

Expression and functional characterization of pathogenesis-related protein family 10 gene, *PgPR10-2*, from *Panax ginseng* C.A. Meyer

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ABSTRACT

A full-length sequence (*PgPR10-2*) of the most abundant transcript from 14-year-old ginseng EST library was isolated and cloned. *PgPR10-2* gene encodes 465 bp open reading frame and its deduced protein contains 155 amino acids. Genomic DNA sequence reveals it contains two exons interrupted by one intron. Expression of *PgPR10-2* gene was especially abundant in roots and its transcripts showed differentially upregulated patterns against several given pathogens and abiotic stimuli. Observed enhanced ribonuclease activity and antifungal activity from tobacco transgenic lines suggest that the possible involvement of *PgPR10-2* in defense-related mechanism via ribonuclease activity against biotic and abiotic stresses.

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1. Introduction

The sessile nature of plant is under constant challenge by an array of biological, chemical, and environmental agents as threats. Every plant is hence forced to evolve its own structural, chemical, inducible defense mechanisms for survival at different level of challenges in order to overcome them. The structural defense involves the protective function of cuticle and lignin, followed by the chemical barriers like phytoanticipins. However, this first line of defense is not sufficient to protect the plant against a host range of constantly evolving pathogen population. Hence, the second line of defense is activated, which involves an array of induced mechanisms like hypersensitive response and the concomitant oxidative burst, accumulation of secondary metabolites, signaling for increased activity of antioxidant enzymes and the induction of pathogenesis-related proteins [1]. Pathogenesis-related proteins (PR proteins) encoded by plants are considered important for their roles in plant defense. They are induced specifically in pathological or related situations. PR proteins are not only accumulated locally

in the infected leaf, but also induced systemically, associated with the development of systemic acquired resistance (SAR) [2].

PR proteins fall into 17 gene families (PR-1 to PR-17) based on their structural difference, serological relationship and biological activity with each group comprising of several members [1–4]. The molecular size of PR proteins varies widely ranging from 7 to 75 kDa [1]. PR-10 is a unique class of small acidic intracellular proteins first identified in parsley [3] and shows amino acid sequence similarity to a large family of food and tree pollen allergens [5]. More than 100 PR-10 and PR-10-related sequences have been identified in numerous dicots and monocots including pea [6], potato [7], white pine [8], bean [9], lupine [10], white spruce [11,12], rice [13], plum [14], and grapevine [15]. Most PR-10 proteins are known to be induced by various biotic and abiotic stresses, and constitutive expression of these proteins is also reported [6–15]. It suggests their role in plant defense and development.

Previously two PR-10 proteins isolated from ginseng callus were shown to have a specific ribonuclease activity [16,17]. However, no reports are available up to now on the isolation and characterization of the genes encoding for the protein from ginseng plant. Here we report the isolation of a full-length cDNA clone encoding *PgPR10-2* from EST library of 14-year-old ginseng root. Korean ginseng (*Panax ginseng* C. A. Meyer) is a perennial herb and belongs to the family *Araliaceae*. It has been cultivated for its highly valued root, which is used traditionally in oriental medicine. The older the ginseng, the higher the value because of the enhanced secondary metabolite contents during long periods of cultivation time (4–5 years). During their life cycle, they have to endure repeated cycles of various environmental stresses. It is also a shade loving crop, giving

Abbreviations: EST, Expressed Sequence Tag; ORF, Open Reading Frame; PR, Pathogenesis-Related Proteins; qRT-PCR, Quantitative Reverse Transcription-Polymerase Chain Reaction; ABA, Absciscic acid; H₂O₂, Hydrogen peroxide; SAR, Systemic Acquired Resistance; JA, Jasmonic Acid; SA, Salicylic Acid.

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ample room for pathogen infection, which may seriously affect the crop yield. In this present study, differential temporal expression patterns of *PgPR10-2* against biotic and abiotic stresses and its ribonuclease activity from *PgPR10-2* overexpressing tobacco plant are examined.

2. Materials and methods

2.1. Plant materials and growth conditions

Korean ginseng (*P. ginseng* C. A. Meyer) cv. “Hwang-Sook” seeds and hairy roots were collected from Ginseng Genetic Resource Bank, Korea. Different organs of ginseng (root, leaf, and stem) were harvested from 2-year-old field grown plants. Ginseng seeds were *in vitro* germinated in Murashige and Skoog (MS) media (Duchefa biochemie, Netherland) at 25 °C under a 16 h photoperiod.

