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ORIGINAL PAPER

Ectopic Expression of a Proteinase Inhibitor I4 (MtPiI4) Gene from *Medicago truncatula* Confers Plant Resistance to *Pseudomonas syringae* pv. Tomato DC3000

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Abstract Proteinase inhibitors (PIs) play an important role in plant responses to biotic and environmental stimuli, but little is known about the role of PIs in mediating plant immune responses to microbial infection. In this study, a gene named *proteinase inhibitor I4* (*MtPiI4*) was isolated from *Medicago truncatula* and characterized as a serpin family gene with a typically conserved DUF716 domain. *MtPiI4* was differentially expressed in seed, root, leaf, stem and flower tissues. Expression of *MtPiI4* was induced by inoculation with a typical bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 strain (*Pst* DC3000). It was also up-regulated by methyl jasmonate (MeJA) treatment. To identify its function in regulating plant immunity against *Pst* DC3000, we constructed transgenic Arabidopsis plants over-expressing *MtPiI4*. Compared to wild type, *35S*::*MtPiI4* plants showed enhanced resistance to *Pst* DC3000. Expression of JA biosynthetic and responsive genes such as *LOX2*, *PDF1.2*, and *VSP1* was depressed in *35S*::*MtPiI4* plants as compared to wild type, suggesting that the JA signaling response was attenuated in *35S*::*MtPiI4* plants upon *Pst* DC3000 exposure. Furthermore, over-expression of *MtPiI4* led to up-regulation of *NPR1* (nonexpressor of pathogenesis-related gene 1—a negative regulator of JA signaling) and down-regulation of *MAPK4* (mitogen-activated protein kinase4—a positive regulator of JA signaling). These results indicate that *MtPiI4* regulation of plant resistance to *Pst* DC3000 is involved in the JA signaling transduction pathway.

Keywords *Medicago truncatula* · *MtPiI4* · Methyl jasmonate · Proteinase inhibitor · *Pseudomonas syringae* pv.tomato DC3000

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Introduction

Proteinase inhibitors (PIs) are a group of plant polypeptides (or proteins) acting against biotic and abiotic stresses (Jongsma and Beekwilder 2011; Kim et al. 2009; Zhang et al. 2008). Based on the active amino acids in their reaction centers, four types of proteases are categorized: serine, cysteine, aspartic and metallo-proteinases (Koiwa et al. 1997). The serine proteinase inhibitors (PiI4 or serpin) are the PIs most frequently identified owning to their diverse biological functions (Fluhr et al. 2012; Roberts and Hejgaard 2008). In practice, plant PiI4 can be further classified into seven subfamilies, namely soybean trypsin inhibitor (Kunitz), soybean proteinase inhibitor (Bowman-Birk), Potato I, potato II, barley trypsin inhibitor, squash inhibitor and steptomyces subtilisin inhibitor (SSI) (Ryan 1989). PIs have been characterized with two basic functions: (1) to prevent uncontrolled proteolysis in cells to ensure the normal function of limited proteolysis, and (2) to protect proteins from foreign proteolytic enzymes (Ryan 1989). While PIs participate in nutrient accumulation due to their high contribution (usually 1–10 %, even 50 %) to total proteins in seeds and vegetative organs (Pearce et al. 1988;

Ryan 1989), the most important characteristic of PIs is their contribution to protection of plants against pathogen and insect attack (John 2011; Schlüter et al. 2010).

Plant PIs have been reported to induce high mortality of insects by binding to insect digestive proteinases to block protein digestion (Dunse et al. 2010; Mosolov and Valueva 2008). In a field trial, co-expression of two types of serpin (potato type I and II proteinase inhibitors) in cotton conferred resistance to insect attack (Dunse et al. 2010). By contrast, *Solanum nigrum* plants with silenced serpin activity displayed a significantly higher degree of damage caused by generalist herbivores than the wild type; however, plant growth and development were not affected by silencing serpins, which suggests that serpins play a major role in mediating insect proteases but not endogenous proteases in the plant itself (Hartl et al. 2010). There are some cases indicating that plant serpins have the potential to block the growth of a variety of pathogenic bacterial and fungal strains (Hu et al. 2007, 2009; Kim et al. 2009). While serpins working in animals play a role in regulating the host immune response rather than interacting directly with pathogens (Law et al. 2006), those in plants work by way of a competitive interaction between plant immunity and pathogen infection (McDowell 2011; Spoel and Dong 2012). To date, despite studies focusing on manipulation of serpins for plant tolerance to exogenous pathogens or insects (Alvarez-Alfageme et al. 2011; Kim et al. 2009), little is known about whether and how plant serpins regulate plants' own intrinsic immune responses against pathogen infection.

Jasmonic acid (JA) and its derivative methyl jasmonate (MeJA) are natural plant signaling molecules playing crucial roles in plant responses to various biotic stresses (Avanci et al. 2010). The positive regulation of PIs by JA signaling has been demonstrated as an effective plant strategy against insect attack (Hartl et al. 2010; Lomate and Hivrale 2012). JA can play a negative role in the plant response to microbes in an antagonistic way via salicylic acid (SA), which makes plant resistance to pathogen infection (Pieterse et al. 2009; Vlot et al. 2009). For instance, *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) utilizes JA signaling to depress SA-mediated defense system to allow its invasion and development in host plants (Brooks et al. 2005; Laurie-Berry et al. 2006; Zheng et al. 2012). The antagonism between SA and JA is differentially regulated by intracellular factors and signaling molecules such as NPR1and MAPK4 in plants (Pieterse et al. 2009). Acting as a major positive regulator in SA signaling, NPR1 is required for repression of JA signaling in the plant response to *Pst* DC3000 (Spoel et al. 2003). As a repressor of SA-induced defensive signaling pathways, MAPK4 is necessary for JA-responsive gene expression in the plant response to *Pst* DC3000 (Petersen et al. 2000). In maize (*Zea mays*), a cysteine proteinase inhibitor has been reported to signal defense against *Ustilago maydis* in a negative way involving SA (van der Linde et al. 2012). Both *AtSepin1* and *AtSRP4* belong to the serpin family of genes identified in *Arabidopsis thaliana*; interestingly, expression of *AtSepin1* was significantly induced by both *Pst* inoculation and cold stress (Fluhr et al. 2012; Roberts and Hejgaard 2008). Similarly, expression of *AtSRP4* was markedly induced after salinity stress (Fluhr et al. 2012). These results indicate that plant serpins play potential roles in regulating the plant response to biotic and abiotic stresses. We recently isolated a serpin gene (here designed *MtPiI4*) from *Medicago truncatula* in response to heavy metal mercury exposure (Zhou et al. 2013). Examination of *MtPiI4* revealed that it could be regulated by pathogen exposure. Here, we describe the functional characterization of *MtPiI4* in the plant response to *Pst* DC3000. Our study revealed that *MtPiI4* was induced by MeJA and inoculation by *Pst* DC3000. Transgenic Arabidopsis over-expressing *MtPiI4* showed enhanced resistance to *Pst* DC3000 and reduced sensitivity to MeJA. This biological response is likely involved in the JA signaling pathway because both *NPR1* and *MAPK4* were regulated in *35S*::*MtPiI4* plants upon *Pst* DC3000 exposure.

