Osmotin-Like Protein Gene from *Panax notoginseng* Is Regulated by Jasmonic Acid and Involved in Defense Responses to *Fusarium solani*

Qin Zhao,^{1,2} Bingling Qiu,^{1,2} Shan Li,^{1,2} Yingpeng Zhang,^{1,2} Xiuming Cui,^{1,2} and Diqiu Liu^{1,2,†}

¹ Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, 650500, Yunnan, China

² Yunnan Provincial Key Laboratory of *Panax notoginseng*, Kunming, 650500, Yunnan, China

Accepted for publication 14 April 2020.

ABSTRACT

Osmotin and osmotin-like proteins (OLPs) play important roles in plant defense responses. The full-length cDNA sequence of an OLP gene was cloned from *Panax notoginseng* using rapid amplification of cDNA-end technology and named *PnOLP1*. A quantitative reverse transcription-PCR analysis showed that the signaling molecules methyl jasmonate, salicylic acid, ethylene, and hydrogen peroxide induced *PnOLP1* expression to different degrees. In addition, the expression level of *PnOLP1* rapidly increased within 48 h of inoculating *P. notoginseng* with the root rot pathogen *Fusarium solani*. Subcellular localization revealed that *PnOLP1* localized to the cell wall. A prokaryotic expression vector containing *PnOLP1* was constructed and transformed into *Escherichia coli* BL21 (DE3), and in vitro antifungal assays were performed using the purified recombinant *PnOLP1* protein. The recombinant *PnOLP1* protein

had strong inhibitory effects on the mycelial growth of *F. oxysporum*, *F. graminearum*, and *F. solani*. A plant *PnOLP1*-overexpression vector was constructed and transfected into tobacco, and the resistance of T2 transgenic tobacco against *F. solani* was significantly enhanced compared with wild-type tobacco. Moreover, a *PnOLP1* RNAi vector was constructed and transferred to the *P. notoginseng* leaves for transient expression, and the decrease of *PnOLP1* expression level in *P. notoginseng* leaves increased the susceptibility to *F. solani*. Thus, *PnOLP1* is an important disease resistance gene involved in the defense responses of *P. notoginseng* to *F. solani*.

Keywords: antifungal activity, elicitors, osmotin-like protein, overexpression in tobacco, root rot

Pathogenesis-related proteins (PRs) are a class of disease-related proteins induced by pathogen infections and abiotic factor stimulation, and PRs participate in plant defense responses through the induced systemic resistance, systemic acquired resistance and other ways. PR proteins are classified into 17 families (PR-1 to PR-17) based on their sequences, serological relationships, and biological activities (Van Loon et al. 2006). The PR-5 gene family is mainly composed of thaumatin, thaumatin-like proteins (TLPs), osmotins, and zeamatin (Hakim et al. 2018). Osmotin and the osmotin-like proteins (OLPs) are prevalent in plants. The *Arabidopsis thaliana* OLP gene (*ATOSM*) corresponds to a single copy gene (Capelli et al. 1997). Moreover, an OLP was purified from the seeds of *Benincasa hispida*, and its genomic clone was isolated and confirmed as a single-copy gene (Shih et al. 2001). The strawberry (*Fragaria × ananassa*) OLP gene, designated *FaOLP2*, represents a multigene family (Zhang and Shih 2007). In addition, the *Solanum nigrum* OLP gene, *SniOLP*, belongs to a small multigene family (Jami et al. 2007).

There are many reports on the induction of OLP genes in different plants by a variety of biotic and abiotic stresses. When 5 mM ethyl gallate was sprayed on tobacco (*Nicotiana tabacum*) plants, some defense-related genes, including *osmotin*, were up-regulated (Goupil et al. 2017). Salicylic acid (SA), abscisic acid (ABA), and mechanical wounding trigger the significant induction of strawberry *OLP2* within 2- to 6-h posttreatment (Zhang and Shih 2007). After a heat-shock pretreatment on germinating seeds of

winter wheat (*Triticum aestivum*), the transcriptome profiling indicated that the expression levels of some stress-related genes, such as heat-shock proteins and osmotins, are up-regulated (Zhang et al. 2016). The expression levels of *OSM1* from pepper (*Capsicum annuum*) and *OSM* from *Olea europaea* can be induced by cold acclimation (Hong et al. 2004). After being infected with *Colletotrichum coccodes*, *Xanthomonas campestris*, and *Phytophthora capsici*, the pepper *OSM1* gene was also rapidly expressed at the transcriptional level (Hong et al. 2004). At 72 h after inoculation with *Aspergillus ochraceus*, proteomics using two-dimensional gel electrophoresis revealed that *Arabidopsis* OSM34 is involved in defense responses (Hao et al. 2015). The *OSMs* in *S. lycopersicum*, *S. tuberosum*, *Lycopersicon cerasiforme*, and *S. commersonii* are greatly induced by *Ralstonia solanacearum* infection (Baichoo and Jaufeerally-Fakim 2017).