2.2. Real time quantitative RT-PCR

Total RNA was isolated from the 14-year-old ginseng root using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The concentration of RNA was measured using a GE nano-value spectrophotometer. To obtain the first strand of cDNA, 5 µg of total RNA were reverse transcribed using Power cDNA kit (Invitrogen) following the instruction given by the manufacturer. qRT-PCR was performed by Rotor-Gene 6000 real-time rotary analyzer using 10 ng of cDNA in a 10 µL reaction volume using SYBR® Green Sensimix Plus Master Mix (Quantace, Watford, England) with the following primers; 5'-GGC AGA AAA GCT GTT CAA GG -3' (forward) and 5'-TTG CAT CTA TCC GGG TCT TC -3' (reverse). The housekeeping gene encoding actin was used as a control in the experiment, which was amplified with the primers; 5'-CGT GAT CTT ACA GAT AGC TTG ATG A-3' (forward) and 5'-AGA GAA GCT AAG ATT GAT CCT CC-3' (reverse). The thermal cycler conditions recommended by the manufacturer were used: 10 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 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). To determine the relative fold differences in template abundance for each sample, the Ct value for *PgPR10-2* 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. The primer efficiencies were determined according to Livak and Schmittgen [18] to validate the $\Delta\Delta Ct$ method used in our experiment. The ΔCt values were plotted across a log of template cDNA diluted over 125-fold range (1:0, 1:3, 1:125; $n = 3$). The slopes were close to zero indicating that the efficiency of gene and the internal control β -actin were equal.

2.3. Application of abiotic and biotic stresses

The *P. ginseng* plantlets were treated with abiotic stimuli about three weeks after zygotic embryo germination. Treatment for 1, 2, 8, 12, 24, and 48 h with the following chemicals [16–15], tested concentrations were based on previously reported works on MS media: 10 mM H₂O₂, 10 mM SA, and 100 µM ABA independently. Hairy roots were also treated with 100 mM NaCl, 0.2 mM JA, and chilling stress, which was applied by keeping the hairy roots at 4 °C. For biotic stressed samples, 4-year-old ginseng roots infected by *Botrytis cinerea* were obtained from Ginseng Genetic Resource Bank, Korea. *Colletotrichum gloeosporioides*, *Phytophthora capsici*, and *Alternaria solani* mycelial plug were excised (3 mm × 3 mm) from the actively growing margin of a colony from PDA (Difco) plates. The fungi are suspended in sterile water and sprayed on

plant leaves. Untreated plants sprayed only with sterile water were taken as a control. After the treatment the plants were covered with polyethylene bags to maintain moisture condition. Plants were harvested at 3, 6, 12, 24, and 48 h post-infection.

2.4. Vector construction and in planta transformation

The entire coding region of *PgPR10-2* gene was amplified from *P. ginseng* cDNA using PCR with specific primers embedded *Nco*I (5'-C CCA TGG ATG GGT GTC CAA AAG ACC GAA ACC C-3') and *Bam*HI (5'-G GGA TCC TAA TTT GCT AGG AGG TAA GCT TCA ACA GC-3') restriction sites. The amplified PCR product was ligated into the same restriction sites between double 35S cauliflower mosaic virus (CaMV) promoter followed by alfalfa mosaic virus enhancer and the nopaline synthase (NOS) terminator in 524Xba cassette vector (NRC Plant Biotechnology Institute, Canada). The 524Xba recombinant vector carrying the insert was digested with *Xba*I and then the expression cassette was ligated into the same restriction sites within pRD400 ([19], identical to pBin 19 possessing *nptII*). The construct was verified by automated DNA sequencing and then transformed into *Agrobacterium tumefaciens* strain GV3130 [20]. Leaf disk explants of *Nicotiana tabacum* cv. Xanthi were co-cultured with *A. tumefaciens* GV3130 containing pRD400 binary vector [21]. Transgenic plants were selected on the basis of their ability to regenerate on the MS medium containing 2 mg/L BA, 0.2 mg/L NAA, 100 mg/L kanamycin sulfate, and 200 mg/L cefotaxime. Upon transfer to MS medium containing 0.2 mg/L NAA, transgenic seedlings developed plantlets. Transgenic tobacco seedlings were survived in selection media contains kanamycin at the concentration of 50 mg/L. The presence of neomycin phosphotransferaseII (*NPTII*) (forward, 5-GAG GCT ATT CGG CTA TGA CTG-3; reverse, 5-ATG CCA TAG CGG CGA GGG CTA-3), *PgPR10-2* (forward 5-CCC ATG GAT GGG TGT CCA AAA GAC CGA AAC CC-3; reverse 5-GGG ATC CTA ATT TGC TAG GAG GTA AGC TTC AAC AGC-3), and NOS terminator (forward, 5-GCG GAG AAT TAA GAG AGT CAC G-3; reverse, 5-GGA TAC CGA GGG GAA TTT ATG G-3) in transgenics were analyzed by PCR from transgenic tobacco leaf tissue samples.

