Involvement of MdWRKY40 in the Defense of Mycorrhizal Apple Against Fusarium Solani

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Abstract

Background: Apple (*Malus domestica* Borkh.) is an important economic crop. The pathological effects of *Fusarium* spp., a group of soilborne pathogens, on the root systems of apple plants was unknown. It was unclear how mycorrhizal apple seedlings resist infection by *F. solani*. The transcriptional profiles of mycorrhizal and non-mycorrhizal plants infected by *F. solani* were compared using RNA-Seq.

Results: Infection with *F. solani* significantly reduced the dry weight of apple roots, and the roots of mycorrhizal apple plants were less damaged when the plants were infected with *F. solani*. They also had enhanced activity of antioxidant enzymes and a reduction in the oxidation of membrane lipids. A total of 1,839 differentially expressed genes (DEGs) were obtained after mycorrhizal and non-mycorrhizal apple plants were infected with *F. solani*. A gene ontology (GO) analysis showed that most DEGs were involved in the binding of ADP and calcium ions. In addition, the enrichment observed from an analysis with the Kyoto Encyclopedia of Genes and Genomes (KEGG) primarily involved plant-pathogen interaction and Glycolysis/Gluconeogenesis, the mitogen-activated protein kinase (MAPK) signaling pathway, and plant hormone signal transduction. Based on a MapMan analysis, an enormous number of DEGs were involved in the response of mycorrhizal plants to stress. Among them, the overexpressed transcription factor *MdWRKY40* significantly improved the resistance of 'Orin' to *F. solani* and the expression of the resistance gene *MdGLU* by binding the promoter of *MdGLU*.

Conclusion: This paper outlines how the inoculation of apple seedlings by arbuscular mycorrhizal fungi reduces the response to stress at the transcription level of the root system following infection with *F. solani*, and *MdWRKY40* played an important role in the resistance of mycorrhizal apple seedlings to infection with *F. solani*.

Background

Apple is one of the most widely produced and economically important fruit crops in temperate regions [1]. Apple replant disease (ARD) is a phenomenon in which the plant growth is severely inhibited after replanting apple trees at the same site for many years [2]. ARD is primarily caused by biological factors [3]. Extensive studies have shown that the major pathogens that lead to ARD are *Cylindrocarpom, Fusarium, Rhizoctonia, Pythium,* and *Phytophthora* [4, 5]. Wang et al [6] found that there was an enormous quantity of *Fusarium* spp. in the Bohai Bay, and the most abundant species was *Fusarium solani* [7, 8]. *Fusarium* tend to exhibit high index of disease in many crops, such as cotton [9], soybeans [10], apple [7] and citrus [11].

*Fusarium* spp. are closely related to agricultural production since they cause a variety of plant diseases and substantially reduce the yield and quality of crops. *F. oxysporum* f. sp. *cubense* is the causal agent of banana wilt disease, whereas *F. oxysporum* f. sp. *cucumerinum* is the causal agent of cucumber Fusarium wilt [12, 13]. Wheat head blight is caused by *F. graminearum* [14], and maize ear rot is caused by *F. verticillioides* or *F. proliferatum* [15]. *Fusarium* spp. can infect the epidermal cells at the roots of
plants and spread to the root tip through the vascular system, which causes an increase in black and brown necrotic spots. This causes serious root tip necrosis and decay [16].

Arbuscular mycorrhizal fungi (AMF) can infect more than 80% of plants to form efficient symbiotic relationships on the roots [17]. AMF can improve the efficiency of utilization of plant nutrients under adverse conditions to improve the growth of host plants [18], enhance the resistance of the host to environmental stress, such as drought and salt stress [19], and have a significant effect on the inhibition of plant diseases [20]. Many studies have indicated that there is an interaction between AMF, pathogens, and plants [21]. An enormous amount of research showed that AMF can effectively provide biological control against soilborne pathogen, such as \textit{F. oxysporum}, \textit{Sclerotium cepivorum} and \textit{Pythium aphanidermatum} [22]. AMF can occupy the growth sites of pathogenic microorganisms in the root systems of host plants and produce wide networks of extraradical mycelia to resist infection by pathogens [23, 24]. AMF can regulate the secondary metabolic pathway of the roots of host plants, promote the synthesis and secretion of terpenes and phenolic compounds, and improve the synthesis of antimicrobial substances, such as phytoalexins and lignin, to control the growth and reproduction of pathogens and even kill them [25, 26]. AMF regulate the synthesis and activation of the plant defense system of endogenous hormones that include salicylic acid (SA), jasmonic acid (JA) [27] and other hormones that are involved in disease resistance, thus, enhancing the response of plant to pathogens [28]. The system of composition of mycorrhizal-root-pathogen plays an active role in the disease resistance process, and plants with mycorrhizae are generally more resistant to soilborne pathogens than plants that lack them [29]. However, it is clear that pre-AMF apple roots respond with physiological and molecular reactions following infection by \textit{F. solani}.

