

Isolation and functional characterization of a pathogenesis-related protein 4 gene from *Panax notoginseng*

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Abstract

Pathogenesis-related (PR) proteins are a large class of proteins that accumulate in plant cells in response to multiple biotic and abiotic stresses. The PR proteins are usually divided into 17 families. Root rot is an important disease of the perennial medicinal plant *Panax notoginseng*, with *Cylindrocarpon destructans* identified as one of the causative pathogens. However, little is known about the PR-encoding genes expressed during the infection of *P. notoginseng* by *C. destructans*. In this study, we isolated a *PR4* gene (*PnPR4*) from *P. notoginseng* on the basis of a genomic analysis. The expression of this gene was significantly up-regulated in the roots of *C. destructans*-infected *P. notoginseng* plants at 72 h post-inoculation (relative to the control level). The expression of *PnPR4* was affected by hydrogen peroxide, gibberellic acid, ethephon, abscisic acid, and salicylic acid. The transient expression of the *PnPR4-GFP* fusion gene in tobacco epidermal cells indicated that PnPR4 was localized in the cell wall (i.e., extracellular protein). In addition, the purified PnPR4 recombinant protein had antifungal activities, with inhibitory effects on *C. destructans*. Furthermore, the *PnPR4* gene was transferred into the model plant tobacco to verify its function. The overexpression of *PnPR4* increased the resistance to *C. destructans*. In conclusion, *PnPR4* contributes to the defense response of *P. notoginseng* to *C. destructans*.

Introduction

Panax notoginseng (Burk) F.H. Chen, which is a perennial herb in the family *Araliaceae*, is a valuable plant in China because of its use in traditional Chinese medicine (Liu et al., 2019). Studies have shown that *P. notoginseng* has a variety of biological effects. For example, it can increase blood circulation, stop bleeding and replenish blood, improve learning and memory, decrease cholesterol levels, and enhance immunity (e.g., anti-tumorigenic and anti-viral effects) (Xie et al., 2018). Recent research indicated it is useful for preventing cardiovascular and cerebrovascular diseases (Liu et al., 2020). Because of its long growth cycle, *P. notoginseng* is typically grown for 3 years before it is used as medicine. Moreover, *P. notoginseng* plants require shade as well as warm and humid conditions, which are conducive to the development of *P. notoginseng* diseases (Fan et al., 2016), including root rot, black rot, round spot disease, gray mold, and diseases caused by *Phytophthora* species (Zheng et al., 2020). Root rot is the most serious disease during the cultivation of *P. notoginseng* plants, with an annual incidence of 5%-20%, which can lead to yield losses exceeding 70% in severe cases (Xia et al., 2016). Root rot decreases the saponin content in the roots, thereby adversely affecting the medicinal value of *P. notoginseng* (Shang et al., 2020). Multiple phytopathogenic fungi, such as *C. destructans*, *Fusarium solani*, and *Fusarium oxysporum*, can cause *P. notoginseng* root diseases (Li et al., 2022).

Chemical pesticides alone are inappropriate for controlling the occurrence of *P. notoginseng* root rot partly because they may contaminate the medicinal materials (i.e., unsafe for human consumption) and pollute the environment (Li et al., 2020a). However, the use of traditional breeding methods to increase *P. notoginseng* disease resistance is inefficient because of the considerable time required and the difficulty in obtaining disease-resistant germplasm resources. Therefore, the identification and thorough

characterization of disease resistance genes in *P. notoginseng* may lead to the development of a sustainable and eco-friendly method to control root rot.

An exposure to biotic and abiotic stresses stimulates pattern-triggered immunity and effector-triggered immunity responses in plants (Ngou et al., 2022). Many defense-related genes are activated, leading to hypersensitive responses and the production of reactive oxygen species (ROS), accumulation of phytoalexins, and synthesis of pathogenesis-related (PR) proteins (Xu et al., 2022). The accumulation of PR proteins is an important part of the mechanism protecting plants from pathogens. According to protein sequence similarities, physicochemical properties, biological activities, and serological relationships, PR proteins have been classified into 17 families (Zribi et al., 2021). Additionally, PR proteins are low-molecular-weight proteins (6–43 kDa) that were first identified in tobacco leaves infected with tobacco mosaic virus and then detected in diverse plant species. They are stable under acidic conditions (pH < 3) and have anti-protease activities. Moreover, they are present in cell vesicles, cell walls, and the extracellular space (van Loon et al., 2006).

The PR protein 4 (PR4) family comprises proteins with a Barwin domain, which was originally identified in a barley basic protein (Svensson et al., 1992). The Barwin domain of the PR4 protein is associated with chitin binding and is also known as a hevein-like domain. Many proteins with a Barwin domain have been identified in various species, including *Nicotiana tabacum*, *Solanum lycopersicum*, *Arabidopsis thaliana*, *Triticum aestivum*, *Zea mays*, *Malus pumila*, and *Theobroma cacao* (Maia et al., 2021). The expression of some *PR4* genes is induced by fungal infections and the encoded protein reportedly has antifungal and ribonuclease activities (Linthorst et al., 1991). In *Hordeum vulgare*, the expression of *PR4* is up-regulated by a salicylic acid (SA) or methyl jasmonate (MeJA) treatment (Hejgaard et al., 1992). In apple, MdPR-4 inhibits the growth of *Botryosphaeria* hyphae and participates in the defense response to pathogen attacks; *MdPR-4* expression is regulated by SA and JA signaling pathways (Bai et al., 2013). The *Panax ginseng* *PR4* expression level increases in response to phytopathogenic fungi, including *Rhizoctonia solani* and *Colletotrichum gloeosporioides*, for up to 72 h post-inoculation (Kim et al., 2014). When *Vaccinium myrtillus* is infected by *Botrytis cinerea*, *PR4* expression peaks at 12 h (Abbey et al., 2023).

In earlier studies, several *PR* genes involved in *P. notoginseng* root rot resistance were isolated, including *PnPR1*, *PR10-2*, and *PR-like* (Zhang et al., 2020; Yang et al., 2017; Li et al., 2021). An infection by the root rot pathogen *F. solani* substantially increases the expression of these *PR* genes in *P. notoginseng* roots. Although *C. destructans* is an important root rot pathogen, the defense response of *P. notoginseng* to an infection by *C. destructans* remains relatively unknown. To clarify the defense mechanism protecting *P. notoginseng* from *C. destructans*, a *PR4* gene (*PnPR4*) was identified following an analysis of *P. notoginseng* genomic data. Moreover, the antifungal activity of a PnPR4 recombinant protein was analyzed. The PnPR4 function during the response to a *C. destructans* infection was determined by conducting a reverse genetics-based analysis of tobacco plants.

Materials and methods

Plant materials

One-year-old *P. notoginseng* plants were collected in Wenshan city, Qiubei county, Yunnan province, China and then cultivated under shade in a greenhouse at Yunnan Agricultural University. Sterile wild-type (WT) tobacco (*N. tabacum*) seedlings were grown in a light incubator and then used for genetic transformations.

Fungal materials

Four pathogens, including *C. destructans*, *F. solani*, and *F. oxysporum*, were isolated from diseased *P. notoginseng* plants with root rot symptoms and then characterized in terms of their morphological and molecular features. The fungal materials (*C. destructans*, *F. solani*, and *F. oxysporum*) were preserved on slants at 4°C. The mycelia on the slants were used to inoculate potato dextrose agar (PDA) medium and carrot agar medium in plates, which were then incubated at 28°C until the mycelial diameter was approximately 3 cm. A mycelial plug was transferred to fresh medium in a new plate and then incubated at 28°C. This step was repeated three times.