2.5. Ribonuclease assay and antifungal activity in transgenic plants

The crude protein extracts were prepared and purified according to Gomes-Filho et al. [22]. Tissues were ground in 0.1 M potassium phosphate buffer, pH 5.7 (1:5, w/v) at 4 °C. The resulting homogenate was filtered through filter paper and centrifuged (VS-550, VISION scientific Co., LTD) at 12,000 rpm for 20 min at 4 °C. Protein concentration was determined using Bradford protein assay kit (BioRad, USA) with bovine serum albumin as the standard [23]. Ribonuclease (RNase) activity towards yeast tRNA was determined [24,25]. Crude extracts adjusted to uniform protein concentration (15 µg) was incubated with 200 µg tRNA in 150 µl of Tris-HCl buffer (100 mM, pH 7.5) at 37 °C for 1 h. The reaction was stopped by adding 350 µl ice-cold 3.4% perchloric acid. The reaction mixture was centrifuged (15,000 rpm, 15 min at 4 °C) to obtain the supernatant after it had been allowed to stand on ice for 15 min. The absorbance of the supernatant was read at 260 nm after suitable dilution. One unit of RNase activity is defined as the amount of enzyme that produces an absorbance increase of one per minute in the acid-soluble supernatant per ml of reaction mixture under the specified conditions. Antifungal activity against *A. solani* and *C. gloeosporioides* was assayed according to the procedure of Ng et al., 2001 [25] using petri plates containing potato dextrose agar. Sterile blank paper disks were placed around a central disk. Crude protein (10 µg) was applied to a disk on plate was incubated at 23 °C for 72 h until mycelial growth from the central disk enveloped

peripheral disks. It produced crescents of inhibition around disks containing samples with antifungal activity.

3. Results and discussion

3.1. Isolation and analysis of PgPR10-2

As part of a genome project to identify genes of the medicinal plant *P. ginseng*, several cDNA libraries consisting of about 20,000 cDNAs were constructed from embryogenic callus, hairy roots, 4-year-old roots, 14-year-old root, leaves, and buds of *P. ginseng* [26]. The most abundant transcript representing about 2.29% of the total cDNA library constructed from 14-year-old ginseng roots was chosen for the present study. Full length genomic clone was identified and sequenced. The translated sequence was identical to

Ribonuclease 2 from ginseng callus (GenBank ID; P80890) and showed 63.2% identity with PR10 from parsley (GenBank ID; P19418) (Suppl. Figs. 1 and 2, Suppl. Table 1). Many PR proteins, which had a functional similarity with ginseng ribonuclease protein, have been grouped under class 10 pathogenesis-related protein [2–5]. Hence, the gene isolated from *P. ginseng* is designated as PgPR10-2 (GenBank ID; GU086324). Although the alignment of the PgPR10-2 amino acid residues indicated that they are not highly conserved, their functional domain is highly conserved among all sequences presented (Suppl. Fig. 2). The phylogenetic study based on the amino acid sequence also supported this conclusion (Suppl. Fig. 1). Secondary structure analysis and molecular modeling for PgPR10-2 were performed by SOMPA [27]. The secondary structure analysis revealed that PgPR10-2 contains 7 α -helices consisting 60 amino acid residues, 3 β -turns consisting of