In this study, the apple stock M9T337 was used as the experimental material to explore the defense mechanism of mycorrhizal apple seedlings. We conducted several experimental evaluations of the pre-inoculation of AMF to help improve the resistance of apple root systems to \textit{F. solani}. We assumed that the presence of symbiotic apple root systems enhances the resistance to \textit{F. solani}. Therefore, we analyzed the molecular mechanism of the AMF-apple interaction in response to the infection by an analysis of the transcriptome. This study provides a basis for understanding the molecular mechanism of mycorrhizal apple against infection by \textit{F. solani}.

**Results**

**Physiological properties of mycorrhizal apple roots**

The plants were harvested 5 days after infection with \textit{F. solani}. The plants that had been colonized by mycorrhizae in the AM (mycorrhizal plants that had not been inoculated with \textit{F. solani}) and AMFs (mycorrhizal plants inoculated with \textit{F. solani}) were 75.98% and 48.85%, respectively, without mycorrhizal colonization in the NM (non-mycorrhizal plants that had not been inoculated with \textit{F. solani}) and NMFs (non-mycorrhizal plants inoculated with \textit{F. solani}) treatments. Infection with \textit{F. solani} significantly reduced the dry weight of plant, and the presence of AMF in plants that had been inoculated with \textit{F. solani}...
significantly reduced the damage of *F. solani* to the plant. The activity of superoxide dismutase (SOD) of the AM was significantly higher than that of the NM treatment, while the activities of other antioxidant enzymes did not differ significantly from the NM treatment. After 5 days of infection with *F. solani*, the SOD and peroxidase (POD) activities of the AMF treatment increased by 12.5% and 56.4%, respectively, compared with the NMF treatment, while there was no significant difference in the activity of catalase (CAT) (Fig. 2A). The contents of malondialdehyde (MDA), H$_2$O$_2$, and O$_2$·$^-$ in the AMF symbiotic plants were 19.8%, 26.1%, and 54.2% lower than those in the non-mycorrhizal plants, respectively (Fig. 2B). We also observed a similar trend in the contents of proline (PRO), soluble protein, and soluble sugar (SOG) in the treated plant roots that correlated with the activities of SOD and POD (Fig. 2C).

**DEG screening**

To investigate the change of transcription levels of apple root M9T337 after inoculation with AMF and *F. solani*, different treatments were assessed. The differentially expressed genes (DEGs) from the four treatments that were upregulated and downregulation are shown in Fig. 3. A total of 809 DEGs were selected in the AMF vs. AM treatment, and 996 DEGs were selected in the NMFs vs. NM treatment. A total of 1,839 DEGs, the most differential genes, were selected for subsequent differential gene analysis in the AMF vs. NMF treatment. The PCA analysis identified a significant separation between the AMF and NMF (Fig. S1).

**GO and KEGG pathway enrichment analysis**

Compared with the NMFs, we obtained 1,839 different genes with 1,212 that were upregulated and 627 genes that were downregulated. In GO enrichment analysis, the genes were annotated and classified by biological processes, cell components and molecular function. In the AMFs vs. NMFs, the greatest number of DEGs were enriched in molecular function, including ADP binding (GO:0043531), calcium ion binding (GO:0005509), hydrolase activity (GO:0004553), vitamin binding (GO:0019842), polygalacturonase activity (GO:0004650), and thiamine pyrophosphate binding (GO:0004650). More DEGs in the cell components were enriched in the cell periphery (GO:0071944) and the cell wall (GO:0005618). In addition, a greater number of DEGs in the biological processes were enriched in the pyridine nucleotide biosynthesis pathway (GO:0019363), carbohydrate catabolic process (GO:0016052), and protein modification (GO:0070647) (Fig. 4A).

To determine the main biological functions and pathway of the DEGs in this study, we utilized a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The extensive DEGs were enriched in AMF vs. NMF to pathways (Fig. 4B), such as plant and pathogen interaction pathways (mdm04626), Glycolysis / Gluconeogenesis (mdm00010), MAPK signaling pathway-plant (mdm04016), Plant hormone signal transduction (mdm04075), Pentose and glucuronate interconversions (mdm00040), and alpha-Linolenic acid metabolism (mdm00592). Up to 47 DEGs were enriched in plant and pathogen interaction pathways (mdm04626). Numerous genes were upregulated, including calcium-binding protein (MD09G1013600, MD06G1143500, MD15G1133000), defense-related gene
(MD17G1215600) (Fig. S2A) in the plant and pathogen interaction pathways, the JA-related gene MD07G1073800 (OPCL1), and the key enzymes (AOS, MD00G1038400) of JA biosynthesis were significantly upregulated (Fig. S2B) in the alpha-Linolenic acid metabolism pathway, and 4-coumarate-CoA ligase (4CL, MD13G1257800), and cinnamoyl-CoA reductase (CCR, MD09G1224200) were significantly upregulated in the phenylpropanoid biosynthetic pathway (Fig S2C).