Chemical treatments and fungal inoculation

The roots of healthy 1-year-old *P. notoginseng* plants were wounded with scissors and then inoculated with *C. destructans*. Specifically, the wounded roots were immersed in a *C. destructans* culture for 30 min. For the chemical treatments, the wounded roots were immersed in 200 µM SA, 100 µM MeJA, 1 mM ethephon (ETH), 1 mM gibberellic acid (GA3), 1 mM abscisic acid (ABA), and 1 mM hydrogen peroxide (H₂O₂) solutions for 30 min. The roots treated with sterile water were used as the control for the fungal inoculation and chemical treatments. The *P. notoginseng* roots were collected at 12, 24, 48, and 72 h after the fungal inoculation and chemical treatments. All samples were immediately frozen in liquid nitrogen and stored at -80°C prior to the subsequent analyses.

Identification of the *P. notoginseng* PR4 gene

The Pfam number for the *PR4* gene is PF00967 (Chen et al., 2020). The *P. notoginseng* reference genome (Yang et al., 2021) was searched using PF00967 and the 'Find Best Homology' module of TBtools to identify *PnPR4*. The *PnPR4* coding sequence (CDS) was obtained by analyzing the genomic sequence and then used to design the following primers for the PCR amplification of *PnPR4* cDNA: *PnPR4*-F (5'-ATGCAAGTTGTACACAT-3') and *PnPR4*-R (5'-TCAGTCTCCGCAGTTCAC-3'). The amplified product was inserted into the pMD18-T vector and sequenced. The accuracy of the sequence was confirmed on the basis of an alignment with the *PnPR4* CDS. The molecular weight and isoelectric point of PnPR4 were predicted using the ExPASy Molecular Biology Server (www.ExPASy.ProtParamtool.html). The TMHMM 2.0 program (DTU Health Tech) was used to predict the presence of a signal peptide and a transmembrane region in PnPR4. The similarity and evolutionary relationships between PnPR4 and other plant PR4 proteins were analyzed using MEGA.

Quantitative real-time PCR

For the quantitative real-time PCR (qRT-PCR) analysis, *PnPR4*-specific primers were designed using the Primer5 software and *PnACT2* (GenBank accession number KF815706.2) was selected as the internal reference gene (Liu et al., 2012). Total RNA was extracted from healthy and uniformly growing (i.e., height) 1-year-old *P. notoginseng* plants. The *P. notoginseng* root tips were wounded using scissors and then treated with *C. destructans*, SA, GA3, ABA, ETH, MeJA, or H₂O₂. The *P. notoginseng* root samples were immediately frozen using liquid nitrogen and stored at -80°C. The RNA was reverse transcribed to synthesize cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, China). The cDNA and the SYBR qPCR master mix and enzyme (Vazyme Biotech, China) were used for the qRT-PCR analysis, which was completed on the QuantStudio™ Real-time PCR system, with four replicates per gene. The QuantStudio™ Design & Analysis software was used to analyze the generated data. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Subcellular localization analysis

The *PnPR4-GFP* fusion gene was constructed to determine the subcellular localization of PnPR4. The *PnPR4* open reading frame (ORF) was inserted into the pCAMBIA1301-GFP vector. *Agrobacterium tumefaciens* cells were transformed with the pCAMBIA1301-*PnPR4-GFP* recombinant plasmid. The positive clones carrying the recombinant plasmid were identified by PCR and then injected into the lower epidermis of the leaves from 4-week-old tobacco plants. After 3–4 days, the subcellular localization of PnPR4 was examined using the UltraVIEW VoX spinning disc confocal microscope (Perkin Elmer, USA).

PnPR4 recombinant protein production and assessment of its antifungal activity

The *PnPR4* ORF without the fragment encoding the N-terminal signal peptide was inserted into the pET-32a vector. *Escherichia coli* BL21 cells were transformed with the pET-32a-*PnPR4* recombinant plasmid. The transformed cells were treated with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce the production of the recombinant protein, which was subsequently purified using a Ni-NTA affinity chromatography column (Sangon Biotech, China).

The antifungal activity of the recombinant protein was evaluated using three fungi (*C. destructans*, *F. oxysporum*, and *F. solani*), which were cultured on PDA medium in plates at 28°C until the colony diameter was approximately 2 cm. Five sterile filter paper discs (0.6 mm diameter) were placed around the colonies, after which 20 μL solutions consisting of 10, 15, or 20 μg recombinant protein were added. Sterile water and the elution buffer (20 μL) were used as the blank controls. All plates were incubated at 28°C for 3–5 days and then photographed using the PowerShot G7X Mark III camera (Canon, Japan). To determine the inhibitory effects of the recombinant protein on fungal growth, the growth-inhibition area was calculated using the Photoshop software.

Generation and screening of transgenic tobacco plants

The *PnPR4* ORF was obtained by digesting the pGEM-T-*PnPR4* plasmid with *SacI* and *BamHI*. The *PnPR4* ORF was then inserted into the pC1300s plant expression vector digested with the same two restriction enzymes. Competent *E. coli* DH5α cells were transformed with the pC1300s-*PnPR4* recombinant plasmid and then the positive clones were confirmed by PCR. The recombinant plasmid was then inserted into *A. tumefaciens* EHA105 cells using a CaCl_2 freeze-thaw method. A leaf disc transformation method was used to infect tobacco with the transformed *A. tumefaciens* EHA105 cells. Transgenic tobacco plants were obtained by tissue culture and by screening for antibiotic resistance. Genomic DNA was extracted from the leaves of the transgenic tobacco plants using the CTAB method to verify the plants were successfully transformed (McCormick et al., 1986). The T_0 generation transgenic tobacco plants verified by PCR were grown in a greenhouse to produce the T_2 generation.

Analysis of the transgenic tobacco plants in terms of *PnPR4* expression and disease resistance

The *PnPR4* transcription levels in the T_2 tobacco lines were analyzed by qRT-PCR. The WT tobacco plants were used as the negative control, whereas the transgenic tobacco line with the highest Ct value was used as the positive control.

To functionally characterize *PnPR4*, the transgenic tobacco lines in which *PnPR4* was highly expressed were examined regarding their resistance to a *C. destructans* infection, with WT plants serving as the control. Fully expanded tobacco leaves with a uniform size were wounded and then inoculated with a 20 μL *C. destructans* spore suspension (10^6 spores/mL). The inoculated leaves were placed on sterile water-soaked filter paper in a foam box, which was sealed with plastic wrap and maintained at 25°C in a light incubator for 1 week. The inoculated leaves were collected and the lesions due to the *C. destructans* infection were examined.

Results

Identification and isolation of the *P. notoginseng* PR4 gene

In this study, we identified a *PR4* gene (gene ID Pno08G001541.t2) in the *P. notoginseng* reference genome on the basis of a Pfam structural domain analysis. The gene, which was named *PnPR4*, was localized to chromosome 8 (chr08:134081584–134083560) (Fig. 1A). The *PnPR4* genomic sequence (1,977 bp) included a 450-bp CDS and a 989-bp intron (Fig. 1B). The *PnPR4* CDS was cloned by PCR and validated by sequencing. The *PnPR4* cDNA sequence was 450 bp long. The complete ORF encoded a protein comprising 149 amino acids. The alignment of the cDNA sequence with the *PnPR4* genomic sequence revealed two exons separated by a 989-bp intron. The encoded PnPR4 protein included the conserved Barwin domain unique to PR4 proteins. The predicted PnPR4 protein characteristics were as follows: molecular weight, 16.45 kDa; isoelectric point, 8.84; instability coefficient, 22.93; aliphatic amino acid index, 75.23; and average hydrophilicity coefficient, -0.097. Moreover, it was predicted to include a signal peptide (amino acids 1–27).

The BLASTp analysis indicated that PnPR4 is highly homologous to a PR4 protein from *P. ginseng*, with a sequence similarity of 84.21%. Additionally, PnPR4 was also very similar to PR4 proteins from various species, including *A. thaliana* (AtPR4), *Camellia sinensis* (CsPR4), *Daucus carota* (DcPR4-1), *M. pumila* (MdPR4), *Solanum tuberosum* (StPR4-1 and StPR4-2), *Triticum durum* (TdPR4-1 and TdPR4-2), and *P. ginseng* (PgPR4). The multiple sequence alignment of PnPR4 and these homologous sequences confirmed that the plant PR4 proteins are highly conserved (Fig. 2A). Several reported PR4 proteins from different plant species and four PR proteins from *P. notoginseng* were included in a phylogenetic analysis. The PR4 proteins were divided into four groups (I, II, III, and IV), with PnPR4 and the highly similar PR4 proteins clustered in group IV, separate from the four *P. notoginseng* PR proteins (Fig. 2B).

