Trichoderma-Induced Ethylene Responsive Factor MsERF105 Mediates Defense Responses in Malus sieversii

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\textit{Trichoderma} can induce plant hormone signal pathways mediating plant defenses, resulting in broad-spectrum resistance to phytopathogens. Herein, \textit{Malus sieversii} seedlings were treated with \textit{Trichoderma} biofertilizer and/or \textit{Alternaria alternata}\ f. sp. \textit{mali}, and transcriptome analysis revealed significant differential expression. There was a high similarity between the transcriptome expression profiles of \textit{Trichoderma}-induced and \textit{A. alternata}-infected \textit{M. sieversii} samples for genes related to jasmonic acid (JA), ethylene, and salicylic acid (SA) signaling pathways. Additionally, \textit{Trichoderma} biofertilizer activated numerous disease-resistant genes (\textit{ERF}, \textit{NAC}, \textit{bHLH}, and \textit{STK}) and defense response genes (\textit{DRP}, \textit{ABC}, and \textit{HSP}). Among transcription factors, members of the \textit{ERF} family were the most differentially expressed (18 \textit{ERFs}), indicating that they may be closely related to defense responses. Among ERFs, differential expression of \textit{MsERF105} was the most significant (upregulated 27.6-fold compared to controls). \textit{MsERF105} was heterologously expressed in PdPap poplar (\textit{Populus davidiana} × \textit{Populus alba} var. \textit{pyramidalis} Louche), and following infection with \textit{A. alternata} (Aal), transgenic PdPap-MsERF105s plants displayed lower malondialdehyde (downregulated 41.4\%) and reactive oxygen species (ROSs) levels, and higher reductase activities, especially superoxide dismutase (SOD; upregulated 77.5\% compared to PdPap-ROK2 plants). Furthermore, the lesion areas of PdPap-MsERF105s leaves were significantly smaller (0.2\%) than those of PdPap-ROK2 leaves (~26.0\%), and the cell membrane integrity was superior for PdPap-MsERF105s leaves. Thus, \textit{MsERF105} enhanced the resistance of PdPap poplar to Aal, presumably because \textit{MsERF105} activates the expression of \textit{PR1} and \textit{PDF1.2}. In conclusion, \textit{Trichoderma} biofertilizer modulated the differential expression of numerous disease resistance genes and defense response genes in \textit{M. sieversii} in response to pathogen attack, and \textit{MsERF105} played important roles in this process.

\textbf{Keywords:} \textit{Trichoderma} biofertilizer, \textit{Malus sieversii}, transcriptome, plant hormone, \textit{Alternaria}
INTRODUCTION

Trichoderma can penetrate and colonize plant roots, triggering plant defense responses, and is commonly used as an inducer of plant defenses against pathogens (Poveda, 2021). The presence of fungal prey and the availability of root-derived nutrients may have been major attractors for the ancestors of Trichoderma to establish themselves in the rhizosphere and thereby facilitate the evolution of positive interactions with plants (Druzhinina et al., 2011). In Trichoderma-root interactions, Trichoderma secretes molecules that trigger induced systemic resistance, such as xylanases, peptaibols, swollenin, and cerato-platanins (Rosa et al., 2012). Eliciting plant response protein 11 (Epi1) secreted by Trichoderma formosa triggers immune responses against Tomato mosaic virus infection in Nicotiana benthamiana, and against Alternaria brassicicola infection in Brassica rapa subsp. chinensis, by activating genes (JAZ1, JAR1,2, CALS, NPR1, PAL1, and ED1) related to defense responses (Cheng et al., 2018). Additionally, 6-pentyl-alpha-pyrone from Trichoderma koningii induces systemic resistance in tobacco against tobacco mosaic virus (TMV), stimulates the activities of pathogenesis-related (PR) enzymes (superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase), and upregulates the expression of defense-related genes (PR-a, PR-b, and PR-10) (Taha et al., 2021).