A homology modeling analysis showed that the *S. nigrum* OLP protein contains an acidic fissure region that may interact with the carbohydrate component of the fungal cell wall (Jami et al. 2007). The osmotin protein permeabilizes the plasma membrane and kills the fungal cells (Ramana Rao et al. 2011). The osmotin (CpOsm) from latex of *Calotropis procera* may induce membrane permeabilization in the spores and hyphae of *Fusarium solani*, as indicated by their uptake of the fluorogenic dye propidium iodide (de Freitas et al. 2011a). In addition, the CpOsm protein is relatively thermostable and retains its antifungal activity over a wide pH range (Ramos et al. 2015). Antimicrobial tests showed that Osm1 derived from *Theobroma cacao* expressed in *Escherichia coli* inhibited the growth of yeast (*Pichia pastoris*) in vitro (Falcão et al. 2016). Recombinant osmotic proteins from tobacco have significant antimicrobial activities, particularly against fungal pathogens causing candidiasis and otitis (*Candida parapsilosis* and *Candida tropicalis*) and two common food pathogens (*Debaryomyces hansenii* and *Torulaspora globosa*) (Viktorova et al. 2017). The osmotins and OLPs are important parts of plant immune system.

Panax notoginseng (Burk) F. H. Chen is an important traditional Chinese herbal medicine, and it is mainly artificially cultivated in

†Corresponding author: D. Liu; diqiu@126.com

Funding: This work was financially supported by two grants received from the National Natural Sciences Foundation of China (81560610) and Yunnan Ten Thousand Talents Plan Young & Elite Talents Project.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online.

The author(s) declare no conflict of interest.

Wenshan Prefecture, Yunnan Province. The growth cycle of *P. notoginseng* is very long and requires warm and damp growing conditions. Therefore, there are a variety of diseases and pests that seriously endanger the growth and development of *P. notoginseng*. The root rot disease is the most serious problem limiting the production of the *P. notoginseng* and mainly caused by *F. solani* (Chen et al. 2016; Miao et al. 2006). It is unfortunate that there is no specific and environmentally friendly approach to control root rot disease in *P. notoginseng* (Tang et al. 2019). In our previous study, exogenous applications of methyl jasmonate (MeJA) on *P. notoginseng* roots increased the resistance to root rot caused by *F. solani* infections, and a unigene encoding the OLP responded to the exogenous MeJA and *F. solani* infection through transcriptome sequence of *P. notoginseng* roots (Liu et al. 2019). In this study, the gene expression and functional analysis of this OLP gene (*PnOLP1*) were performed to further understand the defense mechanisms involved in *P. notoginseng*'s response to *F. solani* exposure.

MATERIALS AND METHODS

Plant and fungal materials. The cultivated *P. notoginseng* has become a highly mixed population during artificial planting over a long period of time. Therefore, the material used in this study is the cultivated *P. notoginseng* population. The healthy 1-year-old cultivated *P. notoginseng* population was collected from a *P. notoginseng* planting base, and then was transplanted in a greenhouse with double shady nets. The sterile nontransgenic tobacco (*N. tabacum* L. 'Xanthi') seedlings were cultured in a light incubator (25°C, 16-h light cycle). Three pathogenic fungi, *F. solani*, *F. oxysporum*, and *F. graminearum*, were preserved and characterized in our laboratory, and the fungi were activated on potato dextrose agar (PDA) medium before being used.

Chemical treatments and inoculation. To analyze the expression pattern of the *PnOLP1* gene, the concentrations of four plant elicitors including the SA, MeJA, ethephon (ETH), and hydrogen peroxide (H₂O₂), were determined by referring to the chemical treatment method in Wang et al. (2019). The *P. notoginseng* seedlings showed normal growth after independent treatment with SA (200 µM/liter), JA (100 µM/liter), ETH (1 mM/liter), and H₂O₂ (1 mM/liter) for 1 month. The *P. notoginseng* roots were dipped in MeJA, ETH, and SA solution with the aforementioned concentrations for 30 min, respectively. The treated roots were collected at 4-, 12-, 24-, 48-, and 72-h posttreatment, respectively. For *F. solani* inoculation, the *P. notoginseng* roots were pretreated with MeJA (100 µmol/liter) for 30 min. After 24 h, the roots were then infected with a suspension of *F. solani* (2 × 10⁶ spores/ml) for 30 min, and then the infected roots were harvested at 4-, 12-, 24-, 48-, and 72-h postinoculation, respectively. The *P. notoginseng* roots pretreated with sterile water were used as a control for the chemical treatments and fungal inoculation. All the treatments and inoculation were performed

with three biological replicates, and the *P. notoginseng* samples were frozen in liquid nitrogen and stored at -80°C before RNA extraction.

Bioinformatics analyses. The full-length cDNA of *PnOLP1* was cloned using a SMART RACE cDNA Amplification Kit (Clontech, U.S.A.). The gene-specific primers for *OLP* are listed in Table 1. Some OLP protein sequences show high homologies with *PnOLP1* were downloaded from the NCBI website after searching protein data with blastp tool. In addition, several plant OLP and osmotin genes were reported to be involved in defense response to stress, and the protein sequences of these genes were also included in the phylogenetic analysis. The unrooted tree was constructed with the neighbor-joining method using MEGA software after the multiple alignment with ClustalX, and the bootstrap values were calculated from 1,000 replicates. The protein localization prediction in plants was analyzed using the PredictProtein tool (<https://www.predictprotein.org/443/>). The signal peptide prediction was performed using the SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The protein disulfide bonds were predicted using the PROSITE online tool (<https://prosite.expasy.org/>).