PnPR4 was responsive to several signaling molecules and its expression was up-regulated by the *C. destructans* infection

According to the qRT-PCR data, *PnPR4* expression was up-regulated in the *P. notoginseng* roots treated with ETH, GA3, H₂O₂, ABA, and SA, which was in contrast to the down-regulated expression of *PnPR4* following the MeJA treatment (Fig. 3A). The *PnPR4* expression level in the roots was highest after the H₂O₂ treatment (peaking at 5.72-times higher than the control level at 72 h) and the GA3 treatment (peaking at 3.30-times higher than the control level at 72 h). For the ETH treatment, the highest *PnPR4* expression level in the roots was detected at 12 h (2.28-times higher than the control level). For the ABA treatment, the *PnPR4* expression level was 2.43-times higher than the control level at 72 h. For the SA treatment, the *PnPR4* expression level was 1.45-times higher than the control level at 24 h. In addition, *PnPR4* expression was induced by the *C. destructans* infection at 72 h post-inoculation (1.94-times higher than the control level) (Fig. 3B).

PnPR4 was localized in the cell wall

The PnPR4 protein was predicted to have a signal peptide (amino acids 1–27), suggesting it may be a secretory protein. The green fluorescence following the transient expression of the *PnPR4-GFP* fusion gene in tobacco cells was observed using a laser confocal microscope. The fluorescence of *PnPR4-GFP* was detected only in the cell wall of tobacco leaf epidermal cells, implying PnPR4 is a cell wall protein (Fig. 4).

The PnPR4 recombinant protein expressed in *E. coli* had antifungal activities

The *PnPR4* ORF without the fragment encoding the signal peptide was subcloned into the pET-32a vector and expressed in *E. coli*. The expected size of the His-tagged PnPR4 recombinant protein was approximately 33.3 kDa. The SDS-PAGE analysis confirmed the 0.5 mM IPTG treatment induced the production of the recombinant protein. The inclusion bodies containing the PnPR4 recombinant protein were solubilized prior to the purification of the recombinant protein using a Ni-NTA chromatography column for the *in vitro* plate assay (Fig. 5A).

The results of the antifungal activity analysis showed that the PnPR4 recombinant protein inhibited the growth of *C. destructans*, *F. oxysporum*, and *F. solani* mycelia (Fig. 5B-D). Relative to the effects of the two blank controls (sterile water and elution buffer), the *F. oxysporum* and *F. solani* growth-inhibition areas were respectively 27.31 and 40.10 mm² after the 10 µg PnPR4 recombinant protein treatment, 132.08 and 110.74 mm² after the 15 µg PnPR4 recombinant protein treatment, and 168.36 and 167.32 mm² after the 20 µg PnPR4 recombinant protein treatment. The *C. destructans* growth-inhibition areas were 29.30, 128.52, and 167.34 mm² after the 10, 15, and 20 µg PnPR4 recombinant protein treatments, respectively (Fig. 5E). Accordingly, the antifungal activities of PnPR4 tended to increase as the protein concentration increased.

Transgenic tobacco plants overexpressing *PnPR4* were highly resistant to *C. destructans*

The *PnPR4* gene was heterologously expressed in the model plant tobacco to investigate its contribution to defense responses. The T₀ generation transgenic tobacco plants were obtained via the genetic transformation mediated by *A. tumefaciens*. A total of 24 transgenic tobacco plants carrying *PnPR4* were identified by a PCR analysis, of which 10 were selected to develop T₂ lines via selfing for two generations. There were no visible phenotypic differences between the T₂ tobacco lines and WT tobacco. Additionally, the qRT-PCR data indicated that *PnPR4* was stably expressed in the T₂ transgenic tobacco lines (Fig. 6A). More specifically, *PnPR4* was most highly expressed in transgenic line OE-7 (relative expression level of 18.42), followed by transgenic lines OE-5, OE-9, OE-11, and OE-14. Conversely, *PnPR4* expression levels were relatively low in lines OE-3, OE-8, OE-12, OE-13, and OE-15. These results indicated that *PnPR4* was stably expressed in the T₂ transgenic tobacco lines (Fig. 6C).

The tobacco lines overexpressing *PnPR4* were inoculated with *C. destructans*, with WT tobacco serving as the control. The inoculated plants were examined for disease symptoms at 7 days post-inoculation. There was mild chlorosis around the inoculation site on the transgenic tobacco leaves, whereas the WT tobacco leaves had severe chlorotic lesions and were rotting (Fig. 6B). The inoculation assay results suggest that the transgenic tobacco plants were more resistant to *C. destructans* than the WT tobacco plants. Thus, the overexpression of *PnPR4* in tobacco confers strong resistance to *C. destructans*.

Discussion

Pathogenesis-related proteins, which are widely distributed in higher plants, are a class of proteins that accumulate when plants are subjected to biotic or abiotic stress. Therefore, PR proteins are critical for plant stress responses. In this study, we identified a PR-encoding gene (*PnPR4*) in *P. notoginseng* by analyzing genomic data. Previously, only one *PR4* gene, which encoded a protein containing the Barwin domain, was detected in the *A. thaliana* genome (Potter et al., 1993). In a more recent study, three *PR4* genes were identified in *Allium sativum* (Anisimova et al., 2021). Some PR-encoding genes in *P. notoginseng* have been cloned, including *PnPR1*, *PnPR10-1*, *PnPR10-2*, and *PnPR-like* (Zhang et al., 2020; Yang et al., 2017; Li et al., 2021). However, the sequences of the encoded PR proteins differ considerably

from the PnPR4 sequence, possibly reflecting the divergence of some of the families in the PR superfamily during long-term evolution.

An analysis of the PnPR4 sequence revealed the presence of an N-terminal signal peptide and a C-terminal Barwin domain. The signal peptide usually mediates the secretion of the protein from the cell, whereas the Barwin domain is associated with the antibacterial activity of the PR4 protein (Caporale et al., 2003). Moreover, the PnPR4-GFP fusion protein was detected only in the tobacco leaf epidermal cell wall, implying PnPR4 functions as a cell wall protein.

The expression of the *PR4* gene is modulated by hormone signaling pathways. For example, in *Brassica juncea*, *PR4* expression levels increase in response to MeJA (Ali et al., 2017). In the oriental hybrid lily 'Sorbonne' (*Lilium* species), *PR4-2* expression is induced by various phytohormones, including MeJA, SA, and ETH (Du et al., 2022). In the present study, *PnPR4* expression was up-regulated to varying degrees by MeJA, ETH, H₂O₂, GA3, ABA, and SA. The expression of *PnPR4* was also induced by *C. destructans*. To effectively defend against pathogens, plants have evolved complex defense mechanisms that "sense" the pathogen and activate appropriate defense responses. The *PnPR4* expression data revealed that the cross-talk among signaling pathways, including hormone signaling pathways, regulates *PnPR4* expression in *P. notoginseng* infected with *C. destructans*.

Some PR4 proteins with antifungal activities can hydrolyze fungal cells (Gupta et al., 2015). Additionally, the RNase and divalent ion-dependent DNase activities of the *T. cacao* TcPR-4b recombinant protein have protective effects against *Moniliophthora perniciosa* (Pereira Menezes et al., 2014). The RNase activity of the *Capsicum chinense* PR-4 protein can degrade the RNA of invasive pathogens (Guevara-Morato et al., 2010). The four PR4 proteins isolated from wheat (*T. aestivum*) can inhibit the mycelial growth and spore germination of various pathogenic fungi, including *B. cinerea* (Caporale et al., 2004). The *Cicer arietinum* PR-4 protein suppresses the growth of *F. oxysporum* *in vitro* because of its RNase and DNase activities (Singh et al., 2018). In the current study, the PnPR4 recombinant protein restricted the mycelial growth of *C. destructans*, *F. solani*, and *F. oxysporum*, indicating that *PnPR4* encodes an antifungal protein.

