

# Molecular characterization of a cysteine proteinase inhibitor, *PgCPI*, from *Panax ginseng* C. A. Meyer

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**Abstract** Phytocystatins are plant-derived cysteine proteinase inhibitors implicated in the endogenous regulation of protein turnover and defense mechanisms against insects and pathogens. A cysteine proteinase inhibitor, *PgCPI*, was isolated and characterized from *Panax ginseng*. Sequence analysis revealed that the coding cDNA sequence of *PgCPI* is 609 base pairs in length with a predicted molecular mass of 22.5 kDa. A GenBank BlastX search revealed that the deduced amino acid sequence of *PgCPI* shares a high-degree homology with cysteine proteinase inhibitors from other plants. In this present study, we analyzed the expression patterns of *PgCPI* against various abiotic and biotic stresses at different time points using quantitative real time-PCR. Enzymatic activity of *PgCPI* was also determined against cysteine proteinase. Our results reveal that *PgCPI* is moderately induced by NaCl, chilling, CuSO<sub>4</sub>, ABA, and jasmonic acid. However, high light, UV, MeJA, and wounding triggered a significant induction (more than fourfold) of *PgCPI* within 12-h post-treatment, especially *PgCPI* prominently accumulated by wounding (24-fold). In addition, increased transcripts of *PgCPI* were investigated in fungal- and nematode-infected roots. These

results suggest that *PgCPI* is involved in defense responses to biotic and abiotic stresses.

**Keywords** Cysteine proteinase · Cysteine proteinase inhibitor · *Panax ginseng* · Biotic stress · Abiotic stress · qRT-PCR

## Abbreviation

EST	Expressed sequence tag
CPI	Cysteine proteinase inhibitor
ORF	Open reading frame
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
ABA	Abscisic acid
MeJA	Methyl jasmonate
UV	Ultraviolet

## Introduction

The cystatins, cysteine proteinase inhibitors (CPIs), constitute a superfamily of proteins which function as reversible inhibitors of papain-like cysteine proteinases (Rawlings and Barrett 1990). The cystatin superfamily has been subdivided into three families based on their sequence homology, the presence and position of intra-chain disulfide bonds, and the molecular mass of the protein. Family-I cystatins (stefins) are about 100 amino acid (a.a) long with no disulfide bonds. Family-II cystatins (cystatin II) are about 150 a.a long with two disulfide bonds, and family-III cystatins (the kininogens) contain three segments homologous to the family-II cystatins (Turk and Bode 1991). In plant kingdom, large number of CPIs have been

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discovered and grouped into a fourth cystatin family, phytocystatins, based on sequence similarities and the absence of disulfide bonds (Abe et al. 1987). Although the primary sequences of phytocystatins are more similar to the family-II cystatins of animals, they are assigned to an independent family (Wang et al. 2008). According to the molecular weight, phytocystatins have been divided into three distinct groups. The first group of phytocystatins is found in rice. They are usually 12–16 kDa in size and show high homology with chicken egg white cystatin (Abe et al. 1987). The second group of phytocystatins is approximately or greater than 23 kDa, such as those found in cabbage, soybean, taro, sesame, and strawberry. They contain a highly conserved N-terminal region which is similar to that of the first group and are tailed by a repetitive peptide at the C-terminus in which variation is possibly caused by gene duplication (Christeller 2005). The third group of phytocystatins is found in potato and tomato of which molecular weight is about 80 kDa (Wang et al. 2008).

Cystatins inhibit cysteine proteinase via direct interaction with the active site. They have been involved in multiple functions such as the response to environmental stimuli, the mobilization of reserve substances, programmed cell death, the processing and degradation of proteins or the regulation of metabolic pathway (Solomon et al. 1999). In addition, functional roles of phytocystatin in the regulation of protein turnover during seed development (Pernas et al. 2000) and plant defense against insect predation and pathogens (Oppert et al. 2003) are also reported. Phytocystatins have shown variable expression patterns during plant development and in response to biotic and abiotic stresses. Expression of the proteinase inhibitor genes is usually limited to specific organs or to particular phases during plant growth; germination (Botella et al. 1996), early leaf senescence (Huang et al. 2001), drought (Waldron et al. 1993) or cold, and salt stresses (Pernas et al. 2000). A similar pattern of gene expression is evoked by wounding or methyl jasmonate (MeJA) (Botella et al. 1996). Several cystatins inhibit the activity of digestive proteases in vitro from coleopteran insects (Zhao et al. 1996).

To the best of our knowledge up to now, no research has been done on *CPI* gene from *Panax ginseng* C. A. Meyer, commonly known as ginseng. Ginseng is one of the most medicinally important perennial herb from the *Araliaceae* family. It has been cultivated for its highly valued medicinal purposes for more than 1,000 years in East Asia countries like China, Korea, and Japan (Vogler et al. 1999). Ginseng plants are more exposed to pathogen infection and other environmental stimuli since they grow in shaded-areas during long-term cultivation time, although ginseng itself also affects various environmental

conditions. Therefore, enhancing the physiological activity of ginseng plant against harsh environmental conditions and pathogen attack is essential by molecular breeding techniques. In a way to achieve this goal, one of cystatin homolog is characterized from ginseng. Full-coding cDNA sequence of *PgCPI* is obtained and its differential expression patterns by several abiotic/biotic stresses show its possible functions in defense mechanism. Crude extracts containing *PgCPI* from ginseng plants are further shown to have enzymatic activity by inhibiting papain, which is generally taken protein for the cysteine proteinase inhibitor function.