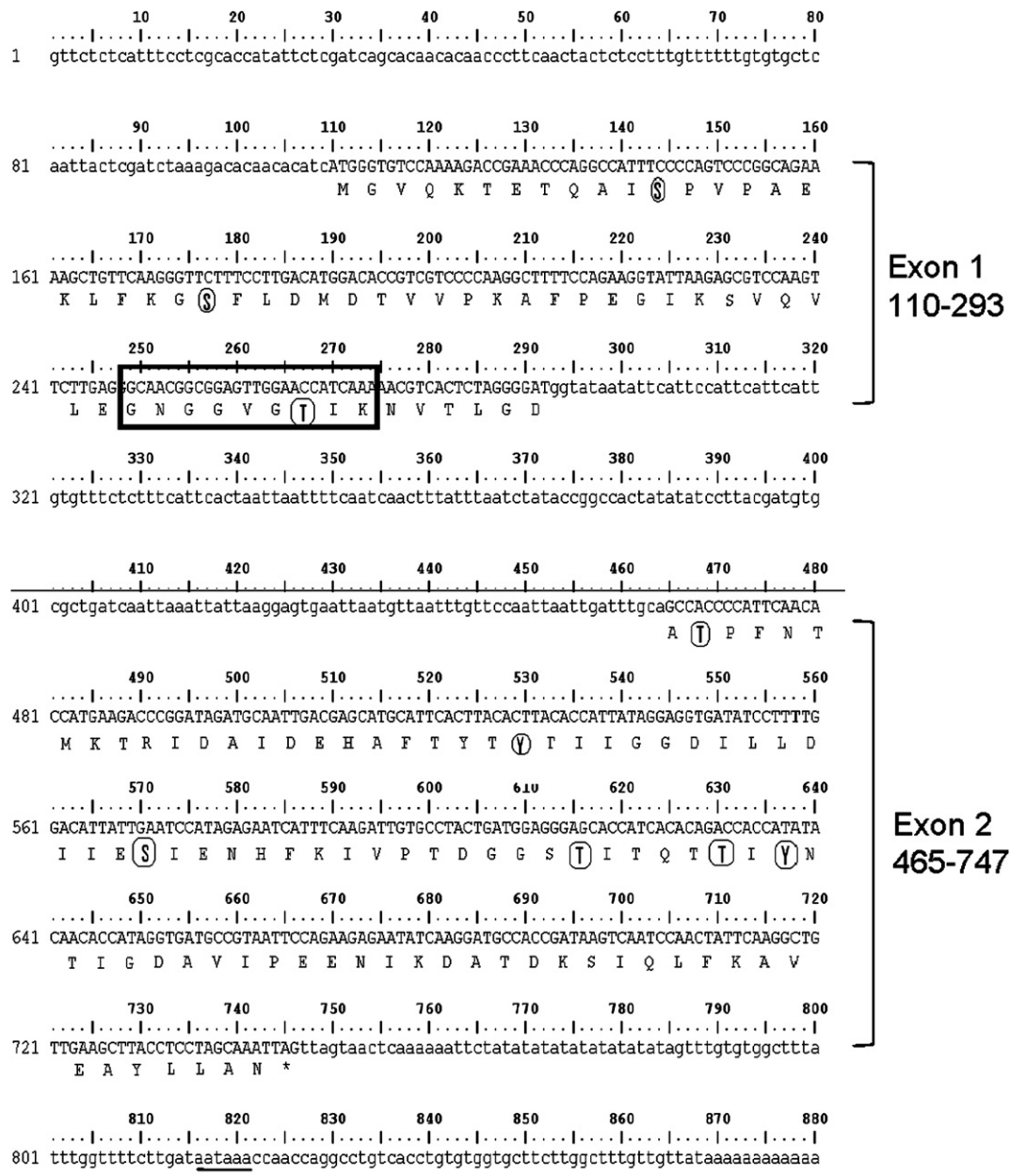


Fig. 1. Genomic DNA sequence of PgPR10-2 from *Panax ginseng*. Capitalized letters indicate the translated regions and small letters indicate the 5'UTRs, 3'UTRs and introns. Exon 1 and Exon 2 regions are marked in the right side and its deduced amino acids are indicated. Circled amino acid indicates putative phosphorylation sites where S is serine, T is threonine and Y is tyrosine. Amino acids in rectangular box indicate the presence of p-loop which is a functional domain in PR10 proteins. Poly A signals (AATAAA) was underlined.

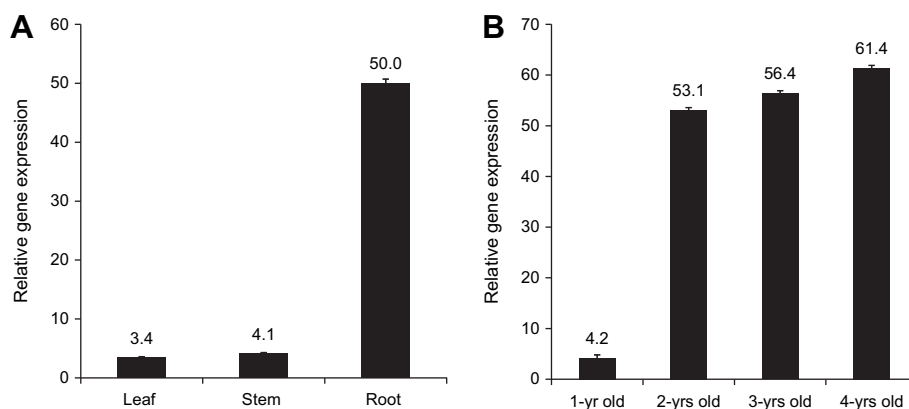


Fig. 2. Differential expression patterns of *PgPR10-2* A: Relative expression of *PgPR10-2* mRNA in organ specific tissues from 2-year-old ginseng plant. B: Relative expression of *PgPR10-2* mRNA in different aged-roots. The Ct value for *PgPR10-2* was normalized to the Ct value for β -actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Data represent means $\pm$ SE for three independent replicates.

16 amino acid residues. This is similar with secondary structure of PR-10 protein of parsley (representative member of PR-10 proteins) (Suppl. Fig. 3).

The *PgPR10-2* cDNA is 885 nucleotides long and has a putative open reading frame of 465 bp (Fig. 1). The ORF encodes a protein of 155 amino acids. Genomic sequence analysis revealed the presence of an intron of 171 bp between 293 bp and 465 bp (Fig. 1). Putative poly A signals (AATAAA) followed by poly A were noticed at 3-end of the sequence. The predicted molecular mass and isoelectric point of protein are 16.6 kDa and 4.56 respectively. The instability index (II) computed to be 23.56, classifies the protein as stable. The aliphatic index, regarded as a positive factor for increased thermostability, was calculated as 96.23 by using ProtParam. Nine putative phosphorylation sites (S is serine, T is threonine and Y is tyrosine) were identified.

3.2. Organ specific differential expression of *PgPR10-2*

The expression patterns of *PgPR10-2* in different organs of *P. ginseng* were examined using qRT-PCR. It is evident that *PgPR10-2* is constitutively expressed in roots, leaves, and shoots with highest transcripts in roots from 2-year-old ginseng plant (Fig. 2A). Strong expression in roots became increased by years (Fig. 2B). It has been reported that, PR-10 proteins are developmentally regulated and constitutively accumulated in different plant tissues and organs [12]. Extensive analysis of *JlOsPR10* in mature tissues of rice revealed a constitutive expression pattern with high levels detected in roots. When various regions of root were analyzed, the highest level of expression was noticed in the maturation zone compared to the root tip and elongation zone [13]. The root specific expression of PR-10 has been reported in many dicot plant species such as alfalfa [28], yellow lupine [29], and pea [30]. Our data indicate a systemic expression of *PgPR10-2* with high levels in roots similar to many other reported PR-10 proteins. It suggests a constitutive defense mechanism to protect the plant before pathogen infection or may be involved in some unknown function during plant development.

