


MdWRKY20-MdPR1 module mediates resistance of apple to *Fusarium solani*

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Abstract

Apple replant disease (ARD) caused by the pathogen *Fusarium solani* is a destructive disease in apple planting areas worldwide, which leads to the decline of apple quality and yield. WRKY transcription factors are involved in the process of plants responding to various environmental stresses, but the function of WRKY TFs in ARD is unclear. In this study, the expression of *MdWRKY20* was significantly increased after infection of apple rootstock 'M9T337' with *F. solani*. Transgenic analysis showed that the resistance of apple callus and *Arabidopsis* to *F. solani* increased after overexpression of *MdWRKY20*. Ectopic expression of *MdWRKY20* also significantly enhanced antioxidant capacity in *Arabidopsis* under treatment with *F. solani*. Then, *MdWRKY20* was found to bind directly to the W-box II of the *MdPR1* promoter and significantly promoted its expression. In summary, *MdWRKY20* plays a positive role in regulating the resistance of apples to *F. solani*.

Citation: Zhao L, Liu Y, Wang M, Xiang L, Wang H, et al. 2025. *MdWRKY20-MdPR1* module mediates resistance of apple to *Fusarium solani*. *Fruit Research* 5: e001 <https://doi.org/10.48130/frures-0024-0033>

Introduction

There is a widespread agricultural problem worldwide, after replanting apples in the same land, abnormal tree growth and development, as well as decreased yield and fruit quality are observed, which is called apple replant disease (ARD)^[1,2]. The most universal symptoms of ARD are stunted growth of sapling, damaged root systems, reduced both in yield and fruit quality^[3,4], which has greatly affected the development of the apple industry. Therefore, it is particularly important to find an effective method to alleviate ARD.

ARD can be caused by many factors, among which biological factors dominated by harmful fungi in soil hold a major position^[5–8]. *Fusarium solani* has been proven to be one of the main pathogens causing ARD in the main production areas of China. Scholars at home and abroad have put forward a variety of preventions and control measures to deal with ARD. Intercropping and crop rotation could alleviate ARD to varying degrees^[9–11], crop rotation has been proven to improve the soil environment and effectively reduce the incidence of plants, but it is difficult to implement in production as it is time consuming. Chemical fumigants such as methyl bromide have been shown to be highly effective against preventing and alleviating ARD^[12,13], but they have been banned due to the serious effects of environmental pollution and ozone destruction^[14–16]. Biological control has become an essential means of agricultural sustainable development because it can regulate soil microecology by competing with pathogenic bacteria for ecological niche^[17–19]. However, biological control by antagonistic microorganisms needs continuous application because of the variety and long-term existence of pathogenic fungi in soil. Therefore, it is difficult to completely eliminate ARD through biological control^[20]. Consequently, it is a new breakthrough point to explore the molecular mechanism of apple defense against *F. solani*.

A variety of abiotic and biological challenges occur at the same time, which puzzles the prevention and control of ARD. External unfavorable factors will trigger the inherent defense mechanism of plants^[21,22]. In this process, transcription factor (TF) plays an

important role. WRKY transcription factors regulated the expression of defense-related genes by combining 'W-box' sites in the promoter region of the target gene, and has been widely studied as a key regulator in the immune response from plants to various biological stresses^[23–25]. *AtWRKY33* can act as a positive regulator of JA- and ET-mediated defense response signals to mediate plant defense against necrotic pathogens *Botrytis cinerea* and *Alternaria brassicicola*^[26]. Overexpression of *GmWRKY136*, 53, and 86 in soybean respectively showed increased resistance to soybean cyst nematode^[27]. In tobacco, *CaWRKY40* homologous gene was regulated by SA, JA, and ET signaling pathways, and coordinated the response to pepper and tobacco to *R. solanacearum* infection and heat stress^[28]. By regulating the signal transduction mediated by SA, JA, ET, and ROS, it was found that *TaWRKY49* negatively regulated the high-temperature seedling-plant resistance to Pst (HTSP) of wheat^[29].

Pathogenesis-Related proteins (PRs) have been proven to participate in plant defense responses^[30–32]. Studies showed that Pathogenesis-Related (PR) proteins can be divided into 17 families^[33]. PRs have been identified to play a critical role in many plant species. In addition, *StPR-1* has been shown to play a positive role in the infection of potato *Phytophthora infestans*^[34]. *PR2* in *Arabidopsis* may act as a regulator of callose and SA-dependent defense responses^[35]. In apple, *MdPR10-1* and *MdPR10-2* were found to be related to resistance to *Alternaria* leaf spot^[36]. Apple Pathogenesis-Related protein *MdPR4* has been shown to be involved in the recognition of chitin and resistance to ARD pathogens^[37]. Overexpression of *MdPR10* significantly reduced the infection of *Valsa mali*, which is the main pathogen causing apple rot^[38].

In apple, it is unknown whether the WRKY transcription factor mediates plant defense mechanisms against *F. solani*. In this study, the infection of *F. solani* increased the expression of *MdWRKY20* in the root of 'M9T337', so *MdWRKY20* was chosen as the research target. The purpose is to analyze the function of *MdWRKY20* and its regulation mode under the infection of *F. solani*. Therefore, we have carried out genetic, biochemical, and physiological analysis to

provide new ideas for apple to defend against *F. solani* and cultivate resistant rootstocks.

Materials and methods

Plant materials and growth conditions

The experimental materials included tissue culture seedlings of apple (*Malus domestica* Borkh rootstock M9T337 with consistent growth state), and the apple callus of 'Orin' was used for pathogen infection test, cultured in subculture medium. Subculture medium: MS, 30 g/L sucrose, 1.5 mg/L 6-benzyladenine (6-BA), 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 7 g/L agar, pH 5.8. Selection medium: MS, 30 g/L sucrose, 1.5 mg/L 6-BA, 0.5 mg/L 2,4-D, 50 µg/mL kanamycin, and 7 g/L agar, pH 5.8, and the medium was placed at 24 °C in darkness.

Seedlings of *Arabidopsis* Columbia (Col-0) were used for genetic transformation tests and were cultured on MS medium: MS, 30 g/L sucrose pH 5.8. Screening medium: MS, 30 g/L sucrose, 50 µg/mL kanamycin pH = 5.8. Seeds were vernalized at 4 °C for 2 d before growth at 23 °C under 16 h light/8 h dark conditions. After taking root in the culture medium, *Arabidopsis* was transplanted into nutrient medium (the volume ratio of vermiculite to nutrient soil was 1:1), and grew under the condition of 14 h light/10 h dark cycle, the temperature was 22 °C and the relative humidity was 60%. *Nicotiana benthamiana* was used in subcellular localization, and it was cultivated and grown under the conditions of 14 h light/10 h dark cycle, with a temperature of 24 °C and relative humidity of 60%.

F. solani was activated in potato dextrose agar (PDA) for 5 d at 28 °C, then the callus were infected with 0.5 cm diameter agar on which hyphae grew evenly. PDA medium without *F. solani* inoculation was treated as a control.

The activated *F. solani* was washed with sterile water to prepare spore suspension with a concentration of 10⁵ CFU/mL, then added to PDB culture medium at a ratio of 1%, then cultured at 28 °C and 160 r/min for about 7 d. After filtering through 8-layer gauze, the spore suspension concentration was calculated using a blood cell counting plate, and the final concentration was adjusted to 10⁵ cells/mL by adding sterile water. Fifty mL spore suspension was added to the nutrient solution, then it was poured into the 'M9T337' seedlings, and the same amount of sterile water was added to the control. The seedlings were cultured in the greenhouse, and the root samples were taken on the 0, 1, 3, 6, 9, and 12 d after inoculation, and then frozen in liquid nitrogen and stored at -80 °C.

The flower, fruit, stem, leaf, and root tissues of apple were collected under natural planting conditions and stored at -80 °C for the expression analysis of *MdWRKY20*.