Transcription factors (TFs) were the “primary switch” to open the gene transcription process. It showed that multiple members of different TF families involved in DEG were expressed, including the WRKY, AP2-ERF, bHLH, NAC, MYB, HB, C2H2, and bHLH families (Fig. 4C). Owing to the extensive expression of the ethylene signaling pathway gene, there is a class of proteins designated ethylene response factors in TFs that are closely related to this signaling pathway, and the differential expression analysis also verified the relevant data. A large number of ERF family members (MD15G1221100, MD07G1248600, MD05G1311400, MD04G1058200, MD09G1114800, MD15G1055200, MD02G1096500, MD05G1080900, MD10G1094700) were upregulated by infection with *F. solani* after 5 days in the sample. Interestingly, the most representative class of TFs was the positive regulatory induction of the WRKY family genes in mycorrhizal plants. Compared with the NMF treatment, *WRKY40, WRKY41, WRKY53, WRKY50, WRKY24, WRKY76, WRKY28,* and *WRKY6* were significantly upregulated.

### MapMan analysis

To have a more comprehensive understanding of the DEGs in interactions of plants and pathogens, we performed a MapMan visual analysis in biotic stress, secondary metabolism, regulation and cellular response (Fig. 5). The results showed that a large number of DEGs were involved in the plants induced with AMF to resist the infection of *F. solani* (Fig. 5A and D). The genes that encode TIR-NBS-LRR and NB-ARC were upregulated by *F. solani*. A large number of genes that are related to hormone signaling, including auxin, brassinolide, abscisic acid, ethylene, JA, and SA; pathogenesis-related proteins, such as PR1 and PR5; and transcription factors, such as WRKY, MYB, ERF and DOF, were upregulated (Fig. 5A). The R protein gene (MD01G1189400, MD11G1218000) in ETI and MdMPK1 and MdMPK18 were upregulated in the mitogen-activated protein kinase (MAPK) signal pathway.

During the process of mycorrhizal plants following infection with *F. solani*, an enormous number of DEGs participated in signal regulation pathways, including auxin, gibberellin, abscisic acid, brassinolide, and cytokinin (Fig. 5C). In response to pathogen intrusion, plants often produce hormone signals related to SA and JA to activate the expression of downstream defense genes to improve plant disease resistance. MD09G1178600 was upregulated, and MD04G1204200 was downregulated in the JA signal pathway, while MD14G1232600 and MD14G1232900 were upregulated in the SA signal pathway. A total of 66 DEGs were annotated as receptor kinases. Twenty-five DEGs were involved in calcium regulation, whereas only five DEGs were downregulated, and others were upregulated.

The biosynthesis of lignin and lignans, flavonoids, phenylpropanoids, and glucosinolates were the primary secondary metabolic pathways. Glucosinolate biosynthesis is thought to produce a chemical
barrier against pathogen infection, with nine genes for the biosynthetic process, of which seven different genes were significantly upregulated. In addition, MD15G1079200, MD09G1169600, MD07G1073800, MD09G1224200, and MD15G1008100 were significantly upregulated in the phenylpropanoid synthesis pathway. The flavonoid synthesis pathway of only MD03G1140400 and MD01G1077200 were upregulated again as different genes, while seven DEGs were downregulated genes. Interestingly, the DEGs were all upregulated in both carotenoids and the simple phenolic synthesis pathway.

**qRT-PCR validation**

Based on the sequencing data from our transcriptional group to validate the RNA-Seq results, 16 upregulated genes and six downregulated genes were verified with quantitative real-time reverse transcriptase-PCR (qRT-PCR) (Fig. 6A). The candidate genes were selected to examine their participation in the process of interaction between plants and pathogens, including a pathogen recognition receptor, signal transduction, transcription factor, and the genes involved in the synthesis of secondary metabolites (Fig. 5). These genes could play an important role in the mycorrhizal plant in response to biological stress reactions by infection with *F. solani*. As shown in Fig. 6B, the trend toward the levels of expression in RNA-Seq and qRT-PCR data was similar, which highlights the reliability of RNA-Seq.

**Overexpression of MdWRKY40 improves the resistance of ‘Orin’ to *F. solani***

We found the highest relative level of expression of *MdWRKY40* (MD15G1039500) qRT-PCR validation (Supplementary Table S3). To validate the resistance of *MdWRKY40* to *F. solani*, MdWRKY40 (MD15G1039500) was chosen to transform the apple callus. Compared with the wild-type (WT) and OE-MdWRKY40 'Orin' callus that had been infected with *F. solani* after 4 days, the diameter of fungal spots in the OE-MdWRKY40 was significantly lower than that in the WT, and the diameter of fungal spots in the WT callus was 0.56-fold that of the diameter of fungal spots in OE-MdWRKY40. To study whether MdWRKY40 regulates the expression of resistance genes, the relative levels of expression of *MdPR1*, *MdPR4*, *MdPR5*, *MdPR8*, *MdECHT*, and *MdGLU* in callus following infection with *F. solani* were measured by qRT-PCR. The levels of expression of *MdGLU* in OE-MdWRKY40 were significantly higher than the levels in wild type (WT) in all the resistance genes tested. This suggests that MdWRKY40 could improve the resistance of callus to *F. solani* by regulating the expression of *MdGLU*.