3.3. Differential expression of *PgPR10-2* in response to biotic and abiotic stresses

In order to understand the molecular mechanisms of stress tolerance adapted by ginseng plants to survive over long years of repeated cycles of biotic and abiotic challenges, the expression of *PgPR10-2* was studied using qRT-PCR analysis. When one-year-old plantlets grown in controlled conditions were challenged with

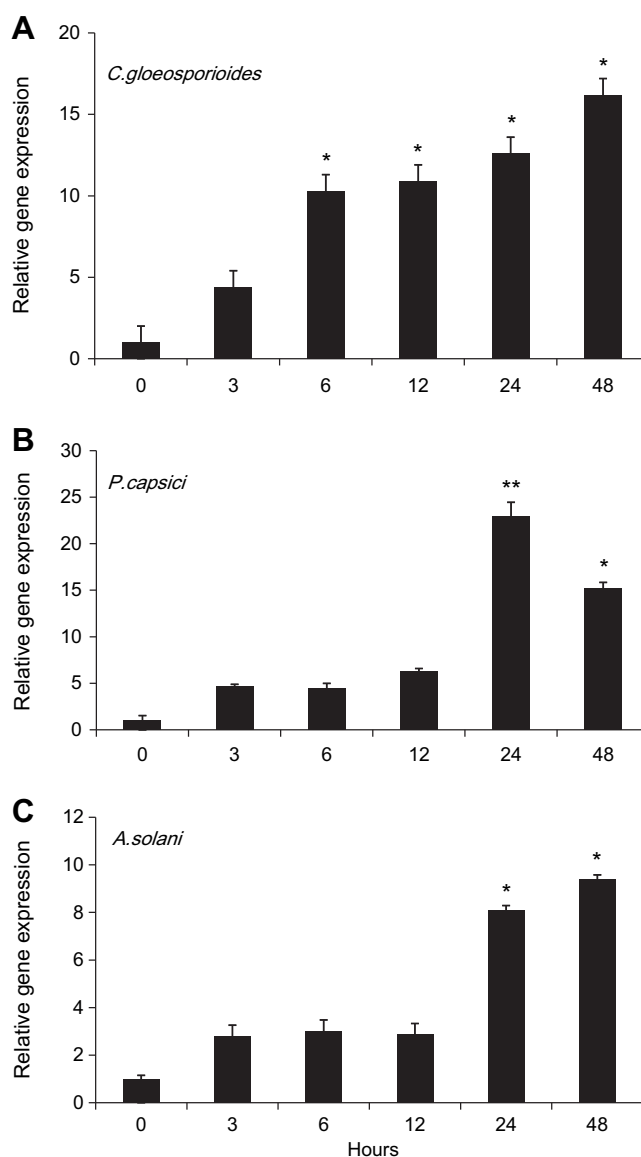


Fig. 3. Temporal expression patterns of the *PgPR10-2* against pathogens. A: *C. gloeosporioides*, B: *P. capsici*, and C: *A. solani*. The Ct value for *PgPR10-2* was normalized to the Ct value for β -actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Data represent means $\pm$ SE for three independent replicates. Averages for pathogens-induced are significantly different compared to the control at $P < 0.05$ for (*) and $P < 0.01$ for (**).

C. gloeosporioides, *P. capsici*, and *A. solani*, a significant increase in the transcripts was observed in leaves compared to the untreated control (Fig. 3 A–C). Starting from as early as 3 h after challenge, there was a continuous increase in expression of transcript up to 48 h of challenge. All the strains were able to induce the expression of *PgPR10-2*. The increased expression pattern of *PgPR10-2* was also observed using *B. cinerea*-infected 4-year-old plant. The accumulation of a number of genes involved in host defense responses was previously reported in ginseng (*Panax quinquefolius*) roots challenged with *Fusarium equiseti* [31]. Fungal infection leads to the activation of several secondary messengers, which in turn culminate in the transcriptional activation of defense-related genes, such as the PR genes [32]. The inducible expression of PR-10 genes in response to biotic stress has been reported in hot pepper [4], *Zea mays* [33] and Western white pine [11]. Altogether, it seems that *PgPR10-2* is a general ginseng pathogen inducible gene and is noticeable that its manipulation might enhance the growth and development of medicinally important ginseng plant.

The differentially induced expression patterns by salicylic acid, H_2O_2 and ABA treatment were analyzed using 3-week-old plantlet

(Fig. 4, left panel). Salicylic acid and H_2O_2 are important components of signal transduction cascades activating plant defense responses against pathogen attack [34,35]. ABA can cause an increased generation of H_2O_2 [36]. H_2O_2 is the most stable among the ROS species, has a direct antimicrobial effect through induction of gene expression, hypersensitive cell death, phytoalexin production, and induced systemic resistance [37]. Moreover, transcripts in hairy roots treated with NaCl, JA, and chilling stresses were also observed to be enhanced (Fig. 4, right panel). Similar induced patterns by stress stimuli including JA, salt, and chilling were observed using two *PR-10* genes in maize [33]. Chilling stress and high salinity result in increased cytosolic Ca^{2+} [38]. A complex signaling network, involving cytosolic Ca^{2+} and H^+ ions, reactive oxygen intermediates, and jasmonate triggers the induction of the defense mechanisms [39]. The enhanced relative expressions shown by the plantlets and hairy roots may be linked to their increased pathogen resistance. Taken together, our results suggest that biotic and abiotic stimulus induces the expression of *PgPR10-2* constitutively in ginseng plants, which suggest an important role in defense mechanism.

