishiguro and Nakamura, 1994) (Song *et al.*, 2010), and since then WRKYs have been identified from a wide variety of plant genome (Rushton *et al.*, 2008; Eulgem *et al.*, 2000; Wu *et al.*, 2005; Mangelsen *et al.*, 2008). From the apple genome, a total of more than 127 WRKYs have been identified (Jin *et al.*, 2016; Meng *et al.*, 2016). Other TF families, which are frequently associated with pathogen resistance, include AP2/ERF (APETALA2/Ethylene-Responsive Factors), bZIP (basic leucine zipper containing domain proteins), MYC (myelocytomatosis related proteins) and MYB (myeloblastosis related proteins). Roles of WRKYs in apple root and soilborne necrotrophic pathogen *Pythium ultimum* interaction has not been investigated.

Plant root diseases caused by soilborne pathogens are a serious problem for many agronomically important crops. Necrotrophic oomycete pathogen *P. ultimum* is a primary component in a pathogen complex inciting apple replant disease (ARD) (Mazzola, 1998). In response to infection by *P. ultimum*, apple rootstock genotypes demonstrated contrasting responses at plant survival rates, biomass reduction, and microscopic features of tissue necrosis patterns (Zhu *et al.*, 2016; Zhu, Zhao*, et al.*, 2018), but the genetic controls of these observed resistance phenotypes are unknown. Comparative transcriptome analyses identified four major TF families, WRKY, MYB, BHLH and AP2/ETF among many other factors, which are believed to actively participate in the defense activation during interaction between apple root and *P. ultimum* (Zhu *et al.*, 2019; Shin, Zheng*, et al.*, 2016). In this study, the expression profiles *MdWRKY33,* a highly-expressed WRKY member in apple root, were studied in detail. Specifically, the genotype-specific induction patterns during apple root interaction with *P. ultimum,* its tissue-specific expression profiles, and its responses to pharmacological treatments or under abiotic stress conditions were analyzed using RT-qPCR. The objective is to gain more insight into the potential roles of *MdWRKY33* as one of the potentially key transcription regulators in apple root-*P. ultimum* pathosystem.

MATERIALS AND METHODS

Plant materials, apple plant micropropagation and plant maintenance: Tissue culture based micropropagation of apple plants was used as a method for providing consistent supply of apple root tissues for repeated root infection assays. Apple plant micropropagation by tissue culture procedure and phenotyping methods on the resistance phenotypes were as described (Zhu, Saltzgiver*, et al.*, 2018). Briefly, apple plants with root tissue induced for four weeks from in vitro micropropagation procedure were used for infection and other treatment. The root tissue of these tissue culture generated apple plants were transplanted to soil medium for one week in-soil acclimation, allowing root tissue to further differentiate before infection assays or abiotic treatments. The selected resistant genotypes such as #161, #58 and susceptible genotypes #115 and #132 belong to a cross population in between Ottawa 3 and Robusta 5. Plants with similar ages and comparable root systems were randomly assigned as controls, or used for pathogen infection, abiotic stresses or pharmacological treatments. Apple flower tissue and immature fruit tissues were collected from ten-year old trees of 'Delicious' cultivar in a Washington State University experimental orchard block.

Inoculum preparation, inoculation and root tissue collection: Inoculum of *P. ultimum* was prepared as previously described (Zhu *et al.*, 2016). In brief, the inoculation of plants with *P. ultimum* was performed by dipping the root system into the inoculum solution (1% methyl cellulose solution) of 2 x 10^3 CFU (colony forming unit) for 5 s and then planting treated seedlings into pasteurized soil medium consisting of construction sands, perlite and vermiculite in 1:1:1: ratio. Control plants were mock inoculated with 1% methyl cellulose solution and then transplanted and maintained the same manner as *P. ultimum* inoculated plants. The pathogen inoculated, and mock inoculated plants were maintained in an environmental growth room at 23 ℃ ± 2 and under a 12/12 h light/dark photoperiod. Plants root tissues

were harvested at 0, 24, 48 and 72 hpi. Root tissues were collected by excavating from soil, washing under tap water and flash-freezing in liquid nitrogen. A mixture of root tissues of 5 plants were collected and pooled as a biological replicate at each timepoint per treatment.

Microscopic observation on the features of necrotic expansion along infected root: The root system was carefully excavated from the pot at selected time points after inoculation. Soils associated with root tissues were gently removed by rinsing under tap water. Root branches were kept in a beaker with autoclaved water until examination under a microscope within two hours. An Olympus SXZ12 dissecting microscope was used to examine the genotype-specific features of root necrotic symptom due to *P. ultimum* infection. A minimum of three plants were examined per treatment, timepoint and genotypes. Images were obtained by an amounted DP73 digital camera with accompanied software suite of Celsense (Olympus, Center Valley, PA).

Treatments of apple root with MeJA, ethylene, BABA or under cold and heat conditions: After four-week of root induction and one-week in-soil acclimation, apple plants from tissue culture micropropagation procedure were treated by pharmacological reagents or under abiotic stress conditions as described below. MeJA (methyl jasmonate) treatments following the method of Li *et al.* (2006), apple roots were dipped in to beaker with 100 µM MeJA (Sigma, Cat# 392707) in 0.1% v/v ethanol for 5 sec. MeJA treated plants were immediately wrapped with a sheet of autoclaved paper towels saturated with water; and root wrapped plants were placed in the upright position into an empty Magenta box with lid loosely covered for the duration of overnight (about 16 hours). Root tissues were collected the next morning. BABA (β-aminobutyric acid) (Sigma, Cat# A44207) treatment was performed followed Koen *et al.* (2014) by dipping the apple plant roots for 5 sec in 300 µM solution dissolved in water. Treated plants were maintained as those with MeJA. Ethylene treatment was carried out by placing plants (with root wrapped with paper towels saturated with water) and placed in a chamber containing ethylene at 10 ppm ethylene for three hours. For cold and heat treatments, micropropagated plants growing in a plastic pot were placed in a chamber at 4 ℃ or a chamber at 37 ℃ for overnight. The control plants were from the same batch of micropropagation procedure and kept under ambient conditions with normal watering schedule. Collected root tissues were flash frozen in liquid nitrogen and stored in -80 ℃ freezer until total RNA isolation.