In recent years, numerous studies have contributed to uncovering the molecular basis of the beneficial effects of Trichoderma on plants. Trichoderma-induced plant transcriptome expression profiles display high similarity to those induced by pathogen infection (Cheng et al., 2018). Furthermore, Trichoderma-induced plant resistance against pathogens is mainly mediated by jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) signaling pathways (Gelsomina et al., 2018). Inoculation with Trichoderma asperellum WKSS02-4-18 strongly induced six defense-related genes in soybean seedlings, namely, those involved in the SA pathway (endoglucanase and chalcone synthase) and the JA/ET pathway (chitinase, defensin precursor, allene oxide synthase, and basic POD genes) (Pimentel et al., 2020). In addition, in tomato induced by Trichoderma erinaceum, SIWRKY31 and SIWRKY37 are upregulated, whereas SIWRKY4 is downregulated, resulting in increased resistance to the vascular wilt pathogen Fusarium oxysporum f. sp. lycopersici (Aamir et al., 2019). Plants treated with Trichoderma harzianum T22 displayed overexpression of transcripts encoding several families of defense-related transcription factors (bZIP, MYB, NAC, ERF, and WRKY), suggesting that the fungus contributes to the priming of plant responses (Coppola et al., 2019). Besides, in our previous, it is found that Trichoderma rossicum T7 (TroT7) and T. harzianum T2 (ThaT2) can enhance the resistance of Malus sieversii to F. oxysporum and Alternaria alternata f. sp. mali (Aalm) (Fang et al., 2019). Thus, Trichoderma can trigger plant defense responses in different pathways.

ERF transcription factors are the largest branch of the AP2/ERF transcription factor superfamily, which is characterized by a conserved AP2 binding domain of 57–66 amino acids (Huang et al., 2020). The AP2/ERF domain contains two conserved elements, namely, YRG and RAYD (Okamura et al., 1997). ERF genes are involved in responses to biotic stresses through plant hormone signaling pathways (Caarl et al., 2016). Similarly, soybean GmERF3 transgenic tobacco can respond to the regulation of many plant hormones, such as SA, JA, and ET (Zhang et al., 2009). In addition, in transgenic tobacco, the transcription factor GmERF3 activated some PR genes expression, and improved the resistance of tobacco to Ralstonia solanacearum, A. alternata (Aalm), and TMV (Zhang et al., 2009). Overexpression of ZmERF105 can improve the resistance of maize to northern leaf blight (Exserohilum turcicum), while the erf105 mutant showed the opposite phenotype. In addition, the activities of SOD and POD in ZmERF105 overexpression lines were significantly higher than those in wild-type plants (Zang et al., 2020). Similarly, overexpression of ZmERF105 enhanced the expression of PR genes such as ZmPR1a, ZmPR2, ZmPR5, ZmPR10.1, and ZmPR10.2 (Zang et al., 2020). AtERF96 overexpression enhances the resistance of Arabidopsis thaliana to necrotic pathogens such as Botrytis cinerea and Pectobacterium carotovorum by upregulating the expression of PDF1.2a, PR-3, PR-4, and ORA59 (Catinot et al., 2015).

In the present study, M. sieversii seedlings were treated with Trichoderma biofertilizer or Aalm, and differentially expressed genes (DEGs) were analyzed by RNA sequencing (RNA-Seq). Gene ontology (GO) function classification, Clusters of Orthologous Groups (COG) function classification, and Kyoto Encyclopedia of Genes and Genomes (KEGG) classification analyses were conducted. In addition, differential expression of transcription factors was explored. The M. sieversii MsERF105 gene was heterologously expressed in Populus davidiana × Populus alba var. pyramidalis Louche (PdpP poplar), and PdpP seedlings were infected with Aalm. The antioxidant ability of transgenic PdpP-MsERF105s plants was evaluated by determining malondialdehyde (MDA) and reactive oxygen species (ROSs) content, reductase (SOD, POD, and CAT) activities, resistance to Aalm, relative lesion area, and cell membrane permeability. Differential expression of defense response genes (PR1 and PDF1.2) was assessed. Based on the above results, the functions of ERFs in defense responses in M. sieversii were evaluated.

MATERIALS AND METHODS

Plant Material and Strains
Malus sieversii seeds from a Xinjiang wild fruit forest were germinated in the laboratory (temperature 28°C and humidity 70%) to obtain 2-month-old seedlings. Apple-spotted defoliation pathogen Aalm was obtained from the Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography. Poplar leaf blight pathogen Aalm was obtained from the State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University, China). Agrobacterium tumefaciens EHA105 and the pROK2 vector were used for the genetic transformation of PdpP poplar. PdpP poplar seedlings
were cultured aseptically in a liquid rooting medium at 25°C (Ji et al., 2017).