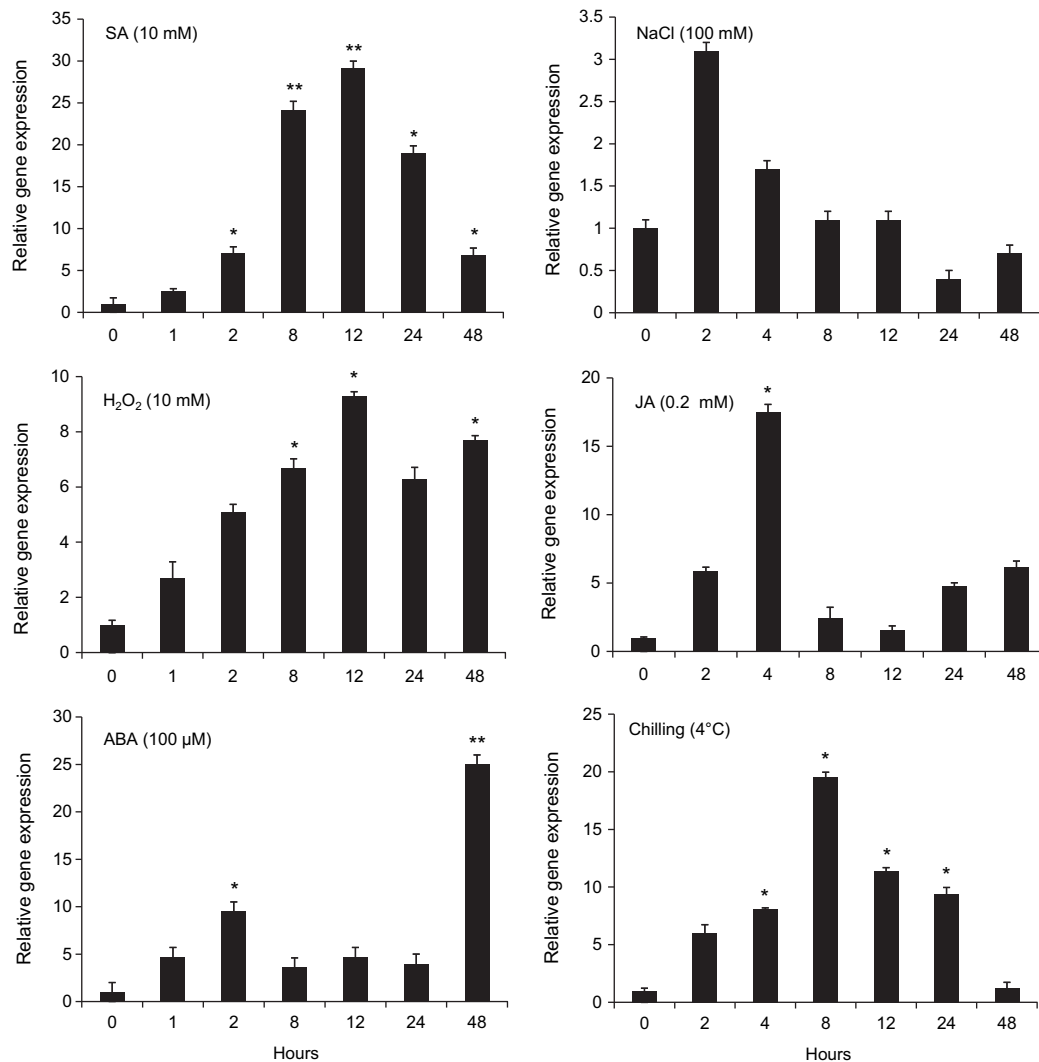


Fig. 4. Temporal expression patterns of *PgPR10-2* gene against abiotic stresses. SA (10 mM), H_2O_2 (10 mM), ABA (100 μ M), NaCl (100 mM), JA (0.2 mM), and chilling (4 °C) were treated to ginseng plantlets by given time intervals. The Ct value for *PgPR10-2* was normalized to the Ct value for β -actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Data represent means $\pm$ SE for three independent replicates. Averages for treated-samples are significantly different compared to the control at $P < 0.05$ for (*) and $P < 0.01$ for (**).

