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A methyl jasmonate induced defensin like protein from *Panax notoginseng* confers resistance against *Fusarium solani* in transgenic tobacco

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Abstract

Plant defensins and defensin like protein (DEFL) form a large family of small cysteine-rich proteins. They are major components of plant immune systems, being involved in host defenses against biotic and abiotic stresses. In this study, a novel defensin like protein (DEFL) gene *PnDEFL1* was isolated from *Panax notoginseng*, a traditional Chinese medicinal herb. The expression patterns of *PnDEFL1* after treatment with methyl jasmonate, salicylic acid, ethephon, and H₂O₂, as well as during *Fusarium solani* infection, were analyzed using reverse transcription qPCR. The up-regulated expression of *PnDEFL1* indicated that it responded to *F. solani* infection and all four defense-related signalling molecules. The *PnDEFL1* gene was further fused with the green fluorescent protein gene in a plant expression vector and transformed into onion (*Allium cepa*) epidermal cells. The laser scanning confocal microscope confirmed that the PnDEFL1 protein localized to the extracellular region. In addition, the recombinant PnDEFL1 protein was expressed in *Escherichia coli* and purified by affinity chromatography. It had antifungal activities against *F. solani*, *F. oxysporum*, *Botrytis dothidea*, and *Sclerotinia sclerotiorum*. The *PnDEFL1* gene was transferred into tobacco (*Nicotiana tabacum*) to verify its function. The overexpression of *PnDEFL1* in tobacco conferred a high resistance to *F. solani* infection. Thus, the *PnDEFL1* gene is involved in the defense responses of *P. notoginseng* to *F. solani* infection.

Additional key words: *Allium cepa*, antifungal activity, *Escherichia coli*, ethephon, green fluorescent protein, H₂O₂, methyl jasmonate, *Nicotiana tabacum*, salicylic acid.

Introduction

In plants, biochemical and physiological responses, and even morphological lesions, occur under stress conditions, hindering normal growth and development, thereby seriously endangering agricultural production. In particular, plants are negatively affected by fungal diseases, and their large-scale occurrence usually leads to serious crop yield reductions. As a long-term response to biotic stresses, plants have gradually acquired a series of complex defense mechanisms, such as the production of plant antimicrobial peptides (AMPs) (De Souza *et al.* 2014). The main plant AMPs are defensins, thionins, lipid transfer proteins, cyclotides, snakins, and hevein-like proteins (Nawrot *et al.* 2014).

Plant defensins were first isolated from the endosperm of wheat (*Triticum aestivum*) and initially classified as novel glucosinolates (Colilla *et al.* 1990). However, they were found to have antibacterial properties and structural similarities with mammalian and insect defensins (Terras *et al.* 1995), and were then renamed “plant defensins”. Plant defensins are cysteine-rich cationic peptides with relative molecular mass of approximately 5 kDa, containing 45 - 50 amino acid residues (Lacerda *et al.* 2014). The typical tertiary structure of a defensin is a CSαβ motif that has an α-helix and three anti-parallel β-folds, and most plant defensins have eight conserved cysteine residues that form four intramolecular disulfide bonds to stabilize their tertiary structures (Fant *et al.* 1998, Gachomo *et al.* 2012). Plant defensins and defensin-like protein (DEFL) genes

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Abbreviations: cDNA - complementary DNA; DEFL - defensin like protein; ETH - ethylene; GFP - green fluorescent protein; hpi - hours post inoculation; IPTG - isopropyl-1-thio-β-D-galactopyranoside; JA - jasmonic acid; MeJA - methyl jasmonate; ORF - open reading frame; PDA - potato dextrose agar; RT-qPCR - reverse transcription quantitative polymerase chain reaction; SA - salicylic acid; RACE - rapid-amplification of cDNA ends; WT - wild type.

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have been isolated from a variety of plants. A total of 13 defensin genes have been identified in the *Arabidopsis thaliana* genome (Thomma *et al.* 2002), and also 317 *DEFLs* (Silverstein *et al.* 2005). In total, 93, 79, and 143 *DEFL* genes have been characterized in rice (*Oryza sativa*), grapevine (*Vitis vinifera*), and wheat (*Triticum kiharae*), respectively (Silverstein *et al.* 2007, Giacomelli *et al.* 2012, Odintsova *et al.* 2019). In addition, the *DEFLs* have the hallmarks of defensin genes, including the conserved cysteine residues, CS $\alpha\beta$ motif or γ -core motif (Silverstein *et al.* 2005, Giacomelli *et al.* 2012). Moreover, the genomic organization of *DEFLs* is highly similar to those of mammalian defensins and plant resistance genes, and the *DEFLs* belong to the defensin supergene family (Silverstein *et al.* 2005).