**MdWRKY40 binds to the MdGLU promoter**

Previous studies showed that WRKY transcription factors usually target W-box motifs to activate or inhibit the level of expression of downstream genes [30]. The promoters of *MdGLU* contain three W-boxes (Supplementary Fig. S4). A yeast one-hybrid test was used to determine whether MdWRKY40 combined with the *MdGLU* promoter. On the media that lacked Trp and His, the optimal concentration to inhibit the expression of HIS3 in the pHIS2 vector was 120 mm 3-AT for proMdECHT-pHIS2 (Fig. 8B). In the Y1H assays, MdWRKY40 interacted with the promoters of *MdGLU*. When WRKY40 was divided into two fragments (N-terminus and C-terminus), we found that the C-terminus that contains the WRKY domain
binds to the *MdGLU* promoter (Fig. 7A and C). An electrophoretic mobility shift assay (EMSA) confirmed that MdWRKY40 could bind to the second W-box in the *MdGLU* promoter but not to the other three W-boxes (Fig. 7D, Supplementary Figure S6). As the concentration of the cold probe increased, the binding weakened, and the addition of the mutant cold probe did not affect the binding. To determine how MdWRKY40 regulates the level of *proMdGLU* activity, luciferase (LUC) reporter assays were performed. MdWRKY40 stimulated the expression of the LUC gene driven by the promoter of *MdGLU* (Fig. 8E). These results indicated that MdWRKY40 activated *proMdGLU*.

**Discussion**

**Mycorrhizal symbiosis improved the resistance of apple seedling to** *F. solani*

The symbiosis of AMF with plant roots is often mutually beneficial. After establishing a symbiotic system with the host plant roots, the AMF will promote root development, change the plant root morphology, and increase the root biomass [31]. A large amount of research has shown that mycorrhizal plants tend to have more developed roots to absorb essential moisture and nutrients for the host plants under adverse conditions [32]. The symbiosis of AMF not only enhances the absorption of soil nutrients by host plant roots but plays an important role in the management of abiotic and biotic stress by the plant [33]. In this study, the root length of M9T337 was significantly reduced after infection by *F. solani*. The pre-inoculation of AMF promoted root growth, indicating that after AMF invaded the plant root system, the soilborne pathogens competed with their ecological niche and reduced their rate of invasion on the root epidermis (Fig. 1). Simultaneously, after the formation of mycorrhizal plants, a developed mycorrhizal network formed a frontal physical barrier with the root system, which helped the plants to better resist the harm caused by pathogens to plants [34, 35]. The mycorrhizal colonization of AMF will lead to the lignification of the root cell wall of the host plant; the degree of lignification of the plant roots was strengthened; the rate of reproduction of pathogen was decreased, and the plant disease was alleviated [36].

*F. solani*, as a plant pathogenic fungus, frequently caused a large amount of necrosis of the roots, resulting in a decline of photosynthetic ability and other pathological changes [7]. When pathogenic fungi infect plants, the resistance of mycorrhizal plant was often induced early to reduce the damage of pathogens to plants [27]. Studies have shown that *Glomus mossae, Rhizophagus irregularis*, and other AMF have been used to varying degrees to reduce plant diseases caused by pathogenic fungi, such as *Fusarium* spp. and *Phytophthora* spp. [37, 38]. Consistent with previous studies, AMF significantly improved the resistance of plants by improving the activities of plant defense enzymes, reducing the oxidation of membrane lipids, and enhancing the plant antioxidant prevention system (Fig. 2). These findings suggest that AMF symbiosis reduced the degree of membrane oxidation, improved the antioxidant activities and permeable regulatory material content to protect the apple root system.