Extraction of fermentation broth extract of *F. solani* and infection test of *Arabidopsis*

1% (v/v) of the target concentration of *F. solani* spore suspension was added to PDB medium, at 28 °C and 160 r/min for about 7 d. Spore suspension filtered with 8-layer gauze, then centrifuged at 12,000 r/min for 10 min, and the collected supernatant was mixed with ethyl acetate at 1:1 for extraction. Shaking it violently three times during this process, and then allowing it to stand until layered. The bottom liquid was collected after layering and excess water absorbed with anhydrous sodium sulfate, then concentrated in a rotary evaporator (36 °C, 80 hPa) to a powder state, and the collected powder was dissolved with methanol, and finally the concentration of fermentation broth extract was 5 mg/mL. Centrifuged at 10,000 r/min and filtered with 0.22 µm filter membrane to remove impurities, and then stored at -80 °C.

Zero, six and 12 mL of fermentation broth extract was added into 300 mL of MS medium and diluted to 0, 100, and 200 mg/L

respectively. After disinfection of *Arabidopsis*, the seeds were planted in solidified MS solid culture medium, then sealed with sealing film, *Arabidopsis* was cultured according to the method mentioned above.

RNA extraction and quantitative reverse transcription PCR

FastPure Plant Total RNA Isolation Kit (Vazyme, Nanjing, China) was employed to extract total RNA from the tissues of 'M9T337' and the callus of the apple. Then, the RNA was reverse transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China), and according to the concentration, 1 pg–1 µg total RNA was extracted for reverse transcription. The qRT-qPCR amplification reactions were performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), and qRT-PCR was then performed on a Real-Time system (Bio-Rad, Hercules, CA, USA). Each sample was repeated three times, normalized with MdActin (CN938023) as an internal control, and the relative quantification of genes were calculated by the cycle threshold (Ct) 2^{-ΔΔCt} method^[39]. Primers used for qRT-PCR analysis are listed in [Supplementary Table S1](#).

Gene cloning and phylogenetic analysis

PCR-amplification (P515, Vazyme, Jiangsu, China) was used to amplify the coding sequence (CDs) of *MdWRKY20* from apple leaves. Primers used for cloning CDs are listed in [Supplementary Table S1](#). Using MEGA version 5.1 software to plot the phylogenetic tree, the *WRKY20* sequence of apple and other species were derived from the NCBI database (www.ncbi.nlm.nih.gov). The phylogenetic tree was created by the adjacency method, and the phylogenetic tree was beautified using the online software ITOL (<https://itol.embl.de/>). DNAMAN (Lynnon Biosoft, San Ramon, CA, USA) software was used to compare protein sequences.

Subcellular localization

The full-length cDNAs without the stop codon of *MdWRKY20* was introduced into the pRI101-GFP vector, and a 35S::*MdWRKY20*-GFP fusion vector was generated. The primer sequence is listed in [Supplementary Table S1](#). The fusion vectors and empty plasmid were then introduced into *Agrobacterium tumefaciens* strain GV3101 and then infiltrated into tobacco leaves. After 2–3 d of infiltration, the GFP signal was observed using confocal microscopy.

Reactive oxygen species measurement diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining of *Arabidopsis* leaves

One mg/L DAB solution and 0.5 mg/mL NBT solution were prepared with 0.01 mmol/L phosphate buffer solution (pH 7.0). After the growth of *Arabidopsis* for about 28 d, leaves with the same growth status were selected and placed in a centrifuge tube, which were dyed with DAB solution for 6 h and NBT solution for 2 h. Incubated at a constant temperature of 28 °C. Then, the leaves were decolorized in 95% alcohol, boiled until chlorophyll was completely degraded, observed and photos taken.

Yeast one-hybrid test (Y1H) assay

Two vectors were used for Y1H assay, the *MdWRKY20* CDs was inserted into pGADT7 vector (Clontech, TaKaRa, Japan), while the promoter fragments of *MdPR1* and *MdPR3* were inserted into the pHis2 vector (BD Biosciences, NJ, China). Then the recombinant plasmids and pGADT7 empty vector were transferred into Y187 (Clontech) yeast cells. The transformed cells were cultured on screening medium (-Trp/-His) with different concentrations of 3-amino-1,2,4-triazole (3-AT) and cultured at 28 °C for about 2–3 d to select the optimal concentration. Compared with the negative control, the binding of *MdWRKY20* protein to the *MdPR1* promoter

enables yeast strains to grow normally under conditions containing appropriate concentrations of 3-AT, while yeast containing PGADT7 empty vector cannot grow normally. Then co-transfect the recombinant plasmid containing pHis2 and pGADT7 of the target gene. The transformed cells were cultured on deficient medium (-Trp/-His/-Leu) containing optimal 3-AT concentration, and cultured at 28 °C for about 2–3 d. Determining the binding ability of transcription factors to promoters based on yeast growth.

Electrophoretic mobility shift assays (EMSA)

The LightShift Chemiluminescent EMSA Kit (Thermo Scientific) was used for EMSA assay. Biotin-labeled probe primers and non-biotin labeled competitive probes were synthesized by Sangon Biotechnology (Shanghai, China) (Supplementary Table S1). Insert the *MdWRKY20* CDs sequence into the pET-32a(+) (His-Tag) expression vector (Novagen, NJ, USA). Express the *MdWRKY20* recombinant protein in *E. coli* DE3 and purify the *MdWRKY20*-His fusion protein using the His tagged protein purification kit (Kangwei).

The specific steps are as follows: the reaction mixture contains probe 1 μ L, H₂O 1 μ L, LightShift 10 \times binding buffer 2 μ L and purified protein 16 μ L. The mixture was kept in the dark at 24 °C for 25 min, after adding loading buffer and mixing, nondenaturing polyacrylamide gel electrophoresis was carried out, and then the DNA-protein complex was transferred to nylon membrane. Then perform UV crosslinking, with one minute on each side. The UV Crosslinker was used for UV crosslinking, with one minute on each side. Chemiluminescence signal detection was performed using the reagents provided in the kit.

Luciferase reporter assay

The CDs of *MdWRKY20* was inserted into pHBT AvrRpm1 vector, and the the promoter of *MdPR1* and *MdPR3* were inserted into pFRK1-LUC-nos vector, respectively. After the protoplasts of 'Orin' callus tissue were extracted, co-transform two plasmids into apple callus protoplasts. The transiently transfected protoplasts were incubated at 24 °C for 6 h and suspended in 100 μ L cell lysis buffer. Added 5 μ L cell extract and 20 μ L 1 mM 4-MUG and cultured at 37 °C for 1 h. Then added 100 μ L 0.2 mol/L sodium carbonate to terminate the reaction. Luciferase reporter analysis system (Promega) was used to determine LUC activity.

Vector construction and genetic transformation

Insert the full-length *MdWRKY20* CDs into pRI101-AN vector containing a GFP tag. Primers are listed in Supplementary Table S1. Then transfer the recombinant plasmid into *Agrobacterium* LBA4404 (AngYuBio, Shanghai, China) cells, and two-week-old 'Orin' callus were infected in the infection solution for about 30 min. Dry the callus with filter paper then cultured them at 24 °C for 2 d in darkness on agar-solidified MS medium without antibiotics, then transferred to selective medium containing 250 mg/L carbenicillin and 50 mg/L kanamycin. PCR amplification was used to verify overexpression of *MdWRKY20*.

In the transformation of *Arabidopsis*, the recombinant plasmid was introduced into the *Arabidopsis* using floral dip method^[40] mediated by *Agrobacterium* LBA3101 (AngYuBio, Shanghai, China). After harvesting *Arabidopsis* seeds, the positive overexpression lines were screened with agar-solidified MS medium containing 50 mg/L kanamycin, the overexpression of *MdWRKY20* was verified by PCR amplification, the T3 homozygous transgenic lines were used for subsequent phenotypic analysis.