The expressions of some defensin genes are regulated by pathogens, abiotic stresses, and several phytohormones. The transcription of the *Capsicum annuum* defensin *CADEFL1* is strongly induced by *Xanthomonas campestris* pv. *vesicatoria* (Do *et al.* 2004). *V. vinifera* *DEFL29* and *DEFL33* are significantly up-regulated after *Botrytis cinerea* infection (Giacomelli *et al.* 2012). *SlyDF1* and *SlyDF2*, two *Solanum lycopersicum* defensin genes, were strongly induced at 1 and 2 d, respectively, after inoculation with *Phytophthora infestans* (Cui *et al.* 2018). The defensin gene *PgD1* of *Picea glauca* is up-regulated by wounding and salicylic acid (SA) treatment (Pervieux *et al.* 2004). The *Nicotiana benthamiana* *NbDef2.2* is induced by wounding and responds strongly to ethylene (ETH) treatment (Bahramnejad *et al.* 2009). The expression of *T. aestivum* defensin 1 (*TAD1*) is induced during cold acclimation (Sasaki *et al.* 2016). In addition, the defensin gene *RiD* from the wild cruciferous plant *Rorippa indica* is up-regulated by application of methyl jasmonate (MeJA) (Sarkar *et al.* 2017).

Plant defensins exist in various plant tissues, including tubers, leaves, flowers, and pods, but most have been found in seeds and roots. Moreover, defensins have broad-spectrum antifungal and antibacterial activities (Franco *et al.* 2006). The defensin protein AFP2 in *Raphanus sativus* has *in vitro* antibacterial activities against *Fusarium graminearum* and *Rhizoctonia solani*, and wheat plants overexpressing *RsAFP2* have increased resistance to both pathogens (Li *et al.* 2011). The recombinant winter wheat defensin *TAD1* protein inhibits the mycelial growth of *Typhula ishikariensis* *in vitro*, while transgenic wheat overexpressing *TAD1* shows resistance to *T. ishikariensis* and *F. graminearum* (Sasaki *et al.* 2016). In addition, class I (*NaD2*) and class II (*NaD1*) defensins from *Nepenthes alata* flowers inhibit the germination of urediniospores and confer resistance to crown rust in oat seedlings when applied as a foliar spray (Dracatos *et al.* 2014). The *Medicago sativa* seed defensin *MsDefl* inhibits the growth of *F. graminearum* *in vitro*, and its antifungal activity was significantly reduced in the presence of Ca²⁺ (Spelbrink *et al.* 2004).

Panax notoginseng is a traditional Chinese medicinal herb with various biological activities, such as reducing swelling, relieving pain, promoting blood circulation, and alleviating blood stasis (Zhang *et al.* 2016). *P. notoginseng*

is mainly distributed in southwestern China, and Wenshan Prefecture of Yunnan Province is the main producing area. During the artificial cultivation of *P. notoginseng*, root rot, black spot, and other fungal diseases severely affect the production and quality of the medicinal materials generated from *P. notoginseng*. Among them, root rot, which is mainly caused by *Fusarium solani*, is the most serious disease (Miao *et al.* 2006, Fan *et al.* 2016). The pretreatment of *P. notoginseng* with 100 μ M MeJA significantly increased its resistance to *F. solani* in our previous study. Therefore, the transcriptome sequencing of *P. notoginseng* roots pretreated with MeJA and then inoculated with *F. solani* was performed [the transcriptome data were submitted to the NCBI (SRA and TSA; accession numbers SRP149049 and GGPQ00000000, respectively). A differentially expressed gene encoding a *DEFL* was induced by the MeJA treatment and responded to *F. solani* infection. Here, to gain a deeper understanding of the defensin's function in the molecular interaction between *P. notoginseng* and *F. solani*, the full-length cDNA sequence of the defensin gene, named *PnDEFL1* (GenBank accession number MK238492), was obtained. In addition, the expression profile, prokaryotic expression, and subcellular localization of *PnDEFL1* were analyzed. *PnDEFL1* was also overexpressed in tobacco to further investigate its function in response to *F. solani* infection.

Materials and methods

Plants, treatments, and inoculation: The *Panax notoginseng* (Burk) F.H. Chen seedlings used in the gene cloning and gene expression analysis were planted and grown in a greenhouse at a temperature of 25 °C and a relative humidity of 60 %. Sterile seedlings of tobacco (*Nicotiana tabacum* L. cv *Xanthi*) were cultured in a climatic cabinet at a temperature of 25 °C and a 16-h photoperiod, and used for genetic transformation. Mycelia of *F. solani*, *Botrytis dothidea*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* that had been preserved at -4 °C were activated on potato dextrose agar (PDA) media before using in a constant temperature incubator (28°C).