Materials and Methods

Plant Materials and Cultivation

Seeds of *M. truncatula* (cv. Jemlog) were surface-sterilized and rinsed thoroughly with sterile water. The seeds were germinated in a growth chamber at 22 °C. The germinating seeds were transferred to ½ Hoagland nutrient solution for hydroponic cultivation for 4 weeks. The growth condition was set at 22 °C with 100 μE m^{-2} s^{-1} photosynthetically active radiation and a 16 h light/8 h dark cycle (Zhou et al. (2008). For *Arabidopsis thaliana*, sterilized seeds (ecotype Col-0) were germinated on Murashige and Skoog (MS) solid medium in a growth chamber under the same conditions described above. After 7 days, seedlings were transferred to half-strength Hoagland nutrient solution and grown for 3 weeks (Song et al. 2012). The prepared seedlings indicated above were used for treatment and analysis.

Plant Inoculation with *Pst* DC3000

Pst DC3000 strain was cultured with KB medium containing 100 mg L^{-1} rifampicin in a shaker at 28 °C. *Pst* DC3000 in the exponential phase was collected and re-suspended in 10 mM $MgCl_2$ for inoculation. Bacterial inoculation on plant leaves was performed using syringe injection as described by Zipfel et al (2004). $MgCl_2$ solution (10 mM) was injected to plants as a negative control. For Arabidopsis, a bacterial suspension containing 0.02 % silwet L-77 was sprayed on leaves until the surface was uniformly wetted (Keith et al. 2003). $MgCl_2$(10 mM) solution containing 0.02 % silwet L-77 was sprayed on plants as a negative control. Plants inoculated with bacteria were kept in the dark for 1 h with plastic wrap to allow

bacteria to grow, and were then transferred to normal growth conditions as mentioned above. For dose-dependent experiments, plants were inoculated with microbial suspensions with different concentrations (OD_{600}=0–2). In time-course experiments, plant samples were harvested after inoculation with bacterial suspension (OD_{600}=0.2) for different times.

MeJA Treatment

Plants were treated with MeJA according to the method described by Brown et al. (2003). MeJA (TCI, Shanghai, China) was dissolved into sterile water with the aid of 5 % ethanol. Before treatment, plants were transferred into a vessel with a fixed volume of 1.5 L that was sealed with plastic wrap. MeJA solution at different concentrations (0–500 μM) was sprayed onto plant leaves. Sterile water containing 5 % ethanol was sprayed on plants as a control.

Plant Transformation

MtPiI4 was PCR-amplified using primers with restriction enzyme sites at the 5′-end of forward and reverse primers by high-fidelity enzymes (fast *pfu*, TransGen). The initial amplified segment was cloned into a T vector (pEASY blunt, TransGen, Beijing, China), sequenced, and digested. The recycled segment was cloned into pCAMBIA1304 under the control of the cauliflower mosaic virus (CaMV) 35S promoter and transformed into *Agrobacterium tumefaciens* strain EHA105. Transformation to Arabidopsis was via the flower dipping method (Clough and Bent 1998). In this study, all lines used were homozygous transgenic lines (T3 generation).

Determination of Bacterial Population in Plant Leaves

Bacterial growth in planta was measured according to the method described by He et al. (2006). Leaf disks were cut with a leaf punch from whole plant leaves inoculated with bacteria. The collected leaf disks were soaked in sterile water for 1 min followed by washing twice. The leaf disks were then ground in 100 μL H_2O and serial dilutions were plated on KB medium containing 25 mg/L rifampicin. The number of bacterial colony forming units (CFU) was counted after 2 days of growth at 28 °C.

Callose Staining

Callose in plant tissue was stained with aniline blue as described previously (Kim et al. 2005). Briefly, plant leaves were harvested after 12 h of bacterial infiltration, cleaned, and soaked into 95 % ethanol to remove the green background. The leaves were stained in 150 mmol L^{-1} K_2HPO_4 (pH 9.5) containing 0.01 % aniline blue for 30 min. Leaves were transferred to 50 % glycerol and examined under a fluorescence microscope (Olympus MVX10, Tokyo, Japan) with epifluorescent illumination (OLYMPUS MVX10). The views of the pictures were randomized. The number of callose deposits was counted and analyzed in triplicate.

Transcript Analysis

Total RNA was extracted from plant tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA). After DNA digestion, 2.0 μg RNA was used as a template for cDNA synthesis (ThermoScript, Life Technologies, Carlsbad, CA). Reverse transcription was performed at 42 °C in a 25-μL reaction mixture including 2.0 μg RNA, 0.5 μg oligo (dT) primers, 12.5 nmol dNTPs, 20 units RiboLock RNase inhibitor and 200 units RevertAid Reverse Transcriptase. First strand cDNA was used as a template to analyze the expression of genes using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using SYBR Green (Bio-Rad) with an Applied Biosystems 7500 Fast Real-Time PCR System (LifeTechnologies™). The relative expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). In some cases, semi-quantitative RT-PCR was performed to detect expression of *MtPiI4*. Basically, the total 25 μL of PCR reaction mixture in Tris-HCl buffer (pH 8.3, 10 mM) comprised 1 μL normalized cDNA template, 10 pmol sense primer, 10 pmol antisense primer, 5 nmol dNTPs, 32.5 nmol Mg^{2+}, and 0.5 U *Taq* DNA polymerase. PCR was performed as follows: 95 °C for 5 min, 30 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The relative abundance of *MtEF1α* was used as an internal standard for cDNA normalization in both RT-PCR and qRT-PCR analysis (Ding et al. 2008). All the primers used in these experiments were showed in Online Resource 1.

