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Isolation and Characterization of Pathogenesis-Related Protein 5 (*PgPR5*) Gene from *Panax ginseng*

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A pathogenesis-related protein (*PgPR5*) gene that isolated from the leaf of *Panax ginseng* was characterized. The ORF is 756 bp with a deduced amino acid sequence of 251 residues. The calculated molecular mass of the matured protein is approximately 27.5 kDa with a predicated isoelectric point of 7.80. A GenBank BlastX search revealed that the deduced amino acid of *PgPR5* shares highest sequence similarity to PR5 of *Actinidia deliciosa* (80% identity, 87% similarity). PgPR5 has a C-terminal and N-terminal signal peptide, suggesting that it is a vacuolar secreted protein. The expression of *PgPR5* under various environmental stresses was analyzed at different time points using real-time PCR. Our results reveal that *PgPR5* is induced by salt stress, chilling stress, heavy metal, UV, and pathogen infection. These results suggest that the PgPR5 could play a role in the molecular defence response of ginseng to abiotic and pathogen attack. This is the first report of the isolation of PR5 gene from the *P. ginseng*.

Keywords : biotic stress, chilling, ginseng, pathogenesis-related protein5, salt stress

Higher plants have developed various defense mechanisms against biotic and abiotic stresses, such as pathogen invasions, wounding, exposure to heavy metal, salinity, cold, and ultraviolet rays (UV). These defense mechanisms include synthesis of pathogenesis-related (PR) proteins. PR is known to function in higher plants against abiotic and biotic stress, especially against pathogen infection. PRs are accumulated after pathogen attack by virus, bacteria, fungi, nematodes, insects and herbivores as well as after wounding and certain abiotic stress conditions (van Loon et al., 2006). PR was first observed in tobacco plants infected with tobacco mosaic virus (TMV) (van Loon and van Kammen, 1970). Since the discovery of PRs in 1970, PRs were identified in many plants species, currently, 17 PR families have been grouped based on amino acid sequences, serological relationship and/or enzymatic or biological activity (van Loon et al., 2006). The specific functions of PRs are not fully understood, although several are postulated to play a role in preventing pathogen invasion.

Several PR5s induced by biotic stress have shown effective antifungal activities, such as the inhibition of fungal spore germination or mycelial growth (Onishi et al., 2006). In addition, transgenic potato plants with PR5 exhibitied delayed development of disease symptoms after inoculation with suspension of *Phytophthora infestans* (Liu et al., 1994).

The PR5 family is also known as thaumatin-like proteins (TLP) due to their homology with the sweet-tasting plant protein thaumatin (Van der Well and Loeve, 1972). The PR5 family consists of TLP and osmotin-like proteins (OLP). PR5s are known to be mainly involved in plant defensive systems that act against infection by pathogens (Vigers et al., 1991). In addition, the family of PR5 comprises proteins with diverse functions, such as roles in development (Salzman et al., 1998), protection against osmotic stress (Singh et al., 1987; Zhu et al., 1995) and freezing tolerance (Zhu et al., 1993). However, the molecular basis of PR5 action in these defensive mechanisms against abiotic stresses remains uncertain.

PR5 can be subgrouped into acidic, extracellular proteins that are characterized by the presence of an N-terminal signal peptide, or basic proteins with additional C-terminal extension targeted vacuole (Stintzi et al., 1993; Sato et al., 1995). They can be further subdivided into large TLPs, with molecular masses of 21-26 kDa and 16 conserved cysteine residues, or small TLPs, with molecular masses of 15-18 kDa and, due to an internal deletion of approximately 58 amino acids (aa), only 10 conserved cysteines. While the more common large TLPs have been identified in moncots, dicots and conifers, small TLPs have primarily been described in monocot crop species (Piggott et al., 2004).

In this study, we isolated a cDNA (*PgPR5*, for *Panax ginseng* pathogenesis-related protein 5) from ginseng which shares significant sequence similarity with *PR5* from other plants. Korean ginseng (*Panax ginseng* C. A. Meyer)

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is cultivated for its highly valued root used for medicinal purposes. The production of ginseng roots required 4-6 year cultivation period, thus it is susceptible to undergo environmental stresses as well as diseases caused by both foliar and soilborne root-infecting fungi (Seifert et al., 2004). They caused to reduce yield by causing premature death of ginseng plants, as well as reduce quality, and thus the price, of ginseng roots. To effectively manage ginseng diseases and other disorders, it is valuable to study and identify functional genes related to defense mechanism in ginseng. However, till now there was no report on characterization of PR in ginseng plant. To understand the defense response of ginseng to stress, we have isolated PR genes from ginseng. In this study, we report on the cloning of PR5 gene from *P. ginseng* and provide analysis on the expression profile of the genes in the defense response to abiotic and biotic stresses.