Quantitative reverse transcription-PCR (qRT-PCR). The qRT-PCR was used to analyze the expression level of *PnOLP1* gene at different time periods after inoculation with *F. solani* or after treatment with MeJA, ETH, H₂O₂, and SA. The internal reference gene is the *P. notoginseng* actin-2 gene (*PnACT-2*, KF815706.1). The methods of RNA extraction and cDNA synthesis were the same as those of Liu et al. (2018). The qRT-PCR reaction system contained 2 µl of cDNA template, 10 µl of 2× Go Taq qPCR Master Mix (Promega, U.S.A.), 6.8 µl of RNase-Free ddH₂O, 0.6 µl of forward primer, and 0.6 µl of reverse primer (Table 1). The amplification conditions and equipment used for PCR were consistent with the qRT-PCR method in Liu et al. (2018). All the qRT-PCR assays included three biological repeats.

Subcellular localization of the PnOLP1 protein. The subcellular localization vector pBINm-gfp5-ER was used in this assay. Gene-specific primers (Table 1) were designed to contain *EcoRV* and *EcoRI* recognition sites. The *PnOLP1* open reading frame (ORF) without the stop codon was obtained by PCR using cDNA reverse-transcribed from RNA of *P. notoginseng* roots. The ORF was subcloned into a pBIN m-gfp5-ER vector, and the recombinant vector was transformed into *Agrobacterium tumefaciens* EHA105. The *Agrobacterium tumefaciens* harboring the pBIN m-gfp5-ER-*PnOLP1* plasmids were obtained by PCR and used to the transiently express in onion epidermal cells. The transformed onion epidermal cells were incubated for 2 days on the coculture medium, and then the PnOLP1-GFP fusion protein was localized using a laser scanning confocal microscope (Nikon A1R, Japan). The detailed protocol was published previously in Liu et al. (2018).

Expression and purification of the recombinant PnOLP1 protein. Gene-specific primers (Table 1) were designed to amplify

TABLE 1. The gene-specific primers

Experiments	Target genes	Primer sequences
RACE	<i>PnOLP1</i> (MN102348)	Forward: 5'TTCCCTCTCATCTCTACAATGGCTT3' Reverse: 5'CGTACTACTTTTCGATGCCTCTTGTA3'
qRT-PCR	<i>PnOLP1</i> (MN102348)	Forward: 5'CACCTATTCCCAACCGCTTC3' Reverse: 5'TAACGGGACAAGTGGCAAGGA3'
	<i>PnACT-2</i> (KF815706.1)	Forward: 5'TCCAAGGGTGAATATGATGATCG3' Reverse: 5'AACCTCTCCAAAGAGAAATTTCTGAGT3'
	<i>Actin</i> (AB158612.1)	Forward: 5'TCCCATTGAGCATGGAATAGTAAGC3' Reverse: 5'TACATGGCAGGTACATTGAAAGTCT3'
Subcellular localization	<i>PnOLP1</i> (MN102348)	Forward: 5'CATATCGCTTCCACTCTTCTAGTCTCTT3' Reverse: 5'GAATTCGTGACAGAAAATGACCTTGAGC3'
Recombinant protein expression	<i>PnOLP1</i> (MN102348)	Forward: 5'GATATCATGCAACCGGGCTCATTCT3' Reverse: 5'GAATTCCTTAGTGACAGAAAATGACCTTGAGC3'
Overexpression in tobacco	<i>PnOLP1</i> (MN102348)	Forward: 5'GGATCCATGGCTTCCACTCTTCTAGTCTCT3' Reverse: 5'GAATTCATTAGTGACAGAAAATGACCTTGAG3'
RNAi	<i>PnOLP1</i> (MN102348)	Forward: 5'ACTGCCCTTCACCGTTTGG3' Reverse: 5'TTGCAACTCTGAGGGCTATT3'

the *PnOLP1* ORF without the 5' terminus that encodes the secretion signal peptide. The truncated *PnOLP1* was then inserted into the multiple cloning site of the pET-32(a) vector. The recombinant pET-32(a)-*PnOLP1* plasmids were further transformed into *Escherichia coli* BL21 (DE3) to express the recombinant protein containing a N-terminal His-tag. The positive clones were incubated in LB broth medium in the presence of ampicillin. Following incubation at 37°C until an optical density reading of 0.6 to 0.8 at 600 nm, the recombinant *E. coli* BL21 were placed on ice for 30 min, and then they were incubated with isopropyl β -D-thiogalactoside at 1 mmol/liter for 7 h at 25°C to induce the protein. The PnOLP1 protein was expressed in the inclusion bodies. An inclusion body solubilizing solution (50 mM Tris-HCl, pH 8.8, 300 mM NaCl, and 8 M urea) was added to the pellet, and the sample was incubated on a shaker (room temperature at 220 rpm) for 1.5 h to denature the protein. The supernatant and precipitate were collected after centrifugation (4°C at 10,000 rpm for 30 min). The denatured recombinant protein in the supernatant was purified using a Ni-NTA-Sepharose column (Sangon Biotech, China). The denatured protein was subjected to dialysis renaturation through a dialysis bag, and the target protein was concentrated using a Millipore ultrafiltration tube (10 kD) having a molecular weight cut-off for the His-PnOLP1 fusion protein of 1/6 to 1/3. The concentrated recombinant protein was used for the in vitro antifungal activity analysis.