Identification of *MdWRKY33* genes from transcriptome data and sequence analyses: Previous comparative transcriptome profiling has been reported (Zhu *et al.*, 2019) and the RNA-seq data was deposited in SRA (Sequence Read Archive) at the NCBI website under the accession number SRP117760. Briefly, the comparative transcriptome analysis was designed including two (2) treatments (mock-inoculation control and *P. ultimum* inoculated), three (3) biological replicates and four (4) timepoints (0, 24, 48 and 72 hpi) after *P. ultimum* inoculation for two genotypes. The inoculation of the roots of a susceptible genotype B.9 and a resistant genotype G.935 were performed using identical preparation of *P. ultimum* inoculum. The sequencing format with paired-end reads of 150 bp were applied using Illumina Solexa HiSeq 3000 platform (Illumina, San Diego, CA). Reads from sequencing libraries were mapped to the nucleotide sequences of predicted coding genes of the *Malus* x *domestica* Whole Genome v3.0.a1 (https://www.rosaceae.org/analysis/162) using the ultrafast, memory-efficient short read aligner Bowtie2-2.2.5, which utilizes a Burrows–Wheeler index. Read count data were obtained for each coding sequence. The DESeq2 program in R (http://www.r-project.org/) performed normalization using geometric mean and the median to normalize the data. DEGs were identified by comparing transcript abundance between mock-inoculated control root tissues and those from *P. ultimum* infected root tissues from the same genotype; and the cutoff values of Log2Fold Change ≥ 1 under the p-adj (adjusted p) values ≤ 0.05. The annotation of these genes was carried out by BLASTP against NR (non-redundant protein sequences) database, and a BLAST database containing genomic sequences for Arabidopsis (*Arabidopsis thaliana*), corn (*Zea mays*), *Medicago truncatula*, rice (*Oryza sativa*), and tomato (*Solanum lycopersicum*). The predicated amino acid sequences for *MdWRKY33* was subjected to analysis using SMART (http://smart.embl-heidelberg.de/) and COBALT for multi-sequence alignment at NCBI site (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?LINK_LOC=BlastHomeLink).

RNA isolation, cDNA synthesis, sequence retrieving, primer design and RT-qPCR: Total RNA isolation and cDNA synthesis were as previously described (Zhu *et al.*, 2016). The nucleotide sequence of each candidate gene

was downloaded from the Genome Database for Rosaceae (GDR, http://www. rosaceae.org/). Primers were designed using the Primer Quest tool (Integrated DNA Technologies) with the following criteria: GC content 45–65%, Tm >50 °C, primer length 20–24 bp, and amplicon size 150–200 bp (Table 2). MdWRKY33-forward primer sequences is TCACCACGTACGAAGGGAAA , and MdWRKY33-reverse primer sequences is TTGATGGCTGCCTTGTGTTC. Total RNA was quantified using a ND 1000 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA quality was verified by agarose gel electrophoresis. Total RNA was treated with DNase I (Qiagen, Valencia, CA) and then purified with RNeasy cleanup columns (Qiagen). Two micrograms of DNase-treated RNA were used to synthesize first-strand cDNA using SuperScript™ II reverse transcriptase (Invitrogen, Grand Island, NY) and poly dT (Operon, Huntsville, AL) as the primer. The cDNA was diluted 20 times and 0.6 μL aliquot was used in a 15 μL quantitative PCR (qPCR) reaction mix: 0.45 μL SYBR Green I dye (Invitrogen, Grand Island, NY), 1x iTaq buffer (Biorad, Hercules, CA), 0.2 mM dNTP (Applied Biosystems, Waltham, MA), 2.5 mM $MgCl_2$, 0.3 units of iTaq DNA polymerase (Biorad, Hercules, CA), and 0.2 μM forward/reverse primer (IDT, Coralville, IA). RT-qPCR was performed in 96-well plates using an CFX real time qPCR detection system (Biorad Lab, Hercules, CA) and the following protocol: cycle conditions of 3 min at 95 ℃ and 40 cycles of 10 s at 95 ℃ and 30 s at 59 ℃. The melting curve for each amplicon was obtained from 60 to 95 ℃ to verify primer specificity. PCR efficiency and correlation coefficient (R^2) for each primer set was calculated by the slopes of standard curves generated in Microsoft Excel 2016 from a 5-fold cDNA dilution series (1:5, 1:25, 1:125, 1:625 and 1:3125). All assays were carried out in two technical and biological replicates with template-free negative controls being performed in parallel. The relative expression level was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The reported values represent the averages fold changes between control and treated samples with SD from the raw values of these assays based on *t*-test.

RESULTS

Apple root necrosis patterns from *P. ultimum* infection as one of the indicators of resistance and susceptibility: Soilborne necrotrophic pathogen *P. ultimum*, a primary component of a pathogen complex inciting ARD, is known to cause root necrosis or even plant mortality; and contrasting patterns of necrosis progression have been reported among tested apple rootstock germplasm (Zhu, Zhao*, et al.*, 2018). As illustrated below, the healthy root branches of mock-inoculation control remained healthy with tissue integrity (intact and no sign of tissue disintegration) and predominantly white color at 7 dpi (days post-inoculation) or later (Figure 1A and B). In response to *P. ultimum* inoculation, localized infection sites with typical symptoms of yellow to brownish necrotic sections often could be detected at 24 hpi (hour post-inoculation), and genotype-specific variation of necrosis progression were demonstrated extensively at 48 hpi or later (Figure 1C and D). For most of susceptible genotypes, wide-spread necrosis with semi-transparent tissues were commonly observed at 48 hpi or later (Figure 1E); and abundant hyphae growth were frequently associated with necrotic tissues (Figure 1C and E). On the other hand, the more resistant genotypes often exhibited the deterred and limited necrosis progression (Figure 1D) at the early stage of 24-48 hpi. Some more noticeable features are the presence of a well-defined “boundaries” or “lines” (Figure 1F, red arrows), which clearly separates the healthy sections (white root tissue and intact appearance) from necrotic tissues (yellow coloration with semi-transparent and collapsing root tissues). Limited or no hyphae were observed along the necrotic sections of roots of resistant genotypes (Figure 1D and F). For genotypes with extreme susceptibility, whole root system can be engulfed by the rapid progression of tissue necrosis in less than 24 h after the initial necrosis was identified, and plant wilting and death can be observed as early as 3 dpi (Zhu, Zhao*, et al.*, 2018).

Identification of *MdWRKY33* during *P. ultimum* infection of apple root by transcriptome analyses: Our recent transcriptome analyses using the large-scale and high-throughput RNA-seq platform have identified many specific transcriptome changes associated with apple root defense activation during *P. ultimum* infection, and the global features of transcriptome changes have been reported previously (Shin, Zheng*, et al.*, 2016; Zhu *et al.*, 2017; Zhu *et al.*, 2019). Among the major transcriptome changes identified TF-encoding genes, in particular the mostly upregulated WRKY-encoding genes, are one of the most noticeable functional groups (Zhu *et al.*, 2019).