In Silico Analysis of Genes

Protein structure was predicted using SMART (http://smart.embl-heidelberg.de/). Protein subcellular location was predicted using WoLF PSORT server (http://wolfpsort.org/). The derived amino acid sequences were download from NCBI (http://www.ncbi.nlm.nih.gov/) and comparisons were analyzed using CLUSTALX 2.0 software. Phylogenetic trees were constructed using the maximum likelihood method in MEGA 5.2. Numbers on internal nodes are the percentage bootstrap support values (1,000 re-sampling). Only values exceeding 50 % are presented.

Statistical Analysis

Each result shown in the figures was the mean of at least three replicated treatments and each treatment contained at least 30 seedlings. Significant differences between treatments were evaluated statistically by standard deviation and one-way analysis of variance (ANOVA). The data between differently treated groups were compared statistically by ANOVA

followed by the least significant difference (LSD) test if the ANOVA result was significant at $P<0.05$. Statistical analyses were performed with SPSS 12.0. Unless indicated, equal amounts of mixed transgenic line (L1 and L2) seeds were used, and seedling samples (including all transgenic lines) were selected randomly for analysis.

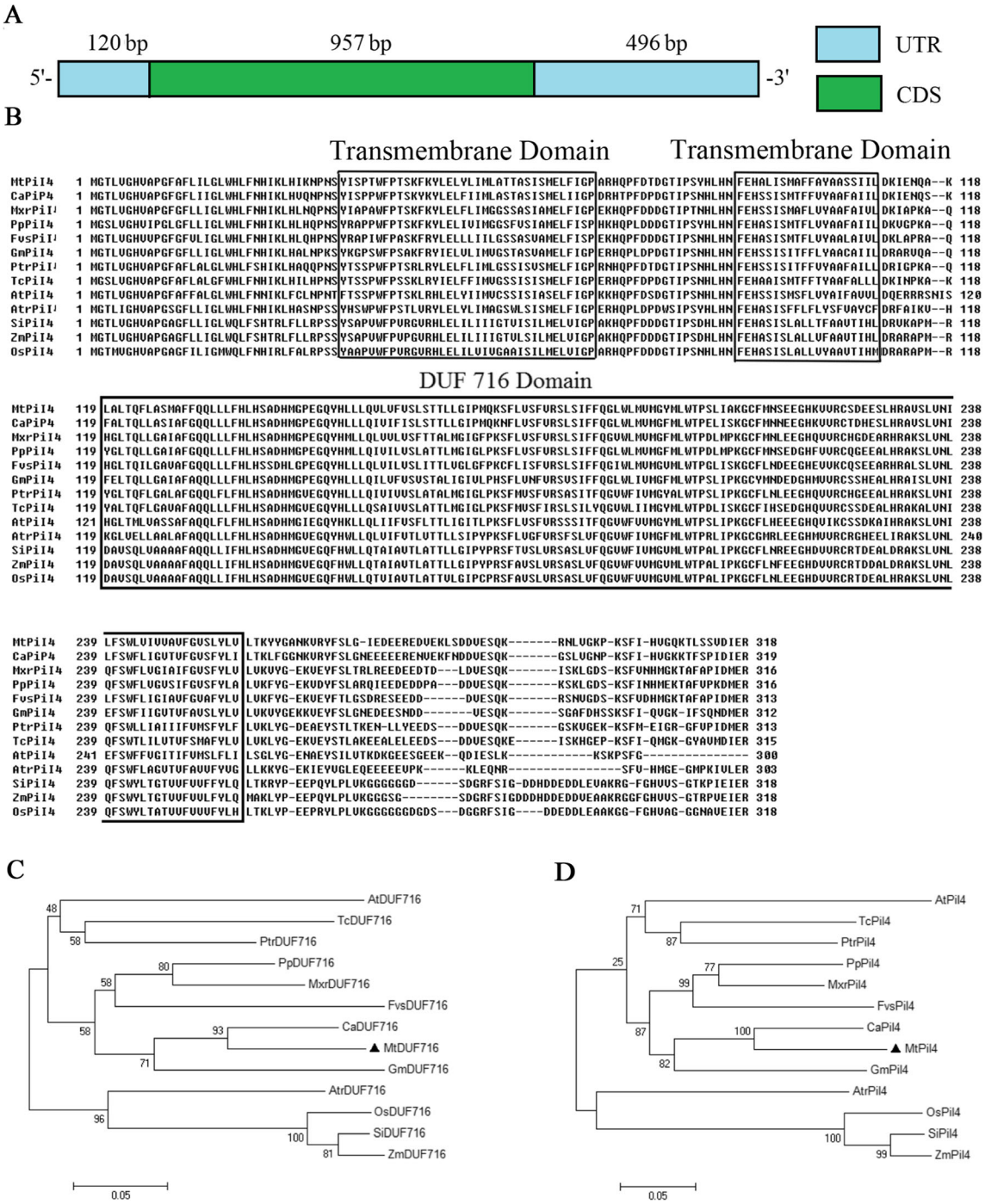

Fig. 1a–d Sequence analysis of *Medicago truncatula proteinase inhibitor I4* (*MtPiI4*). **a** Schematic structure of *MtPiI4* cDNA. *UTR* Untranslated region, *CDS* coding sequence. **b** Alignment of amino acid sequences of deduced MtPiI4 protein and comparison to orthologs from other plant species. The *black box* indicates a conserved DUF716 domain (domain of unknown function 716) and two transmembrane domains. **c** Phylogenetic relationship of the DUF716 domain of PiI4s from other plant species. **d** Phylogenetic relationship of PiI4s from other plant species. The phylogenetic trees were constructed using the maximum likelihood method in MEGA 5.2. Numbers on internal nodes are the percentage bootstrap support values (1,000 re-samplings). Only values exceeding 50 % are shown. At *Arabidopsis thaliana*, Tc *Theobroma cacao*, Ptr *Populus trichocarpa*, Mxr *Malus x robusta*, Fvs *Fragaria vesca* subsp. *vesca*, Ca *Cicer arietinum*, Mt *Medicago truncatula*, Gm *Glycine max*, Atr *Amborella trichopoda*, Os *Oryza sativa*, Si *Setaria italica*, Zm *Zea mays*