Materials and Methods

RNA purification and construction of a cDNA library. Total RNA was isolated from callus using the aqueous phenol extraction procedure as described by Morris et al., (1990). Poly (A)+ RNA was isolated by oligo (dT) cellulose column using the Poly(A) Quick mRNA isolation kit (Stratagene, US). A cDNA synthesis kit was used to construct library according to the manufacture's instruction manual (Clontech, US). Size-selected cDNA was ligated into λ TriplEx2 vector and was packaged *in vitro* using Gigapack III Gold Packaging Extract kits (Stratagene, US).

Nucleotide sequencing and sequence analysis. pTriplEx phagemids were excised from the λpTriplEx2 and used as templates for sequence analysis. The 5' ends of cDNA inserts was sequenced by an automatic DNA sequencer (ABI prism 3700 DNA sequencer, Perkin-Elmer, USA). Homologous sequences of PR5 EST are searched against the GenBank databases using a BLASTX algorithm. A pTriplEx phagemid for PR5 cDNA was excised from the λpTriplEx2 and used as templates for sequence analysis. Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi).

These deduced amino acid sequences were searched for homologous proteins in the databases using BLAST network services at the NCBI. We used ClustalX with default gap penalties to perform multiple alignment of PR5 isolated in ginseng and previously registered in other species. Based on this alignment, a phylogenetic tree was constructed according to the neighbor-joining method using the MEGA3 programs. The protein properties are estimated using ProtParam (Gasteiger et al., 2005) and the hydropathy value is calculated by the method of Kyte and Doolittle (1982).

Plant, environmental stress and pathogen. *P. ginseng* cv. "Hwang-Sook" seeds (provided by Ginseng Genetic Resource Bank) were used and cultured 3-week-old plantlets were used for the treatments and nucleic acid extractions, as described previously (Kim et al., 2008). For chemical stress treatments, the plantlets were placed for various periods in Murashige and Skoog (MS) medium containing indicated concentrations of chemicals; 100 mM NaCl, 500 μM $CuSO_4$, 500 μM $CdSO_4$. Chilling stress was applied by exposing the plantlets at 4 °C. In all cases, stress treatments were carried out on the MS media and 10 plantlets were treated with each stress for 1, 4, 8, 24, 48, or 72 hours.

The fungal strains, *Botrytis cinerae* (KACC 41298), *Colletotrichum gloeosporoides* (KACC 40003) *Rhizoctonia solani* (KACC 40101), *Phythium ultimunm* (KACC 41062), were obtained from Korean Agricultural Collection Center, South Korea. The isolates of fungi were grown for three days at 25 °C on potato dextrose agar to obtain mycelium for the inoculation of ginseng seedlings. For the infection experiments, mycelial plug excised from the actively growing margin of a colony were suspended in sterile water and sprayed. After inoculation, plants were kept at 100% humidity to attain moisture condition. Plants were harvested at 0, 6, 24, 48, and 72 h post-infection. Control plants were held in a growth room at 25 °C under a 16h photoperiod. The stressed plant materials from all completed treatments were immediately frozen in liquid nitrogen and stored at −70 °C until required.

Real time quantitative RT-PCR. Total RNA was extracted from seedlings of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 200 ng of total RNA was used as a template for reverse transcription using oligo$(dT)_{15}$ primer (0.2 mM) and AMV Reverse Transcriptase (10 U/μl) (INTRON Biotechnology, Inc., South Korea) according to the manufacturer's instructions. Real-time quantitative PCR was performed using 100 ng of cDNA in a 10-ul reaction volume using SYBR® Green SensimixPlus Master Mix (Quantace, Watford, England). Specific primers for *PgPR5*, 5'-AAC CGA CTG CAA CTT CGA CT-3' and 5'-GGC ACA TTA AAC CCA TCC AC-3' were used for perform real time-PCR. The thermal cycler conditions recommended by the manufacturer were used as follow: 10 min at 95 °C, followed 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C 20 s. The fluorescent product was detected at the last step of each cycle. Amplification, detection, and data analysis were carried out with a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than the background fluorescence at the initial exponential phase of PCR amplification. To determine the relative fold