For the chemical treatments, the concentrations of four stress-related signalling molecules, SA, MeJA, ethephon, and H₂O₂, were determined by referring to *Lilium regale* and *Juglans sigillata* treatment methods (Zhang *et al.* 2017, Liu *et al.* 2018), and then modified based on growth observations. The *P. notoginseng* seedlings were grown under normal conditions for 4 weeks after independent treatments with SA (200 μ M), JA (100 μ M), ethephon (1 mM), and H₂O₂ (1 mM). The roots of healthy one-year-old *P. notoginseng* seedlings were wounded with scissors, and then the wounded *P. notoginseng* seedlings were treated with signalling molecule solutions using the dip-root method for 30 min. The roots were collected after at 4, 12, 24, 48, and 72 h after treatment. For the fungal inoculations, the wounded roots of healthy one-year-old *P. notoginseng* seedlings were soaked with a solution of MeJA (100 μ M, treatment group) or sterile water (control group) for 30 min. At 24-h after the pretreatment, the two

groups of *P. notoginseng* seedlings were inoculated with fresh spore suspensions of *F. solani* (10^6 spores dm^{-3}) for 30 min. The roots were collected at 4, 12, 24, 48, and 72 h post-inoculation (hpi). Moreover, to eliminate individual differences, the root samples used for each chemical treatment and inoculation were collected from three different healthy *P. notoginseng* plants, and the roots were mixed and thoroughly ground in liquid nitrogen before RNA extraction.

Rapid-amplification of cDNA ends (RACE) and bioinformatics analysis: The full-length cDNA was amplified using a *SMART RACE* cDNA amplification kit (Clontech, Palo Alto, USA), and the gene-specific primers (Table 1 Suppl.) were designed based on the unigene sequence of *DEFL* using *Primer Premier 5* software. The mRNA was isolated from 100 μg of *P. notoginseng* total RNA using a *NucleoTrap*® mRNA midi kit (Macherey-Nagel, Düren, Germany), and the cDNA synthesis, as well as RACE-PCR, were performed according to the manufacturer's protocols (Clontech). The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, USA) and transformed into *Escherichia coli* DH5 α competent cells. The recombinant plasmid was selected for bidirectional sequencing. The overlap and assembly of unigene sequence and RACE products, as well as the identification of open reading frame (ORF), were performed using the *NCBI* website (<https://www.ncbi.nlm.nih.gov/>). The other bioinformatics analysis methods used were previously described in Li *et al.* (2014).

Quantitative reverse transcription-PCR (RT-qPCR) was used to analyze the expression patterns of *PnDEFL1* in *P. notoginseng* roots during *F. solani* infection and after MeJA, ETH, H_2O_2 , and SA treatments. The *P. notoginseng* actin 2 gene (*PnACT2*, GenBank accession number KF815706.1) was used as the internal control for the standardization of different RNA samples. Gene-specific primers for the *PnDEFL1* and *PnACT2* were designed based on the corresponding cDNA sequences using *Primer Premier 5* software (Table 1). The detailed RT-qPCR scheme was the same as described in Liu *et al.* (2013a). Relative gene expressions were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001). The expression of the *PnDEFL1* gene in wounded *P. notoginseng* roots without treatment or inoculation was considered as the control, and its relative expression value was designated as one. The RT-qPCR assay was carried out with three replicates.

Subcellular localization of PnDEFL1: The pGEM-T easy-*PnDEFL1* and pBIN m-gfp5-ER (Zhang *et al.* 2017) plasmids were digested with the restriction enzymes *NdeI* and *SmaI*, and then ligated with T4 DNA ligase (Promega). The *PnDEFL1* gene was fused with the green fluorescent protein (*GFP*) gene present in the pBIN m-gfp5-ER vector under the control of the CaMV 35S promoter to generate the *PnDEFL1-GFP* fusion protein. The pBIN m-gfp5-ER-*PnDEFL1* recombinant plasmid was then transformed into the *Agrobacterium tumefaciens* strain

EHA105 and plated onto Lurie brot (LB) solid medium containing 50 mg dm^{-3} kanamycin and 20 mg dm^{-3} rifampicin. The *A. tumefaciens* positive clones harbouring recombinant plasmids were confirmed by PCR and used for transient expression in onion (*Allium cepa*) epidermal cells. The detailed procedure of the *A. tumefaciens*-mediated genetic transformation and GFP observations with laser confocal microscopy (*AIR*, Nikon, Tokyo, Japan) were previously described in Liu *et al.* (2018).