**Mycorrhizal plants respond to** *F. solani* infection
The plant-AMF-pathogen interaction is a complex network system that involves multiple gene expression and complex regulation [39]. After the pathogens invade plants, the plant will increase the expression of resistance genes and promote the transmission of signal substances and plant metabolism to respond quickly to pathogenic infections [40]. Transcriptome data provide the expression of information and the level of expression for genes that are commonly used to identify the differential stresses of functional plants to biological and non-biological factors [41, 42]. To further understand the biological function of the DEGs, we performed GO, KEGG Pathway significant enrichment and MapMan visualization analyses to determine key differential genes for the main physiological and biochemical metabolic pathways and signal transduction pathways. There were significant morphological differences between the AM and NM roots after pre-inoculation with AMF (Fig. 1). In addition, there were significant differences between gene expression and the DEGs between the AMF and NMF after the infection of *F. solani* (Fig. 3). In the GO enrichment analysis, more DEGs were concentrated in molecular function, with the most upregulated genes found in ADP binding (GO:0043531) and calcium ion binding (GO:0005509) pathway (Fig. 4A). The shock of calcium ion concentration in the cytoplasm of root epidermal cells was a central signal factor for plant symbiosis with AMF. The calcium-dependent protein kinase activates downstream calcium/calcium-dependent protein kinases to induce the expression of genes related to symbioses [43]. In the presence of pathogens, AMF constantly compete with pathogens to invade sites on the plant root system, and thus, increase the calmodulin protein genes, such as CML37 and CML38 (Fig. 5). The plant cells sense that calmodulin was activated in a relative stress response to protect the cells from a high infiltration environment [44, 45].

The KEGG pathway significant enrichment analysis found that the selected DEGs were primarily enriched in the plant-pathogen interaction pathway metabolic pathway, MAPK signaling pathway, and plant hormone signal transduction pathways (Fig. 4B). In the process of the interactions of plants with pathogens, a series of defense mechanisms were formed, and the metabolic routes described above were closely related to the defense responses of plants. The related genes of apple-AMF symbionts were analyzed using a MapMan analysis to more comprehensively show the changes of genes in related pathways between mycorrhizal and non-mycorrhizal plants by infection with *F. solani*. Cells respond to stress through signaling transduction and the intracellular regulation of plant physiological and biochemical reactions, while the MAPK signaling transduction pathway is in the center of cell signal transduction system [46]. Huang et al. [47] found that the inoculation of AMF resulted in a significant upregulation of *MdMAPK*, along with the functional response of *MdMAPKs* to plant hormone signaling and stress responses under drought stress. JA played an important role in the interactions of mycorrhizae to protect the plants against pathogens [48]. In this study, the JA-related gene MD07G1073800 (OPCL1) was significantly upregulated in the alpha-Linolenic acid metabolism pathway. SA, as a hormone directly involved in the plant immune system, could induce the plant synthesis of the PR proteins. We found a significant upregulation of SA-related genes and numerous PR genes. These findings suggest that SA-related and JA-related genes may be activated and promote the synthesis of compounds to resist *F. solani*. The MAPK signaling pathway and the plant hormone signal transduction were essential for the
resistance of mycorrhizal plants to pathogens, but the deeper molecular mechanisms still require further research.

TFs respond to mycorrhizal plant resistance to F. solani

TFs play an important role in the process of the plant response to the environment [49, 50]. Extensive studies have shown that the family of transcription factors, such as WRKY, AP2, NAC, MYB, and GRAS, are involved in the regulation of expression of defense-related genes (Fig. 4C). In this study, a large number of AP2, WRKY and MYB transcription factor family genes were involved in the response of mycorrhizal plants against infection with F. solani. Only one downregulated expressed gene of the WRKY transcription factor was detected in the transcription, and the others increased significantly. The log2 FoldChange of MdWRKY76, MdWRKY53, and MdWRKY40 were more than 2.5-fold (Fig. 6). The results of qRT-PCR were consistent with the RNA-Seq data, and WRKY40 was expressed up to 3.69-fold (Supplementary Table S3).

The overexpression of VvWRKY18 enhances the resistance of Arabidopsis thaliana to Botrytis cinerea through activation of the STILBENE SYNTHASE (STS) genes [51]. The overexpression of HbWRKY40 induced resistance to Colletotrichum gloeosporioides in tobacco [52]. An evolutionary tree analysis found that MdWRKY40 belongs to WRKY ña (Supplementary Figure S7). We hypothesize that WRKY40 played a positive role when mycorrhizal plant roots were infected by F. solani. Therefore, we performed a functional validation of MdWRKY40 (Fig. 7). We found through its overexpression in “Orin” callus that the overexpression of MdWRKY40 enhanced the resistance to F. solani and improved the level of transcription of MdGLU. Through YIH, EMSA and LUC report analyses, the binding of MdWRKY40 to the MdGLU promoter was identified and is thus, involved in the resistance of apple to infection by F. solani (Fig. 8).

Conclusions

The apple seedlings with AMF significantly reduced the deleterious effects of F. solani to the root system, improved the antioxidant enzyme activities, and reduced the degree of membrane lipid oxidation. Most of the DEGs involved in plant pathogen interaction, glycolysis / gluconeogenesis, and hormone signal transduction pathway were identified through GO and KEGG analyses. The mycorrhizal and non-mycorrhizal apple plants were subjected to oxidative stress after inoculation with F. solani. However, compared with non-mycorrhizal plants, an enormous number of resistance genes were involved in the stress response process to reduce the amount of oxidative damage. MdWRKY40 improved its expression by binding with the MdGLU promoter to participate in mycorrhizal apple seedlings against the infection of F. solani.