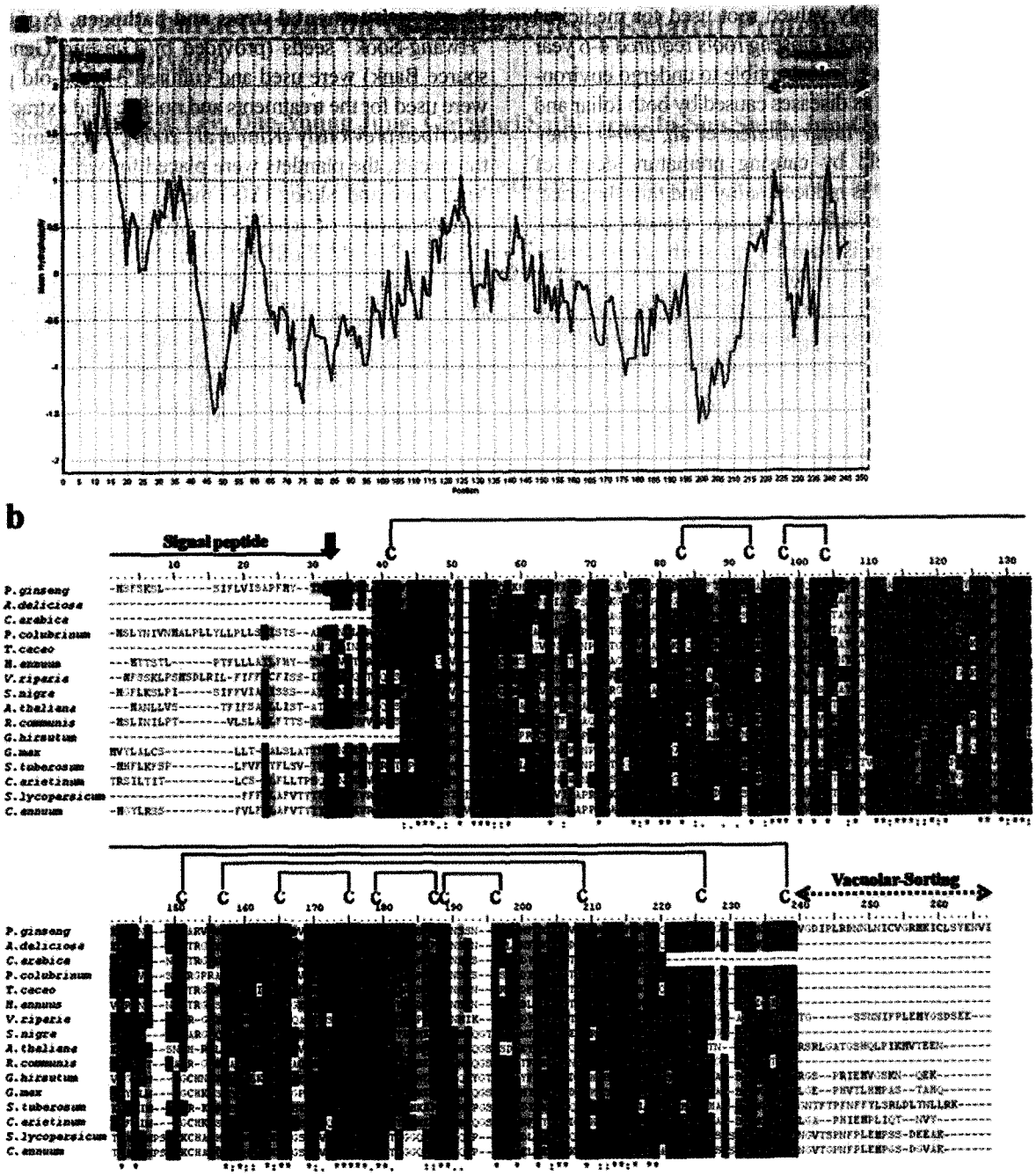

Fig. 1. Sequence analysis of *PgPR5* gene. **a**, Hydropathic index analysis. The putative signal peptide and C-terminal extension peptide are indicated. The cleavage site between the signal peptide and mature protein is indicated by the arrow. Hydrophobic domains are indicated by positive numbers; hydrophilic domains are above line and hydrophilic domains are below; **b**, Multiple alignment of the deduced amino acid sequences of *PgPR5* with PR5s from other plants; *Actinidia deliciosa* TLP (ABQ42566), *Coffea arabica* TLP (ABW76502), *Piper colubrinum* OLP (ABX71220), *Theobroma cacao* OLP (AAV34889), *Helianthus annuus* PR5 (AAM21199), *Vitis riparia* TLP (AAD55090), *Sambucus nigra* TLP (AAK59275), *Arabidopsis thaliana* OLP (NP192902), *Ricinus communis* OLP (EEF51136), *Gossypium hirsutum* OLP (AAQ64678), *Glycine max* PR5b (BAH01715), *Solanum tuberosum* TLP (AAU95246), *Capsicum annuum* TLP (AAK97184), *Cicer arietinum* TLP (CAA09228), *Solanum lycopersicum* OLP (Q01591). Hyphen was inserted within amino acid sequence to denote gap. Shadow box means well conserved residues, *represents conserved amino acid and: represents very similar amino acid. The positions of the eight disulfide bonds are marked. The cleavage site is indicated by the arrow. **c**, Phylogeny of the PR5 proteins from *P. ginseng* and other species. Phylogenetic analysis is based on the deduced amino acid sequences of PR5 genes from plant species. Neighbor-joining method was used and the branch lengths are proportional to divergence, with the scale of 0.05 representing 5% changes. **d**, Comparison of secondary structures of PR5s by SOPMA. The helix, sheet, turn and coil are indicated in order from the longest to the shortest.