Preparation of *Trichoderma* Biofertilizer

Spores of TroT7 and ThaT2 were separately inoculated into potato dextrose agar (PDA) medium and cultured at 28°C for 1 week. Both *Trichoderma* strains were identified in a previous study (Ji et al., 2021). Spores were collected and diluted to 10^8 spores/ml. The number of spores was counted using a blood cell counting board. Next, 10 ml of spore suspension was added to 300 g solid fermentation medium (thickness 1–1.5 cm) and mixed. The solid fermentation medium for *Trichoderma* comprised rice husk (19.9% w/w), wheat bran (5% w/w), (NH_4)_2SO_4 (0.1% w/w), and H_2O (75% w/w). The tray was then sealed with preservative film. TroT7 and ThaT2 were cultured at 28°C for 1 week, spores of both *Trichoderma* strains were mixed (1:1) as *Trichoderma* biofertilizer, and the concentration of conidia in the biofertilizer was 10^8 spores/ml.

Collection of Samples for RNA-Seq

Two-month-old *M. sieversii* seedlings were subjected to four different treatments, three biological replicates each treatment were set. The samples treated without *Trichoderma* biofertilizer and Aalm, as negative control, were collected at 0 h, the other samples were collected at 6 and 48 h post-induction (hpi), and all samples were stored at −80°C for RNA-Seq. For the Tri treatment, seedlings were irrigated with 50 ml *Trichoderma* biofertilizer (10^8 spores/ml), one leaf of each seedling was stabbed, and inoculated without Aalm mycelium. For the Aalm treatment, seedlings were irrigated with 50 ml sterilized water, and one leaf of each seedling was stabbed and inoculated with 0.6 cm disks containing Aalm mycelium, which was cultured in PDA medium at 28°C for 10 days, and 0.6 cm disks were cut with a hole punch. For the Tri + Aalm treatment, seedlings were irrigated with 50 ml *Trichoderma* biofertilizer (10^8 spores/ml), and one leaf of each seedling was stabbed and inoculated with 0.6 cm disks containing Aalm mycelium. For the negative control, seedlings were irrigated with 50 ml sterilized water, one leaf of each seedling was stabbed and inoculated without Aalm mycelium.

RNA-Seq

Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Jiang and Zhang, 2003), following analysis of RNA quality using a NanoDrop 2000C instrument at 0, 3, 6, 12, and 24 hpi, and untreated seedlings served as controls, three technical replicates were performed for each cDNA sample. Primers for RT-qPCR (Supplementary Table 1) were designed by using Primer Premier 6.0 software (PREMIER Biosoft, San Francisco, CA, United States).

Expression of Genes Involved in Plant Hormone Signal Pathway Using RT-qPCR

Following the three treatments (Tri, Aalm, and Tri + Aalm) at 28°C and 70% humidity, the treatment of samples was the same as that described for RNA-Seq, three plants (biological replicates) each treatment were set. Leaf samples were collected at 6 and 48 hpi, and untreated seedlings served as controls, leaf samples were collected at 0 hpi. Total RNA was extracted using the CTAB method, and reverse-transcribed into cDNA for quantitative real-time polymerase chain reaction (RT-qPCR). The gene-encoding *MsActin* (MZ605395) in *M. sieversii* was used as an internal reference. Three technical replicates were performed for each cDNA sample. Primers for RT-qPCR (Supplementary Table 1) were designed by using Primer Premier 6.0 software (PREMIER Biosoft, San Francisco, CA, United States).

Expression of ERF Family Genes Using RT-qPCR

The method is the same as given in the section “Expression of genes involved in plant hormone signal pathway using RT-qPCR.” Following the three treatments (Tri, Aalm, and Tri + Aalm) at 28°C and 70% humidity, three plants (biological replicates) each treatment were set. Leaf samples were collected at 0, 3, 6, 12, and 24 hpi, and untreated seedlings served as controls. Three technical replicates were performed for each cDNA sample. Primers for RT-qPCR (Supplementary Table 1) were designed by using Primer Premier 6.0 software (PREMIER Biosoft, San Francisco, CA, United States).