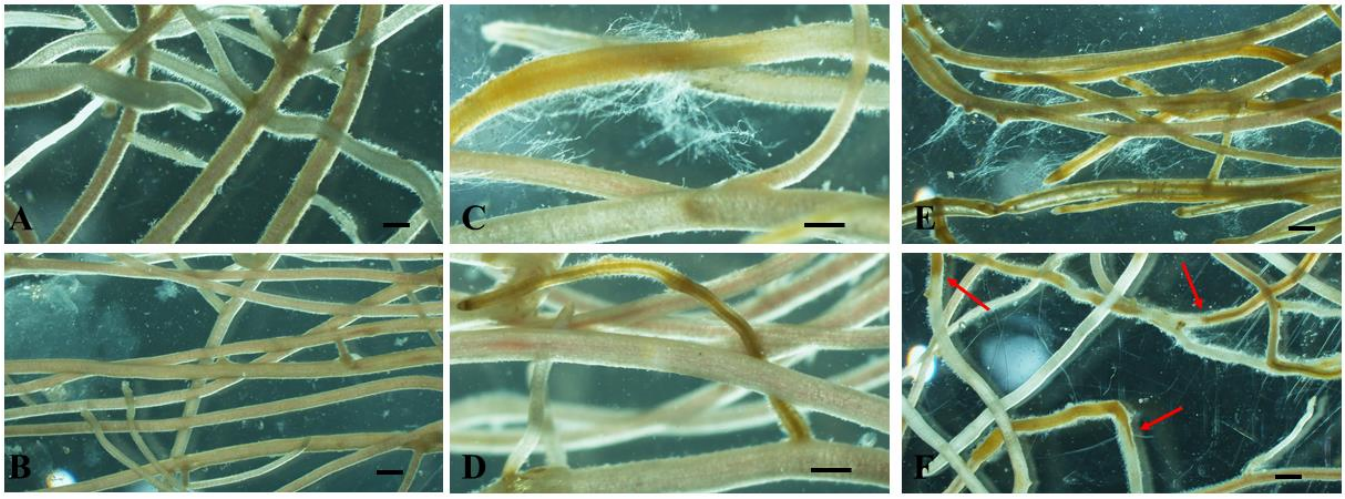

Figure 1. Representative images of necrotic symptoms along infected apple roots between resistant or susceptible genotypes at early (24-48 hpi) and late stages (after 48 hpi). **A.** Mock inoculated apple root of a susceptible genotype #121 at 7 dpi. **B.** Mock inoculated apple root of a resistant genotype #78 at 7 dpi. **C.** A necrotic root section with yellow-brownish coloration for a susceptible apple rootstock genotype observed between 24-48 hpi, and profuse growth of pathogen hyphae at the infected section. **D.** An infected root branch of a resistant genotype with typical yellow-brownish coloration among healthy uninfected root branches between 24-48 hpi, no or rare hyphae observed in most cases. **E.** Widespread necrosis among entire root system of a susceptible genotype at later infection stage 48-96 hpi; with easily identifiable tissue collapse and semi-transparent appearance. **F.** Deterred necrosis and presence of "boundaries" or "lines" separating healthy and necrotic section at 48-96 hpi. Size of the bars at the lower right corner of each image represent 500 μM.

The values of mapped reads representing transcript abundance for specific apple *WRKY* genes are summarized in Table 1. Among several apple WRKY genes, *MdWRKY33* showed consistent upregulation with a highest expression level in response to *P. ultimum* infection. In addition, both *MdWRKY33* and *MdWRKY53* exhibited the genotype-specific expression patterns at the early stage of infection at 24 hpi in a resistant genotype of G.935 compared to that for a susceptible genotype B.9 (Zhu *et al.*, 2016). This observation suggested a possible correlation between earlier or quicker induction of both WRKYs and resistance to *P. ultimum* infection. Two *WRKY22*s were only induced at the early stage and then suppressed by *P. ultimum* infection. For its high expression level, the stronger induction at the earlier stage of infection and preferentially in the resistant genotypes of G.935, *MdWRYK33* was selected for a more detailed analysis to understand its potential roles in apple root resistance to *P. ultimum* infection.

Sequence features of *MdWRKY33* is well conserved with those of AtWRKY33: Analysis of MdWRKY33 amino acid sequence by SMART (http://smart.embl-heidelberg.de/) revealed that it contains two WRKY motifs (Figure 2A and B), positioned slightly toward C-terminus compared to those in Arabidopsis AtWRKY33. Overall amino acid sequences shared 45% identity and 54% similarity between these two genes, though a higher level of divergence exists at the N terminus half of the sequences. Although N terminus is a less homologous section, five SP clusters (clustered proline-directed serine) (Figure 2B, highlighted in green color were identified), which are the potential sites for phosphorylation by MAPKs in Arabidopsis (Andreasson *et al.*, 2005; Mao *et al.*, 2011; Menke *et al.*, 2005), were conserved within a stretch of less than 50 amino acid residues. At the C-terminus, a putative zinc ligands (C–X4–5–C–X22–23–H–X1–H) was identified (Figure 2B, highlighted in blue) (Bakshi and Oelmüller, 2014). Additionally, the cloned genomic sequences from apple rootstock genotypes used in this study, which cover *MdWRKY33* genes showed that there is less than 1% mismatches at nucleotide level within its coding region, as it was compared with the publicly available apple genome sequences using model cultivar 'Golden Delicious' (Velasco *et al.*, 2010; Daccord *et al.*, 2017); and the numbers and sizes of introns were well conserved (data not shown). Therefore, the identified sequence features indicated that *MdWRKY33* in apple genome is most likely an ortholog of *AtWRKY33*.

Table 1. Mapped reads for selected apple *WRKY* genes based on previous transcriptome analyses.

Annotation [a]	Gene ID [b]	Variety	Values of mapped reads [c]						Fold change [d]
			24 hpi		48 hpi		72hpi		
			ck	pu	ck	pu	ck	pu	
MdWRKY 33	MDP0000708692	B.9	1076	2323	1336	4681	1087	3514	2.2-3.6-3.2
	MD04G1167700	G.935	1826	12132	1446	3860	2257	3196	6.6-2.7-1.4
MdWRKY 53	MDP0000157124	B.9	148	219	117	367	102	334	1.5-3.1-3.4
	MD06G1104100	G.935	111	666	80	181	135	186	6.0-2.3-1.4
MdWRKY 22.1	MDP0000602139	B.9	249	385	1026	248	266	219	1.5-(-4.1)-(-1.2)
	MD01G1071300	G.935	209	620	763	714	575	224	3.0-(-1.06)-(-2.6)
MdWRKY 22.2	MDP0000909869	B.9	120	148	198	289	121	84	1.2-1.5-(-1.4)
	MD07G1131400	G.935	98	174	198	227	261	99	1.8-1.1-(-2.3)

Reads from each sequencing library were mapped to the nucleotide sequences of predicted coding genes of the apple (*Malus* x *domestica)* whole genome sequences; Differentially expressed genes (DEGs) were selected based on significantly different expression level (>2-fold, p<0.01) between mock- and *P. ultimum*-inoculated root tissues, for at least one of the three time points (24, 48 and 72 hpi) in either genotype. [a] Annotation of the putative function for these WRKY genes was based on BLASTP against NR (refer Materials and Methods for details). [b] Gene IDs were assigned from two versions of apple genome sequences; top is from Valesco et al., 2010 (Velasco *et al.*, 2010), and the bottom one is from a more recent version of apple genome sequences Deccord et al, 2014 (Daccord *et al.*, 2017). [c] Numbers of mapped reads represent the average of normalized values for three biological replicates, where "ck" denotes mock inoculation as control tissues; and "pu" indicate the value for *P. ultimum* inoculated. [d] Three numbers, separated by hyphens, in the last column titled "fold change" indicated the values of fold changes between mock inoculation control and *P. ultimum*-infected tissues at three time points of 24, 48 and 72 hpi, symbol of "-" in parenthesis indicates the downregulation comparing to control tissue. Several other identified WRKYs from this transcriptome analysis with lower values of mapped reads or with only transient response at one or two timepoints were not included in this table.