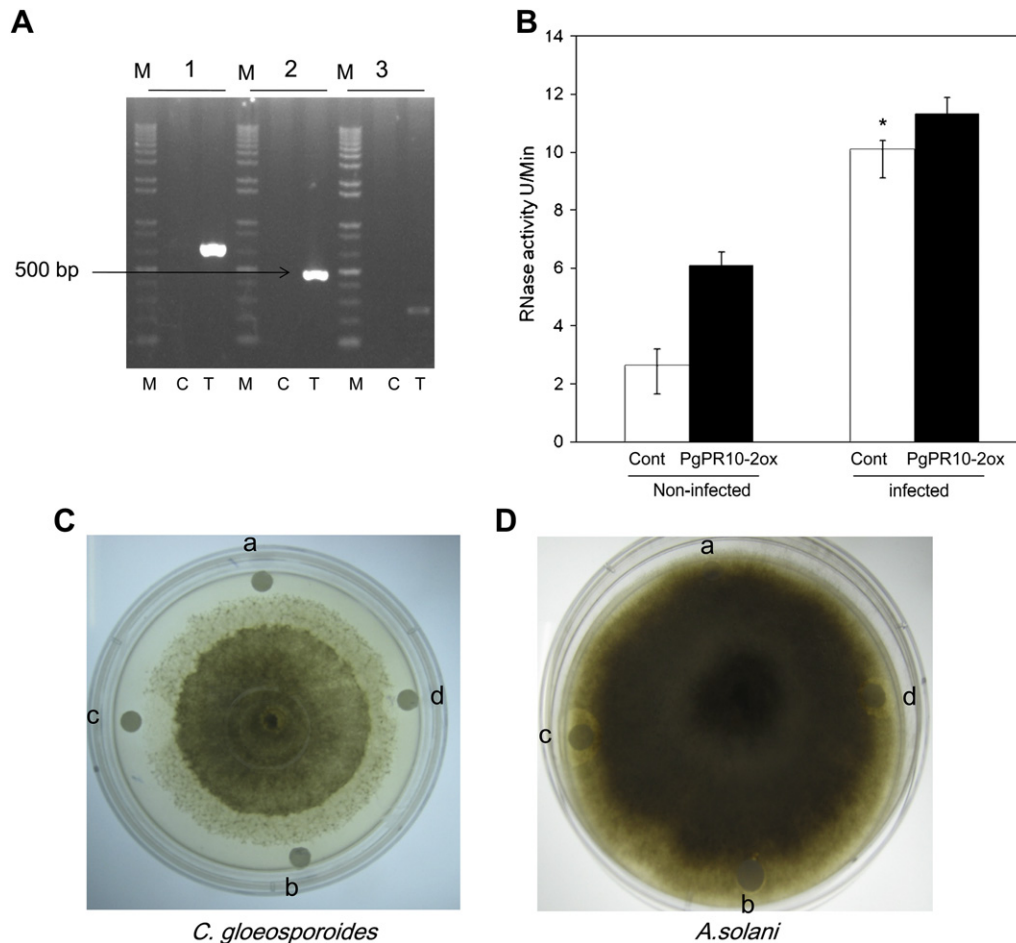


Fig. 5. *PgPR10-2ox* shows ribonuclease activity and increased antifungal activity. **A:** Confirmation of gene insertion in *PgPR10-2* overexpressing (*PgPR10-2ox*) tobacco by PCR using NPTII specific primers, neomycin phosphotransferaseII specific primers (1), *PgPR10-2* gene specific primers (2), and NOS, nopaline synthase terminator specific primers. M: size marker. **B:** Ribonuclease activity in wild type and transgenic tobacco leaf extracts before and after fungal infection. **C and D:** Antifungal activity is increased in *PgPR10-2ox* tobacco plant. a: water (50 μ L), b: crude extract from wild type tobacco (in 50 μ L), c and d: crude extract from *PgPR10-2ox* tobacco line; 100 μ L (c) and 50 μ L (d). Averages for infected-samples are significantly different compared to the non-infected control at $P < 0.05$ for (*).

3.4. RNase activity and antifungal activity of *PgPR10-2ox*

To examine the functional role of *PgPR10-2* in planta, full coding sequence of *PgPR10-2* was overexpressed (*PgPR10-2ox*) in tobacco using conventional *A. tumefaciens*-mediated transformation method [20] under the control of CaMV 35S promoter and NOS terminator. Several independent transgenic lines were established by growing resistant callus on selective media. Upon transfer to MS medium containing 0.1 mg l⁻¹ NAA, transgenic seedlings were developed into plantlets. The integration of the *PgPR10-2*, NPTII and NOS terminator gene was confirmed by PCR analysis. The PCR product showed clear bands for the transgenic plant, but no bands were observed in the wild type (Fig. 5A).

PgPR10-2 belongs to the general group of pathogenesis-related proteins, but it is exceptional as most of their primary structure does not share much similarity with others at protein level [3]. It is grouped under PR-10 since its functional domains are conserved as demonstrated by three dimensional (3D) structure [12]. Based on similarities between PR proteins and ribonucleases from ginseng, it has been suggested that this group of protein may have RNase activity. RNase activity from the crude extracts of *PgPR10-2* overexpressing transgenic tobacco plant was checked using yeast tRNA [24,25]. The endogenous RNase activity was detected in non-transgenic tobacco plant itself, but it was increased to 3.6-fold in

PgPR10-2ox (Fig. 5B). Pathogen infection was shown to increase transcript level of *PgPR10-2* (Fig. 3, Suppl. Fig. 4). In accordance with the increased transcripts, RNase activity was also increased in infected tobacco plant (Fig. 5B). Increased RNase activity was reported in transgenic *Arabidopsis* plants expressing ZmPR10.1 when infected with *P. syringae* [33].