Statistical analysis

At least three replicates were set for each experiment to ensure accuracy, and SPSS version 20.0 (IBM, Inc, Armonk, NY, USA) was used for statistical analysis. The results were compared by one-way ANOVA and Duncan test. Using GraphPad Prism version 9 (San

Diego, CA, USA) for graph analysis, and $p < 0.05$ was statistically significant.

Results

Transcription of *MdWRKY20* was affected by *F. solani* infection

Apple rootstock 'M9T337' was infected by *F. solani*. Visible symptoms can be seen that the slow growth and partial browning of leaves appeared after 9 d post-infection (dpi) (Fig. 1a). On 12 dpi, the infection rate was as high as 92.5% and the mortality rate reached 37.5% (Supplementary Fig. S1). Eight WRKY family target genes were selected for transcriptional analysis according to previous research results^[41]. The transcription of WRKY-TFs was induced by *F. solani* to different degrees (Fig. 2b–i). The infection of *F. solani* induced the expression of *MdWRKY2/4/20/25* and inhibited the expression of *MdWRKY10/33/58*, but had no obvious relationship with the transcription of *MdWRKY44*. Among them, it was found that the transcription level of *MdWRKY20* changed most significantly. Therefore, it was speculated that the transcription of *MdWRKY20* may correspond to the infection of *F. solani*.

Phylogenetic analysis and subcellular localization analysis of *MdWRKY20*

MdWRKY20 was expressed in every tissue of apple, with the highest expression in root and fruit (Supplementary Fig. S2). To examine the evolutionary relationship of *MdWRKY20*, the phylogenetic tree was generated by using the full-length amino acid sequences of *Arabidopsis* WRKYs and various species of Group I WRKYs (Fig. 2a). *MdWRKY20* belongs to group 1, along with several other Group I WRKY20 proteins. The subcellular localization results showed that green fluorescence could be observed throughout the whole cell in the empty GFP, whereas the green fluorescence of *MdWRKY20*-GFP was confined to the nucleus (Fig. 2b).

Overexpression of *MdWRKY20* improves the resistance of 'Orin' callus to *F. solani*

Recombinant pRI101-AN vector carrying *MdWRKY20* was transformed into 'Orin' apple callus, and the callus tissue overexpressing *MdWRKY20* obtained (Fig. 3a). Compared with the wild type (WT), it was observed that fungal elongation of the strain in *MdWRKY20*-OE callus were significantly reduced four days after inoculation with *F. solani* (Fig. 3b), and the diameter of the plaque extension decreased by 56.32%. The expression of *MdWRKY20* in transgenic callus were significantly higher than WT (Fig. 3c). This result indicates that *MdWRKY20* has a positive effect in the response of apple callus to *F. solani* infection.

Overexpression of apple *MdWRKY20* promotes the expression of PR protein gene

To further investigate the mechanism by which *MdWRKY20* and PRs enhance the tolerance of apple callus to *F. solani*, the expression levels of PR genes in apple callus overexpressing *MdWRKY20* and WT were measured after 7 d of inoculation with *F. solani*. It was found that there was no significant change in the expression of *MdPR2*, *MdPR3*, and *MdPR4* in the *MdWRKY20*-OE lines, but the expression of *MdPR1* was significantly higher than that in WT under stress conditions (Fig. 4a–d). Therefore, the results indicated that *MdWRKY20* may play a role by positively activating the expression level of *MdPR1*.

MdWRKY20 binds to the promoters of *MdPR1*

To verify the specificity of *MdWRKY20* binding to the promoters of *MdPR1* after *F. solani* infection, Y1H assay was carried out. It was

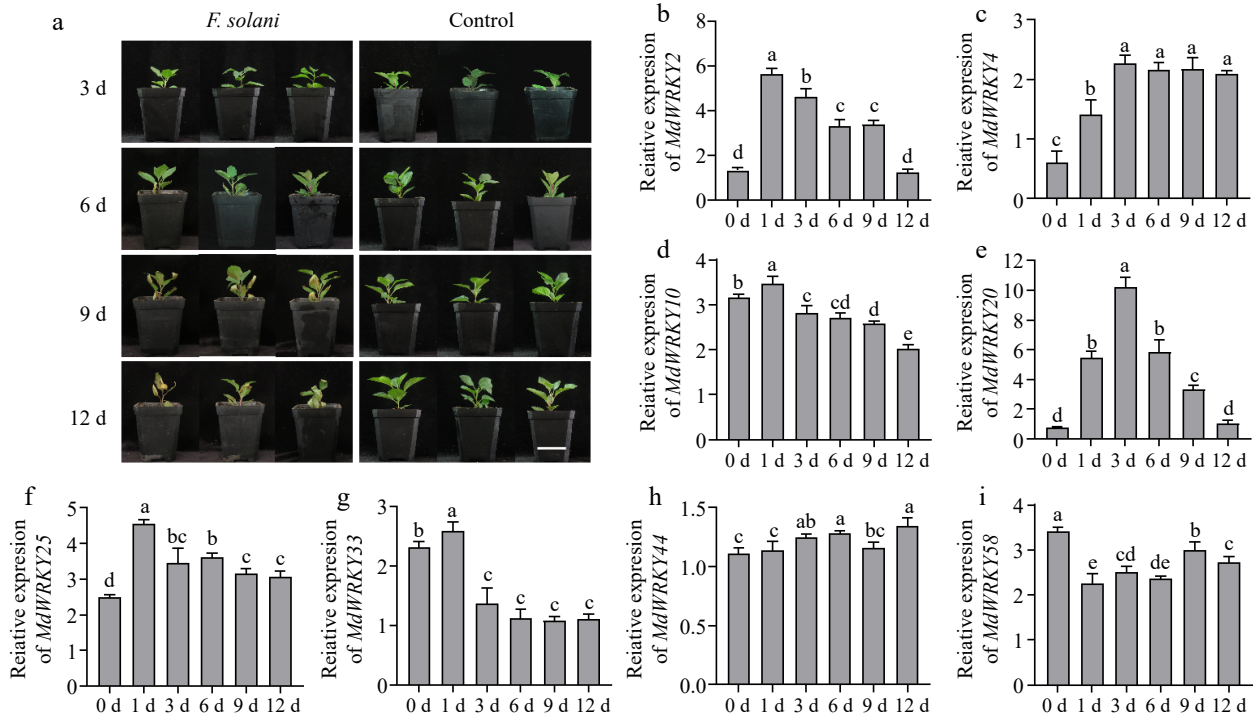


Fig. 1 Response of apple rootstock 'M9T337' to *F. solani* infection and expression levels of MdWRKY-TFs after *F. solani* infection detected by qRT-PCR. (a) Images of 'M9T337' taken on the 3, 6, 9 and 12 dpi after infection by *F. solani*. Scale bar = 5 cm. (b)–(i) qRT-PCR was used to detect the expression level of *MdWRKY2*, *MdWRKY4*, *MdWRKY10*, *MdWRKY20*, *MdWRKY25*, *MdWRKY33*, *MdWRKY44*, *MdWRKY58*. For (b)–(i), values were means \pm s.d. of three independent biological replicates. Bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

found that *MdWRKY20* interacted with the *MdPR1* promoter, which was screened at a suitable 3-AT concentration of 100 mM (Fig. 5a). By analyzing the *cis*-acting elements in the promoter sequences, it was found that there are two speculated W-box motifs in the *MdPR1* promoter (Fig. 5c). Therefore, EMSA demonstrated that *MdWRKY20* could bind to the W-box II motif in the promoter of *MdPR1* (Fig. 5d). With the increase of cold probe concentration, the binding between *MdWRKY20* to the *MdPR1* promoter was weakened (Fig. 5d). To further explore the effect of *MdWRKY20* on the activity of the *MdPR1* promoter, LUC activity assay was performed, and the results showed that *MdWRKY20* has transactivation activity towards the *MdPR1* promoter (Fig. 5e).