Methods

Plant growth and plant infection
Apple stock M9T337 was grown in an illuminated greenhouse at 20-30 °C (daytime) and 0-15 °C (night) with a relative humidity of 55-65%. And the apple stock M9T337 was obtained from Shandong Horticultural Techniques Services Co. Ltd., in Tai’an, Shandong. And the ‘Orin’ apple calli was provided by Prof. Xue-sen Chen of State Key Laboratory of Crop Biology, China. ‘Orin’ apple calli (*Malus domestica* cv. ‘Orin’) were cultured in subculture medium that contained MS, 2.5 mg·L$^{-1}$ 2,4-D, and 0.5 mg·L$^{-1}$ 6-indole-3-butyric acid at room temperature (24 °C) in the dark, and the subculture medium was renewed every 15 days for genetic transformation. The hyphae of *F. solani* were inoculated in sterilized PDA liquid media and cultured in a shaker at 28 °C for one week. The hyphae were filtered with sterile gauze, and the number of plates was counted. The spores were adjusted to 10$^5$·mL$^{-1}$ with sterile water. Arbuscular mycorrhizal fungi use clover as their host, *Trifolium repens* seedling was provided by Shandong Agricultural University Seed Industry Co. Ltd, in Tai’an, China. And the plants were enriched and propagated in sterilized media for three months. A mixture of vermiculite, spores (spore density 28·g$^{-1}$), hyphae and root segments infected with *Paraglomus* sp. SW1 (CGMCCNO. 20744) inoculum. The systems of mycorrhizal plant roots were grown for 4 weeks using *Paraglomus* sp. SW1.

We established four treatments: (1) NM: non-mycorrhizal plants that had not been inoculated with *F. solani*; (2) AM: mycorrhizal plants that had not been inoculated with *F. solani*; (3) NMFs: non-mycorrhizal plants inoculated with *F. solani*; and (4) AMFs: mycorrhizal plants inoculated with *F. solani*. After the apple stock M9T337 plantlets grew for 4 weeks with 1% AMF inoculum, 50 ml of a solution of *F. solani* spores was used to treat them. After 5 days, the apple roots were collected for transcriptome sequencing, real-time RT-PCR verification, and an analysis of the enzymes involved in resistance.

**Rate of mycorrhizal colonization**

The rate of mycorrhizal colonization was determined as described by Bohrer [10]. A 1 cm root segment was digested with a solution of 10% KOH at 90°C for 20 min, acidified with 2% HCl for 5 min, stained with 0.05% triphenyl blue lactic acid glycerin solution (lactic acid/glycerin =1/1) at 90°C for 30 min, and decolorized overnight with a solution of lactic acid glycerin (lactic acid/glycerin/water = 1/1/1 [v/v/v]). The root segments were observed under a microscope. The rate of infection of each root segment was assessed by the number of root mycorrhizal structures in each section and expressed as 0, 10, 20, ..., 100% of the root. The mycorrhizal colonization rate (%) was calculated as follows:

\[
\text{Mycorrhizal colonization rate} = \frac{\sum (0 \times \text{root segment number} + 10\% \times \text{root segment number} + 20\% \times \text{root segment number} + \ldots + 100\% \times \text{root segment number})}{\text{Total root segment number}}.
\]

**RNA Extraction, cDNA library construction, and sequencing**

The roots of each three processed replicates were used with kits to extract the total plant root RNA and the integrity and total RNA was tested with an Agilent 2100 bioanalyzer (Agilent Technologies). The cDNA library was obtained by RT-PCR. After the library had been examined, Illumina sequencing was performed. To ensure the quality and reliability of the data analysis, it is necessary to filter the original data, including the removal of reads with an adapter, and remove the reads that contain N. N indicates that the base
information cannot be determined. The low quality reads were then removed. The base number of Qphred is ≤ 20, which comprises ≥ 50% of the whole read length. Moreover, the contents of Q20, Q30, and GC in the clean data were calculated. All follow-up analyses were conducted by Novogene Co., Ltd. (Beijing, China) for clean data high quality analysis. The sequence quality was determined by FastQC and Cutadapt. The filtered readings were mapped to the apple reference genome.

**Transcriptomic data analysis**

The program featureCounts was used to calculate the readings that mapped to each gene. The FPKM of each gene was calculated based on the length of gene, and the reading that mapped to the gene was calculated. Deseq2 software (1.20.0) was used to analyze the differential expression between the two groups. We selected the DEGs with |log2Fold-Change| ≥ 1 and a P-value < 0.05 after multiple correction for subsequent analyses. The DEGs were analyzed with GO and KEGG enrichment analysis of the DEGs by cluster profiler (3.4.4). The different genes were visualized using MapMan software.