Construction of the Plant Expression Vector pROK2-MsERF105 and Poplar Transformation

The *MsERF105* gene was cloned by PCR using a sense primer (5′-ATCGTCTCAGGAGTTTCTAAATATGGCATCAGAACG-3′) containing an XbaI (Promega, WI, United States) site (underlined) and an antisense primer (5′-CGATGGTACCTCAAATATACCCAGCGGGAGGATAC-3′) containing a KpnI (Promega, United States) site (underlined). The resulting *MsERF105* PCR product and the pROK2 vector were double digested with XbaI and KpnI at 37°C for 4 h, and ligated using T4 DNA ligase (Promega, United States) at 4°C for 12 h, to generate the pROK2-MsERF105 construct. *MsERF105*
was transferred into PdPap poplar using the A. tumefaciens-mediated transformation system, and empty vector pROK2 was transferred in PdPap to generate the PdPap-ROK2 control. The method was described in detail in our previous study (Ji et al., 2016).

The Resistance of Transgenic PdPap-MsERFs Plants to Aal
Aal was cultured in a PDA medium at 28°C for 10 days, and 0.6 cm disks were cut with a hole punch. Leaves from PdPap-MsERF105s and the control PdPap-ROK2 plants were stabbed with a needle, covered with 0.6 cm disks, and cultured at 28°C for 5 days, three biological replicates each treatment were set. The relative area of disease spots was calculated by the Chalkiness 1.0 program (China). First, infected leaves were scanned to obtain digital images, the software accurately determined the location and profile of disease spots, and the relative area of disease spots was evaluated (Zheng and Wu, 2008).

Determination of MDA Content and Reductase Activity
Following treatment of PdPap-MsERF105s and the control PdPap-ROK2 plants with 0.6 cm disks containing Aal mycelia, leaves were collected at 0, 3, 6, 12, 24, 48, and 72 hpi, 0.1 g was weighed using an analytical balance, ground in a pestle and mortar with liquid nitrogen, and the leaf powder was added to extraction solution (from the kit mentioned below) and centrifuged at 1,100 × g for 10 min, three biological replicates each treatment were set. The supernatant was used to determine the MDA content using an MDA-2-Y kit (Suozhou Comin Biotechnology Co., Ltd., China). The activities of SOD, POD, and catalase (CAT) were determined using dedicated kits (POD = A084-3; SOD = A001-1; CAT = A007-1; Nanjing Jiancheng Bioengineering Institute, China), three biological replicates each treatment were set.

Leaf Staining for ROS Quantification and Plant Membrane Integrity
Evans blue and nitrotetrazolium blue chloride (NBT) were dissolved in 10 mM phosphate-buffered saline (pH 7.8), and diaminobenzidine (DAB) were dissolved in 50 mM Tris- acetate buffer (pH 5.0) (Hernández et al., 2001), and the final concentration of the staining solution was 1 mg/ml. NBT and DAB staining solutions were used immediately after preparation. Following treatment of PdPap-MsERF105s and the control PdPap-ROK2 plants with 0.6 cm disks containing Aal mycelia for 3 days, leaves were cut and stained with each of the three staining solutions as described in our previous study (Ji et al., 2021), and 10 biological replicates of each treatment were set.

Differential Expression of Defense Response Genes in PdPap-MsERFs Plants
Following treatment of PdPap-MsERF105s and the control PdPap-ROK2 plants with 0.6 cm disks containing Aal mycelia, leaves were collected at 0, 3, 6, 12, 24, 48, and 72 hpi, and the expression levels of two PR1 genes and two PDF1.2 genes were determined by RT-qPCR, three biological replicates and technical replicates each treatment were set, respectively. The gene encoding PdActin in PdPap poplar served as an internal reference. The method was the same as described above for RT-qPCR analysis of ERF family genes. All primers are listed in Supplementary Table 1.

Statistical Analysis
Statistical analysis was performed using SPSS 17.0 (IBM Corp., Armonk, NY, United States). Data are expressed as the mean of three or more independent replicates ± SD. Analysis of variance was conducted using Duncan’s test, and p ≤ 0.05 was considered significant.