Tissue-specific expression of *MdWRKY33* and its transcriptional responses to abiotic stresses, pharmacological treatments: Tissue-specific expression patterns of *MdWRKY33* were examined using five types of tissues including fruit, flower, leaf, healthy (non-inoculated) root and *P. ultimum* infected roots. As shown in Figure 3A, lower levels of *MdWRKY33* transcripts were observed in the reproductive tissues of fruit and flower, in relation to higher expression levels in the vegetative tissues of leaf and root. The inducibility of *MdWRKY33* from *P. ultimum* infection was demonstrated as the infected root tissue (root + P) showed almost three times higher expression level comparing to non-infected control root tissue. It is known that, in addition to the roles in defense activation against necrotrophic pathogens, members of WRKY family are responsive to abiotic stress conditions and hormonal or other chemical signals (Zhang *et al.*, 2016; Ren *et al.*, 2010).

The transcriptional responses of *MdWRKY33* in apple root tissues were examined under abiotic stresses of cold (4 ℃) and heat condition (37 ℃), treatments by four chemicals, with some of these reagents are known to be involved in plant defense activation or defense priming. Our data indicated that cold treatment caused the most noticeable upregulation of *MdWRKY33*, with about 12 folds increases of detected transcript abundance in the root of two apple rootstock genotypes.

Heat seemed to have less dramatic effect on the expression of *MdWRKY33* with about 1-2 folds increase of expression levels compared to plants growing under ambient temperature (~22-24 ℃). Chemical treatments induced the moderate upregulation of *MdWRKY33* expression, at about 2-4 folds increases based on the comparison between detected transcript levels in control and treated apple root tissues. An interesting note is that both genotypes, a resistant #161 and susceptible # 132, showed the relative similar pattern of responses to these treatments.

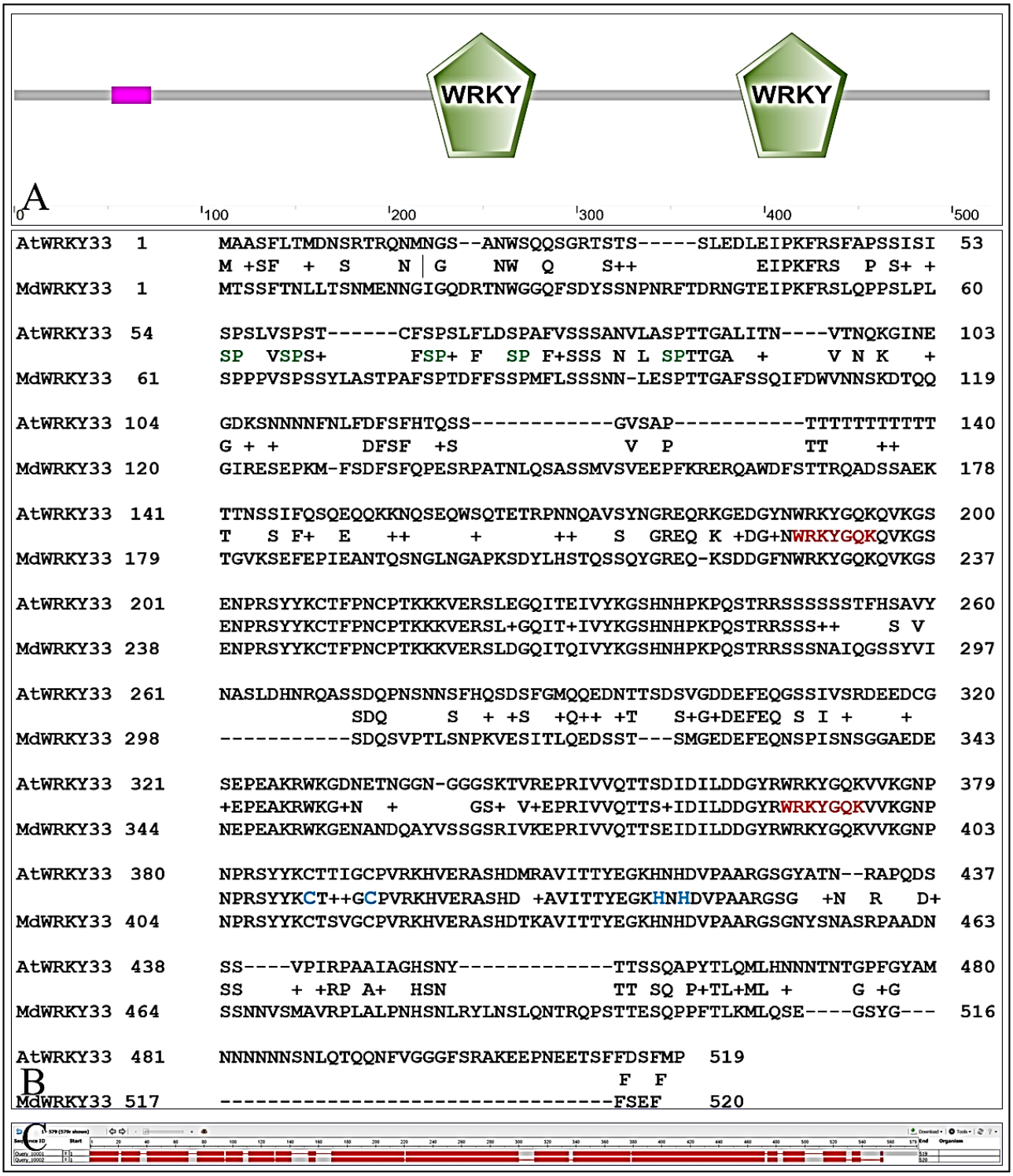

Figure 2. Sequence features and overall homology between *MdWRKY33* and a well-studied Arabidopsis homologous gene AtMdWRKY33. Nucleotide and protein sequences were downloaded from publicly available data base. **A.** Two WRKY domains were identified and their positions for *MdWRKY33* using SMART program. **B.** Overall protein sequence comparison demonstrated the alignment of conserved core WRKY sequences highlighted in red, and SP as potential phosphorylation sites were highlighted in green, putative zinc finger motif was highlighted in blue, respectively. **C.** Graphic demonstration of overall sequence similarity between *MdWRKY33* and *AtWRKY33*.