Expression and purification of recombinant PnDEFL1 protein: The pGEM-T easy-*PnDEFL1* plasmids were digested with *NdeI* and *SmaI*, and then the *PnDEFL1* ORF, without the stop codon, was subcloned into the C-terminus of the pET-32a vector to generate a 6 $\times$ His-tagged fusion protein in *Escherichia coli*. *E. coli* strain BL21 (DE3) containing the pET-32a-*PnDEFL1* plasmid was grown in liquid LB medium supplemented with ampicillin (50 mg dm^{-3}) in a shaker at 37 °C. When the absorbance at 600 nm reached 0.6 - 0.8, isopropyl-1-thio- β -D-galactopyranoside (IPTG, 1 mM final concentration) was added to the LB medium, and the *E. coli* cells were further cultured in a shaker at 25 °C. The *E. coli* cells were collected after induction for 8 h. The cells were first lysed by lysozyme, and then the supernatant and the precipitate were separately sampled. The recombinant PnDEFL1 protein was purified from the supernatant using a *Ni-NTA* column according to manufacturer's instructions (Sangon, Shanghai, China).

Antifungal assay of recombinant PnDEFL1 protein: Four fungi, *F. solani*, *F. oxysporum*, *B. dothidea*, and *S. sclerotiorum* were activated on PDA solid medium at 28 °C. The activated mycelia were inoculated on the center of the PDA solid medium, and when the radii of the fungal colonies were ~2 cm, sterilized filter-paper discs (0.6 mm in diameter) were placed uniformly around the fungal colonies. Then, 5, 10, and 15 μg of purified recombinant PnDEFL1 protein were added independently to the filter paper. In addition, 0.01 cm^3 of ddH_2O and buffer were added as blank controls. All the culture dishes were placed in an incubator at 28 °C for 3 d, and then, the antifungal activity of the PnDEFL1 recombinant protein against the four fungi was recorded using a digital camera (Canon, Tokyo, Japan). The zones of growth inhibition around the disks were calculated using *Adobe Photoshop v. 7.0*, and three replications of the antifungal assay were performed.

Overexpression vector construction and tobacco genetic transformation: The plant vector for overexpressing *PnDEFL1* was constructed using the pCAMBIA2300s vector (Liu *et al.* 2013b). The ORF of *PnDEFL1* was obtained by digesting pGEM-T easy-*PnDEFL1* with *BamHI* and *EcoRI* and was then ligated into the digested pCAMBIA2300s vector using the same restriction enzymes. The ligation product was transformed into *E. coli* DH5 α competent cells, and positive clones harbouring the recombinant plasmid pCAMBIA2300s-*PnDEFL1* were selected using PCR. The recombinant plasmid pCAMBIA2300S-*PnDEFL1* was further

transformed into the competent cells of *A. tumefaciens* strain LBA4404 using the freeze-thaw method (Holsters *et al.* 1978). The tobacco (*N. tabacum* L. cv. Xanthi) leaf discs were transformed using the LBA4404 bacterial suspension containing the recombinant vector pCambia2300S-*PnDEFL1* (Horsch *et al.* 1985).

Transgenic tobacco analysis: The tobacco seedlings were regenerated through tissue culture, and the positive transgenic tobacco plants were screened using PCR. The genomic DNA of T0 generation of tobacco plants was the template for PCR with *PnDEFL1*-specific primers (Table 1), and the wild type (WT) genomic DNA was the negative control. Moreover, the positive *PnDEFL1* transgenic tobacco plants were cultured in a greenhouse to develop T2 generation tobacco lines, which were confirmed using PCR with *PnDEFL1*-specific primers. The expression of *PnDEFL1* in the T2 generation transgenic tobacco was analyzed using RT-qPCR. The tobacco *actin* gene (AB158612.1) was used as the internal control for the standardization of different RNA samples, and WT tobacco was used as the negative control. The transgenic

tobacco line with the highest Ct value was taken as a positive control, and the value of *PnDEFL1* expression in the positive control was designated as one.

The sterile T2 tobacco lines, as well as the WT plants, were cultured in a half strength Murashige and Skoog medium for 2 months in an artificial climatic incubator (25 °C, a 16-h photoperiod), and several tobacco lines with relatively high *PnDEFL1* transcription were selected to estimate the resistance to *F. solani* infection. The fully expanded tobacco leaves were partially wounded and inoculated with 0.02 cm³ of *F. solani* spore suspension (10⁶ spores cm⁻³). The infected leaves were placed on a damp filter paper and placed in a light incubator. One week after inoculation, the infected leaves were collected and lesions caused by *F. solani* infection were recorded. In addition, the wounded roots of sterile transgenic and WT tobacco were immersed in the *F. solani* spore suspension (2 × 10⁶ spore cm⁻³) for 30 min and then transferred into a half strength Murashige and Skoog culture medium. The tobacco symptoms were recorded at one week after inoculation.