RESULTS

Differential Expression Following Treatment of M. sieversii With Trichoderma Biofertilizer and/or Aalm
Following treatment with Trichoderma and/or Aalm, the gene expression pattern of M. sieversii was significantly different from that of controls, and the three replicate treatments yielded highly similar results, especially the three control replicates, which shared > 98% similarity (Figure 1A and Supplementary Table 2). In addition, the gene expression patterns of M. sieversii treated with Trichoderma biofertilizer and/or Aalm at the same timepoint were also similar (Figure 1A). Furthermore, the number of differential genes in M. sieversii treated with Trichoderma biofertilizer or Aalm was less than that following treatment with both, and more genes were upregulated than downregulated under all treatment conditions (Figure 1B).

Trichoderma Biofertilizer Induces Plant Hormone Signal Transduction Pathways in M. sieversii
Following treatment of M. sieversii with Trichoderma biofertilizer, GO function analysis showed that the identified DEGs were linked to response to stimulus, immune system process, and signal transducer activity categories (Figure 2A). Meanwhile, COG function classification also indicated the DEGs were closely related to signal transduction mechanisms (130 DEGs, 7.99% of all COG annotation genes) and defense mechanisms (23 DEGs, 1.41% of all COG annotation genes; Figure 2B). Finally, KEGG classification analysis indicated that the DEGs were involved in plant hormone signal transduction (40 DEGs, 5.12%) and plant-pathogen interactions (21 DEGs, 2.69%; Figure 2C), such as Auxin/indole-3-acetic acid (AUX/IAA), JA, and SA signal transduction pathways. Following treatment of M. sieversii with Trichoderma biofertilizer and/or Aalm, the expression patterns of related DEGs were relatively similar to each other. Compared with Aalm treatment, Trichoderma biofertilizer induced higher expression levels (Figures 2D,E), which indicates that Trichoderma biofertilizer may activate JA and SA signal transduction pathways by upregulating the expression of MYC2 and NPR1.

REFERENCE

Ji et al. (2021)
FIGURE 1 | The differential expression genes of *Malus sieversii* treated with *Trichoderma* biofertilizer and Aalm. (A) The correlation analysis of gene expression pattern. T1-3 (Con): the control; T4-6 (Aalm6): *M. sieversii* treated with *A. alternata* f. sp. *mali* for 6 h; T7-9 (Tri6): *M. sieversii* treated with *Trichoderma* biofertilizer for 6 h; T10-12 (Tri + Aalm6): *M. sieversii* treated with *Trichoderma* biofertilizer and *A. alternata* f. sp. *mali* for 6 h; T13-15 (Aalm48): *M. sieversii* treated with *A. alternata* f. sp. *mali* for 48 h; T16-18 (Tri48): *M. sieversii* treated with *Trichoderma* biofertilizer for 48 h; and T19-21 (Tri + Aalm48): *M. sieversii* treated with *Trichoderma* biofertilizer and *A. alternata* f. sp. *mali* for 48 h. (B) The number of differential expression genes.

**Differentially Expressed Genes Related to Defense Responses in *M. sieversii***

Following treatment of *M. sieversii* with *Trichoderma* biofertilizer and/or Aalm, expression levels of numerous resistance genes and defense response genes were significantly different (Figure 3). Resistance genes included *STK*, *LRR-RLK*, *AP2/ERF*, *NAC*, *bHLH*, *MYB*, and *HSF*. Differences in expression of these resistance genes were more significant after treating *M. sieversii* for 6 h than 48 h (Figure 3A). *AP2/ERF*, *NAC*, *bHLH*, *MYB*, *bZIP*, and leucine zipper genes were upregulated and downregulated after treatment for 6 h. These transcription factors can positively or negatively regulate downstream gene expression. Defense response genes included *DPR*, *ABC*, *HSP*, and *PR*, and the expression levels of the defense response genes were significantly upregulated after treatment for 6 and 48 h (Figure 3B). Thus, following treatment with *Trichoderma* biofertilizer and Aalm, expression levels of transcription factors were altered, and defense response genes were activated in response to pathogen attack.