Overexpression of *MdWRKY20* improves the germination rate and *F. solani* resistance of *Arabidopsis*

To further explore the function of *MdWRKY20*, the expression vector was constructed to transform *Arabidopsis* and obtained *MdWRKY20*-OE *Arabidopsis* (Supplementary Fig. S3). Inoculate *Arabidopsis* on MS medium, which contains extracts of fermentation broth extract of *F. solani* with concentrations of 0, 100, and 200 mg/L, and observe the phenotype of *Arabidopsis* after 7 d. It could be seen that the growth of *Arabidopsis* were uniform and flourishing under control conditions. Under the treatment of *F. solani*, the growth of *Arabidopsis* were inhibited to varying degrees. When the concentration of extracts of fermentation broth extract of *F. solani* is 100 mg/L, the growth of Col were significantly inhibited, while plants overexpressing *MdWRKY20* were less inhibited (Fig. 6d). When the concentration of extracts of fermentation broth extract of *F. solani* reached 200 mg/L, the germination rate of Col seed decreased and the growth rate slowed down and the leaves turned purple (Supplementary Fig. S4), while the transgenic lines showed

better tolerance, mainly reflected in more lush leaves and healthier growth state (Fig. 6f). By contrast, the *MdWRKY20*-OE lines showed better resistance to *F. solani* infection.

ROS level, antioxidant enzyme activity, and MDA content of *Arabidopsis*

Further, the ROS level, antioxidant enzyme activity and MDA content of *Arabidopsis* leaves under control and *F. solani* treatment were detected (Fig. 7). The results showed that under control conditions, there were no significant differences in MDA content, ROS levels, and antioxidant enzyme activity among transgenic *Arabidopsis* and Col. However, after treatment with *F. solani*, the ROS level and MDA content of *MdWRKY20*-OE *Arabidopsis* were significantly lower than Col, and the antioxidant enzyme activities were significantly higher than that of Col (Fig. 7a–f). The results of histochemical staining showed that, unlike Col, the accumulation of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) in *MdWRKY20*-OE lines were lower, which showed that transgenic plants had better antioxidant capacity (Fig. 7g, h).

Discussion

F. solani is a common plant pathogenic fungi, which has been proved to be one of the main pathogens causing ARD^[42]. Replant disease is widespread in orchards worldwide. It is a disease based on the soil microbial community and harmful to plant physiology and morphological response, especially in Rosaceae plants. This disease frequently occurs in warm and humid environments, which causes great losses. Therefore, we are committed to finding a new method to effectively alleviate ARD.

Various fluctuating abiotic environmental factors accompany the growth process of plants, such as drought, salinity, threshold temperatures, nutrient starvation, and so on^[43–46]. These factors

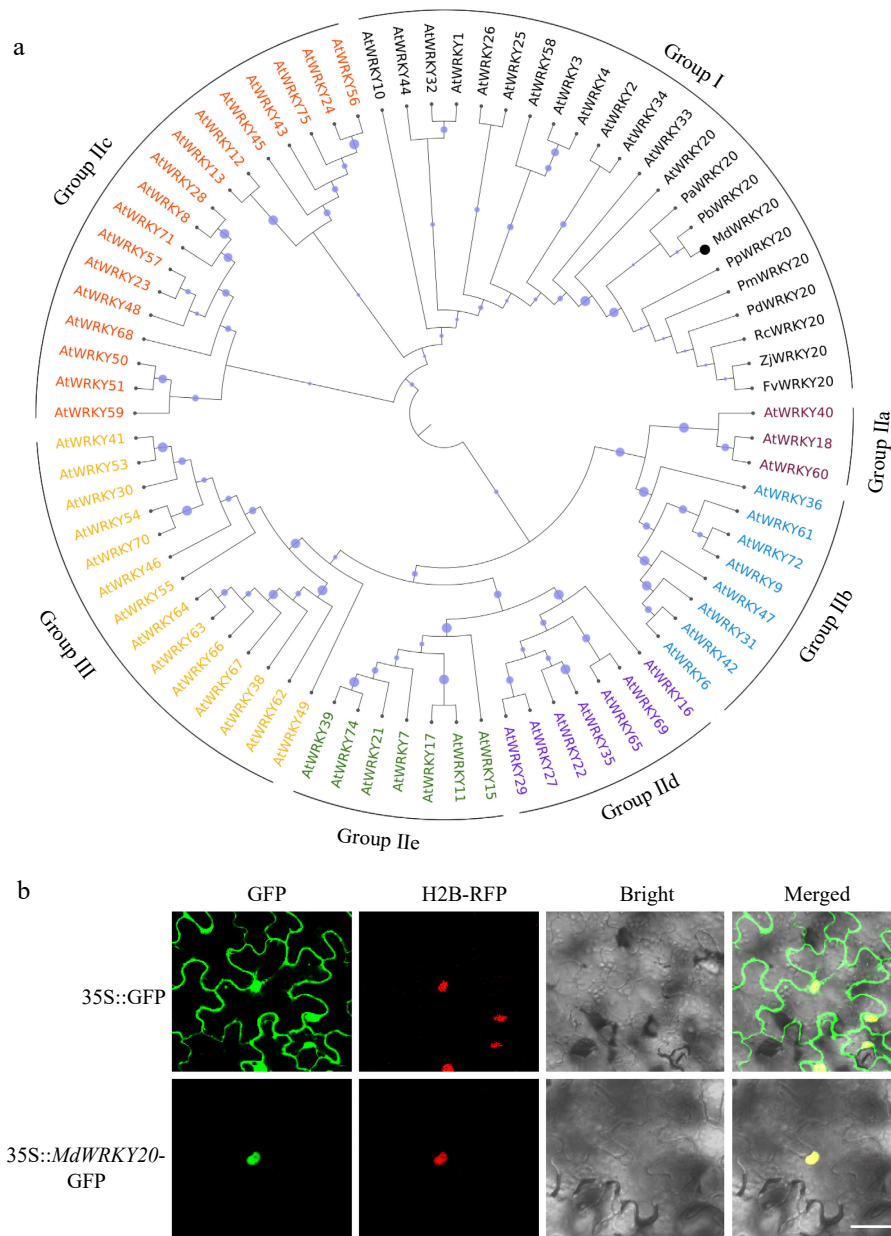


Fig. 2 Phylogenetic analysis and subcellular localization analysis of *MdWRKY20*. (a) Phylogenetic relationship and subgroup classification of *MdWRKY20*, *AtWRKY*, and other Group I WRKYs proteins. The WRKY20 sequence of apple and other species was derived from NCBI database (www.ncbi.nlm.nih.gov), and the WRKY protein sequence of *Arabidopsis* was derived from TAIR database (www.arabidopsis.org). The phylogenetic tree was created using MEGA software (version 5.1) by the adjacency method. *MdWRKY20* is highlighted with black circles in the figure. (b) 35S::*MdWRKY20*-GFP vectors were transformed into the leaves of *Nicotiana benthamiana*. The nucleus were labeled with red fluorescent protein H2B. Three biological repeats were performed. Scale bar = 50 μ m.

may occur in many stages of plant growth and may limit the growth and development of plants, which has attracted wide attention because of the negative impact on agricultural production. Plants have their own strategies to deal with various stresses. They have evolved complex mechanisms at different levels to cope with the changing external environment.

At the molecular level, transcription factors play a vital role in this process. WRKY transcription factors have been widely reported in immunity triggered by various microbial-related molecular patterns. For example, in *Brassica napus*, *BnWRKY33* upregulates the expression of genes regulated by salicylic acid (SA) and jasmonic acid (JA), positively regulating resistance to *Sclerotinia sclerotiorum* pathogens^[47]. In addition, *SpWRKY6* reduced cell membrane

damage by regulating ROS level and expression level of PR genes^[48]. In this study, it was found that the expression of *MdWRKY20* was upregulated after infecting apple rootstocks with *F. solani*, indicating that *MdWRKY20* may be related to apple rootstock's defense against *F. solani* infection, but the regulation mechanism of apple under *F. solani* infection needs to be further explored and supplemented.