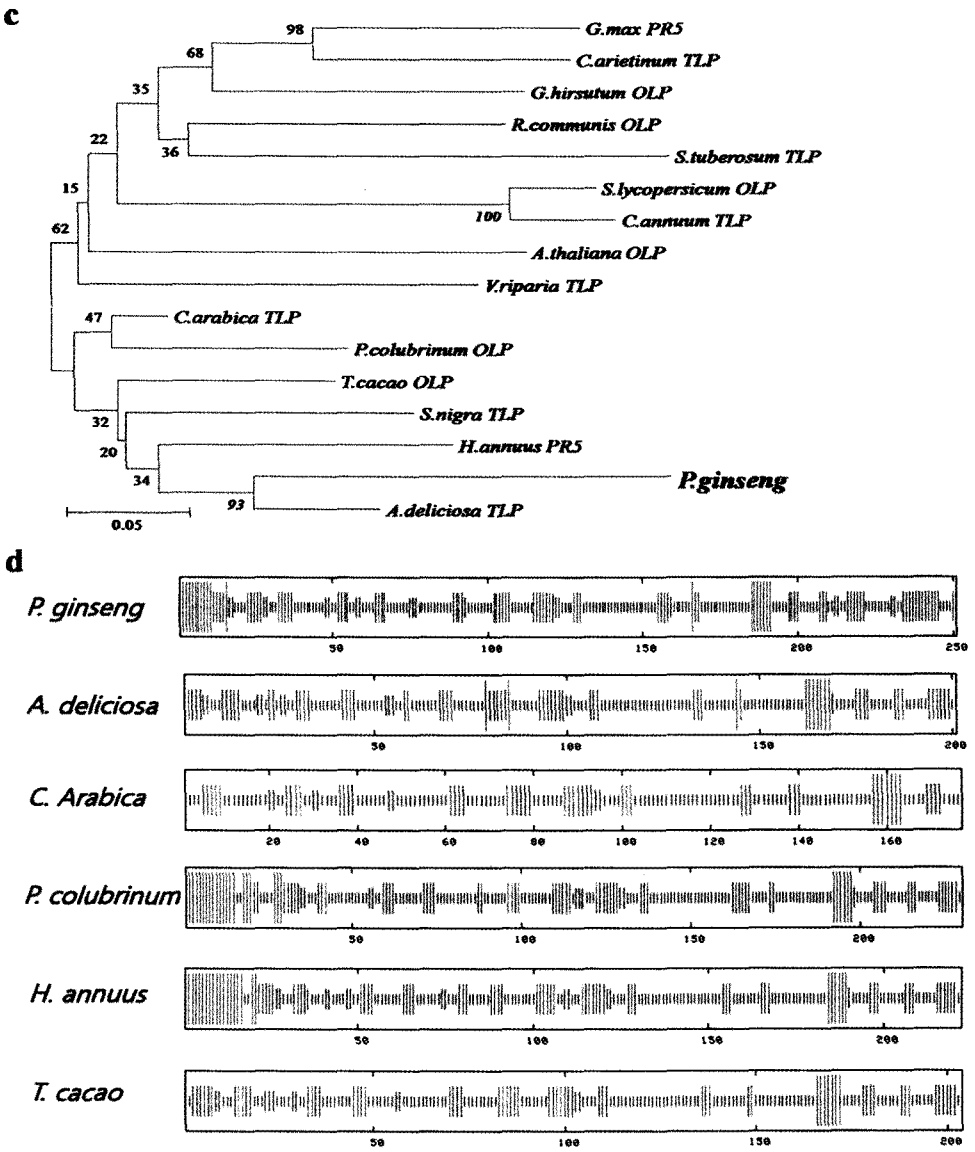

Fig. 1. Continued.

differences in template abundance for each sample the Ct value for *PgPR5* was normalized to the Ct value for β-actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Three independent experiments were performed.

Results

Isolation and amino acid sequence analysis of a *PgPR5*. From our expressed sequence tags (EST) analysis of a cDNA library, which was prepared with the callus of *P. ginseng*, we identified a cDNA clone encoding a PR5 gene. We named this gene *PgPR5* (*P. ginseng* pathogenesis-related protein 5); the sequence data of *PgPR5* have been deposited in GenBank under accession number GQ452234. The *PgPR5* cDNA was 854 nucleotides long and had the putative open reading frame of 756 bp. This ORF encodes a pathogenesis-related protein of 251 aa, beginning at the initiation codon ATG (Position 32) and ending at the stop codon TAA (Position 787) of the cDNA, with the predicted molecular weight of 27.5 kDa and isoelectric point of 7.80. The instability index (II) is computed to be 30.10. This classifies the protein as stable. The aliphatic index, regarded as a positive factor for increased thermostability, was calculated as 66.41 by using ProtParam (Gasteiger et al., 2005). In the deduced amino acid sequence of PgPR5 protein, the total number of negatively charged residues (Asp+Glu) was 17, while the total number of positively charged residues (Arg+Lys) was 19.

The SignalP program (Bendtsen et al., 2004) predicted that the N-terminus would be cleaved out at between Ala22