**Differential Expression of ERF Transcription Factor Family Genes**

ERF transcription factors are closely related to plant defense responses. Following treatment with *Trichoderma* biofertilizer for 6 h, the number of differentially expressed genes belonging to the AP2/ERF superfamily was more than other transcription
FIGURE 2 | Trichoderma biofertilizer activates the plant hormone signal transduction pathways. (A) GO function analysis after M. sieversii treated with Trichoderma fertilizer for 6 h. (B) COG function classification of consensus sequence after M. sieversii treated with Trichoderma fertilizer for 6 h. (C) KEGG classification analysis after M. sieversii treated with Trichoderma fertilizer for 6 h. (D) DEGs involved in AUX/IAA, JA, and SA, after M. sieversii treated with Trichoderma fertilizer for 6 h. (E) Verify expression of genes in panel (D) using RT-qPCR. The data were counted by RT-qPCR, ANOVA was conducted using Duncan’s method, and $p \leq 0.05$ was considered significant. The gene sequences have been deposited in GenBank, c23659.graph_c1 (MZ736861), c36601.graph_c0 (MZ736862), c18973.graph_c0 (MZ736863), c40102.graph_c0 (MZ736864), c33528.graph_c0 (MZ736865), c43172.graph_c2 (MZ736866), c19865.graph_c0 (MZ736867), c40416.graph_c0 (MZ736868), c38512.graph_c1 (MZ736869), and c36229.graph_c0 (MZ736870). COG: Clusters of Orthologous Groups, DEGs: differentially expressed genes, GO: gene ontology, JA: jasmonic acid, KEGG: Kyoto Encyclopedia of Genes and Genomes, RT-qPCR: quantitative real-time PCR, SA: salicylic acid.

factor families, including total of 19 AP2/ERF superfamily genes (Figure 4A). We, therefore, speculated that ERF family genes are widely involved in responding to pathogen attack in M. sieversii. The transcriptome data revealed that 11 ERF genes were differentially expressed, and expression levels of MsERF105 were the highest among ERF family genes (Figure 4B). RT-qPCR analysis of ERF family genes also confirmed that Trichoderma biofertilizer strongly induced ERF genes expression, but mostly the expression of MsERF105 (Figure 4C). Thus, MsERF105 was selected for heterologous expression in transgenic PdPap plants to assess gene function.

Antioxidant Ability of Transgenic PdPap-MsERF105 Plants
Pathogen attack can cause an elevation in ROS, resulting in severe damage to plants. Herein, transgenic PdPap-MsERF105s plants were treated with Aal, and the MDA
were hpi, and SOD activity peaked at 48 hpi. Additionally, values in PdPap-MsERF105s plants compared to controls after 6 hpi, the activities of SOD and CAT were significantly increased determined. The results showed that following treatment with 63.8 nmol/min/g for all the three replicates, compared with only 55.0 nmol/min/g, compared with only 64.2 nmol/min/g for controls (Figure 5B). POD activity was also significantly increased compared to controls at 12 hpi, and it peaked at 48 hpi. Again, values for all the three replicates were > 63.8 nmol/min/g, compared with only 55.0 nmol/min/g for the controls (Figure 5C). CAT activity increased over time, values were > 268.2 nmol/min/g for all the three replicates, and they were increased by ~37.9% compared to the controls (194.5 nmol/min/g; Figure 5D). After infection by Aal for 3 days, DAB and NBT staining showed that PdPap-MsERF105s leaves were lighter brown or blue in color compared to those of controls (Figures 5E,F), indicating that PdPap-MsERF105s leaves contained less ROS than control leaves after pathogen attack.

Resistance of Transgenic PdPap-MsERF105s Plants to Aal

To further verify the resistance ability of PdPap-MsERF105 plants, leaves were inoculated with Aal mycelium disks. The results revealed no lesions on the leaves of all the three PdPap-MsERF105s replicates at 5 days postinfection (dpi), but there were large lesions on leaves of the controls at 5 dpi, and the relative lesion area was 26% (Figure 6A). In addition, following infection by Aal for 5 days, Evans blue staining results showed that PdPap-MsERF105s leaves were lighter blue than those of the controls (Figure 6B), indicating that the cell membrane integrity of PdPap-MsERF105s plants was better than that in the controls after pathogen attack.