**Measurement of root physiological parameters**

The root physiological parameters included the activities of SOD, CAT, and POD, proline content, PRO, SUG, MDA, and the contents of H$_2$O$_2$ and O$_2$·−. Each physiological parameter was measured using kits obtained from Suzhou Kemeing Biotechnology Co., Ltd. (Suzhou, China). All the measurements were performed three times, and the average was calculated for further analysis.

**Function validation of MdWRKY40**

The pRI 101-AN expression vector was cleaved by SalI and BamHI enzymes; the product was subjected to electrophoresis with a 1.0% agarose gel, and the strip was recovered. The CDs and PRI101-AN vectors of MdWRKY40 gene were ligated with T4 DNA ligase at 16 °C for more than 8 h and transformed into E. coli. The recombinant plasmid PRI101-MdWRKY40 was obtained by screening and sequencing. The constructed overexpression vector was transformed into Agrobacterium tumefaciens LBA4404. The positive A. tumefaciens was obtained by PCR and used to infect ‘Orin’ callus. The infected calli were cultured on MS solid media at 24°C in the dark for 1-2 days. It was then transferred to a screening medium that contained 50 mg·L$^{-1}$ kanamycin and 250 mg·L$^{-1}$ carbenicillin. The overexpression of MdWRKY40 was confirmed by PCR.

**Yeast one-hybrid test**

The target gene fragment was ligated with the yeast single hybrid vectors pGAD T7 and pHIS2, respectively, and transformed into Escherichia coli DH5α. The positive clones were screened by PCR and sequenced. A yeast one-hybrid test was operated according to the instructions of TaKaRa’s East transformation system 2. The yeast one-hybrid strain was Y187.

**Electrophoretic mobility shift assays**
The sequence of *MdWRKY40* was inserted into the pET-32a (+) expression vector (Novagen, Madison, WI, USA). The recombinant MdWRKY40 protein was expressed in *E. coli* BL21 (DE3), and the fusion protein MdWRKY40-His was purified by a His-Tagged Protein Purification Kit (CW BIO, Beijing, China). The biotinylated probe was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The fusion protein, probe, and binding buffer were mixed in a centrifuge tube and incubated at 24 °C for 30 min. After 50% glycerol and 5×loading buffer were added to the sample, non-denaturing acrylamide gel electrophoresis was performed, and the protein-nucleic acid strip was transferred to film placed on a nylon film. After the completion of UV crosslinking, the preheated Blocking Buffer closure was added, then HRP Conjugate and 20 mL new Blocking Buffer (ThermoFisher, Shanghai) were incubated at room temperature for 15 min. The Washing Buffer (ThermoFisher, Shanghai) was used after washing and developing.

**LUC activity**

*MdWRKY40* full length CDS inserted into the pHBT-AvrRpm 1 carrier and promoter segments of *MdGLU* into the pFRK1-LUC-nos carrier. Both plasmids were converted simultaneously from the protoplasm of apple callus and then expressed for 6 h at 24 °C. Subsequently, the protoplasm was suspended in 100 µL of cell lysate. The 5 µL cell extract and 20 µL 1 mmol·L\(^{-1}\) 4-MUG were incubated at 37 °C at 1 h, and the 100 µL of 0.2 mol·L\(^{-1}\) sodium acetate was added to the termination reaction. The LUC activity was determined using the Luciferase Reporting Analysis System (Promega, Madison, WI, USA).

**qRT-PCR**

The primers were designed for qRT-PCR by Primer 6.0 software ((Premier Biosoft, Palo Alto, CA, USA). The internal reference gene was actin. The reaction system in the PCR of each primer was SYBR Green Mix 5 µL, primer (10 µM) 0.3 µL, cDNA 1 µL, and dd H\(_2\)O 3.4 µL. The PCR procedures were 50°C 2 min, 95°C 10 min, 95°C 15 s, 65°C 60 s, 72°C (30 cycles), and 72°C 10 min. The primers were synthesized by Sangon Biotech Co., Ltd. The relative quantitative method used the 2\(^{-\Delta\Delta CT}\) method [53]. Each sample had three biological replicates. The primer sequences are shown in Supplementary Table S2.

**Data analysis**

The experimental data was expressed as the means and standard errors (SE) of three biological replicates. SPSS v. 19 (IBM, Inc., Armonk, NY, USA) was used to compare the means for soil physicochemical and microbial diversity data with Duncan’s least significant difference (P=0.05). We determined all the data in GraphPad Prism 8 (San Diego, CA, USA). The annotations of the DEGs were based on the databases of GO, KEGG, and MapMan software.