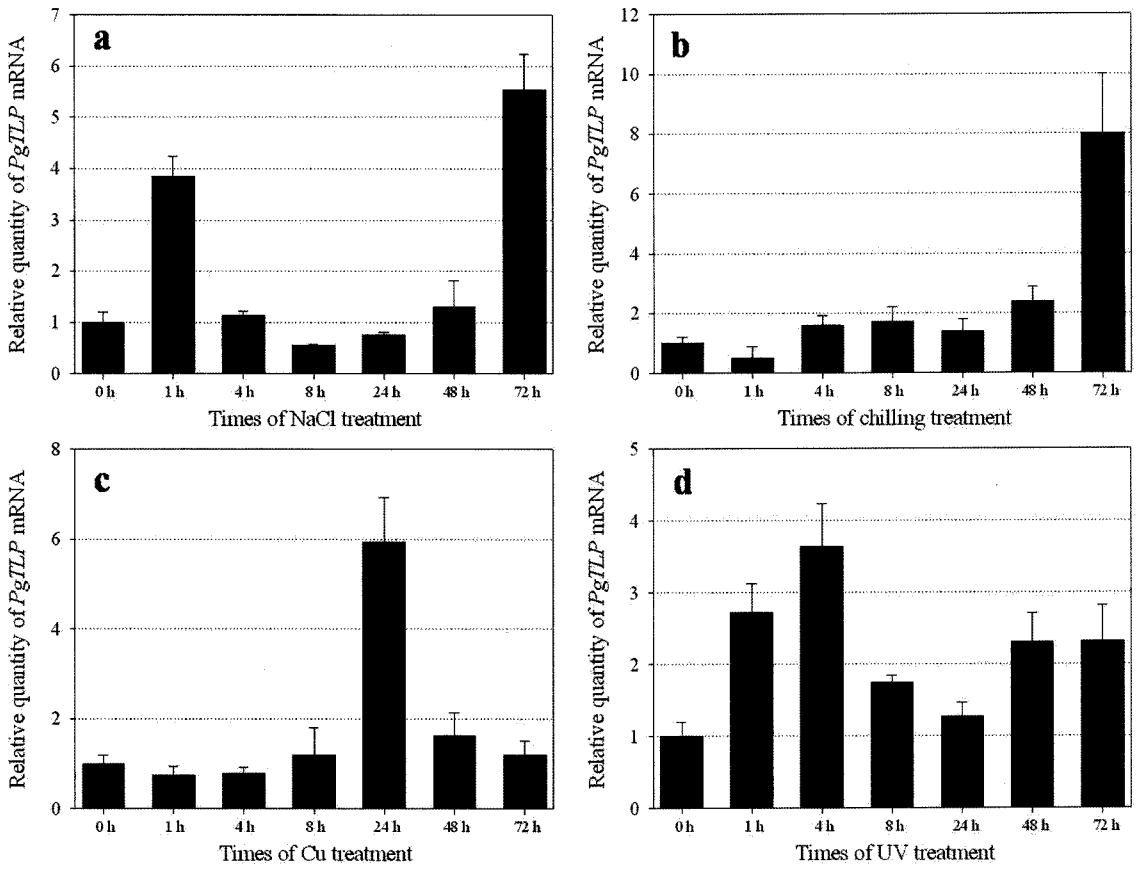

Fig. 2. Relative quantities of *PgPR5* mRNA at various time points (h) post-treatment with abiotic stresses. **a**, NaCl, **b**, Chilling, **c**, $CuSO_4$, **d**, UV. The error bars represent the standard error of the means of three independent replicates.

and Ala23 by translational processing (Fig. 1). The ORF of the *PgPR5* cDNA encodes a pre-proprotein, consisting of a predicted signal peptide (22 aa), and a putative C-terminal extension (28 aa). After processing, the mature polypeptide is 201 aa long, with a calculated molecular mass of 21.9 kDa and an estimated pI of 7.53. The hydrophilicity profile of estimated PR5 protein is shown Fig. 1a and N-terminal and C-terminal peptide exhibited hydrophobic pattern. The presence of a C-terminal and N-terminal signal peptide suggest that it is a vacuolar secreted protein.

Homology analysis. A GenBank BlastX search revealed that the deduced amino acid of *PgPR5* shares a high degree homology with PR5 from other plants. Fig. 2a shows the alignment of PgPR5 with similar proteins from other plants. PgPR5 shows the highest sequence similarity to TLP of *Actinidia deliciosa* (80% identity, 87% similarity) followed by TLP of *Coffea arabica* (77% identity, 86% similarity), OLP of *Piper colubrinum* (75% identity, 85% similarity), OLP of *Theobroma cacao* (73% identity, 83% similarity), and PR5 of *Helianthus annuus* (73% identity, 82% similarity). Additionally, it has above 75% similarity with other TLP and OLP from several plants. Alignment of the PR5 indicated that amino-acid residues are highly conserved and all 16 cysteine residues that are present in PR5 are conserved in PgPR5 (Fig. 1b). Phylogenetic analysis further confirms that PgPR5 belongs to the PR5 family (Fig. 1c).