Differential Expression of Defense Response Genes in PdPap-MsERF105 Plants

To explore whether MsERF105 could activate the expression of downstream defense response genes, the expression levels of two PR1s (PR1-1 and PR1-2) and two PDF1.2 proteins (PDF1.2-1 and PDF1.2-2) were determined. The results showed that following infection by Aal, expression levels of PR1-1 were significantly higher than the controls, with an increase after 12 hpi and a peak at 48 hpi. For PdPap-MsERF105-3, the increase was 7.41 (22.89)-folds (Figure 7A). PR1-2 expression levels were also increased in all the three PdPap-MsERF105s replicates after 24 hpi (Figure 7B). The expression levels of two PDF1.2 genes were upregulated after 24 hpi, and increased over time, and the expression levels of PDF1.2-1 were the highest (24.4-folds) in PdPap-MsERF105-2 (increased by 18-fold compared with the control PdPap-ROK2 (Figure 7C). Expression levels of PDF1.2-2 were the highest (24.8-fold) in PdPap-MsERF105-2, an increase of ~28-fold compared with the control PdPap-ROK2 (Figure 7D).

DISCUSSION

Trichoderma genus, the widely used biocontrol agents, can induce plant hormone signal pathways, and thereby endow plants with broad-spectrum resistance to phytopathogens (Manigundan et al., 2016). Herein, *M. sieversii* seedlings were treated with *Trichoderma* biofertilizer and/or Aalm, and transcriptomes showed significant differences between treatments. *M. sieversii*
防御机制。此外，治疗后许多DEGs都参与了信号传导并表达，探索了它们的功能。

我们随后调查了哪种基因在生物制剂和Aalm治疗的幼苗中差分表达。更多DEGs与单独使用任一种剂的幼苗相比。因此，我们推测Trichoderma生物制剂可以强烈诱导Erf家族成员的表达。

**图4**| 11种Erf家族转录因子基因的表达差异。**A** | Trichoderma生物制剂和Aalm治疗的幼苗中差分表达的转录因子家族成员。**B** | Trichoderma生物制剂和Aalm治疗的幼苗中差分表达的Erf家族成员。**C** | 杂交图显示了不同条件下的Erf家族成员的转录因子。

种苗与Trichoderma生物制剂和Aalm相比，治疗了更多的DEGs。我们进一步探究了它们的作用和功能。

基因按COG和KEGG分类，表明许多DEGs都参与了信号转导和防御机制。此外，治疗了与Trichoderma生物制剂和Aalm相比，有更多的DEGs。我们推测了这些基因的差分表达和功能。

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基因按COG和KEGG分类，表明许多DEGs都参与了信号转导和防御机制。此外，治疗了与Trichoderma生物制剂和Aalm相比，有更多的DEGs。我们推测了这些基因的差分表达和功能。

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FIGURE 5 | Antioxidant ability of PdPap-MsERF105. (A) The MDA content. (B) The SOD activity. (C) The POD activity. (D) The CAT activity. (E) DAB staining of PdPap leaves. (F) NBT staining of PdPap leaves. ANOVA was conducted using Duncan’s method, and $p \leq 0.05$ was considered significant. CAT: catalase, DAB: diaminobenzidine, MDA: malondialdehyde, NBT: nitrotetrazolium blue chloride, POD: peroxidase, SOD: superoxide dismutase.
FIGURE 6 | The resistance of PdPap-MsERF105s to Aal. (A) The lesion on PdPap-MsERF105s and PdPap-ROK2 after being infected by Aal. (B) The cell membrane integrity of PdPap-MsERF105s and PdPap-ROK2 after being infected by Aal. ANOVA was conducted using Duncan’s method, and $p \leq 0.05$ was considered significant.

FIGURE 7 | The differential expression of PR1 and PDF1.2 in transformant PdPap-MsERF105s. Following infected by Aal, the expression levels of (A) PR1-1, (B) PR1-2, (C) PDF1.2-1, and (D) PDF1.2-2 were determined by RT-qPCR. ANOVA was conducted using Duncan’s method, and $p \leq 0.05$ was considered significant. RT-qPCR: quantitative real-time PCR.
Verticillium dahliae (Liu et al., 2017). Thus, MsERF105 may play an important role in the defense responses of M. sieversii.