Fig. 1. A multiple alignment and phylogenetic tree of the amino acid sequences of *PnDEFL1* and several known defensins or defensin like proteins (DEFLs). The multiple alignment was completed using *ClustalX* (A). The signal peptide is labeled with a horizontal line, and the highly conserved cysteine residues are labeled with triangles. Defensins included in the alignment are two *Nicotiana glauca* defensins (NaD1 and NaD2) (Dracatos *et al.* 2014), four DEFLs from *Vitis vinifera* (VvDEFL1, VvDEFL13, VvDEFL22, and VvDEFL35) (Giacomelli *et al.* 2012), *Cucumis melo* CmDEFL1 (XP_008454663.1), and *Momordica charantia* McDEFL1 (XP_022139709.1), respectively. The phylogenetic tree was constructed using the *Mega 6* software with the neighbour-joining method (B). The scale bar equals the distance of 10 changes per 100 amino acid positions, and the numbers on each node are bootstrap values of 1 000 replicates.

Statistical analyses: The above experiments were repeated three times. The relative expressions of *PnDEFL1* in *P. notoginseng* and T2 tobacco lines and the zones of fungal growth inhibition are shown as means and standard deviations. The Student's *t*-test was performed using the SPSS software to reveal statistical differences between the treatments or inoculations and controls.

Results

In this study, a *DEFL* gene *PnDEFL1* was isolated from *P. notoginseng*. The 5' and 3' RACE PCR products were 301 bp and 487 bp, respectively, and the overlap and assembly of unigene sequence and RACE products generated a cDNA of 702 bp, in which an ORF of 234 bp was identified. Moreover, the ORF encodes a complete protein sequence of 77 amino acid residues that is highly homologous to plant *DEFLs*. The calculated molecular mass of the deduced protein PnDEFL1 is 8.63 kDa, with a theoretical pI of 7.51. At the nucleotide level, the *PnDEFL1* gene shared a 74 % identity with the *Helianthus annuus DEFL19* gene (XM_022160565.1). At the protein level, the deduced protein sequence of

PnDEFL1 shared a 58 % identity with DEFL1 of *Cucumis melo* (XP_008454663.1) and a 54 % identity with *Momordica charantia* DEFL1 (XP_022139709.1).

The class I and II defensins derived from the flowers of *N. alata* (Dracatos *et al.* 2014), four kinds of DEFLs from *V. vinifera* (Giacomelli *et al.* 2012), and two homologous sequences (CmDEFL1 and McDEFL1) from the protein BLAST analysis were chosen for the multiple sequence alignment and phylogenetic tree analysis. PnDEFL1 had a predicted signal peptide positioned at its N-terminus like other defensins, and in addition, the hallmarks of defensins, the CS α β and γ -core motifs, were present (Fig. 1A). The majority of the plant defensins or DEFLs contain eight highly conserved cysteine residues. For the phylogenetic tree, VvDEFL35 and NaD1 were grouped at the bottom of the tree, while PnDEFL1, the other DEFLs, and NaD2 clustered into another group (Fig. 1B). PnDEFL1 was more homologous with the class I defensin (NaD2) than the class II defensin (NaD1).

The transcriptions of *PnDEFL1* in *P. notoginseng* roots after independent treatments with four different signalling molecules were analyzed using RT-qPCR. The expression of *PnDEFL1* was rapidly induced by MeJA, ETH, H₂O₂, and SA treatments (Fig. 2A-D), but

Fig. 2. The expression profiles of *PnDEFL1* in roots of *Panax notoginseng*. The *PnDEFL1* expressions after treatments with four signalling molecules including methyl jasmonate (A), salicylic acid (B), ethylene (C), and H₂O₂ (D), as well as after inoculation with *Fusarium solani* (E) were analyzed by reverse transcription quantitative PCR. Means $\pm$ SEs of three biological replicates, and the statistical differences between the treatments and the controls were analyzed by the Student's *t* test (* - $P < 0.05$, ** - $P < 0.01$).

the expressions of *PnDEFL1* were different. The greatest expression occurred after the ETH treatment, followed by MeJA, SA, and H₂O₂ treatments. Moreover, the highest relative expressions of *PnDEFL1* were reached at 24, 12, 24, and 12 h for the MeJA, SA, ETH, and H₂O₂ treatments, respectively. Compared with the sterile water treatment, the *PnDEFL1* expression was highly induced (~12 times) by the exogenous application of MeJA at 24 h. After *F. solani* inoculation, the *PnDEFL1* expression in the sterile water pre-treatment group rapidly increased; however, the *PnDEFL1* expression in MeJA pre-treatment group were even greater than that in the water pre-treatment group (Fig. 2E). In particular, at 24 h, the relative expression value in the MeJA pre-treatment group was ~2.1 times greater than in the water pre-treatment group (Fig. 2E). Furthermore, the overall *PnDEFL1* expressions were greater in the MeJA group than in the water group until 72 h.