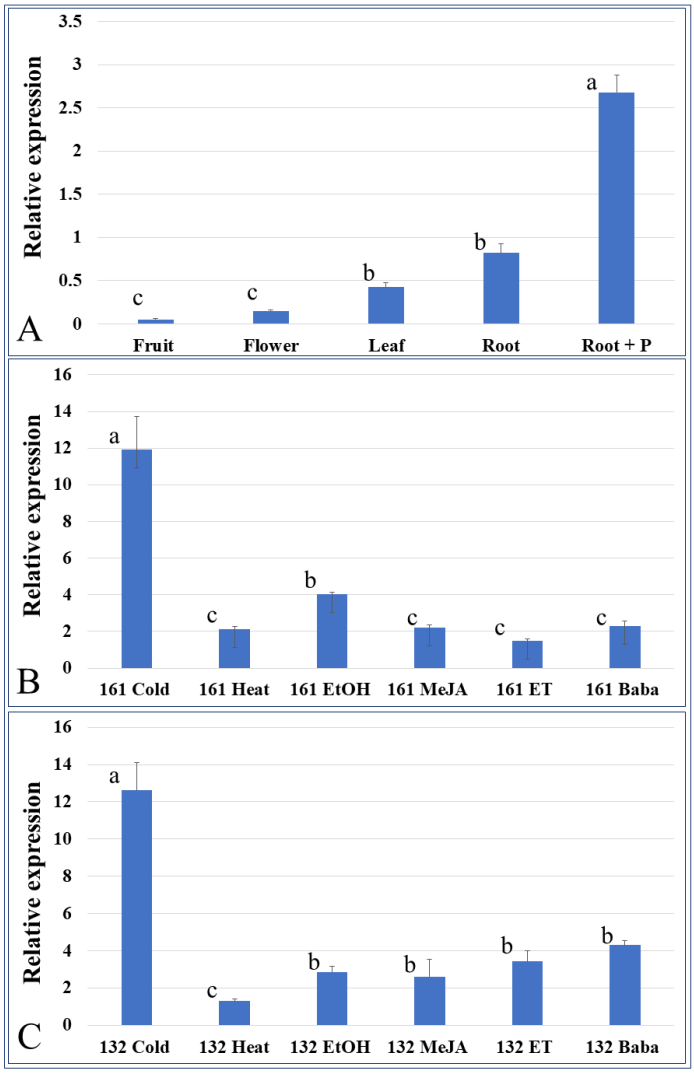

Figure 3. Expression patterns of *MdWRKY33* among apple plant tissue types and under abiotic stress conditions or in response to chemical treatments. **A.** Expression of *MdWRKY33* in different tissue types including apple fruit, flower and leaf and root tissues. were collected from the ten-year old trees in the experimental orchard. Root tissues and infected root tissues were collected from micropropagated plants. **B.** Transcriptional responses to abiotic stresses and pharmacological treatments using a resistant apple rootstock #161; **C.** Transcriptional responses to abiotic stresses and pharmacological treatments using a susceptible apple rootstock #132. Micropropagated apple plants for each genotype were randomly selected for various treatments. Label on X axis denote the tissues types or various treatments. Values on Y axis indicate the relative expression level based on RT-qPCR detection. Values represent the average of two technical repeats for each of the two biological replicates. Different letters indicate statistical differences (P = 0.05) according to the t test. Or "values marked with the same letter are not significantly different (*P* = 0.05).

Genotype-specific induction patterns of *MdWRKY33* in apple roots due to *P. ultimum* infection: The genotype-specific induction patterns of *MdWRKY33* in response to infection from *P. ultimum* were examined using four pairs of apple rootstock genotypes based on their resistance or susceptibility to *P. ultimum* reported previously (Zhu *et al.*, 2016; Zhu, Zhao, *et al.*, 2018). Expression data indicated that *MdWRKY33* was uniformly upregulated per genotype and timepoint due to *P. ultimum* infection, based on fold change of measured transcript abundance between *P. ultimum*-inoculated and its respective mock inoculation controls. These results validated the essential roles of *MdWRKY33* during defense activation under the pathogenic pressure from *P. ultimum* (Figure 4). One noticed trend appeared to be that a quicker and stronger activation of

MdWRKY33 at the early stage 24 hpi occurred often in the root of resistant genotypes such as #58, #63 and G.935, except #161. In contrast, the stronger level of upregulation appeared to occur in the later stage of 48 and 72 hpi in the root of the susceptible genotypes, such as B.9, #115 and #121. These observed induction patterns were consistent with the previous results from comparative transcriptome analysis between B.9 and G.935 (Zhu *et al.*, 2019). The results appeared to support the notion that earlier and stronger activation of *MdWRKY33* may be a significant factor contributing to the observed resistance phenotypes, but the existence of an opposite pattern between the pair of #161 and #132 suggested that other genetic elements also critically impact apple root resistance phenotypes to *P. ultimum* infection.

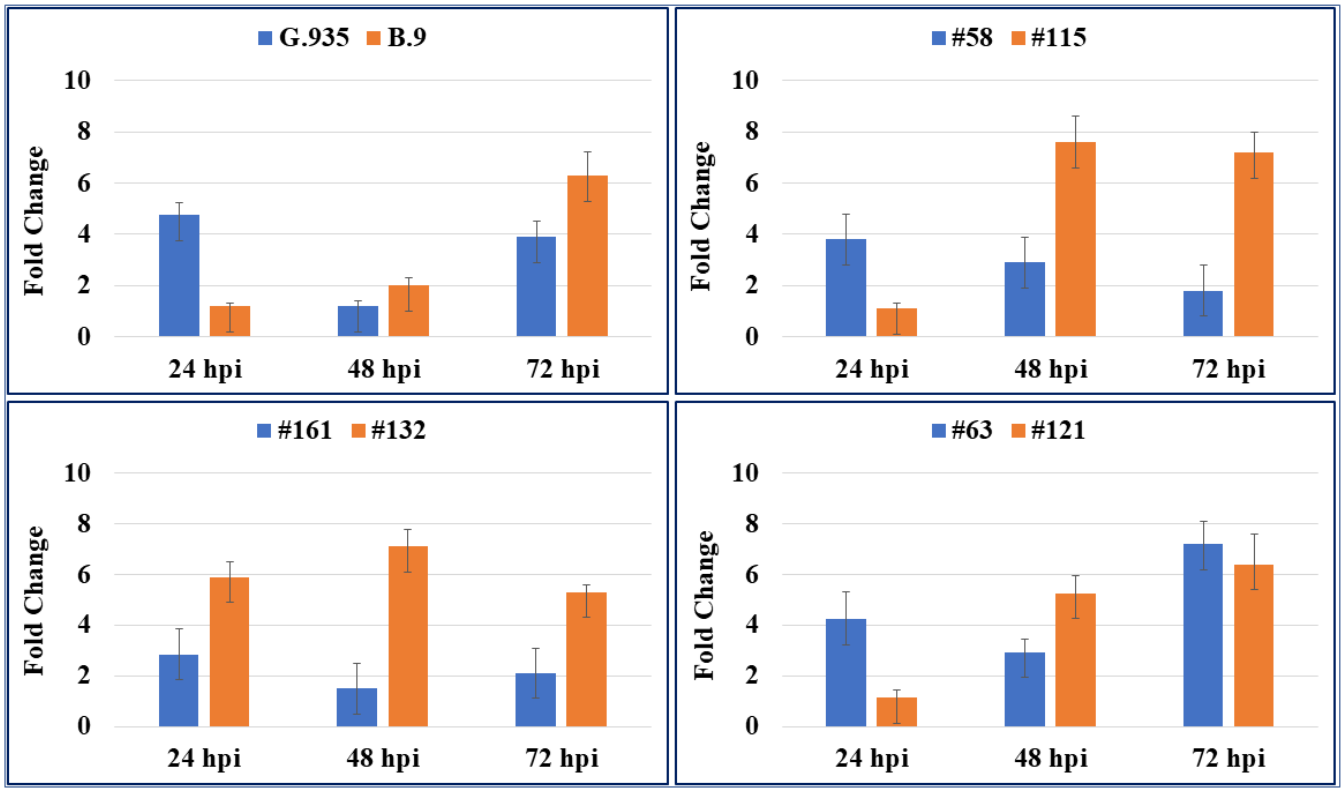

Figure 4. Genotype-specific induction patterns of *MdWRKY33* expression in apple root in response to infection by *P. ultimum*. Four pair of apple rootstock genotypes, with the resistant genotypes in blue color and the susceptible genotypes in orange color, demonstrated distinguishable pattern of expression based on RT-qPCR analysis. Higher induction levels at early stage of infection were associated with most of the tested resistant genotypes, but higher levels of expression were observed in the later stages of infection, 48 to 72 hpi. Different letters indicate statistical differences (P = 0.05) according to the t test.