The functions of many PR4 proteins have been verified via reverse genetics. For example, the overexpression of wheat *PR4a* and *PR4b* in transgenic tobacco plants reportedly decreases the susceptibility to *Phytophthora nicotianae*, with T₄ generation plants exhibiting strong resistance to *P. nicotianae* (Fiocchetti et al., 2008). Compared with WT plants, the grape (*Vitis vinifera*) *PR4b* knockout lines are more susceptible to *Pseudomonas* species that cause downy mildew (Li et al., 2020b). The overexpression of the two *PR4* genes (*PaPR4-a* and *PaPR4-b*) cloned from spruce (*Picea asperata*) in tobacco leads to increased ROS-scavenging enzyme activities as well as resistance to *Lophodermium piceae* (Zhao et al., 2022). Because of the lack of an effective genetic transformation system for *P. notoginseng*, in this study, we functionally characterized *PnPR4* using tobacco plants. Compared with the WT controls, the transgenic tobacco plants overexpressing *PnPR4* were significantly more resistant to *C. destructans*, implying *PnPR4* is a disease resistance-related gene in *P. notoginseng*, with an important role in the response to the root rot pathogen.

Conclusion

In this study, the *PnPR4* gene was isolated from *P. notoginseng* and revealed to encode a cell wall protein. The expression of *PnPR4* was significantly up-regulated by *C. destructans* and hormone signaling molecules. The PnPR4 recombinant protein had clear antifungal effects on root rot pathogens. Moreover, the overexpression of *PnPR4* in tobacco conferred increased resistance to *C. destructans*. Thus, *PnPR4* is a disease resistance-related gene that protects *P. notoginseng* against root rot pathogens.

Declarations

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Author contributions

Methodology, data curation, experiment execution, and writing-original draft preparation: Hao Sun. Methodology, data curation, and experiment execution: Manqiao Li. Investigation and software: XuYan Liu. Conceptualization: Kuixiu Li. Material collection and methodology: Diqui Liu. Methodology and supervision: Shengchao Yang. Methodology, supervision, writing-reviewing, and editing: Guanze Liu. All authors contributed to the article and approved the submitted version.

Data availability

All datasets generated or analyzed during this study are included in the manuscript.

Conflict of interest The authors declare that they have no conflict of financial or non-financial interest. This study is original, has not been published previously, and is not under consideration for publication elsewhere.

References

1. Liu, S. Z., Cheng, W., Shao, J. W., Gu, Y. F., Zhu, Y. Y., Dong, Q. J., Bai, S. Y., Wang, P., & Lin, L. (2019). Notoginseng Saponin Rg1 Prevents Cognitive Impairment through Modulating APP Processing in A β 1-42-injected Rats. *Current medical science*, 39(2), 196–203. <https://doi.org/10.1007/s11596-019-2019-1>
2. Xie, W., Meng, X., Zhai, Y., Zhou, P., Ye, T., Wang, Z., Sun, G., & Sun, X. (2018). Panax Notoginseng Saponins: A Review of Its Mechanisms of Antidepressant or Anxiolytic Effects and Network Analysis

- on Phytochemistry and Pharmacology. *Molecules (Basel, Switzerland)*, 23(4), 940.
<https://doi.org/10.3390/molecules23040940>
3. Liu, H., Lu, X., Hu, Y., & Fan, X. (2020). Chemical constituents of *Panax ginseng* and *Panax notoginseng* explain why they differ in therapeutic efficacy. *Pharmacological research*, 161, 105263.
<https://doi.org/10.1016/j.phrs.2020.105263>
 4. Fan, Z. Y., Miao, C. P., Qiao, X. G., Zheng, Y. K., Chen, H. H., Chen, Y. W., Xu, L. H., Zhao, L. X., & Guan, H. L. (2016). Diversity, distribution, and antagonistic activities of rhizobacteria of *Panax notoginseng*. *Journal of ginseng research*, 40(2), 97–104. <https://doi.org/10.1016/j.jgr.2015.05.003>
 5. Zheng, K., Guo, L. P., Zhang, X. B., Wang, C. X., Qu, Y., Liu, W., Zhou, T., Zhang, Y. Y., Cui, X. M., & Yang, Y. (2022). *Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica*, 47(6), 1438–1444. <https://doi.org/10.19540/j.cnki.cjcmm.20211221.101> (In Chinese)
 6. Xia, P., Guo, H., Zhao, H., Jiao, J., Deyholos, M. K., Yan, X., Liu, Y., & Liang, Z. (2016). Optimal fertilizer application for *Panax notoginseng* and effect of soil water on root rot disease and saponin contents. *Journal of ginseng research*, 40(1), 38–46. <https://doi.org/10.1016/j.jgr.2015.04.003>
 7. Shang, J. H., Sun, W. J., Zhu, H. T., Wang, D., Yang, C. R., & Zhang, Y. J. (2020). New hydroperoxylated and 20,24-epoxylated dammarane triterpenes from the rot roots of *Panax notoginseng*. *Journal of ginseng research*, 44(3), 405–412. <https://doi.org/10.1016/j.jgr.2019.01.008>
 8. Li, T. T., Yang, J., Huo, Y. Y., Zeng, Z. Y., Huang, H. Y., Xu, F. R., & Dong, X. (2022). Control of pathogenic fungi on *Panax notoginseng* by volatile oils from the food ingredients *Allium sativum* and *Foeniculum vulgare*. *Letters in applied microbiology*, 75(1), 89–102.
<https://doi.org/10.1111/lam.13706>
 9. Li, Y., Dai, S., Wang, B., Jiang, Y., Ma, Y., Pan, L., Wu, K., Huang, X., Zhang, J., Cai, Z., & Zhao, J. (2020a). Autotoxic Ginsenoside Disrupts Soil Fungal Microbiomes by Stimulating Potentially Pathogenic Microbes. *Applied and environmental microbiology*, 86(9), e00130-20.
<https://doi.org/10.1128/AEM.00130-20>
 10. Ngou, B. P. M., Ding, P., & Jones, J. D. G. (2022). Thirty years of resistance: Zig-zag through the plant immune system. *The Plant cell*, 34(5), 1447–1478. <https://doi.org/10.1093/plcell/koac041>
 11. Xu, G., Moeder, W., Yoshioka, K., & Shan, L. (2022). A tale of many families: calcium channels in plant immunity. *The Plant cell*, 34(5), 1551–1567. <https://doi.org/10.1093/plcell/koac033>
 12. Zribi, I., Ghorbel, M., & Brini, F. (2021). Pathogenesis Related Proteins (PRs): From Cellular Mechanisms to Plant Defense. *Current protein & peptide science*, 22(5), 396–412.
<https://doi.org/10.2174/1389203721999201231212736>
 13. van Loon, L. C., Rep, M., & Pieterse, C. M. (2006). Significance of inducible defense-related proteins in infected plants. *Annual review of phytopathology*, 44, 135–162.
<https://doi.org/10.1146/annurev.phyto.44.070505.143425>
 14. Svensson, B., Svendsen, I., Højrup, P., Roepstorff, P., Ludvigsen, S., & Poulsen, F. M. (1992). Primary structure of barwin: a barley seed protein closely related to the C-terminal domain of proteins