**Abbreviations**

**RNAseq**

RNA sequencing

**DEGs**

Differentially expressed genes
GO
Gene ontogeny

KEGG
Kyoto Encyclopedia of Genes and Genomes

MAPK
Mitogen-activated protein kinase

ARD
Apple replant disease

AMF
Arbuscular mycorrhizal fungi

AM
Mycorrhizal plants that had not been inoculated with *F. solani*

AMFs
Mycorrhizal plants inoculated with *F. solani*

NM
Non-mycorrhizal plants that had not been inoculated with *F. solani*

NMFs
Non-mycorrhizal plants inoculated with *F. solani*

SOD
Superoxide dismutase

POD
Peroxidase

CAT
Catalase

MDA
Malondialdehyde

PRO
Proline

SOG
Soluble sugar

PCA
Principal components analysis

SA
Salicylic acid

JA
Jasmonate acid

AOS
Allene oxide synthase

TFs
Transcription factors
ETI
Effector-Triggered immunity

qRT-PCR
Quantitative real-time reverse transcriptase-PCR

WT
Wild-type

OE
Over-expression

LUC
Luciferase

CML
Calmodulin-like protein

EMSA
Electrophoretic mobility shift assay

Declarations

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Authors’ contributions

MW, CY and ZM are the experimental designers and executors of this study; XC and XS participate in the experimental guidance; MW, WT and LX participates in the data processing and paper writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication
Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The raw sequence data are available in the NCBI Sequence Read Archive (SRA) repository. The accession number is PRJNA752816, and SRA RunSelector as follows: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA752816. All data supporting the conclusions of this article are included in the article and its additional files.

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Figures

Figure 1
Effects of different treatments on (A) plant growth, (B) fresh weight, (C) dry weight, (D) mycorrhizal colonization, (E) root length, (F) root area, (G) root volume of apple seedlings. Different letters represent significant differences at $P < 0.05$.

**Figure 2**

Effects of different treatments on the physiological indicators of apple seedlings. (A) defensive enzyme activity, (B) reactive oxygen species content, (C) osmotic regulator substances. Different letters represent significant differences at $P < 0.05$. 
Figure 3

Numbers of differentially expressed genes. (A) the experimental design and comparisons, (B) Venn diagram, (C) Diagram illustrating the number of total, upregulated, and downregulated genes in the roots of mycorrhizal plants that were infected with Fusarium solani (AMFs) or not infected (AM) with F. solani and the roots of non-mycorrhizal plants infected with F. solani (NMFs) or not infected (NM).
Figure 4

GO enrichment analysis (A), KEGG enrichment analysis (B), and the Transcription factor (C) numbers of DEGs. The scale bar represents the log2FC (AMFs/NMFs) of DEGs. Red and blue indicate upregulated and downregulated genes, respectively. DEGs, differentially expressed genes; GO, gene ontology; KEGG, the Kyoto Encyclopedia of Genes and Genomes.
Figure 5

Impact of AMF pre-colonization on gene expression in apple roots infected with Fusarium solani. MapMan graphs of (A) biotic stress, (B) secondary metabolism, (C) regulation, (D) cellular response. The scale bar represents the log2FC (AMFs/NMFs) of the DEGs. Red and blue indicate upregulated and downregulated genes, respectively. AMF, arbuscular mycorrhizal fungi; DEGs, differentially expressed genes; NMF, not infected with mycorrhizal fungi.
Figure 6

qRT-PCR verification of the genes in biotic stress process. A total of 22 genes were selected for qRT-PCR analysis from the RNA-Seq data. (A) A heatmap was generated based on the fold-change values for RT-qPCR and the color scale for values was shown at the top. (B) Scatterplots with the correlation between RNA-Seq and qRT-PCR data. qRT-PCR, quantitative real-time reverse transcriptase PCR.
Figure 7

Functional characteristics of ‘Orin’ callus that overexpressed MdWRKY40. (A) qRT-PCR detection of the expression of MdWRKY40 in MdWRKY40-OE transgenic calli. WT represents wild-type calli. OE represents MdWRKY40-OE transgenic lines. (B) Diameters of spots in different apple calli after 4 days of infection with Fusarium solani. (C) Phenotype of WT, empty vector and MdWRKY40 overexpressing, transgenic apple calli inoculated with F. solani after 4 days. (D) Transcript levels of MdPR2, MdPR4, MdPR5, MdPR8,
MdCHIT and MdGLU in OE-MdWRKY40 and control calli infected with F. solani. The error bars indicate the mean values ± standard error (SE) of three independent experiments. OE, overexpression; qRT-PCR, quantitative real-time reverse transcriptase PCR. **P < 0.01.

Figure 8

MdWRKY74 bound to the MdGLU promoter. (A) MdWRKY40 was divided into two fragments, N (N) terminus, and C (C) terminus. (B) The optimal concentration of 3-AT was determined by cloning proMdGLU into the pHIS2 vector. (C) MdWRKY40 interacted with MdGLU promoter fragments as per the Y1H assay. (D) An EMSA analysis revealed that MdWRKY40 binds to the W-box II of the MdGLU promoter. (E) Luciferase reporter (LUC) assays showed the MdWRKY40-mediated activation of proMdGLU.

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