Secondary structure analysis and molecular modeling for PgPR5 were performed by SOMPA. The secondary structure analysis revealed that PgPR5 consists of 21α-helices, 19β-turns jointed by 74 extended strands, and 137 random coils (Fig. 1d). This result is highly similar to the secondary structure of OLP of *P. colubrinum*, which contains 28α-helices, 10β-turns jointed by 61 extended strands, and 131 random coils and PR5 of *H. annuus*, which contains 26α-helices, 14β-turns jointed by 58 extended strands, and 124 random coils.

Temporal expression of *PgPR5* gene in response to stresses. The expression of *PgPR5* at different time points after various treatments was analyzed by real-time PCR. Fig. 2a shows the expression of *PgPR5* against salt stress. At 1 h post-treatment, gene expression was 3.84-fold of control and then gradually decreased to lower level during the following 4-24 h. However, *PgPR5* expression dramatically increased to 5.53-fold at 72 h post-treatment. Fig. 2b

shows the expression pattern of *PgPR5* in response to chilling stress. The level of *PgPR5* mRNA maintained control level and then dramatically increased to 8-fold at 72 h post-treatment. Copper treatment induced an increase in the PgPR5 transcript level to maximum accumulation (5.94-fold) at 24 h (Fig. 2c). Under UV radiation stress, the *PgPR5* transcript level gradually increased and peaked at 4 h time-point (3.64-fold) and expression decreased after 8 h post-treatment and maintained 2.3-fold until 72 h (Fig. 2d).