- encoded by wound-induced plant genes. *Biochemistry*, 31(37), 8767–8770.
<https://doi.org/10.1021/bi00152a012>
15. Maia, L. B. L., Pereira, H. D., Garratt, R. C., Brandão-Neto, J., Henrique-Silva, F., Toyama, D., Dias, R. O., Bachega, J. F. R., Peixoto, J. V., & Silva-Filho, M. C. (2021). Structural and Evolutionary Analyses of PR-4 SUGARWINs Points to a Different Pattern of Protein Function. *Frontiers in plant science*, 12, 734248. <https://doi.org/10.3389/fpls.2021.734248>
 16. Linthorst, H. J., Danhash, N., Brederode, F. T., Van Kan, J. A., De Wit, P. J., & Bol, J. F. (1991). Tobacco and tomato PR proteins homologous to win and pro-hevein lack the "hevein" domain. *Molecular plant-microbe interactions : MPMI*, 4(6), 586–592. <https://doi.org/10.1094/mpmi-4-586>
 17. Hejgaard, J., Jacobsen, S., Bjørn, S. E., & Kragh, K. M. (1992). Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. *FEBS letters*, 307(3), 389–392. [https://doi.org/10.1016/0014-5793\(92\)80720-2](https://doi.org/10.1016/0014-5793(92)80720-2)
 18. Bai, S., Dong, C., Li, B., & Dai, H. (2013). A PR-4 gene identified from *Malus domestica* is involved in the defense responses against *Botryosphaeria dothidea*. *Plant physiology and biochemistry: PPB*, 62, 23–32. <https://doi.org/10.1016/j.plaphy.2012.10.016>
 19. Kim, Y. J., Lee, H. J., Jang, M. G., Kwon, W. S., Kim, S. Y., & Yang, D. C. (2014). Cloning and characterization of pathogenesis-related protein 4 gene from *Panax ginseng*. *Russ J Plant Physiol.* 61, 664–671. <https://doi.org/10.1134/S1021443714050100>
 20. Abbey, J., Jose, S., Percival, D., Jaakola, L., & Asiedu, S. K. (2023). Modulation of defense genes and phenolic compounds in wild blueberry in response to *Botrytis cinerea* under field conditions. *BMC plant biology*, 23(1), 117. <https://doi.org/10.1186/s12870-023-04090-5>
 21. Zhang, Y. P., Chen, H. J., Zheng, L. L., Wang, Z. E., Cui, X. M., Ge, F., & Liu, D. Q. (2020). Expression characteristics and functional analysis of PnPR1 in the gene associated with the course of **Panax notoginseng**. *Northwestern Botanical Journal*. 12, 2000-2007. (In Chinese)
 22. Yang, D., Bao, Y., Chen, L. M., Cui, X. M., & Li, K. Z. (2017). Cloning, expression and functional analysis of PR10-2 gene associated with the course of *Panax notoginseng*. *China Journal of Chinese Materia Medica*. 16, 3106-3111. <https://doi.org/10.19540/j.cnki.cjcmm.20170714.007> (In Chinese)
 23. Li, S., Wang, Z., Tang, B., Zheng, L., Chen, H., Cui, X., Ge, F., & Liu, D. (2021). A Pathogenesis-Related Protein-Like Gene Is Involved in the *Panax notoginseng* Defense Response to the Root Rot Pathogen. *Frontiers in plant science*, 11, 610176. <https://doi.org/10.3389/fpls.2020.610176>
 24. Chen, C., Chen, H., Zhang, Y., Thomas, H. R., Frank, M. H., He, Y., & Xia, R. (2020). TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Molecular plant*, 13(8), 1194–1202. <https://doi.org/10.1016/j.molp.2020.06.009>
 25. Yang, Z., Liu, G., Zhang, G., Yan, J., Dong, Y., Lu, Y., Fan, W., Hao, B., Lin, Y., Li, Y., Li, X., Tang, Q., Xiang, G., He, S., Chen, J., Chen, W., Xu, Z., Mao, Z., Duan, S., Jin, S., ... Yang, S. (2021). The chromosome-scale high-quality genome assembly of *Panax notoginseng* provides insight into dencichine biosynthesis. *Plant biotechnology journal*, 19(5), 869–871. <https://doi.org/10.1111/pbi.13558>

26. Liu, D. Q., He, X., Li, W., Chen, C., & Ge, F. (2012). A β -1,3-glucanase gene expressed in fruit of *Pyrus pyrifolia* enhances resistance to several pathogenic fungi in transgenic tobacco. *Eur J Plant Pathol.* 135, 265–277. <https://doi.org/10.1007/s10658-012-0083-5>
27. McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., & Fraley, R. (1986). Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. *Plant cell reports*, 5(2), 81–84. <https://doi.org/10.1007/BF00269239>
28. Potter, S., Uknes, S., Lawton, K., Winter, A. M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E., & Ryals, J. (1993). Regulation of a hevein-like gene in *Arabidopsis*. *Molecular plant-microbe interactions : MPMI*, 6(6), 680–685. <https://doi.org/10.1094/mpmi-6-680>
29. Anisimova, O. K., Shchennikova, A. V., Kochieva, E. Z., & Filyushin, M. A. (2021). Pathogenesis-Related Genes of PR1, PR2, PR4, and PR5 Families Are Involved in the Response to *Fusarium Infection in Garlic (Allium sativum L.)*. *International journal of molecular sciences*, 22(13), 6688. <https://doi.org/10.3390/ijms22136688>
30. Caporale, C., Facchiano, A., Bertini, L., Leonardi, L., Chilosi, G., Buonocore, V., & Caruso, C. (2003). Comparing the modeled structures of PR-4 proteins from wheat. *Journal of molecular modeling*, 9(1), 9–15. <https://doi.org/10.1007/s00894-002-0103-z>
31. Ali, S., Mir, Z. A., Tyagi, A., Bhat, J. A., Chandrashekar, N., Papolu, P. K., Rawat, S., & Grover, A. (2017). Identification and comparative analysis of Brassica juncea pathogenesis related genes in response to hormonal, biotic and abiotic stresses. *Acta Physiol Plant.* 39, 268, <https://doi.org/10.1007/s11738-017-2565-8>
32. Du, W., Chai, N., Sun, Z., Wang, H., Liu, S., Sui, S., Luo, L., & Liu, D. (2022). Full-Length Transcriptome Characterization and Functional Analysis of Pathogenesis-Related Proteins in *Lilium* Oriental Hybrid 'Sorbonne' Infected with *Botrytis elliptica*. *International journal of molecular sciences*, 24(1), 425. <https://doi.org/10.3390/ijms24010425>
33. Gupta, R., Lee, S. E., Agrawal, G. K., Rakwal, R., Park, S., Wang, Y., & Kim, S. T. (2015). Understanding the plant-pathogen interactions in the context of proteomics-generated apoplastic proteins inventory. *Frontiers in plant science*, 6, 352. <https://doi.org/10.3389/fpls.2015.00352>
34. Pereira Menezes, S., de Andrade Silva, E. M., Matos Lima, E., Oliveira de Sousa, A., Silva Andrade, B., Santos Lima Lemos, L., Peres Gramacho, K., da Silva Gesteira, A., Pirovani, C. P., & Micheli, F. (2014). The pathogenesis-related protein PR-4b from *Theobroma cacao* presents RNase activity, Ca(2+) and Mg(2+) dependent-DNase activity and antifungal action on *Moniliophthora perniciosa*. *BMC plant biology*, 14, 161. <https://doi.org/10.1186/1471-2229-14-161>
35. Guevara-Morato, M. A., de Lacoba, M. G., García-Luque, I., & Serra, M. T. (2010). Characterization of a pathogenesis-related protein 4 (PR-4) induced in *Capsicum chinense* L3 plants with dual RNase and DNase activities. *Journal of experimental botany*, 61(12), 3259–3271. <https://doi.org/10.1093/jxb/erq148>
36. Caporale, C., Di Berardino, I., Leonardi, L., Bertini, L., Cascone, A., Buonocore, V., & Caruso, C. (2004). Wheat pathogenesis-related proteins of class 4 have ribonuclease activity. *FEBS letters*, 575(1-3),