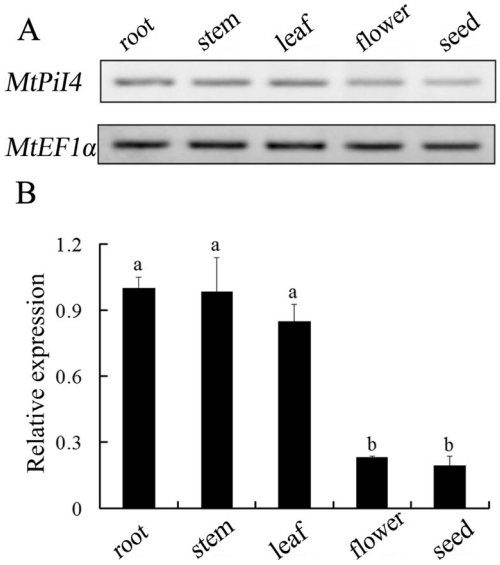

Fig. 2 *MtPiI4* expression in different tissues of *M. truncatula*. Total RNA was extracted from different tissues (root, stem, leaf, flower and seed) of plants cultured hydroponically for 4 weeks. The relative expression of *MtPiI4* was analyzed using **a** semi-quantitative RT-PCR and **b** qRT-PCR. *Error bars* Standard deviation of the mean. Means with different lower case letters indicate significantly different gene expression ($P < 0.05$). *MtEF1α* was used for cDNA normalization

Results

Isolation and Analysis of *MtPiI4* from *M. truncatula*

The full-length cDNA sequence of *MtPiI4* was isolated from the *M. truncatula* genome using a RT-PCR-based strategy. The *MtPiI4* cDNA sequence was 1,676 bp in length with an open reading frame (ORF) of 957 bp, a 5′-UTR of 120 bp, and a 3′-UTR of 496 bp (Fig. 1a; Online Resource 2). No intron was found in the DNA sequence of *mtPiI4*. The accession numbers of the full cDNA sequence of *MtPiI4* deposited with NCBI and *M. truncatula* database (http://www.medicagohapmap.org/) are XM_003594077 and Medtr2g021690, respectively. The deduced protein of *MtPiI4* consists of 318 amino acids, with a predicted molecular weight of 36 kDa and a pI of 6.73. MtPiI4 protein was predicted to contain a DUF716 domain (domain of unknown function 716) (Fig. 1b). This domain is conserved in plants, and clusters with its orthologues from other plant species (Fig. 1c). Further analysis revealed that MtPiI4 proteins contain two typical transmembrane domains (Fig. 1b; Online Resource 3). A phylogenetic analysis showed that MtPiI4 is related most closely to a PiI4 protein (XP_

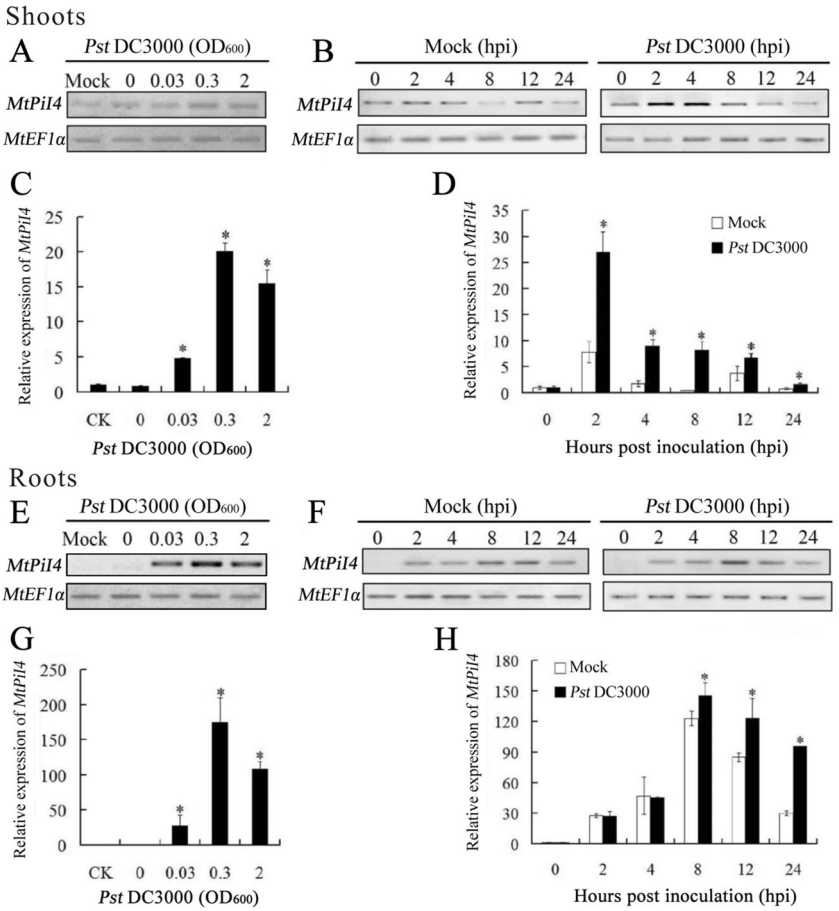

Fig. 3a–h Expression pattern of *MtPiI4* in wild type *M. truncatula* in response to *Pst* DC3000. Four-week-old seedlings were inoculated with *Pst* DC3000 at different concentrations (OD_{600}= 0–2) for 2 h or inoculated with *Pst* DC3000 at a specific concentration (OD_{600}=0.3) for different times (0–24 h). Expression of *MtPiI4* in shoots (**a**–**d**) and roots (**e**–**h**) was analyzed using semi-quantitative RT-PCR (**a**, **b**, **e**, **f**) and qRT-PCR (**c**, **d**, **g**, **h**). *Asterisks* indicate that mean values are significantly different between the control and treatment ($P<0.05$). *MtEF1α* was used for cDNA normalization

004486117) from *Cicer arietinum*, with 82 % identity (Fig. 1d). Thus, *MtPiI4* most likely belongs to the serpin family.