Onion epidermal cells were independently infected with *A. tumefaciens* carrying the pBIN m-gfp5-ER-*PnDEFL1* and the empty pBIN m-gfp5-ER vector (positive control), and the green fluorescence signal was observed using a laser confocal microscope (Fig. 3). In the positive control, the green fluorescence was distributed throughout the onion cells harbouring the empty pBIN m-gfp5-ER vector. The green fluorescence of onion cells harbouring the pBIN m-gfp5-ER-*PnDEFL1* vector was specifically distributed in the cell wall, indicating that PnDEFL1 is an extracellular protein that localized in the plant cell wall.

The PnDEFL1 protein without a signal peptide was expressed in *E. coli*, and the size of the PnDEFL1 recombinant protein was approximately 25 kDa, which was consistent with the predicted size of the histidine-tagged fusion protein (Fig. 4A). The PnDEFL1 fusion protein was

purified by nickel affinity chromatography. The antifungal assay showed that the PnDEFL1 recombinant protein inhibited the mycelial growth of *F. solani*, *F. oxysporum*, *B. dothidea*, and *S. sclerotinia* *in vitro* (Fig. 4B-E). In addition, greater amount of the PnDEFL1 protein exhibited more pronounced inhibitory effects on the four pathogenic fungi growth (Fig. 4F).

The positive *PnDEFL1* transgenic tobacco plants were grown in a greenhouse until T2 generations were obtained. No significant differences were observed between the *PnDEFL1* transgenic and WT tobacco in growth and morphogenesis. The expressions of *PnDEFL1* in T2 generation transgenic tobacco leaves were detected using RT-qPCR. The *PnDEFL1* transcripts only accumulated in nine transgenic tobacco lines (D-2, -4, -5, -7, -12, -16, -17, -18, and -23), but not in the WT, and the expressions in these transgenic lines differed (Fig. 5A). The expressions in the transgenic tobacco lines D-2, -7, -18, and -23 were relatively low, with values below 20; however, the expressions in the lines D-4, -5, -12, -16, and -17 were much greater than in other lines.

The *PnDEFL1* transgenic and WT tobacco leaves were inoculated with *F. solani* spore suspensions. After 7 d of incubation, the leaves of WT tobacco showed extremely severe chlorosis and decay over a large area; however, the *PnDEFL1* transgenic tobacco leaves showed almost no symptoms (Fig. 5B). In addition, the *F. solani* spore suspension was used to inoculate the wounded roots of *PnDEFL1* transgenic and WT tobacco. After being cultured in a climatic cabinet for 7 d after inoculation, the WT tobacco roots were seriously rotted and blackened, and the leaves were withered and yellowed (Fig. 5C). On the contrary, the roots of *PnDEFL1* transgenic tobacco exhibited limited blackening, and the plants showed

Fig. 3. Subcellular localization analysis indicates that the PnDEFL1 protein was localized in the cell wall. The *PnDEFL1*-GFP fusion gene and *greenfluorescent protein* (GFP, control) were transiently expressed in onion epidermal cells mediated by *Agrobacterium tumefaciens*. The onion epidermal tissue was treated with a 30 % sucrose solution to induce plasmolysis, and then a confocal laser microscopy was used to display GFP (green fluorescence), bright field (bright), and combination (merged) views, respectively.