71–76. <https://doi.org/10.1016/j.febslet.2004.07.091>

37. Singh, A., Jain, D., Tyagi, C., Singh, S., Kumar, S., & Singh, I. K. (2018). In silico prediction of active site and in vitro DNase and RNase activities of Helicoverpa-inducible pathogenesis related-4 protein from *Cicer arietinum*. *International journal of biological macromolecules*, 113, 869–880. <https://doi.org/10.1016/j.ijbiomac.2018.03.027>
38. Fiocchetti, F., D'Amore, R., De Palma, M., Laura, B., Caruso, C., Caporale, C., Antonino, T., Gennaro, C., Francesco, S., & Marina, T. (2008). Constitutive over-expression of two wheat pathogenesis-related genes enhances resistance of tobacco plants to *Phytophthora nicotianae*. *Plant Cell Tiss Organ Cult.* 92, 73–84. <https://doi.org/10.1007/s11240-007-9306-4>
39. Li, M. Y., Jiao, Y. T., Wang, Y. T., Zhang, N., Wang, B. B., Liu, R. Q., Yin, X., Xu, Y., & Liu, G. T. (2020b). CRISPR/Cas9-mediated *VvPR4b* editing decreases downy mildew resistance in grapevine (*Vitis vinifera* L.). *Horticulture research*, 7, 149. <https://doi.org/10.1038/s41438-020-00371-4>
40. Zhao, W., Liu, L., Li, C., Yang, C., Li, S., Han, S., Lin, T., & Liu, Y. (2022). Cloning and Characterization of Two Novel PR4 Genes from *Picea asperata*. *International journal of molecular sciences*, 23(23), 14906. <https://doi.org/10.3390/ijms232314906>

Figures

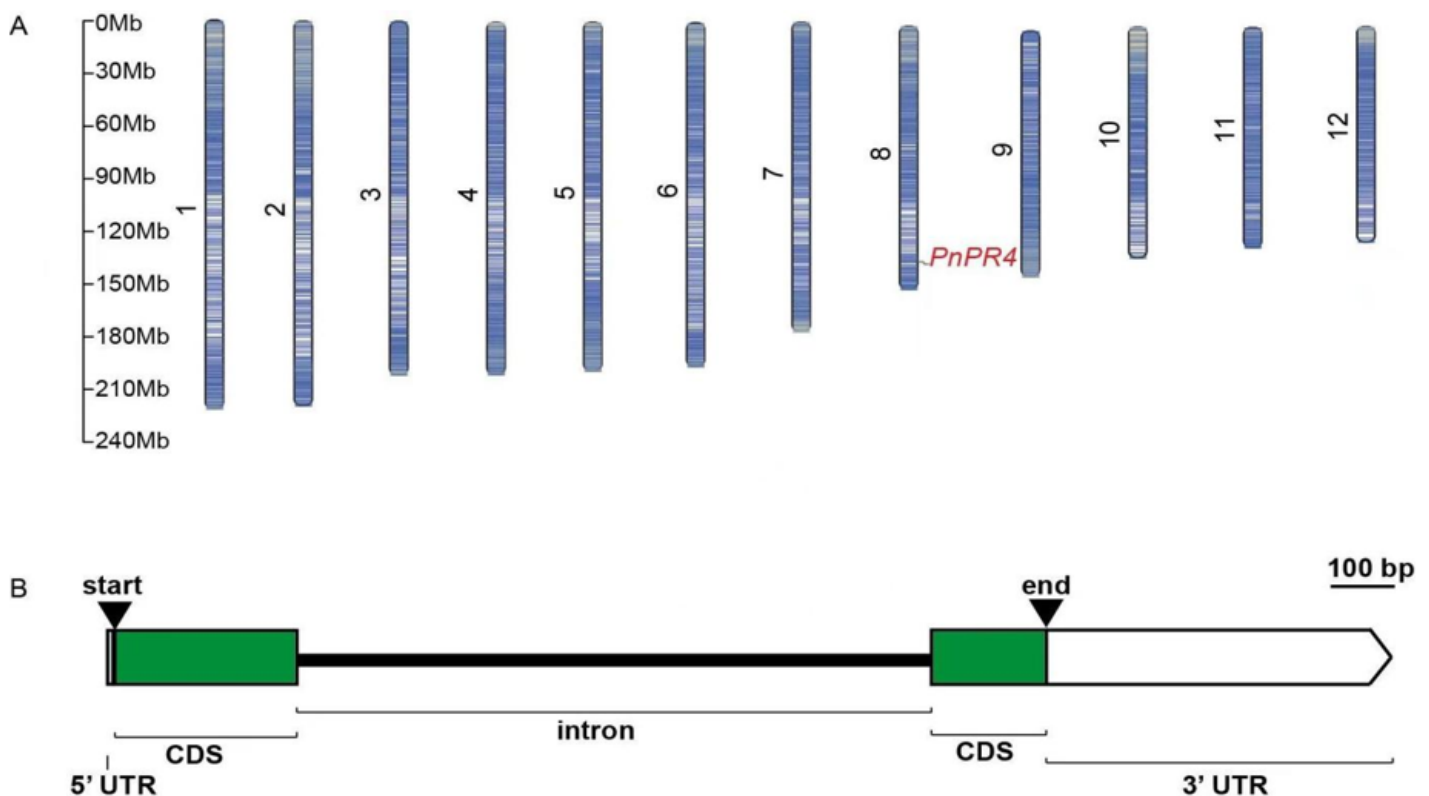


Figure 1

The chromosomal localization and gene structure of *PnPR4*. **A.** The *PnPR4* was localized on chromosome 8 (chr08:134081584-134083560). **B.** The gene structure of *PnPR4*.