MtPiI4 is Expressed Constitutively in Various Tissues of *M. truncatula*

To determine the expression pattern of *MtPiI4*, the relative abundance of *MtPiI4* transcripts in different plant tissues was analyzed using semi-quantitative RT-PCR and qRT-PCR. *MtPiI4* was expressed ubiquitously in different tissues including roots, stems, leaves, flowers, and germinating seeds (Fig. 2). Strong expression was observed in root, stem, and leaf tissues, whereas expression in flowering tissues and germinating seeds was weak, indicating that transcripts of *MtPiI4* were detectable throughout most of the lifecycle of *M. truncatula*, although its expression level varied in different tissues.

MtPiI4 is Induced by *Pst* DC3000 Inoculation and MeJA in *M. truncatula*

To investigate the response of *MtPiI4* to pathogen infection, total RNA was extracted from shoots and roots of 4 week-old *M. truncatula* seedlings inoculated with *Pst* DC3000 and analyzed using RT-PCR. Compared to uninoculated plants (CK, control), *Pst* DC3000 infection induced a significant increase in *MtPiI4* transcripts in shoots in a dose-responsive manner (Fig. 3a, c). In a time-course experiment, *MtPiI4* was induced rapidly within 2 hpi (hours post inoculation) and the higher level remained up to 12 hpi (Fig. 3b, d). In roots, a similar pattern of *MtPiI4* expression was observed after pathogen infection (Fig. 3e–h). The *MtPiI4* transcripts increased initially at 4 hpi, peaked at 8–12 hpi, and then began to decline (Fig. 3h). At any level of *Pst* DC3000 inoculation, a basic level of *MtPiI4* transcripts was detected. This was observed particularly in root tissues.

MeJA has been suggested as an important regulator of plant PIs (Hartl et al. 2010; Lomate and Hivrale 2012). To investigate the possible effect of MeJA on expression of *MtPiI4*, shoots of *M. truncatula* plants were treated with MeJA at different concentrations and time intervals. As shown in Fig. 4, treatment with MeJA induced a marked increase in *MtPiI4* transcripts in shoots and roots. Compared to the control, 50 μM MeJA was able to induce 2.5-fold higher expression of *MtPiI4* than in the control (Fig. 4c). MeJA-induced *MtPiI4* transcripts were also analyzed over the time of MeJA application (Fig. 4b, d, f, h). The pattern of *MtPiI4* expression

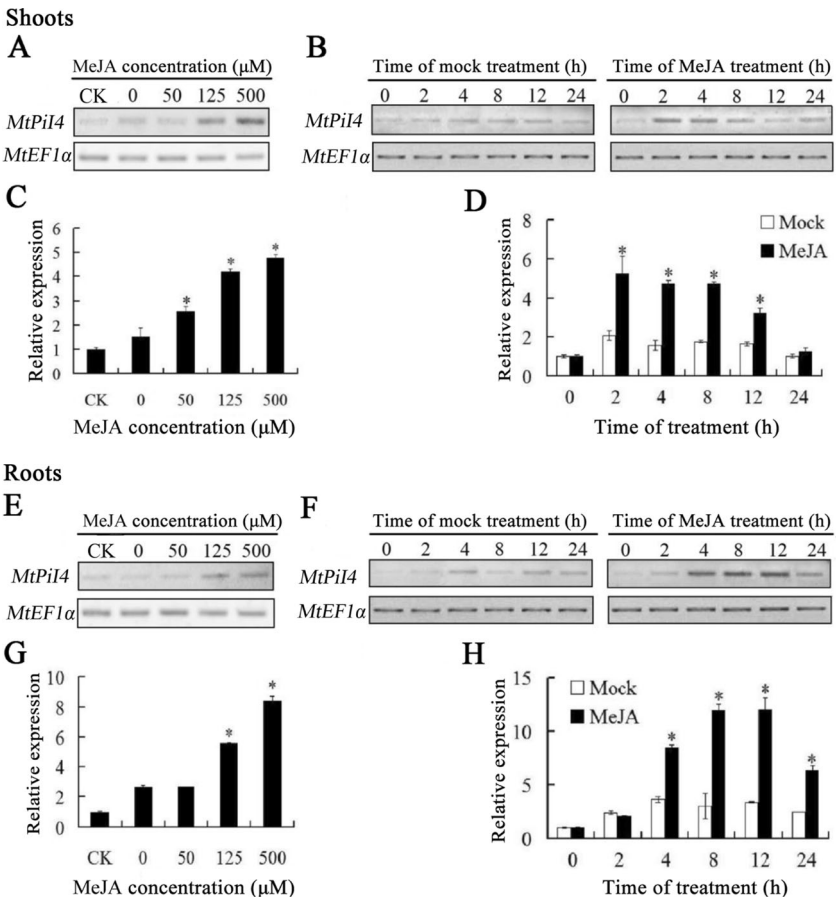

Fig. 4a–h Expression pattern of *MtPiI4* in wild type *M. truncatula* in response to methyl jasmonate (MeJA). Four-week-old seedlings were treated with MeJA at different concentrations (0–500 μM) for 4 h or treated with 500 μM of MeJA for different times (0–24 h). Expression of *MtPiI4* in shoots (**a**–**d**) and roots (**e**–**h**) was analyzed using semi-quantitative RT-PCR (**a**, **b**, **e**, **f**) and qRT-PCR (**c**, **d**, **g**, **h**). *Asterisks* indicate that mean values are significantly different between the control and treatment ($P<0.05$). *MtEF1α* was used for cDNA normalization

in the presence of MeJA was very similar to that of *Pst* DC3000 inoculation.