In vitro antifungal assay of the recombinant PnOLP1 protein. *F. oxysporum*, *F. graminearum*, and *F. solani* hyphae were inoculated on PDA medium and cultured at 28°C for 2 to 3 days. Then the sterile filter paper (6 mm in diameter) was placed evenly on the edge of the colony, and 5, 10, and 15 μ g of recombinant PnOLP1 protein solution was placed sequentially on the filter paper. The amount of the recombinant protein was measured with the Bradford method using a Bradford Protein Assay Kit (Sangon Biotech, China). The negative controls were the sterile water and phosphate buffer (pH 8.0). After the fungi were incubated at 28°C for 2 to 3 days, the growth states of the three pathogenic fungal hyphae were recorded. The zones of fungal growth inhibition around the disks were measured using Adobe Photoshop version 7.0, and the antifungal assay was repeated three times.

Generation and selection of PnOLP1 transgenic tobacco. The *PnOLP1* ORF was obtained using gene-specific primers (Table 1) and then ligated into the pGEM-T vector. The pGEM-T-*PnOLP1* vector and the plant expression vector pCambia2300s were digested with restriction endonucleases *Eco*RI and *Bam*HI, respectively, and then the gel-recovered fragments were ligated using T4 DNA ligase. The ligated product was transferred into *E. coli* DH5 α , and the positive clones containing pCambia2300s-*PnOLP1* were screened by PCR. The pCambia2300s-*PnOLP1* recombinant vector was transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method, and then the positive clones were obtained by PCR. The pCambia2300s-*PnOLP1* plasmids in *Agrobacterium tumefaciens* LBA4404 were transferred into WT tobaccos by the tobacco leaf disc method. The methods of screening transgenic tobacco and obtaining T2 transgenic tobacco were similar to those of Liu et al. (2018).

Resistance identification of the T2 generation of transgenic tobacco. The qRT-PCR was used to assess the transcription levels of *PnOLP1* in the T2 tobacco lines, and the reference gene was the tobacco *actin* gene (AB158612.1) (Table 1). In addition, the three lines of T2 transgenic tobacco with high *PnOLP1* expression levels were selected for to determine their resistance to *F. solani* infection. The same size wounds were gently created on the tobacco leaves with sandpaper, and 100 μ l of *F. solani* spore suspension (2×10^6 spores/ml) was inoculated on the wound sites. All the inoculated leaves were placed on wet filter paper to maintain moisture and cultured in a light incubator (25°C, 16-h light/day). After incubating for 7 days, the disease symptoms were recorded with a digital camera (Canon, Japan). Additionally, sterile WT tobacco and *PnOLP1* transgenic tobacco plants of a uniform size were selected to receive the

F. solani inoculations. The tobacco roots were wounded with scissors, and the treated tobacco roots were immersed in a spore suspension (2×10^6 spores/ml). The inoculated tobacco plants were cultured for 12 days in a light incubator, and the disease symptoms were recorded using the digital camera. The inoculation experiment was repeated three times.

Transient expression PnOLP1 RNAi construct in P. notoginseng leaves. The *PnOLP1*-specific primers with attB adapter (Table 1) were designed to amplify the RNAi fragment (520 bp). A Gateway BP Clonase II Enzyme Mix (Invitrogen, U.S.A.) was used to perform a recombination reaction between the *PnOLP1* RNAi PCR product and the pHellsgate2 vector, and then the BP reaction mixture was transformed into the *E. coli* DH10B. The pHellsgate2-*PnOLP1* recombinant plasmids were further transformed into *Agrobacterium tumefaciens* LBA4404, and the positive clones were identified by PCR, which were used to infect the *P. notoginseng* leaves.

The same size wounds were gently created on the healthy *P. notoginseng* leaves with sand paper, and then the leaves placed on the damp filter paper. The *Agrobacterium tumefaciens* suspension (50 μ l) containing the pHellsgate2-*PnOLP1* recombinant plasmid was added to the wound sites, and the infected leaves were cultured in a light incubator for 24-h in order to transiently express the *PnOLP1* RNAi construct in *P. notoginseng* leaves. In addition, the infection with nontransformation *Agrobacterium tumefaciens* suspension was performed with the same method as a control. The infected leaves were further inoculated with *F. solani* spore suspension (2×10^6 spores/ml) at the wound sites, and then were cultured in a light incubator (25°C, 16-h light/day). The disease symptoms of *P. notoginseng* leaves were recorded using the digital camera after 72 h, and the leaves were collected and frozen in liquid nitrogen for RNA extraction. The *PnOLP1* expression level in *P. notoginseng* leaves after transient expression of *PnOLP1* RNAi construct was analyzed by qRT-PCR.

Statistical analyses. The transcription levels of *PnOLP1* in *P. notoginseng* and T2 tobacco lines, lesion size caused by *F. solani* infection as well as the fungal growth inhibition zones, are shown as means with standard deviations. Student's *t* test was performed using SPSS software to reveal the statistical differences between the treatments or inoculations and the controls.