The DNA binding domain of WRKY protein can activate or inhibit its expression by binding to the W-box element of the target gene promoter^[49]. In *Arabidopsis*, *AtWRKY33* mediated resistance targets *NCED3* and *NCED5* directly^[40]. In addition, the W-box element in the promoter of WRKY protein can also be targeted by other WRKY proteins. For example, the synergistic effect of *WRKY70* and *WRKY54*

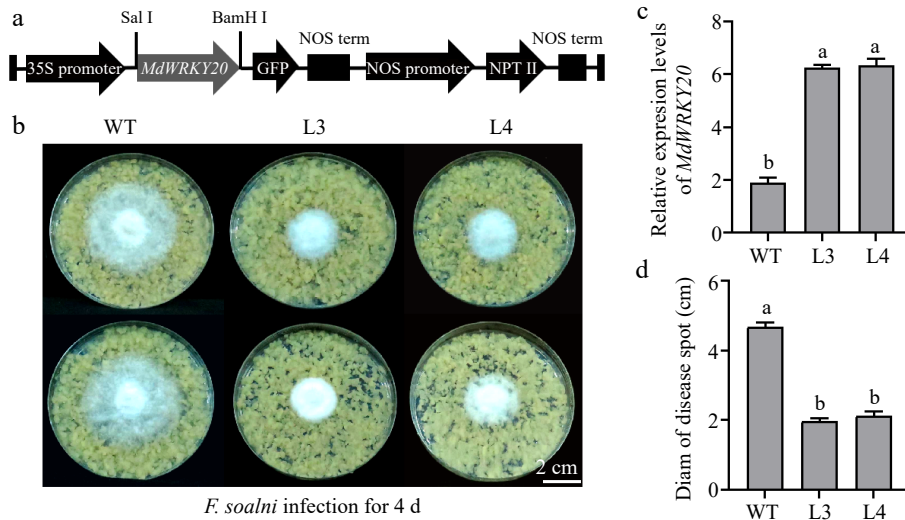


Fig. 3 Functional characteristics of 'Orin' callus overexpressing *MdWRKY20*. (a) *MdWRKY20* CDs was inserted into PRI101-AN vector with CaMV 35S promoter and green fluorescent protein (GFP) sequence. (b) Phenotype of wild type (WT) and *MdWRKY20*-OE callus 4 d after the infection of *F. solani*. (c) RT-PCR based validation of *MdWRKY20*-OE in 'Orin' callus. (d) The diameter of the plaque extension of different callus after infection with *F. solani*. (c), (d), Values are means \pm s.d. of three independent biological replicates. Bars not labeled with the same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

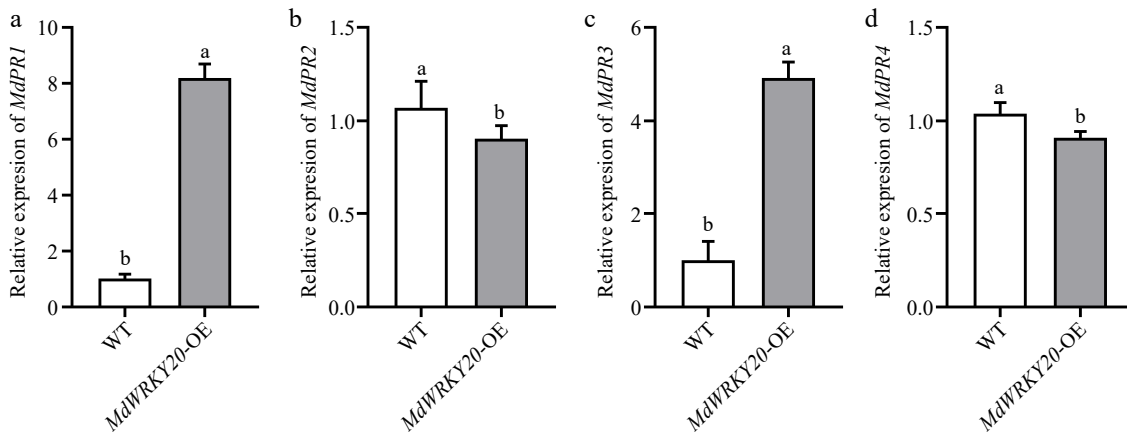


Fig. 4 qRT-PCR analysis of *MdPR1*, *MdPR2*, *MdPR3*, and *MdPR4* expressions in *MdWRKY20*-OE and wild type apple callus with *F. solani* infection. Values are means \pm s.d. of three independent biological replicates. Bars not labeled with the same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

has a negative impact on the response of *Arabidopsis* to *Pectobacterium carotovorum* and *Botrytis cinerea*^[50]. After the callus were inoculated with *F. solani*, it was observed that the diameter of plaque extension of the callus with overexpression of *MdWRKY20* was obviously smaller than that of the wild type. Transgenic *Arabidopsis* also showed increased resistance. *MdWRKY20* may exert its function by binding to the W-box of downstream target genes and activating its expression.

Studies have shown that the PR gene was one of the most promising candidate genes for the cultivation of crop varieties resistant to multiple stresses^[51–53]. The overexpression of PR genes in plants alone or in combination greatly improved the level of plant defense response to various pathogens^[14,54,55]. Under stress conditions, the expression of *MdPR1* was significantly upregulated after overexpression of *MdWRKY20* in apple callus compared with wild type. Based on the analysis of Y1H, EMSA, and LUC, we have confirmed that *MdWRKY20* participate in the defense response to *F. solani* infection by promoting the expression of *MdPR1*.

Active resistance refers to the host defense response caused by pathogen infection, and reactive oxygen species (ROS) burst is the earliest defense response produced by plants when pathogens invade^[56]. Pathogen infection would unbalance ROS metabolism in plants and increase ROS production, and lead to the destruction of cell membrane structure^[57]. In this study, after *Arabidopsis* were infected with *F. solani*, the H_2O_2 and O_2^- production rates in the leaves of Col were significantly increased, which were significantly higher than that in the *MdWRKY20*-OE lines. Appropriate increase of ROS plays an active role in plants' response to pathogen infection, but when ROS is excessive, it will lead to membrane lipid peroxidation, decreased enzyme activity, and cell metabolism disorders, which may eventually lead to the death of host cells^[58,59]. Defense-related enzymes such as SOD, POD, and CAT could remove excessive ROS^[60,61] from plant leaves. The present results showed that the contents of SOD, POD, and CAT in *MdWRKY20*-OE *Arabidopsis* leaves were significantly increased after *F. solani* infection, while the increase range of the Col were not significant. This may be due to the cell membrane damage of Col being severe, while transgenic

lines could respond to the infection of *F. solani*, so their cell membrane damage was relatively light. The MDA content in leaves of *Arabidopsis* increased after infection with *F. solani*, but the MDA

content in *MdWRKY20*-OE lines were significantly less than the Col, which also proved that the overexpression of *MdWRKY20* had strong tolerance to *F. solani* infection.