Ectopic Over-Expression of *MtPiI4* in Arabidopsis Conferred Plant Resistance to *Pst* DC3000

To identify the role of *MtPiI4* in regulating plant response to pathogen infection, we constructed transgenic Arabidopsis (Col-0) plants over-expressing *MtPiI4* driven by the CaMV 35S promoter. The *35S*::*MtPiI4* transgenic lines were screened and identified from the T0 to the T3 generation. Two homozygous *35S*::*MtPiI4* transgenic lines were obtained. Transgenic plants carrying *35S*::*MtPiI4* have expression levels that are 105- to 351-fold *MtPiI4* more than the wild-type (WT) (Fig. 5).

The 35S::*MtPiI4* plants inoculated with *Pst* DC3000 showed a phenotype of enhanced resistance to the disease (Fig. 6a) and reduced bacterial population relative to wild type (Fig. 6b). Callose formation in plants has been suggested as an important biomarker of defense priming in plants against pathogen attack (Luna et al. 2010). Aniline blue staining showed

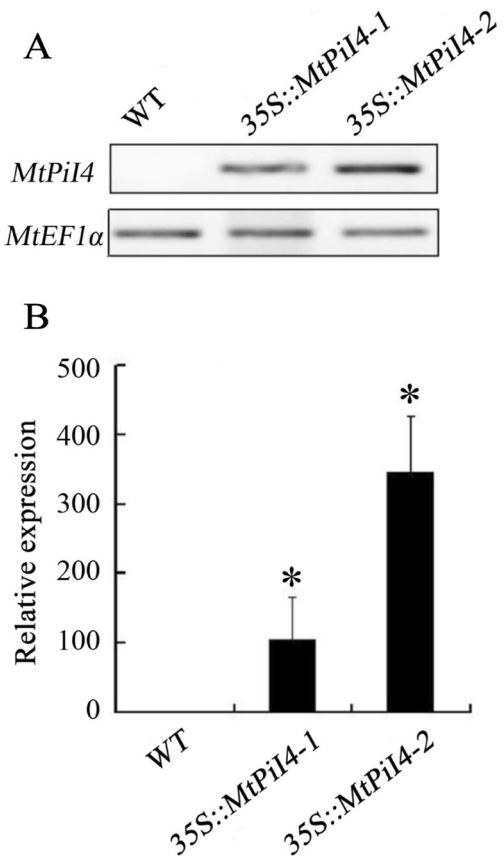

Fig. 5a,b Analysis of *MtPiI4* expression in *35S*::*MtPiI4* transgenic Arabidopsis lines. Transcripts of *MtPiI4* in 3-week-old seedlings were analyzed using **a** semi-quantitative RT-PCR and **b** qRT-PCR. *Asterisks* indicate that mean values are significantly different between the wild type and *35S*::*MtPiI4* lines ($P<0.05$). *MtEF1α* was used for cDNA normalization

that more callose was deposited in the leaves of 35S::*MtPiI4* plants with *Pst* DC3000 than in wild type leaves (Fig. 6c, d).

Over-Expression of *MtPiI4* Alters Expression Patterns of JA Biosynthetic and Responsive Genes in Arabidopsis

LOX2 (*LIPOXYGENASE2*) is responsible for JA biosynthesis, while *VSP1* (*VEGETATIVE STORAGE PROTEIN1*) and *PDF1.2* (*PLANT DEFENSIN1.2*) were identified as typically JA-responsive genes (Avanci et al. 2010). Under normal conditions (without external MeJA provision), expression of *LOX2* and *PDF1.2* was very low and *VSP1* was undetectable in WT and *MtPiI4* transgenic plants (Fig. 7a–c). When exposed to 500 μM MeJA, wild-type plants showed a progressive increase in expression of *LOX2*, *PDF1.2* and *VSP1*. However, 35S::*MtPiI4* plants showed reduced transcripts of these genes compared to WT; significant depression of these genes was found 4 or 8 h after MeJA treatment.

To identify further the impact of over-expression of *MtPiI4* on JA signaling under pathogen infection, we analyzed the transcripts of *LOX2*, *PDF1.2* and *VSP1* in *35S*::*MtPiI4* plants inoculated with *Pst* DC3000. Compared to wild-type, expression of *LOX2* was always lower in *35S*::*MtPiI4* plants before or after pathogen exposure (Fig. 8a). The lowest expression of *LOX2* was determined at 2 hpi, and expression of *LOX2* increased progressively thereafter.

Expression of *PDF1.2* was higher in *35S*::*MtPiI4* plants than in wild-type before *Pst* DC3000 inoculation, but its expression pattern with *Pst* DC3000 was similar to that with MeJA treatment (Fig. 8b). Slightly higher expression of *VSP1* was detected in *35S*::*MtPiI4* plants than in wild-type without *Pst* DC3000 exposure; however, expression of *VSP1* at 2 hpi was drastically depressed compared to wild-type (Fig. 8c). During the following hpi, there was no difference in *VSP1* expression between *35S*::*MtPiI4* and wild-type plants. We further examined transcripts of *NPR1*, a negative regulator and *MAPK4*, a positive regulator of JA signaling (Pieterse et al. 2009; Spoel et al. 2003). Our analysis showed that *NPR1* expression was higher, whereas *MAPK4* expression was lower in *35S*::*MtPiI4* plants compared to wild type (Fig. 8d, e). Taken together, these results indicate that manipulation of *MtPiI4* was able to alter expression of genes involved in the JA synthetic and signaling pathway.