Fig. 4. (See the next page.) The expression of the recombinant PnDEFL1 protein in *Escherichia coli* BL21 (DE3) was analyzed through SDS-PAGE (A). M - protein marker; 1 - total protein of *E. coli* BL21 (DE3) containing pET-32(a) empty vector induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG, 1 mM) for 7 h; 2 - total protein of *E. coli* BL21 (DE3) containing pET-32(a)-*PnDEFL1* recombinant vector without induction; 3, 4, and 5 - total proteins of *E. coli* BL21 (DE3) containing pET-32(a)-*PnDEFL1* cultured for 3, 5, and 7 h with the induction by 0.5 mM IPTG, respectively; 6, 7, 8, and 9 - total protein of *E. coli* BL21 (DE3) containing pET-32(a)-*PnDEFL1* cultured for 3, 5, 7, and 8 h with the induction by 1 mM IPTG, respectively. The SDS-PAGE analysis of the His-tagged recombinant PnDEFL1 protein purified from *E. coli* (B). M - protein marker; 1 - total protein of *E. coli* BL21 (DE3) containing pET-32(a)-*PnDEFL1* recombinant vector without induction; 2, 3, 4, 5, 6, and 7 - purified PnDEFL1 protein eluted with 50, 100, 150, 200, 250, and 300 mM imidazole buffer, respectively. The recombinant PnDEFL1 protein has an evident antifungal activity to *F. solani* (C), *F. oxysporum* (D), *S. sclerotium* (E), and *B. dothidea* (F). In addition, the fungal growth inhibition analysis shows the antifungal activities increased with the increase of recombinant PnDEFL1 protein (G). The experiment was repeated three times to obtain an average size of fungal growth inhibition, and the statistical differences were analyzed by the Duncan's multiple range test.

almost healthy growth state (Fig. 5C). Thus, the *PnDEFL1* transgenic tobacco lines were much more resistant to *F. solani* infection than the WT tobacco, which suggested that the overexpression of *PnDEFL1* in tobacco conferred a strong resistance to *F. solani*.

Discussion

Plant defensins belong to the plant AMPs (Broekaert *et al.* 1997), which are important parts of the plant innate immune system. They have an inhibitory effect on pathogenic microorganisms, such as bacteria and fungi, and play an important role in plant defense responses (Zasloff 2002). In the present study, a novel defensin-like protein gene *PnDEFL1* was investigated and isolated from *P. notoginseng*. The full-length cDNA of *PnDEFL1* is 702 bp and contains an ORF of 234 bp. The sequence analysis included several defensins and DEFLs from different plant species and showed that DEFLs have a diverse primary sequence. However, DEFLs contain the highly conserved structural features that are hallmarks of defensins, such as the CS α β and γ -core motifs, and a specified number of

cysteine residues. Previous genomic studies indicated that plant *DEFLs* and defensins constitute a supergene family, and the *DEFLs* are real defensins (Graham *et al.* 2004, Silverstein *et al.* 2005).

The bioinformatics analysis indicated that there is an N-terminal signal peptide predicted in the *PnDEFL1* protein, and its subcellular localization was investigated using its fused expression with *GFP*. Although the green fluorescence of the empty vector with *GFP* was distributed throughout the cell wall and protoplasm, the fluorescence signal of the fusion gene *PnDEFL1-GFP* was detected only in the cell wall, which indicated that *PnDEFL1* is localized in the cell wall. Like *PnDEFL1*, some other defensins and DEFLs are extracellular proteins. The two rice defensin genes *OsDEF7* and *OsDEF8* have been fused with *GFP*, and the fusion proteins localized in the extracellular compartment (Weerawanich *et al.* 2018). In addition, the *V. vinifera* defensin antibacterial peptide Vv-AMP1 fused with *GFP* accumulated also in the extracellular region (De Beer and Vivier 2008). However, *N. alata* *NaD1* has been immunolocalized in vacuoles (Lay *et al.* 2003). The *A. halleri* defensin *AhPDF 1.1* remained in the intracellular rather than the extracellular compartment

Fig. 5. The *PnDEFL1* expressions and resistance analyses of T2 generation *PnDEFL1* transgenic tobacco. The *PnDEFL1* expressions in the T2 generation of transgenic tobacco were detected by reverse transcription quantitative PCR, and the *PnDEFL1* transcripts accumulated in all the transgenic tobacco plants but not in the wild type (WT) (A). The D-2, -4, -5, -7, -12, -16, -17, -18, and -23 are T2 generation transgenic tobacco lines. Leaf-inoculation-assay revealed the enhanced resistance of *PnDEFL1* transgenic tobacco lines against *Fusarium solani* (B). Root-inoculation-assay revealed the enhanced resistance of *PnDEFL1* transgenic tobacco lines against *Fusarium solani* (C).

on the way to the lytic vacuole (Oomen *et al.* 2011). The different localizations of defensins in plant cells suggest that defensins are involved in multiple physiological processes.