To observe *PgPR5* gene expression against pathogen infection, the fungal strains, *B. cinerea*, *C. gloeosporioides*, *P. ultimum*, *R. solani* were treated (Fig. 3). *Botrytis* infection induced *PgPR5* level to 2-fold at 6 h, and it didn't show significant induction until 72 h (2.41-fold). Upon *Colletotrichum* inoculation, the transcription of *PgPR5* gradually increased until 72 h post-treatment (4.02-fold). Similarly, during *Phythium* infection, the *PgPR5* transcript level was highest at 48 h (4.53-fold) and then slightly decreased to 3.8-fold at 72 h. *Rhizoctonia* infection caused a rapid induction in *PgPR5* expression within 6 h post-treatment (4.81-fold), and then expression level was maintained at steady-state level until 72 h post-treatment (4.19-fold).

Discussion

In this study, we described the first report of the isolation and characterization of the gene encoding PR5 from *P. ginseng*. Searches of the BlastX database suggested that *PgPR5* cDNA belongs to large group of PR5 genes. A comparison of the deduced amino acid sequences of PgPR5 with other PR5s reveals a striking set of similarities. Most large group of PR5s possess 16 conserved cysteine residues and form eight disulfide bonds; *PgPR5* also has 16 cysteines (Fig. 1b). These 16 cysteines will form eight disulfide bonds, representing a very thermostable and pH-stable compound (Breiteneder, 2004).

Previous studies reported that the extracellular PR5s tend to be acidic, whereas vacuolar PR5s tends to be basic (Stintzi et al., 1993). In this view, basic PgPR5 might be a vacuolar protein, and we find the typical features of signal peptides using a SIGNALP program. A putative N-terminal elongation is known as a signal peptide sequence used for secretary transport (Sato et al., 1995). The *PgPR5* has a hydrophobic signal peptide on the N-terminus, corresponding to the predicted cleavage site of the putative polypeptide (Fig. 1). In fact, most PR5 proteins have signal peptides on the N-terminus, with a highly conserved alanine residue at the cleavage site, of various amino acid lengths (Fig. 1b).

The basic and neutral isoforms, which are retained in the vacuole of the cell, have an additional C-terminal extension, that is absent in the acidic extracellular form. These short C-terminal propeptides have been shown to contain information for the vacuolar targeting of several PR proteins in plants and may be removed during or after transport to the plant vacuole (Melchers et al., 1993; Sato et al., 1995). The PgPR5 had a C-terminal elongation, 28 aa in length, possibly used for vacuolar targeting (Fig. 1b).

The results of our study suggest that PgPR5 possessing both peptides could be localized in vacuoles of the ginseng cells through the directing effect of the elongated C-terminal. Therefore, we suggested that the mature polypeptide of PgPR5 would consist of 201 aa, and have a mass of 21.9 kDa and a pI of 7.53, which is typical of 'large PR5' commonly found in dicot species. SnOLP, PR5 from *Solanum nigrum*, consists 247 aa in length, include N- and C-terminal propeptides, and its mature protein is 207 aa with a calculated molecular weight of 22 kDa and pI of 7.28 (Campos et al., 2002), which coincides with our PgPR5. Atosm, basic OLP from *A. thaliana*, also consist N- and C-terminal extension sequence (Capelli et al., 1997). In soybean plant, neutral PR5, GmOLPb had C-terminal elongation, 17 aa in length, possibly employing in vacuolar targeting (Tachi et al., 2009). In contrast, GmOLPa (Onishi et al., 2006) and P21 protein (Graham et al., 1992), acidic PR5s from same plant, had no C-terminal polypeptide.

Secondary structure showed PgPR5 is similar with two OLP rather than those of other three plants (Fig. 1d). Nevertheless, as shown in Fig. 1b and c, TLP and OLP have no strike distinguishable difference. The family of PR5s is diverse and expression of PR5 isoforms is different in same plant, and each PR5s may play a distinctive role in the stress response (Tachi et al., 2009). The reason why plants produce a large number of diverse PRs is not clear. One likely cause could be evolutionary pressure towards the protection of the plant against different pathogens and abiotic stresses, since different PR5 genes are activated by different signals (Campos et al., 2002). Therefore, characterization in detail the expression and structure of many isoforms of PR5s are needed to better understand the roles and functions of PR5s. PgPR5 is homologous with other plant PR5s, have conserved sequences, but has a distinguishable C-terminal extension. Since expression of many *PR5s* is induced by biotic and abiotic stresses, we treated many stresses to test *PgPR5* expression. Salt-inducible expression of *PgPR5* is common trait with some plant *PR5*s. Gene expression of soybean neutral *PR5*, including C-terminal elongation, also induced at early time, but stimulated during 18-72 h under 100 mM NaCl stress treatment in soybean plant (Tachi et al., 2009). Tobacco (Singh et al., 1985) and a tomato PR5 (King et al., 1988) were shown to accumulate in salt stressed cells. The expression of PR5 genes from potato (Zhu et al., 1995) and pepper (Hong et al., 2004) were activated by low