Discussion

Plant PIs serve as defensive molecules mainly against exogenous pathogens or insects (John 2011; Kim et al. 2009), but the regulatory role of PIs in plant immunity remains obscure. This study identified a new PI gene *MtPiI4* from *M. truncatula*. Several lines of evidence indicate that *MtPiI4* was able to resist *Pst* DC3000 infection by repressing JA

signaling. First, the cDNA sequence of *MtPiI4* showed similarity to the serine PI family genes that are well known for their anti-pathogen and anti-insect activities. Second, both *Pst* DC3000 inoculation and MeJA treatment induced expression of *MtPiI4* in *M. truncatula*, which may be attributed to the existence of MeJA-responsive and defense-responsive elements in the promoter region of *MtPiI4* (Online Resource 4). Third, ectopic over-expression of *MtPiI4* in Arabidopsis improved plant resistance to *Pst* DC3000 and attenuated plant sensitivity to MeJA. Finally, over-expression of *MtPiI4* in Arabidopsis resulted in repressed JA signaling through contrasting regulation of *NPR1* and *MAPK4* in plants exposed to *Pst* DC3000.

MtPiI4 contains the DUF716 domain in its predicted protein. This domain is a hallmark of a family with functionally uncharacterized membrane proteins restricted to eukaryotes (Okada et al. 2011). *TMEM45A*, which codes for a DUF716 protein in humans, is involved in anti-viral responses (Gerber et al. 2013; Justesen et al. 2000). Reduced expression of *TMEM45A* promoted a progression of ductal carcinoma to invasive breast cancer (Lee et al. 2012). Studies in mammals indicate that *TMEM45A* can stimulate host immunity, implicating it in defense against viral disease. The present study showed *MtPiI4* involvement in regulation of plant immunity against bacterial invasion and possible association with suppression of the JA signaling pathway.

MtPiI4-mediated plant resistance to *Pst* DC3000 infection can be supported by the observation that over-expression of *MtPiI4* in Arabidopsis attenuated disease symptoms, reduced bacterial populations, and increased callose deposition. *Pst* DC3000 can hijack the JA signaling pathway to weaken plant

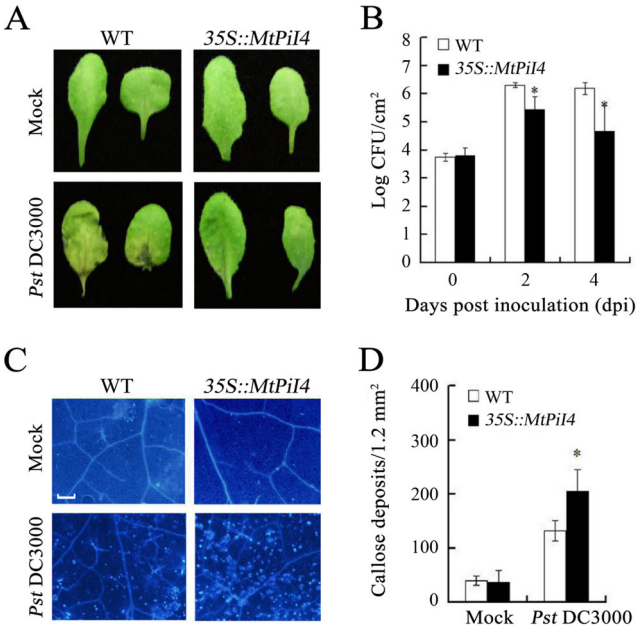

Fig. 6a–d Effect of *MtPiI4* over-expression on resistance of Arabidopsis leaves to *Pst* DC3000. Seven-day-old rosette leaves of plants were inoculated with *Pst* DC3000. **a** Leaf symptoms of WT (wild-type) and *35S*::*MtPiI4* Arabidopsis at 2 days post inoculation (dpi) with *Pst* DC3000. Symptoms of each treatment were examined on cotyledons (*right*) and the 1st euphylla (*left*). **b** In planta bacterial counting in the leaves of WT and *35S*::*MtPiI4* Arabidopsis at 0–4 days post inoculation (dpi). **c**, **d** Callose deposition in leaves of WT and *35S*::*MtPiI4* Arabidopsis at 2 dpi. *White bar* in **c** 1 mm

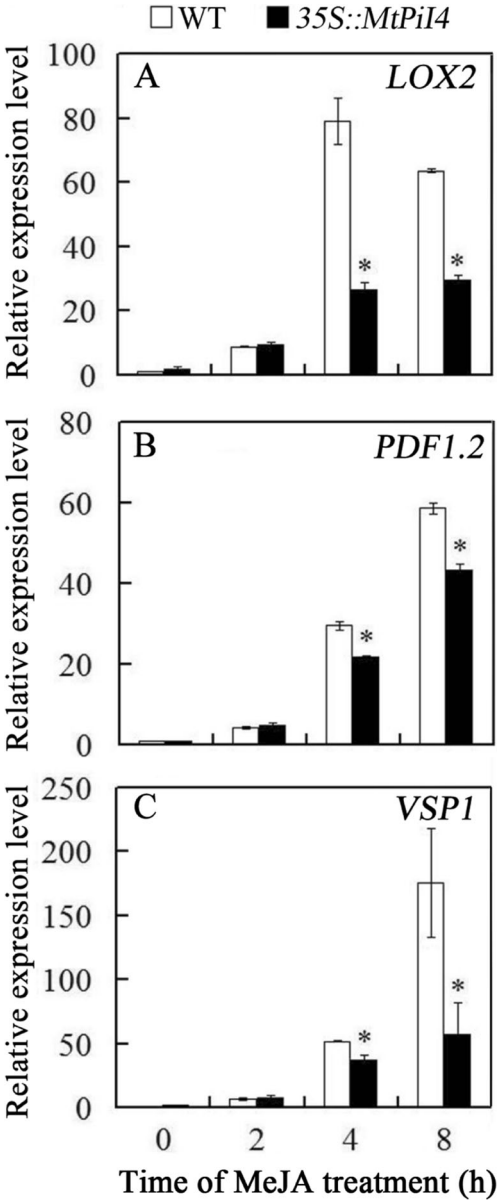

Fig. 7 Expression analysis of jasmonic acid (JA) biosynthetic and responsive genes in WT and *35S*::*MtPiI4* Arabidopsis treated with MeJA. Three-week-old seedlings were exposed to 500 μM MeJA for 0–8 h. Leaves were sampled at the indicated time. Total RNA was isolated from the samples and analyzed by qRT-PCR. The graphs indicate the fold-induction of the genes in MeJA-treated plants at the time point relative to the control (WT with 0 μM MeJA). *Error bars* Standard deviation of the mean of three treatments ($n=3$). *Asterisks* indicate that mean values are significantly different between the *35S*::*MtPiI4* plants and WT ($P<0.05$). *MtEF1α* was used for cDNA normalization