RESULTS

Molecular cloning and bioinformatics analysis of PnOLP1. In this study, an OLP gene, *PnOLP1* (GenBank accession number MN102348), was isolated from *P. notoginseng*. The *PnOLP1* cDNA was 1,098 bp, and it contained an ORF of 744 bp, a 5' untranslated region (UTR) of 43 bp, and a 3' UTR of 311 bp. *PnOLP1* encoded a protein of 247 amino acid residues with a calculated molecular mass of 26.4 kDa and theoretical isoelectric point of 7.21. PredictProtein predicted the protein to have a signal peptide of 22 amino acid residues at the N-terminus, and it was predicted to be a secreted protein. The mature PnOLP1 was predicted to have 225 amino acid residues with a calculated molecular mass of 24.1 kDa and theoretical isoelectric point of 7.31. PredictProtein predicted the secondary structure of the PnOLP1 protein to contain an α -helix, β -sheet, and random coil, with percentages of 9.72, 15.79, and 74.49%, respectively. Moreover, there may be 8 disulfide bonds in PnOLP1 protein structure according to the bioinformatics prediction result with PROSITE. The deduced PnOLP1 protein was highly homologous with known plant OLPs, and it shared an 82, 78, and 79% identity with *Quercus suber* (XP_023907784.1), *Ziziphus jujuba* (NP_001310784.1), and *Erythranthe guttata* (XP_012827362.1) OLPs, respectively.

In addition, a phylogenetic tree of 20 plant OLPs and osmotins was constructed, that consists of two big branches (Fig. 1). The upper branch is composed of PnOLP1 and 11 homologous OLPs from different species, which is clearly divided into two groups. The OLPs from *Brachypodium distachyon* (XP_003564582.1),

Hordeum vulgare (BAK00635.1), *Zea mays* (NP_001147098.2), *Oryza sativa* Japonica group (BAG87528.1), and *O. sativa* Indica group (ADI43216.1) fall into the monocotyledonous group. The dicotyledonous group contains seven OLPs from *E. guttata* (XP_012827362.1), *Gossypium raimondii* (XP_012446484.1), *G. hirsutum* (XP_016750563.1), *Herrania umbratica* (XP_021278215.1), *Z. jujuba* (NP_001310784.1), *Q. suber* (XP_023907784.1), and *P. notoginseng*, respectively. The lower branch consists of six OLPs and two osmotins that have been reported to be involved in defense response to stress.

***PnOLP1* responded to several signaling molecule treatments and was up-regulated during *F. solani* infection.** The *PnOLP1* expression level in *P. notoginseng* roots was induced by MeJA, ETH, H₂O₂, and SA treatments (Fig. 2A to D). Compared with the control, the MeJA treatment rapidly induced the expression of *PnOLP1*, reaching the maximum level at 4 h. For the ETH, H₂O₂, and SA treatments, the highest *PnOLP1* expression levels were reached at 48 h. Our previous study indicated that a pretreatment with MeJA increased the resistance of *P. notoginseng* to *F. solani* infection, and based on the qRT-PCR analysis in the present study, the transcriptional level of *PnOLP1* was higher in the MeJA-pretreated *P. notoginseng* roots during *F. solani* infection than in the sterile water-pretreated roots (Fig. 2E). The maximum *PnOLP1* expression was recorded at 48-h postinfection in the *P. notoginseng* roots pretreated with MeJA, and the relative gene expression value was roughly 2.8

times as much as the control (sterile water-pretreated without inoculation). In brief, the four signaling molecules induced the expression level of *PnOLP1* in *P. notoginseng* roots, and the MeJA-pretreatment greatly up-regulated *PnOLP1* expression during *F. solani* infection. These data indicated that *PnOLP1* is involved in *P. notoginseng* defense responses against *F. solani*.

***PnOLP1* protein is localized in the plant cell wall.** It was predicted that the protein PnOLP1 was localized on the plant cell wall. *Agrobacterium tumefaciens* containing the *PnOLP1-GFP* fusion gene was used to transform onion epidermal cells to confirm the localization of PnOLP1. The green fluorescence in the onion cells transformed with the *PnOLP1-GFP* was specifically distributed on the cell wall (Fig. 3). In contrast, the green fluorescence of the empty vector was uniformly distributed throughout the onion epidermal cells. Thus, PnOLP1 is localized in the plant cell wall.

The recombinant PnOLP1 protein expressed in *E. coli* possesses antifungal activities to *P. notoginseng* root rot disease pathogens. A truncated form of *PnOLP* lacking the secretory signal sequence was cloned into the prokaryotic expression vector pET32a and heterologously expressed in *E. coli* to obtain a recombinant PnOLP1 protein. The *E. coli* BL21 cells with pET32a-*PnOLP1* were induced with isopropyl β-D-thiogalactoside at 1 mmol/liter for 7 h at 25°C, and the SDS-PAGE analysis indicated that large amount of recombinant protein with approximately 40 kDa molecular mass was induced

Fig. 1. Phylogenetic tree of PnOLP1 and 19 other plant osmotin-like proteins (OLPs). The tree was created using the neighbor-joining method with MEGA3.1. The scale bar equals a distance of 10 changes per 100 amino acid positions, and the numbers on each node represent bootstrap values of 1,000 replicates.

(Supplementary Fig. S1a). The Ni-NTA column was used to bind the PnOLP1 recombinant protein, and then the purified protein was eluted with 100 mM imidazole elution buffer (Supplementary Fig. S1b). Furthermore, the antifungal assay showed that the PnOLP1 recombinant protein inhibited the mycelial growth of the *P. notoginseng* root rot disease-related pathogens *F. oxysporum*, *F. solani*, and *F. graminearum* (Fig. 4A to C). Compared with the blank control (sterile water), the fungal growth inhibition scores produced by 5, 10, and 15 µg/ml of recombinant PnOLP1 protein against *F. oxysporum* were 23.92, 28.44, and 63.14 mm², respectively (Fig. 4D). The inhibition scores for the same amounts of protein against *F. solani* were 20.58, 33.03, and 51.02 mm²,

respectively, while they were 29.06, 37.89, and 64.81 mm², respectively, against *F. graminearum* (Fig. 4D). Thus, the PnOLP1 recombinant protein had the strongest inhibitory activity against the mycelial growth of *F. graminearum*, and the antifungal activities increased along with the recombinant PnOLP1 protein level.