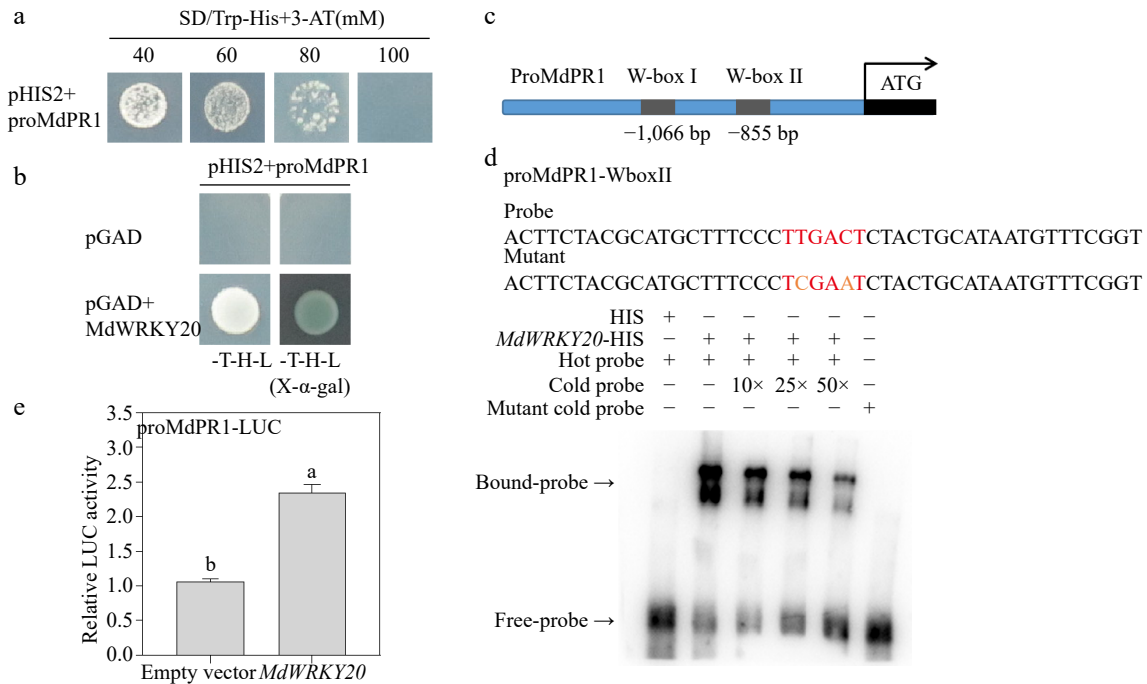


Fig. 5 *MdWRKY20* binds to the *MdPR1* promoter. (a) The optimal 3-AT concentration was determined by cloning proMdPR1 into the pHIS2 vector. (b) *MdWRKY20* interacted with *MdPR1* promoter fragments as per the Y1H assay. (c) The W-box I and W-box II elements were used in the EMSA. (d) EMSA analysis revealed that *MdWRKY20* binds to the W-box II of the *MdPR1* promoter (e) Luciferase reporter (LUC) assays showed the *MdWRKY20*-mediated activation of proMdPR1.

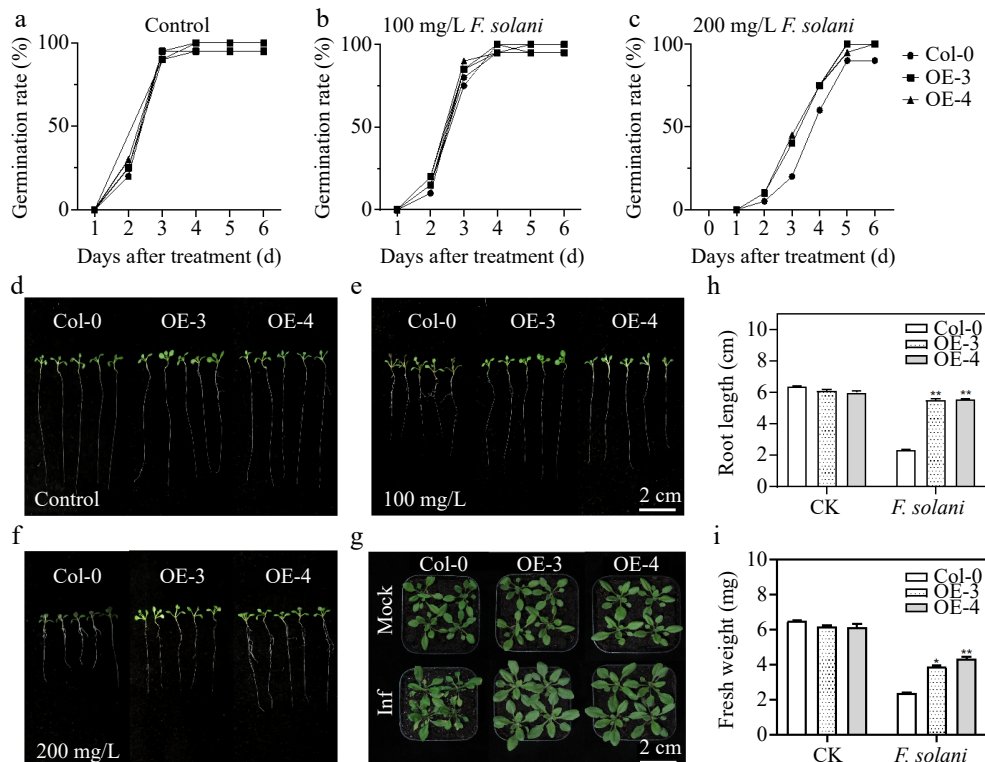


Fig. 6 Germination percentage and phenotypic analysis of *Arabidopsis* under control and *F. solani* treatment. The germination rates of *Arabidopsis* seeds treated with (a) 0 mg/L, (b) 100 mg/L, and (c) 200 mg/L fermentation extract of *F. solani*. The phenotype of *Arabidopsis* was recorded by taking photos on the (d)–(f) 7 dpi, and (g) 21 dpi treated with (d) 0 mg/L, (e) 100 mg/L, and (f) 200 mg/L fermentation extract of *F. solani*. For (d)–(f) bars = 2 cm. (h) Root length of *Arabidopsis* after 7 d of treatment with control and *F. solani*. (i) Fresh weight of *Arabidopsis* after 7 d of treatment with control and *F. solani*. *0.01 $\leq p < 0.05$, **0.001 $< p \leq 0.01$.