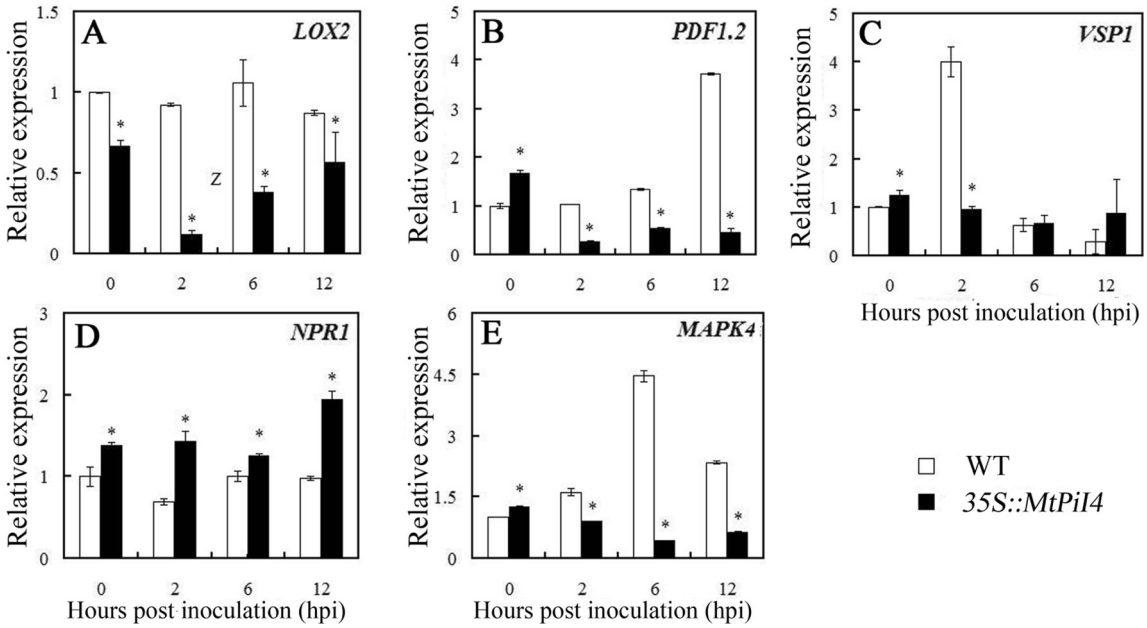

Fig. 8 qRT-PCR analysis of JA biosynthetic and responsive genes in *35S*::*MtPiI4* Arabidopsis plants exposed to *Pst* DC3000. Three-week-old seedlings of WT and *35S*::*MtPiI4* plants were inoculated with *Pst* DC3000 for 0–12 h. Leaves were sampled at different time points (0–12 hpi). Total RNA was isolated from the samples and analyzed by qRT-PCR. The graphs indicate the induction fold of the genes in *Pst* DC3000-exposed plants at the time point relative to the control (WT without *Pst* DC3000 exposure). *Error bars* Standard deviation of the mean three treatments (n=3). *Asterisks* indicate that mean values are significantly different between the *35S*::*MtPiI4* plants and WT (P<0.05). *MtEF1α* was used for cDNA normalization

defenses to bacterial invasion (Katsir et al. 2008). When plants were treated with exogenous MeJA, wild-type Arabidopsis showed higher levels of *LOX2*, *PDF1.2*, and *VSP1* transcripts, whereas expression of these genes were lower in *35S*::*MtPiI4* plants. Furthermore, transgenic Arabidopsis over-expressing *MtPiI4* showed lower abundance of *LOX2*, *PDF1.2*, and *VSP1* transcripts relative to wild-type under *Pst* DC3000 exposure. These results suggest that *Pst* DC3000 infection activated JA responsive genes, while *MtPiI4* conferred plant resistance to *Pst* DC3000 infection via attenuation of JA-responsive genes.

In Arabidopsis, AtMC9 (*Arabidopsis thaliana* metacaspase 9) and RD21 (RESPONSIVE TO DESI CCATION 21) were identified as targets of AtSerpin1 (Lampl et al. 2010; Vercammen et al. 2006). AtSerpin1 controls pathogen-induced programmed cell death (PCD) by directly connecting RD21 in vivo (Lampl et al. 2013). Interestingly, RD21 boosted immunity to the necrotrophic fungal pathogen *Botrytis cinerea*, but not to *Pst* DC3000 (Shindo et al. 2012). AtMC9, which is a type II metacaspase, acts as an important component for mediating the *Pst* DC3000-induced PCD process in plants (Bollhöner et al. 2013). Interaction between AtSerpin1 and AtMC9 occurs in apoplastic space, where priming of host innate immunity is initiated (Vercammen et al. 2006). AtSerpin1-mediated PCD was reported to link SA-dependent defense networks (Belenghi et al. 2007; Coll et al. 2011; Kim et al. 2013). Our results suggest that MtPiI4 appears to be a homologue of AtSerpin1 (Fig. 1d). Additionally, expression of *NPR1* and *MAPK4*, two important regulators of SA-dependent systemic acquired resistance (SAR) and pathogen-induced PCD response (Taj et al. 2010; Yoshimoto et al. 2009), can be differentially regulated by *MtPiI4* over-expression (Fig. 8). However, whether *MtPiI4* functions in a similar way to *AtSerpin1*, or whether both *MtPiI4* and *AtSerpin1* coordinate to mediate their download genes and defense responses remains to be investigated. In conclusion, we have demonstrated here that expression of a new serpin gene *MtPiI4* from *M. truncatula* enhanced plant resistance to *Pst* DC3000 infection by mediating some components in the JA and SA responsive signaling pathways. This gene could ultimately be applied to *M. truncatula* or other crop species to improve their pathogen resistance.

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Conflict of Interest The authors declare that they have no conflict of interest.

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