DISCUSSION

Root is the foundation of plant architecture as well as the entire plant physiology, with the essential functions encompassing anchorage, absorption, and biosynthesis. Root, as an underground organ, is also constantly challenged by complicated biological, physical, and chemical factors in soils surrounding it (De Coninck *et al.*, 2015; Benfey, 2012). Because of the persistent existence of soilborne pathogens, effective management of root diseases is vital for the productivity and sustainability of many crops. Defining the phenotypic features of apple root responses to *P. ultimum* infection is essential for dissecting the genetic controls and exploitation of root resistance traits. However, the hidden nature of root system hampers the non-disruptive observation of root pathogenesis, and the tiny stature of young apple root branches requires innovative approaches to observe and document the detailed

symptom development. The microscopic details of genotype-specific responses to *P. ultimum* infection were part of a systematic phenotyping effort (Zhu, Saltzgiver, *et al.*, 2018; Zhu, Zhao, *et al.*, 2018), and the representative images illustrated the contrasting defense response patterns, such as genotype-specific necrosis progression, in apple roots from *P. ultimum* infection.

The study on the time course of molecular interactions between apple root and *P. ultimum* revealed that the most intense responses in apple root occurred at 48 hpi based on the results from two consecutive transcriptome analyses (Shin, Zheng, *et al.*, 2016; Zhu *et al.*, 2019). Therefore, the stronger induction of *MdWRKY33* at the earlier stage of infection at 24 hpi in the root of a resistance genotypes implicated its critical roles in initiating the defense activation against *P. ultimum* infection. Direct contribution of specific WRKYs in plant defense against necrotrophic pathogens has been investigated in several model pathosystems, though little was known in apple root-*P. ultimum* pathosystem until these transcriptome analyses (Shin, Zheng, *et al.*, 2016; Zhu *et al.*, 2019). For its higher expression levels and elevated inducibility in resistant genotype of G.935, the roles of *MdWRKY33* in apple root resistance to *P. ultimum* infection deserve a more careful examination. Among more than 100 WRKY-encoding genes in apple genome (Jin *et al.*, 2016; Meng *et al.*, 2016) , only a handful of WRKY genes were identified by transcriptome analyses for their possible participation in apple root defense responses (Shin, Zheng, et al., 2016; Zhu et al., 2019). Additionally, the predicted *MdWRKY33* amino acid sequence is mostly homologous to that of Arabidopsis counterpart *AtWRKY33*, whose functional identity in resistance response to infection by *Botrytis cenera* is well studied (Birkenbihl *et al.*, 2012). A careful examination on *MdWRKY33* on its the sequence features, transcriptional regulations upon pathogen infection, and responses towards other external factors could provide valuable insights for interpreting its functional roles as a potentially major transcriptional switch for regulating defense activation and resistance traits to *P. ultimum* infection.

Sequence features at both nucleotide and amino acid levels strongly support the denotation that *MdWRKY33* is an ortholog to a well-investigated *AtWRKY33* in Arabidopsis. Whether or not it plays a similarly critical role in apple root resistance requires further investigation. Within the transcription machinery over a biological process, TF is simply one node in a large transcriptional regulation network, and its function can be impacted by multiple factors including its subcellular localization, post-translational modification such as phosphorylation and even the promoter sequences of target genes (Ishihama and Yoshioka, 2012; Samad *et al.*, 2017; Zhou and Memelink, 2016). The existence of multiple SP clusters (clustered proline-directed serine) within MdWRKY33 sequences, similar to those of AtWRKY33, indicated it is a likely substrate of phosphorylation by MAPKs, such as MPK3/MPK6 in Arabidopsis and under the similar cellular regulation mechanisms (Andreasson *et al.*, 2005; Mao *et al.*, 2011; Menke *et al.*, 2005). Phosphorylated TFs regulate the expression of downstream genes such as those functioning to generate various classes of antimicrobial secondary metabolites (Patra *et al.*, 2013; Chezem and Clay, 2016; Zhou and Memelink, 2016). Plant hormone such as JA and ET and multiple external signals also modulate the activities of TFs (Wasternack and Strnad, 2019). In Arabidopsis, ET biosynthetic genes ACS2 and ACS6 are the direct targets of *AtWRKY33*, and it can also bind to 1576 target loci and regulated the expression of 318 genes (Datta *et al.*, 2015; Liu *et al.*, 2015). In the pathosystem of apple roots interaction with *P. ultimum*, plant hormone signaling related to ET and JA is an integral part of apple root defense activation upon *P. ultimum* infection (Shin, Lee, *et al.*, 2016; Zhu *et al.*, 2019). Therefore, the potential influences of *MdWRKY33* by other internal or external factors in regulating defense activation in apple roots need to be defined.

It is well established that a given TF can respond to multiple internal developmental cues and/or external environmental stimuli (Chen *et al.*, 2015; Jensen and Skriver, 2014). Although *MdWRKY33* was originally identified from apple root-*P. ultimum* interaction, understanding its responses to other non-pathogenic factors will be valuable for possible exploitation of *MdWRKY33* in apple root disease resistance. The contrasting responses to cold and heat conditions indicated that distinct mechanisms were operational to mitigate these stress signals. The overall similarity of the response patterns between two apple rootstock genotypes suggested similar cellular processes were employed to deal with abiotic stresses and treatments by chemical factors, despite their contrasting responses to *P. ultimum* infection. EtOH was initially set as an

experimental control for MeJA treatment, because MeJA needs 0.1% ethanol to be dissolve in water. Although 0.1% ethanol was not the original target of chemical reagents, it evidently exhibited inducibility on the expression of *MdWRKY33*. BABA has been shown to be a potent inducer of defense priming in several model pathosystem (Baccelli and Mauch-Mani, 2016; Martinez-Medina *et al.*, 2016). In the resistance genotype #161, application of BABA showed two-fold increase of its transcript abundance in apple root as compared to four-fold increase in root of susceptible genotype #132. Similar to BABA, ET showed higher inducibility on *MdWRKY33* expression in the susceptible genotype than that in resistant genotype. It is possible that the susceptible genotype is more prone to agitation from these external conditions or treatments, though the molecular basis for such hypersensitivities is unknown. More study will be required to establish the potential correlation between the inducing activities from these chemicals on *MdWRKY33* expression and possible implications of applying them in managing disease resistance in apple root.