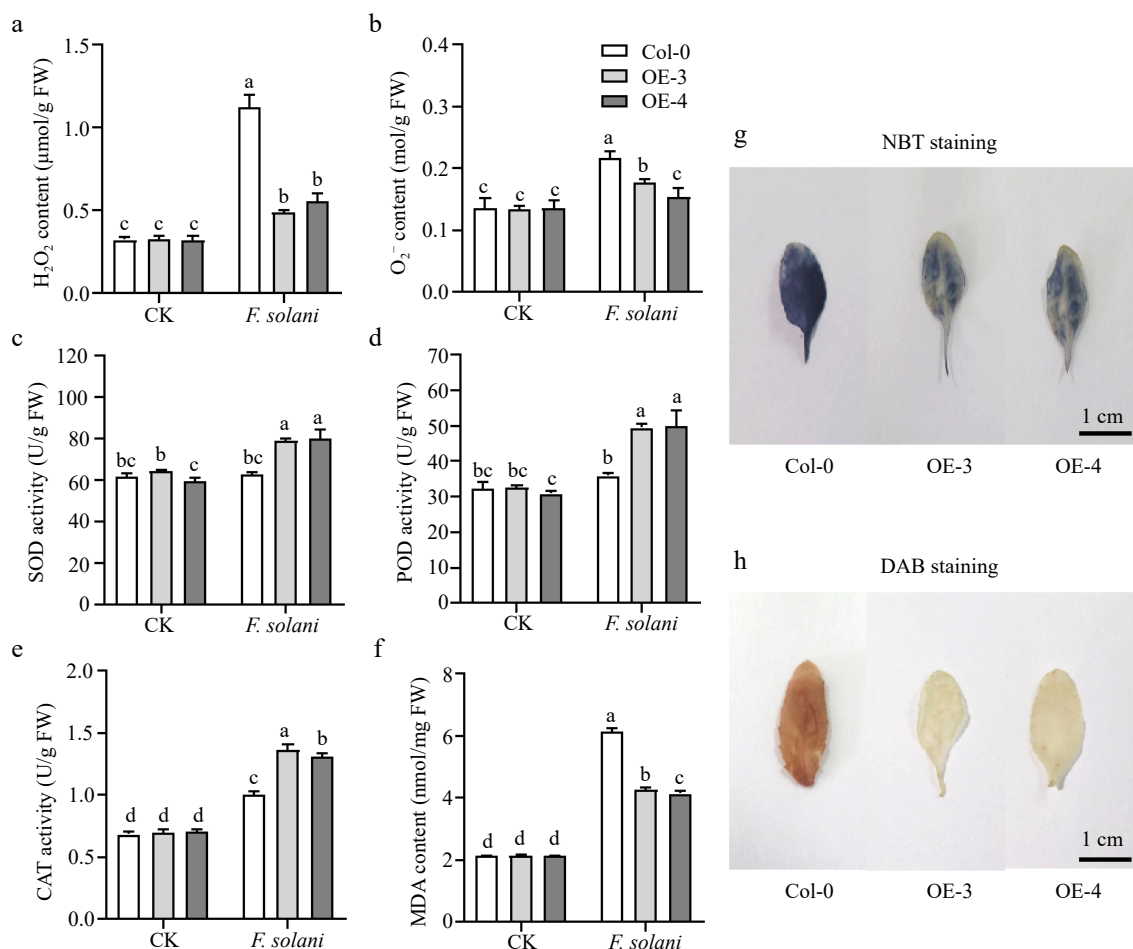


Fig. 7 Effects of *F. solani* treatment on several parameters of *Arabidopsis* leaves. The influence of *F. solani* treatment on (a), (b) active oxygen levels, (c)–(e) the activity of antioxidant enzymes, and (f) the content of malondialdehyde in *Arabidopsis* leaves. The (g) NBT staining, and (h) DAB staining of *Arabidopsis* leaves. For (a)–(f), values were means \pm s.d. of three independent biological replicates. Bars not labeled with the same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests. For (g) and (h), the deeper the color, the more content of O₂⁻ or H₂O₂ in plant tissues. Bars = 1 cm.

Conclusions

In this study, *MdWRKY20* was isolated from the apple rootstock 'M9T337' and its role in resisting *F. solani* infection reported. The plants with high expression of *MdWRKY20* showed enhanced resistance to *F. solani* infection. *MdWRKY20* promoted its expression by combining with the promoters of disease course related proteins *MdPR1* to improve the resistance of plants, which was the positive regulatory factor for resistance of apple to *F. solani*. This work has provided a scientific basis for the prevention and control of ARD and can be used to introduce durable resistance to replant disease in apples.

Authors contributions

The authors confirm contribution to the paper as follows: experiment design: Mao Z, Yin C; research performed: Zhao L, Liu Y, Wang M and Xiang L; data analysis: Wang H; manuscript preparation: Jiang H, Chen X. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (Grant No. 32072510), China Agriculture Research System of MOF and MARA (Grant No. CARS-27), Taishan Scholar Funded Project (No. ts20190923, No. tsqn202408119), Key RandD program of Shandong Province (Grant No. 2022TZXD0037), and the National Key Research and Development Program (Grant No. 2023YFD2301003).

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary information accompanies this paper at (<https://www.maxapress.com/article/doi/10.48130/frures-0024-0033>)

Dates

Received 9 September 2024; Revised 30 September 2024; Accepted 12 October 2024; Published online 6 January 2025

References

- Mazzola M, Gu YH. 2000. Impact of wheat cultivation on microbial communities from replant soils and apple growth in greenhouse trials. *Phytopathology* 90:114–19
- Somera TS, Mazzola M. 2022. Toward a holistic view of orchard ecosystem dynamics: a comprehensive review of the multiple factors governing development or suppression of apple replant disease. *Frontiers in Microbiology* 13:949404
- Tewoldemedhin YT, Mazzola M, Labuschagne I, McLeod A. 2011. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. *Soil Biology and Biochemistry* 43:1917–27
- Rumberger A, Merwin IA, Thies JE. 2007. Microbial community development in the rhizosphere of apple trees at a replant disease site. *Soil Biology and Biochemistry* 39:1645–54
- Wang G, Yin C, Pan F, Wang X, Xiang L, et al. 2018. Analysis of the fungal community in apple replanted soil around Bohai Gulf. *Horticultural Plant Journal* 4:175–81
- Liu X, Xu S, Wang X, Xin L, Wang L, et al. 2022. *MdBAK1* overexpression in apple enhanced resistance to replant disease as well as to the causative pathogen *Fusarium oxysporum*. *Plant Physiology and Biochemistry* 179:144–57
- Yin C, Xiang L, Wang G, Wang Y, Shen X, et al. 2017. Phloridzin promotes the growth of *Fusarium moniliforme* (*Fusarium verticillioides*). *Scientia Horticulturae* 214:187–94
- Xiang L, Wang M, Pan F, Wang G, Jiang W, et al. 2021. Transcriptome analysis *Malus domestica* 'M9T337' root molecular responses to *Fusarium solani* infection. *Physiological and Molecular Plant Pathology* 113:101567
- Wang H, Zhao L, Jiang W, Zhang R, Chen R, et al. 2021. Effects of *Allium fistulosum*-*Brassica juncea*-*Triticum aestivum* rotation a year on the soil microbial environment and the subsequent growth of young apple trees. *Scientia Horticulturae* 290:110549
- Manici LM, Kelderer M, Caputo F, Nicoletti F, De Luca Picione F, et al. 2015. Impact of cover crop in pre-plant of apple orchards: relationship between crop health, root inhabiting fungi and rhizospheric bacteria. *Canadian Journal of Plant Science* 95:947–58
- Mazzola M, Hewavitharana SS, Strauss SL. 2015. *Brassica* seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. *Phytopathology* 105:460–69
- Chen R, Jiang W, Wang H, Pan F, Fan H, et al. 2021. Effects of different fumigants on the replanted soil environment and growth of *Malus hupehensis* Rehd. seedlings. *HortScience* 56:491–99
- Jiang W, Chen R, Zhao L, Qin L, Fan H, et al. 2022. Chemical fumigants control apple replant disease: microbial community structure-mediated inhibition of *Fusarium* and degradation of phenolic acids. *Journal of Hazardous Materials* 440:129786
- Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* 444:323–29
- Zasada IA, Halbrendt JM, Kokalis-Burelle N, LaMondia J, McKenry MV, et al. 2010. Managing nematodes without methyl bromide. *Annual Review of Phytopathology* 48:311–28
- Park MG, Lee BH, Yang JO, Kim BS, Roh GH, et al. 2021. Ethyl formate as a methyl bromide alternative for fumigation of citrus: efficacy, fruit quality, and workplace safety. *Journal of Economic Entomology* 114(6):2290–96
- Martin FN. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology* 41:325–50
- Lahlali R, Ezrari S, Radouane N, Kenfaoui J, Esmaeel Q, et al. 2022. Biological control of plant pathogens: a global perspective. *Microorganisms* 10:596
- Jiang W, Chen R, Zhao L, Duan Y, Wang H, et al. 2023. Isolation of phloridzin-degrading, IAA-producing bacterium *Ochrobactrum haematophilum* and its effects on the apple replant soil environment. *Horticultural Plant Journal* 9(2):199–208
- Hirsch M, Burges PL, Migueliz L, Villarreal NM, Marina M. 2024. Isolation of beneficial bacteria from strawberry (*Fragaria × ananassa*, Duch). Potentialities for fungal disease control and plant growth promotion. *Plant Growth Regulation* 102:135–52
- Berendsen RL, Vismans G, Yu K, Song Y, de Jonge R, et al. 2018. Disease-induced assemblage of a plant-beneficial bacterial consortium. *The ISME Journal* 12:1496–507
- Yang Y, Shah J, Klessig DF. 1997. Signal perception and transduction in plant defense responses. *Genes & Development* 11:1621–39
- Manzoor MA, Xu Y, Lv Z, Xu J, Wang Y, et al. 2023. Fruit crop abiotic stress management: a comprehensive review of plant hormones mediated responses. *Fruit Research* 3:30
- Wu W, Fu P, Lu J. 2022. Grapevine WRKY transcription factors. *Fruit Research* 2:10
- Zhang K, Liu F, Wang Z, Zhuo C, Hu K, et al. 2022. Transcription factor WRKY28 curbs WRKY33-mediated resistance to *Sclerotinia sclerotiorum* in *Brassica napus*. *Plant Physiology* 190:2757–74
- Abbruscato P, Nepusz T, Mizzi L, Del Corvo M, Morandini P, et al. 2012. *OsWRKY22*, a monocot WRKY gene, plays a role in the resistance response to blast. *Molecular Plant Pathology* 13:828–41
- Zheng Z, Qamar SA, Chen Z, Mengiste T. 2006. *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *The Plant Journal* 48:592–605
- Yang Y, Zhou Y, Chi Y, Fan B, Chen Z. 2017. Characterization of soybean WRKY gene family and identification of soybean WRKY genes that promote resistance to soybean cyst nematode. *Scientific Reports* 7:17804
- Dang F, Wang Y, Yu L, Eulgem T, Lai Y, et al. 2013. *CaWRKY40*, a WRKY protein of pepper, plays an important role in the regulation of tolerance to heat stress and resistance to *Ralstonia solanacearum* infection. *Plant, Cell & Environment* 36:757–74
- Wang J, Tao F, Tian W, Guo Z, Chen X, et al. 2017. The wheat WRKY transcription factors *TaWRKY49* and *TaWRKY62* confer differential high-temperature seedling-plant resistance to *Puccinia striiformis* f. sp. *tritici*. *PLoS One* 12:e0181963
- Ferreira RB, Monteiro S, Freitas R, Santos CN, Chen Z, et al. 2007. The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* 8:677–700
- Rogers EE, Ausubel FM. 1997. *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in PR-1 gene expression. *The Plant Cell* 9:305–16
- Bonasera JM, Kim JF, Beer SV. 2006. PR genes of apple: identification and expression in response to elicitors and inoculation with *Erwinia amylovora*. *BMC Plant Biology* 6:23
- Christensen AB, Cho BH, Næsby M, Gregersen PL, Brandt J, et al. 2002. The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins. *Molecular Plant Pathology* 3:135–44
- Zaynab M, Peng J, Sharif Y, Al-Yahyai R, Jamil A, et al. 2021. Expression profiling of pathogenesis-related Protein-1 (*PR-1*) genes from *Solanum tuberosum* reveals its critical role in phytophthora infestans infection. *Microbial Pathogenesis* 161:105290
- Oide S, Bejai S, Staal J, Guan N, Kaliff M, et al. 2013. A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *New Phytologist* 200:1187–99
- Zhang Q, Xu C, Wei H, Fan W, Li T. 2021. Two pathogenesis-related proteins interact with leucine-rich repeat proteins to promote *Alternaria* leaf spot resistance in apple. *Horticulture Research* 8:219
- Zhou Z, Zhu Y, Tian Y, Yao J, Bian S, et al. 2021. *MdPRA*, a pathogenesis-related protein in apple, is involved in chitin recognition and resistance response to apple replant disease pathogens. *Journal of Plant Physiology* 260:153390
- Wang W, Nie J, Lv L, Gong W, Wang S, et al. 2021. A *Valsa mali* effector protein 1 targets apple (*Malus domestica*) pathogenesis-related 10 protein to promote virulence. *Frontiers in Plant Science* 12:741342
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–08

41. Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. 2006. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* 1(2):641–46
42. Xiang L. 2021. *Apple replant disease key factor in old orchards and mechanism of rootstock response to its infection*. Thesis. Shandong Agricultural University, China. pp. 51–55
43. Xiang L, Zhao L, Wang M, Huang J, Chen X, et al. 2021. Physiological responses of apple rootstock M.9 to infection by *Fusarium solani*. *HortScience* 56:1104–11
44. Chen L, Song Y, Li S, Zhang L, Zou C, et al. 2012. The role of WRKY transcription factors in plant abiotic stresses. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1819:120–28
45. Wang A, Wang L, Liu K, Liang K, Yang S, et al. 2022. Comparative transcriptome profiling reveals the defense pathways and mechanisms in the leaves and roots of blueberry to drought stress. *Fruit Research* 2:18
46. Li S, Chen H, Yu H, Li Y, Wang L. 2023. Responses and adaptations of fruit trees to high temperatures. *Fruit Research* 3:23
47. Liu Z, Ma Z, Li J, Bian N, Guo Z, et al. 2023. Interfering small ubiquitin modifiers (SUMO) exhibits apple's enhanced tolerance to nitrogen deficiency. *Fruit Research* 3:24
48. Liu F, Li X, Wang M, Wen J, Yi B, et al. 2018. Interactions of *WRKY15* and *WRKY33* transcription factors and their roles in the resistance of oilseed rape to *Sclerotinia* infection. *Plant Biotechnology Journal* 16:911–25
49. Cheng X, Zhao Y, Jiang Q, Yang J, Zhao W, et al. 2019. Structural basis of dimerization and dual W-box DNA recognition by rice WRKY domain. *Nucleic Acids Research* 47:4308–18
50. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. 2000. The WRKY superfamily of plant transcription factors. *Trends in Plant Science* 5:199–206
51. Li J, Zhong R, Palva ET. 2017. *WRKY70* and its homolog *WRKY54* negatively modulate the cell wall-associated defenses to necrotrophic pathogens in *Arabidopsis*. *PLoS One* 12:e0183731
52. Chen YL, Lin FW, Cheng KT, Chang CH, Hung SC, et al. 2023. XCP1 cleaves Pathogenesis-related protein 1 into CAPE9 for systemic immunity in *Arabidopsis*. *Nature Communications* 14:4697
53. Luo X, Tian T, Feng L, Yang X, Li L, et al. 2023. Pathogenesis-related protein 1 suppresses oomycete pathogen by targeting against AMPK kinase complex. *Journal of Advanced Research* 43:13–26
54. Kalde M, Nühse TS, Findlay K, Peck SC. 2007. The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. *Proceedings of the National Academy of Sciences of the United States of America* 104:11850–55
55. Breen S, Williams SJ, Outram M, Kobe B, Solomon PS. 2017. Emerging insights into the functions of pathogenesis-related protein 1. *Trends in Plant Science* 22:871–79
56. Feki K, Tounsi S, Jemli S, Boubakri H, Saidi MN, et al. 2024. Genome-wide identification of PR10 family members and expression profile analysis of *PvPR10* in common bean (*Phaseolus vulgaris* L.) in response to hormones and several abiotic stress conditions. *Plant Growth Regulation* 102:279–95
57. Qi J, Song CP, Wang B, Zhou J, Kangasjärvi J, et al. 2018. Reactive oxygen species signaling and stomatal movement in plant responses to drought stress and pathogen attack. *Journal of Integrative Plant Biology* 60:805–26
58. Castro B, Citterico M, Kimura S, Stevens DM, Wrzaczek M, et al. 2021. Stress-induced reactive oxygen species compartmentalization, perception and signalling. *Nature Plants* 7(4):403–12
59. Mittler R, Zandalinas SI, Fichman Y, Van Breusegem F. 2022. Reactive oxygen species signalling in plant stress responses. *Nature Reviews Molecular Cell Biology* 23:663–79
60. Dat J, Vandenabeele S, Vranová E, Van Montagu M, Inzé D, et al. 2000. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS* 57:779–95
61. Deng Q, Wu J, Chen J, Shen W. 2020. Physiological mechanisms of improved smut resistance in sugarcane through application of silicon. *Frontiers in Plant Science* 11:568130



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