Further antifungal activity was tested using *PgPR10-2ox* tobacco plant. Clear antifungal activity zone was noticeable with crude extracts of *PgPR10-2ox* tobacco plant against *C. gloeosporoides* (Fig. 5C) and *A. solani* (Fig. 5D). Previously, Lam and Ng [40] reported antifungal activity using *PgPR10-2* homologous gene from *Panax notoginseng*. Taken together, it is clear that genetically engineered ginseng might improve cultivars with better resistance to a variety of biotic and abiotic stress agents. Systemic expression of *PgPR10-2* with increased levels in response to biotic and abiotic stimulus is an indicator of the defense-related protein function. Studies involving mutant lines and purified recombinant protein will shed more light to understand the function of this gene in ginseng.

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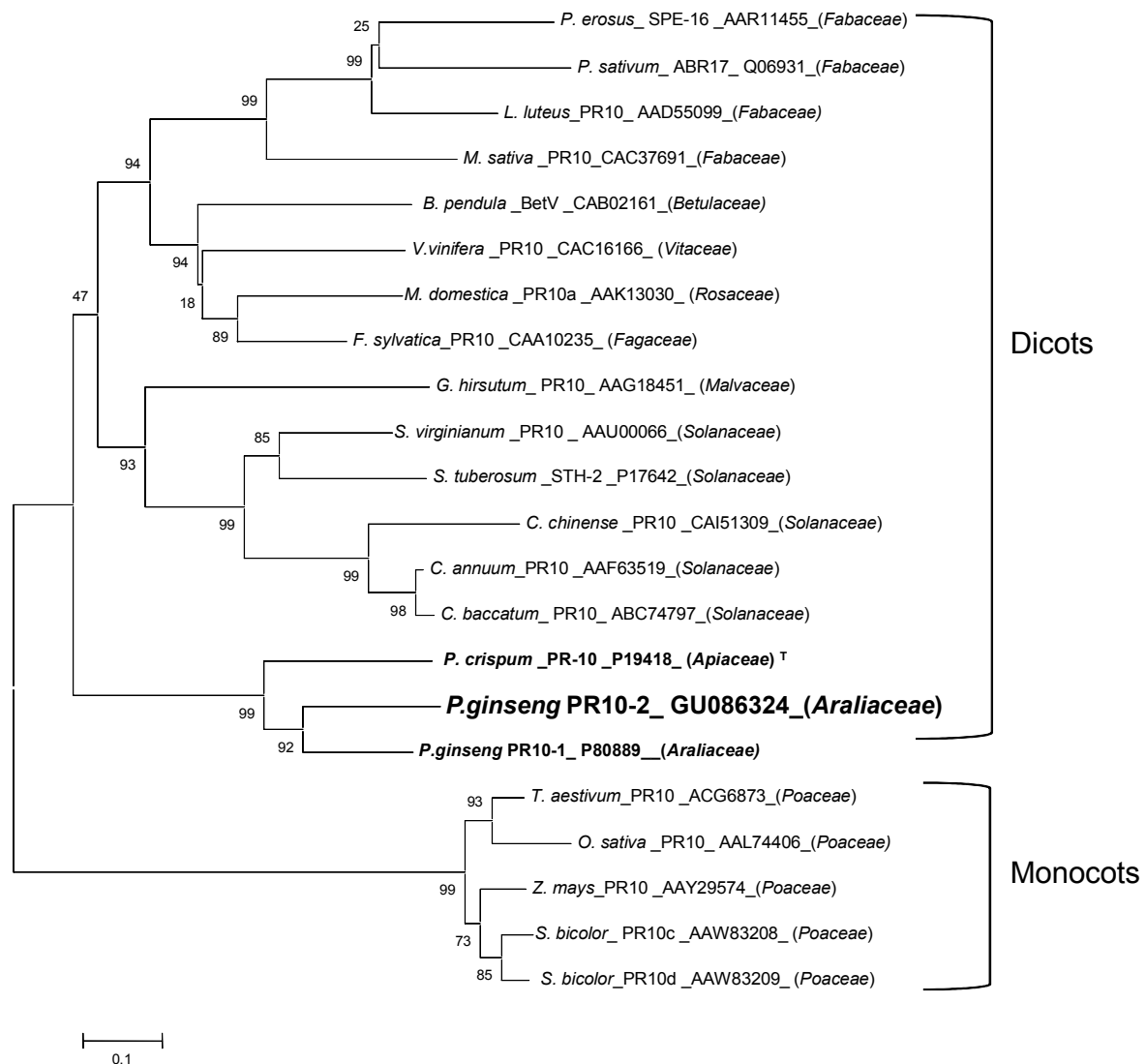
Technology Development Program of Agriculture and Forestry, Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pmpp.2010.05.001.

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Supplementary Fig. 1

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