Overexpression of *PnOLP1* conferred a high level of resistance to *F. solani* infection. To further understand the biological function of *PnOLP1*, the plant overexpression vector pCambia2300s-*PnOLP1* was constructed, and more than 100 tobacco leaf discs were transformed by *Agrobacterium tumefaciens*. A total of 45 T0 transgenic tobacco plants were screened using PCR with *PnOLP1*-specific primers. In addition, 12

Fig. 2. Expression profile of *PnOLP1* in *Panax notoginseng* roots. *P. notoginseng* roots after independent exposures to **A**, methyl jasmonate (MeJA) at 100 µmol/liter, **B**, ethephon (ETH) at 1 mmol/liter, **C**, H₂O₂ at 1 mmol/liter, and **D**, salicylic acid (SA) at 200 µmol/liter were collected at 4-, 12-, 24-, 48-, and 72-h posttreatment, respectively. **E**, Moreover, the *P. notoginseng* roots respectively pretreated with sterile water and MeJA at 100 µmol/liter were inoculated with *Fusarium solani* spore suspension (2×10^6 spores/ml), the roots were collected at 4-, 12-, 24-, 48-, and 72-h postinoculation, respectively. The relative transcription levels of *PnOLP1* were assessed by qRT-PCR, and the results are shown as the average values calculated from three replicates.

Fig. 3. Subcellular analysis of the transient expression of the *PnOLP1*-GFP fusion gene in onion epidermal cells revealed the location of PnOLP1 in the cell wall. The *PnOLP1*-GFP fusion gene and GFP (control) were independently transiently expressed in onion epidermal cells transformed with *Agrobacterium tumefaciens* independently harboring the two constructs. After transformation for 48 h, the epidermal cells were viewed using a confocal laser microscope showing green fluorescence (GFP), bright field illumination (bright), and combined (merged) views.

transgenic tobacco plants were randomly selected to develop the T2 transgenic tobacco lines (O2, O5, O6, O7, O13, O16, O19, O20, O22, O25, O26, and O33). The qRT-PCR analysis indicated that *PnOLP1* was overexpressed in all the transgenic lines (Fig. 5A). The CaMV 35S promoter of the overexpression vector directed the stable expression of *PnOLP1* in the transgenic plants. Based on the qRT-PCR, the tobacco lines O2, O6, and O7, having high *PnOLP1* expression levels, were selected for further study.

To evaluate the resistance levels of *PnOLP1* transgenic tobacco, the young leaves of three transgenic tobacco lines and WT tobacco were inoculated with *F. solani* spore suspensions at wound sites. After 7 days of inoculation, the leaves of the transgenic lines showed slight decaying and yellowing around the inoculation sites (Fig. 5B), while more evident yellowing and rot, over an area of 300 mm², was presented in the WT tobacco leaves (Fig. 5C). In addition, the roots of WT and transgenic tobacco seedlings were inoculated with *F. solani*, and the disease symptoms are shown in Figure 5D. The roots of WT tobacco were severely blackened, rotted, and easily detached. Additionally, whole leaves withered, and the stems and leaves near the roots turned yellow and rotten. However, the transgenic lines showed mild symptom, such as a slight blackening of roots, and they grew normally (Fig. 5D). Thus, the overexpression of *PnOLP1* in tobacco enhanced its resistance to *F. solani* infection.

Transient expression of *PnOLP1* RNAi vector in *P. notoginseng* leaves increased susceptibility to *F. solani*. The stable genetic transformation system for *P. notoginseng* is not established so far, therefore, the *PnOLP1* RNAi construct was transiently expressed in the *P. notoginseng* leaves in order to analyze its function. The *PnOLP1* RNAi construct was expressed in the leaves mediated by *Agrobacterium tumefaciens*, which was followed by the *F. solani* inoculation. The result indicated that both the *PnOLP1* RNAi construct expressed leaves and the control leaves showed evident rot after inoculation with *F. solani*; however, the lesion

size of the *PnOLP1* RNAi construct transiently expressed *P. notoginseng* leaves was significantly bigger than that of control (Fig. 6A and B). Furthermore, the qRT-PCR showed that the *PnOLP1* expression level in *P. notoginseng* leaves expressing the *PnOLP1* RNAi construct was about 2.2 times lower than that of the control after inoculation with *F. solani* for 72 h (Fig. 6C). It is evident that the decreased *PnOLP1* expression level in *P. notoginseng* leaves increased the susceptibility to *F. solani* infection.