To explore the functions of MsERF105 in defense responses, the protein was heterologously expressed in PdPap poplar. Following treatment with Aal, the MDA content was determined, and NBT and DAB staining indicated that the antioxidant ability of transgenic PdPap-MsERF105s plants was superior to that of PdPap-ROK2 plants, presumably because MsERF105 enhanced the activity of reductases (SOD, POD, and CAT). Similarly, under cold stress, when Cynodon dactylon CdERF1 was heterologously expressed in Arabidopsis plants, the MDA content was reduced, and the activities of SOD and POD were elevated (Hu et al., 2020). Furthermore, the activities of SOD and POD in ZmERF105-overexpressing lines were markedly higher than in wild-type maize lines after infection with E. turcicum (Zang et al., 2020). Thus, MsERF105 may help to clear ROS after pathogen attack.

Following treatment with Aal mycelia, lesions on PdPap-MsERF105s leaves were significantly smaller than those on PdPap-ROK2 leaves, and cell membrane integrity in PdPap-MsERF105s leaves was better than in PdPap-ROK2 leaves. This suggests that MsERF105 enhanced the resistance of PaPap poplar to Aal, possibly because MsERF105 activates the expression of PR1 and PDF1.2. Additionally, overexpression of AcERF2 induced the accumulation of transcripts of plant defense-related genes (PR1, PR2, and PR5), and increased Arabidopsis resistance to the pathogens Pseudomonas syringae pv. tomato DC3000 and B. cinerea (Sun et al., 2018). Overexpression of ZmERF105 in soybean enhanced the expression of several PR genes, including ZmPR1a, ZmPR2, ZmPR5, ZmPR10.1, and ZmPR10.2, following infection with E. turcicum (Zang et al., 2020). These results indicate that MsERF105 plays a positive modulatory role in response to pathogen infection in M. sieversii.

In conclusion, Trichoderma biofertilizer not only activated JA and SA signaling pathways but also induced the differential expression of numerous disease resistance genes and defense response genes, especially ERF transcription factor family members. Heterologous expression of MsERF105 significantly enhanced the antioxidant and antipathogen abilities of transgenic PdPap poplar. The findings suggest that MsERF105 is crucial for the response to pathogen attack in M. sieversii.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The raw transcriptome data have been deposited in the SRA database. The accession numbers of Con transcriptome data are SRR16148375, SRR16148374, and SRR16148363, the accession numbers of T6 transcriptome data are SRR16148361, SRR16148360, and SRR16148359, the accession numbers of A6 transcriptome data are SRR16148358, SRR16148357, and SRR16148356, the accession numbers of TA6 transcriptome data are SRR16148373, SRR16148372, and SRR16148355, the accession numbers of T48 transcriptome data are SRR16148371, SRR16148370, and SRR16148369, the accession numbers of A48 transcriptome data are SRR16148368, SRR16148367, and SRR16148366, and the accession numbers of TA48 transcriptome data are SRR16148365, SRR16148364, and SRR16148362. And the transcriptome assembly data have been deposited in the SRA database, and the accession number is GJMU 00000000. BioProject number is PRJNA 736527, and BioSample number is SAMN 19651934.

AUTHOR CONTRIBUTIONS

SJ: conceptualization, formal analysis, investigation, methodology, and roles/writing – original draft. ZL: conceptualization, data curation, formal analysis, funding acquisition, methodology, supervision, and writing – review and editing. YW: conceptualization, data curation, funding acquisition, methodology, project administration, supervision, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.708010/full#supplementary-material

Supplementary Figure 1 | Poplar transformation PdPap-MsERF105s construction. (a) Resistant buds of transformant PdPap-MsERF105s; (b) asexual reproduction of PdPap-MsERF105s; and (c) transcription detection of MsERF105 gene in transformant PdPap-MsERF105s by RT-qPCR. The data were counted by RT-qPCR, ANOVA was conducted using Duncan’s method, and \( p \leq 0.05 \) was considered significant. RT-qPCR: quantitative real-time PCR.

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