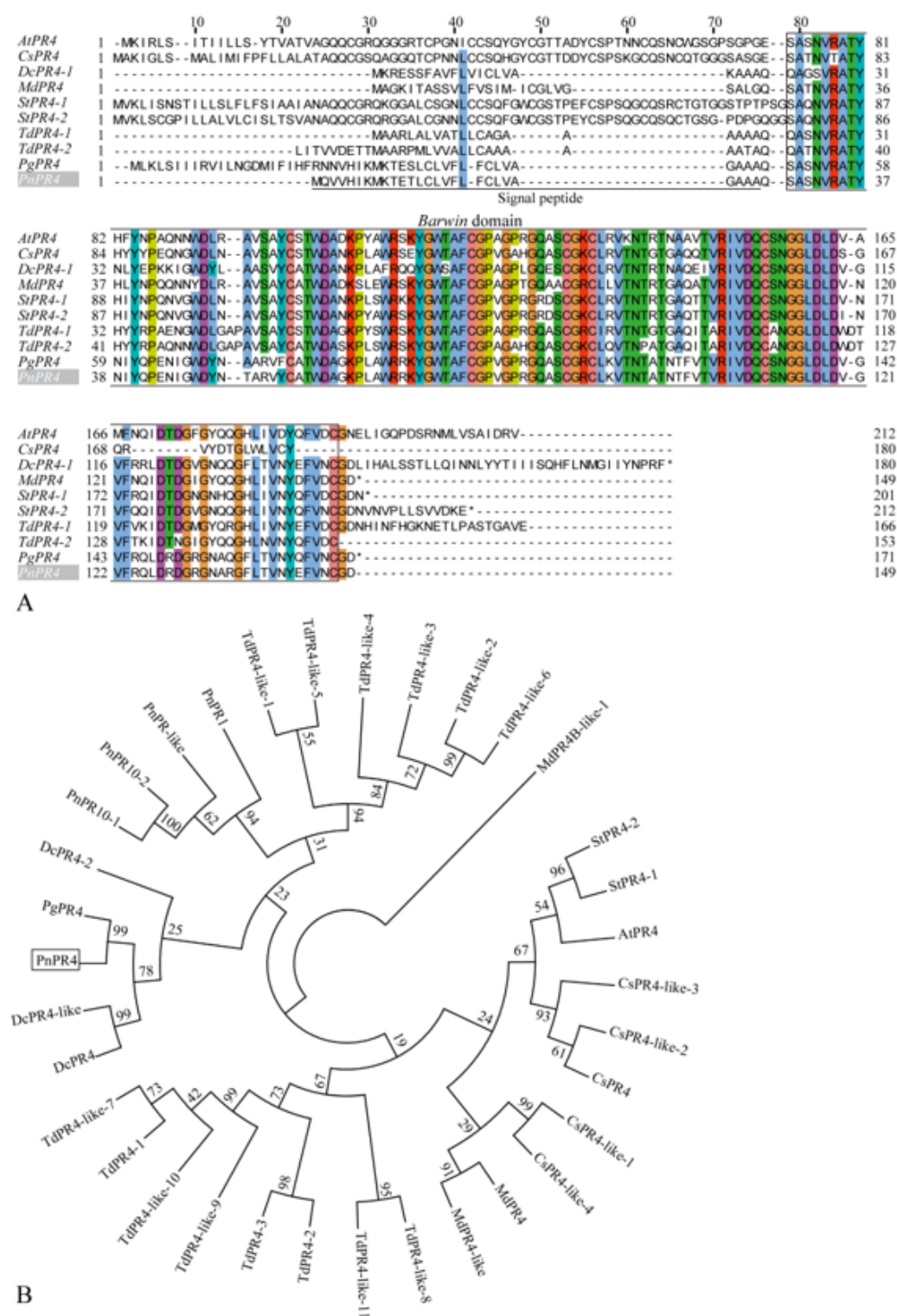


Figure 2

Multiple sequence alignment and phylogenetic tree of PnPR4 and some known PRs. **A.** The PnPR4 was aligned with PR4 proteins from different species. **B.** The evolutionary tree analysis of PnPR4 and PR4

proteins from different species.

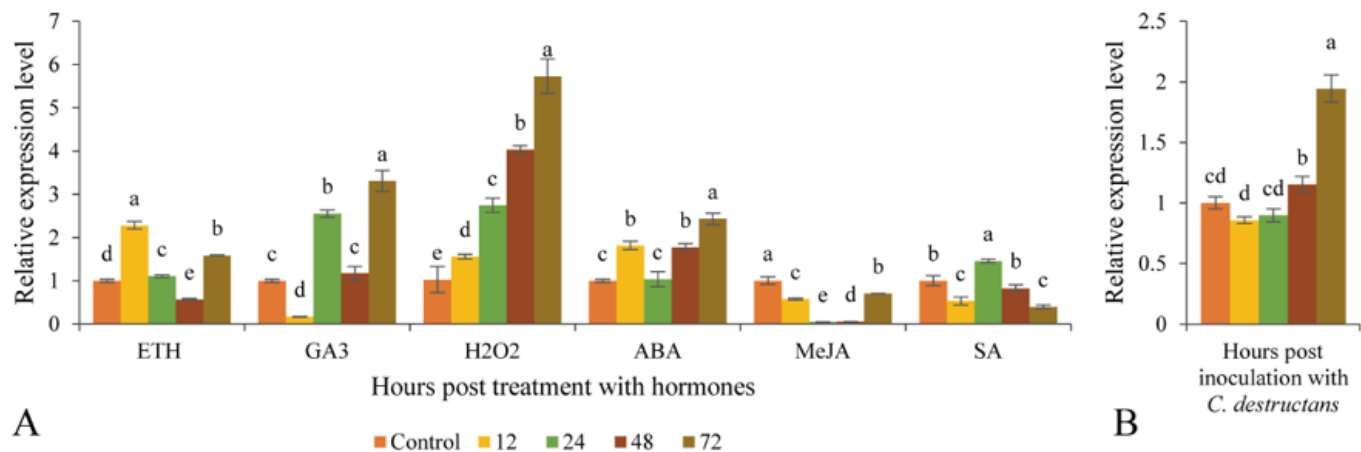


Figure 3

The expression of *PnPR4* in *P. notoginseng* roots. **A.** The relative expression levels of *PnPR4* after treatment with six signaling molecules (ETH, GA3, H₂O₂, ABA, MeJA, and SA) were analyzed by qRT-PCR. **B.** The expression characterization after inoculation with *C. destructans*.

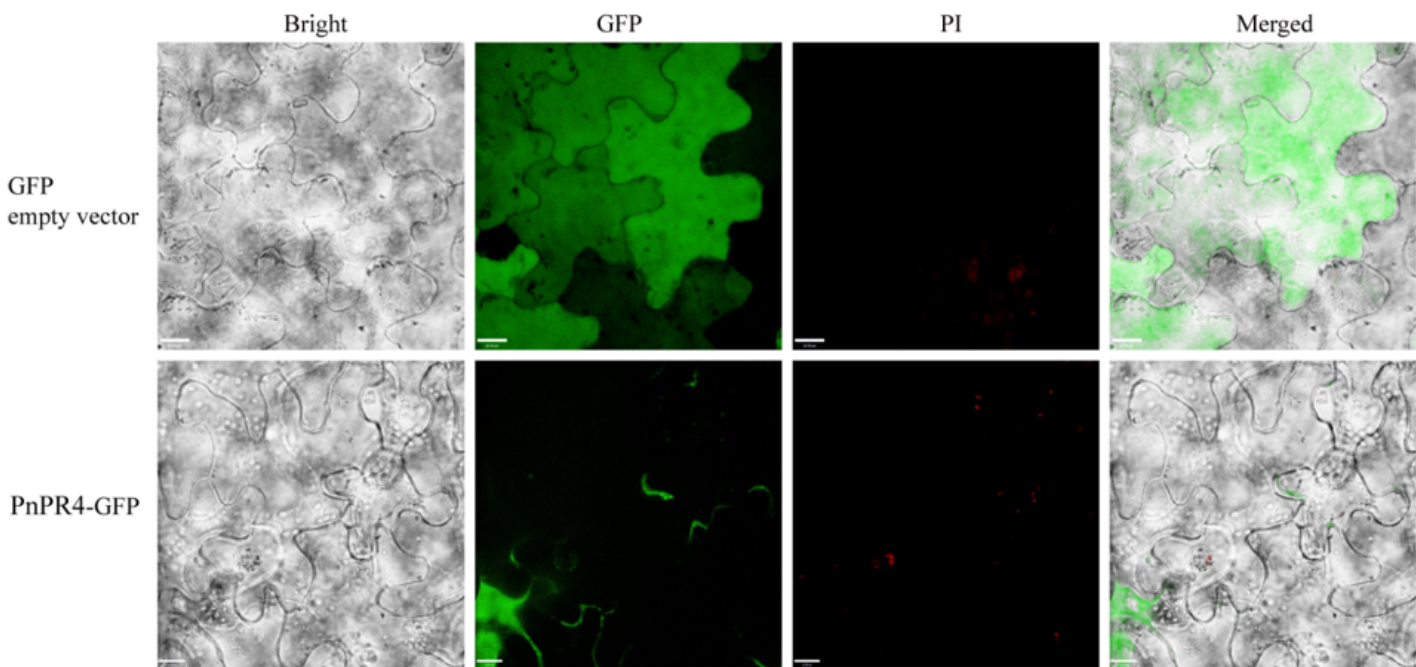


Figure 4

Subcellular analysis of transient expression of *PnPR4-GFP* fusion gene in tobacco epidermal cells showing that *PnPR4* was localized in the cell wall. *PnPR4-GFP* fusion gene and *GFP* (empty vector) were transiently expressed in tobacco epidermal cells, and onion epidermal cells were transformed by *Agrobacterium*-mediated transformation method.