DISCUSSION

Osmotins and OLPs belong to the PR5 family, and they play important roles in the plant defense system against a variety of biotic and abiotic stresses (Cao et al. 2015; Hakim et al. 2018). In this study, an OLP gene was isolated from the famous Chinese medicinal plant *P. notoginseng*. The deduced PnOLP1 protein has a signal peptide at the N-terminus like some osmotins and OLPs from *S. dulcamara*, *Hordeum vulgare*, and *Hevea brasiliensis* (Newton and Duman 2000; Reiss et al. 2006; Tong et al. 2016). In addition, the proteins in the PR5 family, including TLPs and osmotins, are cysteine-rich proteins. For example, barley TLP 1-8 has 10 or 16 cysteine residues (Reiss et al. 2006). A total of 17 cysteine residues are distributed in the PnOLP1 protein sequence, and the bioinformatics prediction result showed that these cysteine residues may form 8 disulfide bonds, that could provide stability to the protein under adverse environmental conditions.

The secretory proteins are conventionally transported through the endoplasmic reticulum to the Golgi and then to the plasma membrane where they are released into the extracellular space (Rabouille 2017). The PnOLP1 was predicted to be a secreted protein and localized on the cell wall in this study, and the subcellular localization result supported the prediction by showing that PnOLP1 is distributed in the onion epidermal cell wall. *Glycine max* OLPa protein with a predicted N-terminal signal peptide is secreted into the extracellular matrix, while GmOLPb protein with a N-terminal signal peptide and the C-terminal vacuolar target peptide is secreted into the vacuolar compartment (Onishi et al. 2006; Tachi et al. 2009). The CAOSM1 mRNA is prevalently localized in the phloem cells of vascular bundles in pepper leaf, however, the subcellular localization indicates that CAOSM1 protein is localized in the plasma membrane of *N. benthamiana* leaf cells (Choi et al. 2013; Hong et al. 2004). In addition, HbOsmotin protein from the latex of *Hevea brasiliensis* is mainly distributed in the luteoid fraction of latex, and the subcellular localization indicates that the protein is distributed in the cell wall of onion epidermal cells (Tong et al. 2016). HbOsmotin primarily remains in the luteoid, moreover, it could be secreted by protoplasts. The plant cell wall is the first obstacle for pathogens to penetrate and infect the plant cells, and it is also a sensor for pathogen invasion. The plant cells recruit their own cell surface pattern recognition receptors to identify the pathogen-associated molecular patterns (PAMP) during pathogen infection (Boller and He 2009), meanwhile, some cell wall proteins show evident antimicrobial activity (Bellincampi et al. 2014). Although the diversified localizations of plant OLP proteins may be associated with their different functions in plant cells, the cell wall-localized PnOLP1 protein may act as a defense protein that interacts with invading pathogens during the early stage of infection.

P. notoginseng is a highly valued medicinal plant in China, but the root rot seriously impairs the production and quality of *P. notoginseng* raw material. Our previous study indicated that exogenous application of MeJA on roots of *P. notoginseng* induced a high level of resistance to the root rot pathogen *F. solani* (Liu et al. 2019). Many genes involved in the plant–pathogen interactions were predominantly up-regulated by MeJA; moreover, these genes showed increased transcriptional levels after inoculation with *F. solani*. This study clearly showed that the *PnOLP1* is a defense related genes responding to the exogenous application of MeJA,

Fig. 4. Antifungal assay using the recombinant PnOLP1 protein. The recombinant PnOLP1 protein has evident antifungal activities to **A**, *Fusarium oxysporum*, **B**, *F. solani*, and **C**, *F. graminearum*. Water and phosphate buffer (pH 8.0) are the negative controls. **D**, In addition, the fungal growth inhibition analysis showed that the antifungal activities increased along with the recombinant PnOLP1 protein level. Results are shown as the average values calculated from three replicates, and the Student's *t* test was used to analyze the statistical differences between the recombinant PnOLP1 protein and the negative control (** *P* < 0.01, * *P* < 0.05).

which hints that the *PnOLP1* is regulated by the JA signaling during defense to *F. solani* infection. In addition, some phytohormone signaling pathways including the ETH and SA are important parts of signal transduction in the plant immune response, and the crosstalk among these different signaling pathways is ubiquitous (Vos et al. 2015). The gene expression pattern analysis indicated that the *PnOLP1* expression was induced by the SA and ETH

treatments. Similarly, the exogenous MeJA/ETH also significantly induced the expression of *OLP* gene from *Ocimum basilicum* and the osmotin gene *SINP24* from germinated tomato seeds (Pluskota et al. 2019; Rather et al. 2015). Furthermore, the transcriptome sequencing data showed that the SA and ETH signaling pathway-related genes were positively regulated in the MeJA treated *P. notoginseng* during response to *F. solani* (Liu et al. 2019),

Fig. 5. Analyses of the T2 generation *PnOLP1* transgenic tobacco lines. **A**, The expression level of *PnOLP1* in the T2 generation of transgenic tobacco was assessed by qRT-PCR. *PnOLP1* was overexpressed in the T2 transgenic tobacco lines. **B**, The leaf inoculation assay revealed the enhanced resistance of *PnOLP1* transgenic tobacco lines against *Fusarium solani*. **C**, Measurements of leaf lesions caused by infection with *F. solani*. **, $P < 0.01$, compared with the wild type (WT) using Student's *t* test. **D**, Enhanced resistance of *PnOLP1* transgenic tobacco seedlings against *F. solani* using the root-dip inoculation method. O2, O6, and O7 represent transgenic tobacco lines.

suggesting that the crosstalk among JA, SA, and ETH signaling pathways is present in the immune reaction to *F. solani*. The molecular mechanism of the *PnOLP1* regulated by the hormone signaling pathways needs to be further explored.