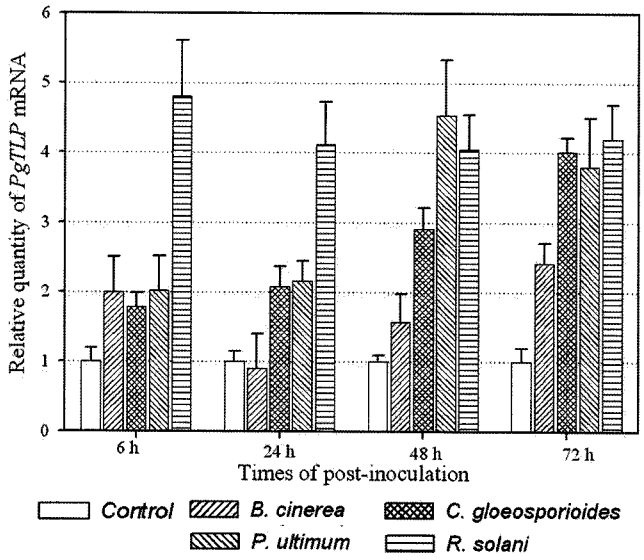

Fig. 3. Relative quantities of *PgPR5* mRNA at various time points (h) post-treatment with biotic stresses, such as *Botrytis cinerae*, *Colletotrichum gloeosporoides*, *Phythium ultimunm*, and *Rhizoctonia solani*. The error bars represent the standard error of the means of three independent replicates.

temperature and high salinity.

In addition, *PgPR5* transcript is induced by copper stress (Fig. 2c) and UV stress (Fig. 2d). There is one report that Rice TLP is also induced by copper stress and UV stress (Rakwal et al., 1999). Other stress-related signaling molecules, such as H_2O_2, ABA, SA and JA, did not induce the accumulation of PgPR5 (data not shown). It is revealed that not only ABA-dependent but also ABA-independent signal transduction pathways exist. Similarly, in case of carrot PR5, induction was by only drought stress, it is also not affected by signaling molecules, such as ABA, SA, and JA (Jung et al., 2005).

We subsequently tested the effect of pathogen infection on *PgPR5* transcription. Two-week old ginseng plants were inculated with *Botrytis cinerae*, *Colletotrichum gloeosporoides*, *Phythium ultimunm*, and *Rhizoctonia solani*. These fungi are known as cause anthracnose, gray mold, and damping-off in ginseng which leads to decrease in yield. *Botrytis* infection did not show significant accumulation, however, three strain inoculation increased the accumulation of transcripts at 72 h after treatment, although *Rhizoctonia* induced rapid increase *PgPR5* transcript (Fig. 3). The induction of PR5s in response to microbial infection has been observed in various plant species. It has been observed that PR5s exhibit some degrees of specificity toward the fungi species upon which they act. Tobacco osmotin could inhibit some species including *Phytophthora*, but had no effect on other species including *Rhizocotonia* (Abad et al., 1996).

Ginseng cultivation suffers difficulties with pathogen infection. By contrast, relatively little is known about the molecular study of ginseng pathogenesis-related genes. In this respect, we describe for the first time the isolation and characterization of PR gene in *P. ginseng*. We identified the expression pattern of *PgPR5* gene against abiotic and biotic stresses. PgPR5 might accumulate abundantly in vacuoles to protect against the imbalance and impart increased tolerance to high-salt, chilling, heavy metal, pathogen stresses. But expression against heavy metal stress and pathogen-specific expression need further investigation. In this study, we could not clarify the functional relationships and physiological roles of *PgPR5* on the basis of the high amino acid sequence similarity to other PR5s. More ginseng PR5 proteins with different features need to be indentified and compared at the structural and functional levels in further analyses. Furthermore, the characterization of transgenic plants overexpressing this *PgPR5* would help clarify the relationships between its structures and functional roles in environmental stress tolerance.

Acknowledgments

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