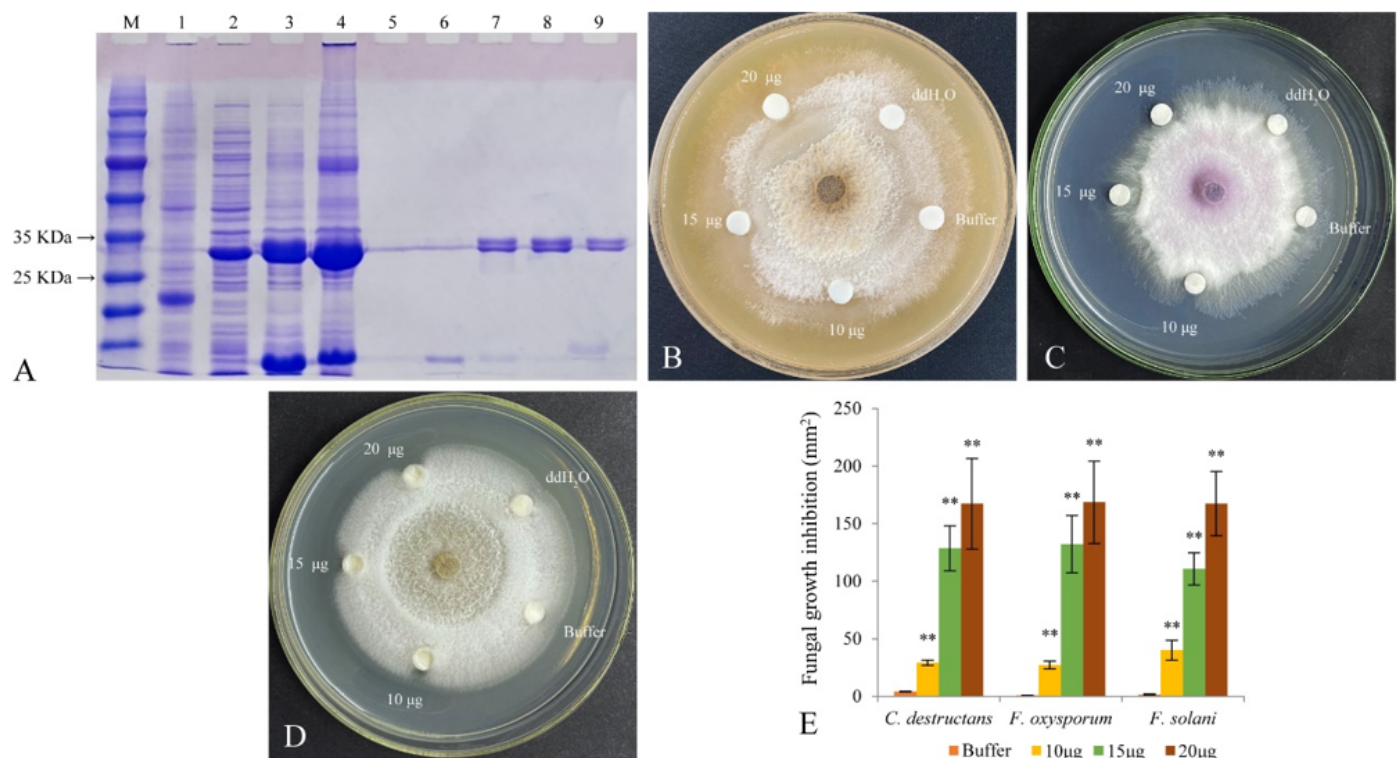


Figure 5

Prokaryotic expression results of PnPR4 and plate antifungal test. **A.** SDS-PAGE electrophoresis analysis of PnPR4 recombinant protein. M, Protein marker; 1. The pET32a empty vector; 2. The PnPR4 recombinant protein induced by 0.5 mM IPTG for 6 h; 3. The protein in supernatant after inclusion body fragmentation; 4. The protein in precipitation after inclusion body fragmentation; 5. The protein in supernatant over column after inclusion body fragmentation; 6. The inclusion body solubilization reagent over column; 7. The protein in eluent I (50 mM) imidazole over column elution; 8. The protein in eluent II (100 mM imidazole) over column elution; 9. The protein in eluent III (100 mM imidazole) over column elution. **B-D.** Plate antifungal test of PnPR4 recombinant protein on *F. oxysporum*, *F. solani* and *C. destructans*. **E.** The colony inhibition area. The results are showed with the average values calculated from three replicates, and the statistical differences are analyzed by Student's *t*-test (***p* < 0.01).

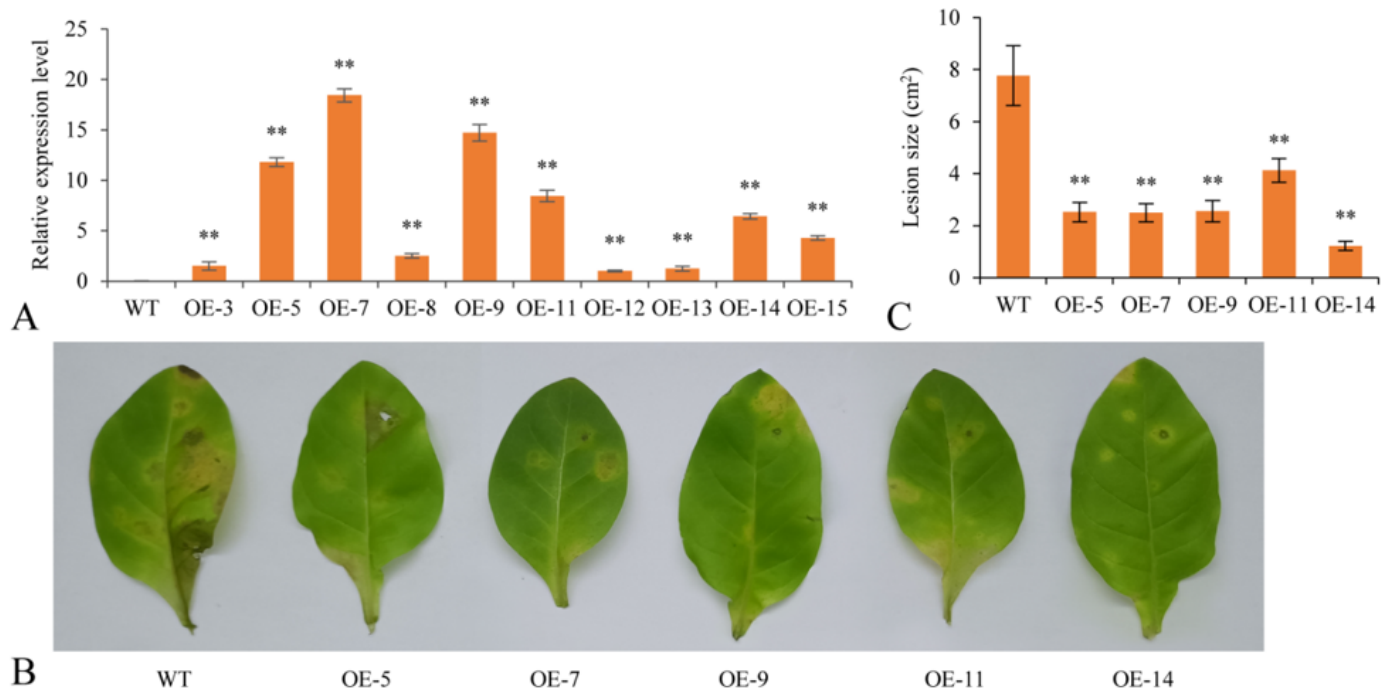


Figure 6

The expression level and resistance analysis of T2 generation *PnPR4* transgenic tobacco. **A.** The expression levels of *PnPR4* in the T2 generation of transgenic tobacco were detected through qRT-PCR, and *PnPR4* was expressed in all transgenic tobacco plants. The OE-3, OE-5, OE-7, OE-8, OE-9, OE-11, OE-12, OE-13, OE-14 and OE-15 were the T2 generation transgenic tobacco lines, and WT was the wild-type tobacco. **B.** The leave inoculation assay showed the enhanced resistance of *PnPR4* transgenic tobacco lines against *C. destructans*. **C.** The lesion sizes in leaves of *PnPR4* transgenic tobacco lines and WT during *C. destructans* infection. The results show the mean of three replicate and statistical differences were analyzed by Student's t-test (** $p < 0.01$).