The uniformity of upregulation of *MdWRKY33* expression per timepoints and genotypes clearly indicated its roles in apple root defense activation against *P. ultimum* infection. Based on studies of other pathosystems, it is well acknowledged that plant resistance to necrotrophic pathogens is a polygenic trait derived from the contribution of multiple genes with minor effect (Wang *et al.*, 2014). The polygenic nature of quantitative resistance reflects the multiplicity of virulence mechanisms from necrotrophic pathogens which interfering diverse host cellular processes (Lorang, 2018). While it is likely that *MdWRKY33* functioned as a key factor contributing to *P. ultimum* resistance in some genotypes, this type of resistance mechanism can be overcome by other virulence factors if the corresponding defense pathways were compromised. Due to this complexity, it is possible that lacking another key functional resistance element (such as the capability of detoxifying pathogen derived phytotoxin) can still lead to susceptibility in #132. It is clear that multiple resistance mechanisms, operating additively or synergistically, are required for the robust resistance behavior as observed with higher plant survival rates among selected resistant genotypes. As *P. ultimum* can grow fast along infected root tissue, the rapidness of defense activation such as the quicker induction of *MdWRKY33* could make a huge variation at the downstream events of defense activation. The data observed in this study, accompanied by the overall transcriptome changes reported previously (Shin, Zheng, *et al.*, 2016; Zhu *et al.*, 2017; Zhu *et al.*, 2019), seem to suggest that the timely activation of *MdWRKY33* is an essential factor, but unlikely the only factor, contributing to the outcomes during interaction between apple root and *P. ultimum* among tested genotypes.

CONCLUSION

Due to the lack of mobility, plants must endure almost constant challenges from biotic and abiotic stresses over their lifetime. Plant roots live in a complex environment with many physical, chemical and biological variables including persistent soilborne pathogens. Proper and rapid transcriptional regulation of gene expression can be crucial for their survival. Transcription factors (TF), through sequence-specific DNA-binding to the promoter of target genes, control one of the primary regulation points on the level and/or duration of transcriptional expression of various functional categories of genes. Based on previous transcriptome data and the expression profiles in multiple apple rootstock genotypes, it appeared that the expression patterns of *MdWRKY33* plays an essential role in contributing to resistance traits during interaction between apple roots and *P. ultimum*. The sequence features of MdWRKY33 suggested that it is an orthologue of the well-characterized Arabidopsis counterpart. Its tissue-specific expression and responses to abiotic and pharmacological treatments added to the evidence for its functional role in defense response to stress conditions in apple vegetative tissues. The uniformity of its upregulation per timepoint and genotype indicated its roles in apple root defense responses to *P. ultimum* infection. Although not universal, the stronger induction at the earlier stage of infection in resistant genotypes suggests it could be a key element contributing to apple root resistance to *P. ultimum* infection. Given the fact of polygenic quantitative resistance to necrotrophic pathogens, it is not surprising that even though #132 showed the earlier and stronger expression of *MdWRKY33,* it still resulted in with susceptibility to *P. ultimum* infection. Therefore, it can be interpreted that the early and strong transcriptional activation of *MdWRKY33* is necessary, but not the single deciding factor, for conferring apple root resistance to this pathogen. Further experiments are needed to identify

other crucial elements contributing to a robust resistance to infection from this necrotrophic pathogen in apple root. In summary, the results from the current study and previous transcriptome data clearly demonstrated the identity and role of *MdWRKY33* as a key factor in apple root defense activation towards *P. ultimum infection*. Transgenic manipulation of *MdWRKY33* expression in apple roots is underway which should provide more definitive evidence for its roles of pathogen resistance.

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REFERENCES

Alves, M. S., S. P. Dadalto, A. B. Goncalves, G. B. de Souza, V. A. Barros and L. G. Fietto. 2014. Transcription Factor Functional Protein-Protein Interactions in Plant Defense Responses. Proteomes, 2: 85-106.

Andreasson, E., T. Jenkins, P. Brodersen, S. Thorgrimsen, N. H. Petersen, S. Zhu, J. L. Qiu, P. Micheelsen, A. Rocher and M. Petersen. 2005. The MAP kinase substrate MKS1 is a regulator of plant defense responses. The EMBO Journal, 24: 2579-89.

Baccelli, I. and B. Mauch-Mani. 2016. Beta-aminobutyric acid priming of plant defense: the role of ABA and other hormones. Plant Molecular Biology, 91: 703-11.

Bakshi, M. and R. Oelmüller. 2014. WRKY transcription factors: Jack of many trades in plants. Plant Signaling & Behavior, 9: e27700.

Benfey, P. N. 2012. Toward a systems analysis of the root Cold Spring Harbor Symposia on Quantitative Biology. pp. 91-96.

Birkenbihl, R. P., C. Diezel and I. E. Somssich. 2012. Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. Plant Physiol, 159: 266-85.

Birkenbihl, R. P., S. Liu and I. E. Somssich. 2017. Transcriptional events defining plant immune responses. Current Opinion in Plant Biology, 38: 1-9.

Buscaill, P. and S. Rivas. 2014. Transcriptional control of plant defence responses. Current opinion in plant Biology, 20: 35-46.

Chen, T., W. Li, X. Hu, J. Guo, A. Liu and B. Zhang. 2015. A Cotton MYB Transcription Factor, GbMYB5, is Positively Involved in Plant Adaptive Response to Drought Stress. Plant Cell Physiol, 56: 917-29.

Chezem, W. R. and N. K. Clay. 2016. Regulation of plant secondary metabolism and associated specialized cell development by MYBs and bHLHs. Phytochemistry, 131: 26-43.

Daccord, N., J.-M. Celton, G. Linsmith, C. Becker, N. Choisne, E. Schijlen, H. van de Geest, L. Bianco, D. Micheletti and R. Velasco. 2017. High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. Nature Genetics, 49: 1099.

Datta, R., D. Kumar, A. Sultana, S. Hazra, D. Bhattacharyya and S. Chattopadhyay. 2015. Glutathione regulates 1-aminocyclopropane-1-carboxylate synthase transcription via WRKY33 and 1-aminocyclopropane-1-carboxylate oxidase by modulating messenger RNA stability to induce ethylene synthesis during stress. Plant Physiology, 169: 2963-81.

De Coninck, B., P. Timmermans, C. Vos, B. P. A. Cammue and K. Kazan. 2015. What lies beneath: Belowground defense strategies in plants. Trends in Plant Science, 20: 91-101.

Eulgem, T., P. J. Rushton, S. Robatzek and I. E. Somssich. 2000. The WRKY superfamily of plant transcription factors. Trends in plant Science, 5: 199-206.

Ishiguro, S. and K. Nakamura. 1994. Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5′ upstream regions of genes coding for sporamin and β-amylase from sweet potato. Molecular and General Genetics MGG, 244: 563-71.

Ishihama, N. and H. Yoshioka. 2012. Post-translational regulation of WRKY transcription factors in plant immunity. Current Opinion in Plant Biology, 15: 431-37.

Jensen, M. K. and K. Skriver. 2014. NAC transcription factor gene regulatory and protein-protein interaction networks in plant stress responses and senescence. IUBMB Life.

Jin, J., F. Tian, D.-C. Yang, Y.-Q. Meng, L. Kong, J. Luo and G. Gao. 2016. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Research: gkw982.

Jin, J., H. Zhang, L. Kong, G. Gao and J. Luo. 2014.

planttfdb 3.0: a portal for the functional and evolutionary study of plant transcription factors. Nucleic Acids Research, 42: D1182-D87.

Koen, E., P. Trapet, D. Brulé, A. Kulik, A. Klinguer, L. Atauri-Miranda, R. Meunier-Prest, G. Boni, G. Glauser, B. Mauch-Mani, D. Wendehenne and A. Besson-Bard. 2014. β-Aminobutyric Acid (BABA)-Induced Resistance in *Arabidopsis thaliana*: Link with Iron Homeostasis. Molecular Plant-Microbe Interactions, 27: 1226-40.