Plant defensins have been isolated from a variety of plants and expressed in different organs and tissues. Plant defensins are induced as a part of the systemic defense response during pathogenic attack and environmental stresses, and defensin genes respond to some signalling molecules, including MeJA, ETH, and SA. After inoculation with *F. oxysporum*, many DEFLs show enhanced expressions in induced resistance-displaying wheat seedlings (Odintsova *et al.* 2019). The *OsDEF* expressions are highly induced by *Aspergillus oryzae* infection (Weerawanich *et al.* 2018). Compared with uninoculated seedlings, defensin *PDF1.2b* was significantly up-regulated in the *A. thaliana* leaves after inoculation with *Alternaria brassicicola* or *Pseudomonas syringae* (Tsfaye *et al.* 2013). Moreover, *AtPDF1* responds to *B. cinerea* through the activation of the ETH/JA signalling pathway (Nguyen *et al.* 2014). In addition, the expression of a defensin gene *J1-1* in pepper was induced by *Colletotrichum gloeosporioides* infection as well as an exogenous MeJA treatment (Seo *et al.* 2014). In this study, *DEFL1* from *P. notoginseng* was induced by applications of MeJA, SA, ETH, or H₂O₂ (Fig. 2A-D). In addition, *PnDEFL1*'s expression was further up-regulated in the MeJA pre-treated *P. notoginseng* during the response to *F. solani* infection, with a maximum expression at 24 hpi (Fig. 2E). Thus, *PnDEFL1* is involved in the defense response of *P. notoginseng* to *F. solani*, which may be mediated by several signalling pathways, including JA.

Multicellular organisms produce small cysteine-rich AMPs as an innate defense against a broad range of pathogens. The plant defensins and DEFLs are cysteine-rich AMPs. Because of a large number of cysteines, it may be difficult to express defensin peptides at high activity *in vitro*; however, their antimicrobial activities have been successfully obtained using heterologous expression systems. Four recombinant VvDEFLs purified from *E. coli* inhibited the conidial germination and mycelial growth of *B. cinerea* (Giacomelli *et al.* 2012). The *Phaseolus vulgaris* defensin protein PvD1 was expressed in *E. coli*, and the purified PvD1 inhibited the growth of *Candida albicans* (Mello *et al.* 2014). Lacerda *et al.* (2016) expressed the recombinant *Pisum sativum* defensin Drr230a in *Saccharomyces cerevisiae*, and the recombinant protein Drr230a showed *in vitro* activities against the fungal growth and spore germination of *C. gossypii* var. *cephalosporioides*. The pepper defensin protein J1-1 inhibited the mycelial growth of *F. oxysporum* and *B. cinerea*, and the recombinant J1-1/GST fusion protein significantly inhibited the growth of *C. gloeosporioides* (Seo *et al.* 2014). In this study, the recombinant protein PnDEFL1 purified from *E. coli* significantly inhibited the mycelial growth of *F. solani*, *F. oxysporum*, *B. dothidea*, and *S. sclerotinia* (Fig. 4). *F. solani* and *F. oxysporum* are both important agents that cause root rot disease in *P. notoginseng*, and the antifungal activity of PnDEFL1 clearly indicated that *PnDEFL1* plays a vital role in the

defense against root rot disease in *P. notoginseng*.

In addition, the biological functions of plant defensins as natural antibiotics were confirmed using reverse genetics. The overexpression of *AtPDF1.1* in *A. thaliana* reduced infection by the non-parasitic pathogen *Cercospora beticola* (De Coninck *et al.* 2010). *Petunia* floral defensins have an *in vitro* antifungal activity against *F. oxysporum*, and transgenic bananas overexpressing *PhDef1* and *PhDef2* increase resistance to *F. oxysporum* (Ghag *et al.* 2012). The plant defensin *NaD1* of *N. alata* has been overexpressed in cotton plants, compared with non-transgenic cv. Coker315, and the transgenic line D1 has enhanced the resistance to *F. oxysporum* and *Verticillium dahliae* (Gaspar *et al.* 2014). The constitutive expression of *J1-1* endowed transgenic pepper plants with a strong resistance to the anthracnose fungus (Seo *et al.* 2014). Moreover, the expression of *Def4.2* from *M. truncatula* in transgenic wheat confers resistance to leaf rust caused by the biotrophic basidiomycete fungus *Puccinia tritica* (Kaur *et al.* 2017). Here, the *PnDEFL1* transgenic tobacco exhibited a much greater resistance to *F. solani* infection than the WT (Fig. 5B,C), which is conferred by the overexpression of *PnDEFL1*.

In conclusion, *PnDEFL1*, a defensin like protein gene, was isolated from *P. notoginseng* and characterized. *PnDEFL1* expression was induced by *F. solani* infection and exogenous signalling molecules. In addition, the recombinant PnDEFL1 protein had significant antifungal activities against four pathogenic fungi. The overexpression of *PnDEFL1* also conferred a high resistance to *F. solani* infection. *PnDEFL1* appears to be a key component of the defense system in *P. notoginseng* during responses to root rot disease.

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