In order to escape the PAMP recognition of plant defense system, pathogens produce the effector proteins that counteract the plant defense responses, meanwhile, the antimicrobial proteins that are the final effectors of plant defense can recognize the pathogen effectors (Giraldo and Valent 2013; Lee et al. 2010). Studies have shown the specific interaction between osmotin and pathogenic plasma membrane, and the osmotin protein is an ultimate effector of plant defense with antifungal activity (Narasimhan et al. 2001, 2005). There is a potential target position of osmotin in the fungal membrane, and when the osmotin reacts with the fungal wall, it allows the osmotin to enter the plasma membrane, which in turn leads to the formation of transmembrane pores and rupture of the fungal membrane (Anžlovar and Dermastia 2003; Hakim et al. 2018; Singh et al. 2013). Moreover, the conserved fungal genes including the *FOR1* (*Fusarium Osmotin Resistance*), *FOR2*, and *FOR3* that determine the cell wall characteristics play an important role in regulating the sensitivity of fungi to plant defense protein osmotin (Lee et al. 2010). This may be the reason why the fungi show different sensitivity to the different plant antimicrobial proteins. The OLP protein purified from *Calotropis procera* latex has strong antifungal activity against *F. solani* and smaller activity against *Colletotrichum gloeosporioides* (de Freitas et al. 2011b). This study confirmed that the protein encoded by the *PnOLP1* gene is an antifungal protein, moreover, the heterologously expressed PnOLP1 recombinant protein in *E. coli* inhibited the mycelial

growth of *F. oxysporum*, *F. graminearum*, and *F. solani* with small difference in activity. All these three *Fusarium* pathogens not only harm the *P. notoginseng* growth and development, but also threaten the quality of original medicinal materials. The high level of antifungal activity against the three *Fusarium* pathogens hints that the PnOLP1 maybe directly interacts with the root rot pathogens as a cell wall defense protein.

Furthermore, the reverse genetics technology was used to verify the function of *PnOLP1* in this study. The transgenic tobacco expressing the *PnOLP1* showed strong resistance to *F. solani*. In addition, the gene silencing was induced by the RNAi vector of *PnOLP1* through transient expression in the *P. notoginseng* leaves, and the reduced *PnOLP1* expression increased the susceptibility to *F. solani* infection. These data clearly indicate that *PnOLP1* is an important resistance gene in the defense responses of *P. notoginseng*. *Fusarium* disease is well known to be an important factor, which causes heavy agricultural losses, and the *PnOLP1* can be transferred into the food and economic plants in order to develop the genetically modified plants with a high level of resistance to *Fusarium* pathogens. The transgenic potato plants expressing an OLP gene (*pA13*) showed increased resistance to the *Phytophthora infestans* (Sanju et al. 2015). The *S. nigrum* OLP and rice *Chi11* were transferred into tomato, and the resistance against *Alternaria solani* and *F. oxysporum* was increased (Kumar et al. 2016). Moreover, the transgenic rice lines expressing the tobacco osmotin gene exhibited a high level of resistance to sheath blight (*Rhizoctonia solani*) (Sripriya et al. 2017). Additionally, there is hope in developing the transgenic plants with broad-spectrum resistance to a variety of different pathogens by cotransformation of *PnOLP1* and other reported disease resistance genes.

Conclusion. This is the first report on OLP gene characterization from *P. notoginseng*. The antifungal activity in vitro suggests that the PnOLP1 protein directly participates in the defense process against the infection of *P. notoginseng* root rot pathogen. Moreover, the *PnOLP1* gene plays an important role in *P. notoginseng* defense response mediated by the JA signaling pathway during *F. solani* infection, in which the SA and ETH signaling pathways may crosstalk the JA signaling in modulating the *PnOLP1* expression. In addition, *PnOLP1* is a new candidate gene for plant disease-resistant genetic engineering.

ACKNOWLEDGMENTS

We thank Lesley Benyon, Ph.D., from Liwen Bianji, Edanz Group China (<http://www.liwenbianji.cn/ac>), for editing the English text of a draft of this manuscript.

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Fig. 6. Analysis of *Panax notoginseng* leaves after transient expressing the *PnOLP1* RNAi construct. **A**, Inoculation experiments showed that the transient expression of *PnOLP1* RNAi construct in the *P. notoginseng* leaves increased the susceptibility to *Fusarium solani* infection compared with the control (the *P. notoginseng* leaves transiently expressing the empty RNAi vector after inoculation with *F. solani* for 72 h). **B**, Measurements of leaf lesions caused by *F. solani* infection. The Student's *t* test was used to analyze the statistical difference (*, $P < 0.05$). **C**, The expression level of *PnOLP1* in the *P. notoginseng* leaves transiently expressing the *PnOLP1* RNAi construct evaluated by qRT-PCR. Control, the *P. notoginseng* leaves infected with *Agrobacterium tumefaciens* suspension containing the pHellsgate2-*PnOLP1* and pHellsgate2 empty vector, respectively; and 72 h, two control samples were inoculated with *F. solani* for 72 h. Results are shown as the average values calculated from three replicates, and the Student's *t* test was used to analyze the statistical differences (** $P < 0.01$). The empty RNAi vector infected samples without inoculation with *F. solani* was the control of statistical analysis.

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