Li, J., G. Brader, T. Kariola and E. Tapio Palva. 2006. WRKY70 modulates the selection of signaling pathways in plant defense. Plant Journal, 46: 477-91.

Liu, S., B. Kracher, J. Ziegler, R. P. Birkenbihl and I. E. Somssich. 2015. Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards *Botrytis cinerea* 2100. elife, 4: e07295.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402-08.

Lorang, J. 2018. Necrotrophic Exploitation and Subversion of Plant Defense: A Lifestyle or Just a Phase, and Implications in Breeding Resistance. Phytopathology, 109: 332-46.

Mangelsen, E., J. Kilian, K. W. Berendzen, Ü. H. Kolukisaoglu, K. Harter, C. Jansson and D. Wanke. 2008. Phylogenetic and comparative gene expression analysis of barley (*Hordeum vulgare*) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. BMC Genomics, 9: 194.

Mao, G., X. Meng, Y. Liu, Z. Zheng, Z. Chen and S. Zhang. 2011. Phosphorylation of a WRKY Transcription Factor by Two Pathogen-Responsive MAPKs Drives Phytoalexin Biosynthesis in Arabidopsis. The Plant Cell, 23: 1639-53.

Martinez-Medina, A., V. Flors, M. Heil, B. Mauch-Mani, C. M. J. Pieterse, M. J. Pozo, J. Ton, N. M. van Dam and U. Conrath. 2016. Recognizing Plant Defense Priming. Trends in Plant Science, 21: 818-22.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in washington. Phytopathology, 88: 930-8.

Meng, D., Y. Li, Y. Bai, M. Li and L. Cheng. 2016. Genome-wide identification and characterization of WRKY transcriptional factor family in apple and analysis of their responses to waterlogging and drought stress. Plant Physiology and Biochemistry, 103: 71-83.

Menke, F. L., H.-G. Kang, Z. Chen, J. M. Park, D. Kumar and D. F. Klessig. 2005. Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Molecular Plant-Microbe Interactions, 18: 1027-34.

Patra, B., C. Schluttenhofer, Y. Wu, S. Pattanaik and L. Yuan. 2013. Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1829: 1236-47.

Ren, X., Z. Chen, Y. Liu, H. Zhang, M. Zhang, Q. Liu, X. Hong, J. K. Zhu and Z. Gong. 2010. ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. Plant J, 63: 417-29.

Riechmann, J. L., J. Heard, G. Martin, L. Reuber, C.-Z. Jiang , J. Keddie, L. Adam, O. Pineda, O. J. Ratcliffe, R. R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J. Z. Zhang, D. Ghandehari, B. K. Sherman and G. -L. Yu. 2000. Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes. Science, 290: 2105-10.

Rushton, P. J., M. T. Bokowiec, S. Han, H. Zhang, J. F. Brannock, X. Chen, T. W. Laudeman and M. P. Timko. 2008. Tobacco Transcription Factors: Novel Insights into Transcriptional Regulation in the Solanaceae. Plant Physiology, 147: 280-95.

Samad, A. F. A., M. Sajad, N. Nazaruddin, I. A. Fauzi, A. M. A. Murad, Z. Zainal and I. Ismail. 2017. MicroRNA and Transcription Factor: Key Players in Plant Regulatory Network. Front Plant Sci, 8: 565.

Shin, S., J. Lee, D. Rudell, K. Evans and Y. Zhu. 2016. Transcriptional regulation of auxin metabolism and ethylene biosynthesis activation during apple (*Malus domestica*) fruit maturation. Journal of Plant Growth Regulation, 35: 655-66.

Shin, S., P. Zheng, G. Fazio, M. Mazzola, D. Main and Y. Zhu. 2016. Transcriptome changes specifically associated with apple (*Malus domestica*) root defense response during Pythium ultimum infection. Physiological and Molecular Plant Pathology, 94: 16-26.

Singh, K. B., R. C. Foley and L. Oñate-Sánchez. 2002. Transcription factors in plant defense and stress responses. Current Opinion in Plant Biology, 5: 430-36.

Song, Y., C. Ai, S. Jing and D. Yu. 2010. Research progress on functional analysis of rice WRKY genes. Rice science, 17: 60-72.

Van Verk, M. C., C. Gatz and H. J. Linthorst. 2009. Transcriptional regulation of plant defense responses. Advances in Botanical Research, 51: 397-438.

Velasco, R., A. Zharkikh, J. Affourtit, A. Dhingra, A. Cestaro, A. Kalyanaraman, P. Fontana, S. K. Bhatnagar, M. Troggio and D. Pruss. 2010. The genome of the domesticated apple (*Malus domestica* Borkh.). Nature Genetics, 42: 833.

Wang, X., N. Jiang, J. Liu, W. Liu and G. L. Wang. 2014. The role of effectors and host immunity in plant-necrotrophic fungal interactions. Virulence, 5: 722-32.

Wasternack, C. and M. Strnad. 2019. Jasmonates are signals in the biosynthesis of secondary metabolites — Pathways, transcription factors and applied aspects — A brief review. New Biotechnology, 48: 1-11.

Wu, K.-L., Z.-J. Guo, H.-H. Wang and J. Li. 2005. The WRKY family of transcription factors in rice and Arabidopsis and their origins. DNA Research, 12: 9-26.

Zhang, H., J. Jin, L. Tang, Y. Zhao, X. Gu, G. Gao and J. Luo. 2011. PlantTFDB 2.0: update and improvement of the comprehensive plant transcription factor database. Nucleic Acids Res, 39: D1114-7.

Zhang, Y., H. Yu, X. Yang, Q. Li, J. Ling, H. Wang, X. Gu, S. Huang and W. Jiang. 2016. CsWRKY46, a WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by regulating a set of cold-stress responsive genes in an ABA-dependent manner. Plant Physiol Biochem, 108: 478-87.

Zhou, M. and J. Memelink. 2016. Jasmonate-responsive transcription factors regulating plant secondary metabolism. Biotechnology Advances, 34: 441-49.

Zhu, Y., M. Saltzgiver and J. Zhao. 2018. A Phenotyping Protocol for Detailed Evaluation of Apple Root Resistance Responses Utilizing Tissue Culture Micropropagated Apple Plants. American Journal of Plant Sciences, 9: 2183.

Zhu, Y., J. Shao, Z. Zhou and R. E. Davis. 2017. Comparative Transcriptome Analysis Reveals a Preformed Defense System in Apple Root of a Resistant Genotype of G.935 in the Absence of Pathogen. International Journal of Plant Genomics, 2017: 8950746.

Zhu, Y., J. Shao, Z. Zhou and R. E. Davis. 2019. Genotype-specific suppression of multiple defense pathways in apple root during infection by *Pythium ultimum*. Horticulture Research, 6: 10-16.

Zhu, Y., S. Shin and M. Mazzola. 2016. Genotype responses of two apple rootstocks to infection by *Pythium ultimum* causing apple replant disease. Canadian Journal of Plant Pathology, 38: 483-91.

Zhu, Y., J. Zhao and Z. Zhou. 2018. Identifying an Elite Panel of Apple Rootstock Germplasm with Contrasting Root Resistance to *Pythium ultimum*. Journal of Plant Pathology & Microbiology, 9:34-39.