## Materials and methods

### Plant materials and growth conditions

Five-week-old ginseng seedlings and 1-year-old ginseng plants were used for the qRT-PCR analysis. Ginseng seeds were germinated in Murashige and Skoog (MS) media (Duchefa Biochemie, The Netherlands) and supplemented with gibberillic acid at room temperature. The 1-year-old plants were grown in pots at 25°C.

### RNA purification and construction of a cDNA library

The total RNA was isolated from the root of 14-year-old ginseng plant, grown in the field (Kumsan in Korea) by aqueous phenol extraction procedure. The poly (A)<sup>+</sup> RNA was isolated via oligo-dT-cellulose chromatography using a mRNA isolation kit (Stratagene, USA). Using a commercial cDNA synthesis kit (Clontech, USA), size-selected cDNA was ligated into  $\lambda$  TriplEx2 vector and was packaged in vitro using Gigapack III Gold Packaging Extract kits (Stratagene, USA). A GigapackIII Gold packaging extract (Stratagene, USA) were utilized in the construction of the cDNA library in accordance with the instruction manual provided by the manufacturer (Clontech, USA). The fractions containing cDNAs larger than 500 base pairs were recovered, and used as a cDNA library construction.

### Nucleotide sequencing and sequence analysis

The pTriplEx phagemids were excised from the  $\lambda$ pTriplEx2 and used as templates for sequence analysis. The 5' ends of cDNA inserts were sequenced by an automatic DNA sequencer (ABI prism 3700 DNA sequencer, Perkin-Elmer, USA). Sequences shorter than 100 base pairs were discarded. Homologous sequences of *CPI* EST are searched against the GenBank databases using a BLASTX algorithm. A pTriplEx phagemid for *CPI* cDNA was

excised from the  $\lambda$ pTriplEx2 and used as template for sequence analysis. The cDNA insert was sequenced using the 5' and 3' sequencing primers by an automatic DNA sequencer (ABI prism 3700, USA). Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi Software Engineering, Brisbane, CA, USA). These deduced amino acid sequences were searched for homologous proteins in the databases using BLAST network services at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

#### Southern blot analysis

Genomic DNA was extracted from the ginseng leaf tissue with a DNA mini kit (GENEAll Biotechnology Inc, South Korea). Approximately 10  $\mu$ g of the DNA was digested with *Eco*RI or *Hind*III, and the digested samples were subjected to electrophoresis on a 1.3% agarose gel. The separated DNA fragments were transferred to a nylon membrane using a Turbo blotter apparatus (Schleicher and Schuell, UK) in 10 $\times$  SSC. The DNA fragments were cross-linked to the membrane by UV-irradiation and then pre-hybridized with DIG Easy Hyb Solution (Roche Applied Science, Germany) containing 100  $\mu$ g of fragmented ginseng DNA for 1 h at 38°C. Hybridization with a DIG-labeled probe was carried out at 38°C overnight and washed with 2 $\times$  SSC, 0.1% SDS; 1 $\times$  SSC, 0.1% SDS and 0.5 $\times$  SSC, 0.1% SDS at 65°C for 20 min, respectively. The immunological detection of the DIG-labeled probe was performed with 1:10,000 anti DIG-AP. The blot was exposed to X-ray film (AGFA, Germany) with CSPD. The *PgCPI* was detected by using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Germany) following the manufacturer protocol.

#### Application of abiotic and biotic stresses

For chemical stresses or plant hormone treatments, the plantlets were placed for various time point in 1/2 MS medium containing indicated concentrations of chemicals; NaCl (100 mM), CuSO<sub>4</sub> (500  $\mu$ M), ABA (100 mM), jasmonic acid (0.2 mM), methyl jasmonate (45.4  $\mu$ M). Chilling was applied by exposing the plantlets at 4°C. To monitor the effect of light stress, plants were kept in the high light with intensity of about 500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. For the UV treatment, the plantlets were irradiated under UV lamps at 1.35  $\mu$ E m<sup>-2</sup> s<sup>-1</sup> (below 280 nm). For mechanical wounding stress, leaves and stems of seedlings were wounded with scalpel. In all cases, stress treatments were carried out on the 1/2 MS media, and ten plantlets were treated with each stress for 1-, 4-, 8-, 12-, 24-, and 48-h. For biotic stresses, *Botrytis cinerea* and nematode

(*Meloidogyne* sp.) infected 4-year-old root knots were obtained from Ginseng Genetic Resource Bank, Korea.

#### Real-time quantitative RT-PCR

RNA was extracted from 4-year-old ginseng plants (biotic stresses) and plantlets (for biotic stresses) using RNeasy kit (Qiagen, Valencia, CA, USA) according to the instruction given by the manufacturer. The concentration of RNA was measured using GE nano-value spectrophotometer. To obtain the first strand of cDNA, 5  $\mu$ g of total RNA was reverse-transcriptased using Power cDNA kit (Invitrogen) following the instructions given by the manufacturer. Real-time quantitative PCR was performed by Rotor-Gene 6000 real-time rotary analyzer using 100 ng of cDNA in a 10- $\mu$ l reaction volume using SYBR<sup>®</sup> Green Sensimix Plus Master Mix (Quantace, Watford, England) using the following primers; 5'-TCT CTC CCG CAT CAA TAA CC-3' (forward) and 5'-TCA GAA CAG TGC CGA GAT TG-3' (reverse). The housekeeping gene-encoding actin gene 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). PCR conditions for each 40 cycles are 95°C for 10 s, 60°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). 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 differences in template abundance for each sample, the Ct value for *PgCPI* was normalized to the Ct value for  $\beta$ -actin and calculated relative to a calibrator using the formula  $2^{-\Delta\Delta Ct}$ . Three independent experiments were performed.

#### Papain inhibitory activity assay

For papain inhibitory activity assay, 1-year-old ginseng plant roots were treated with MeJA (45.4  $\mu$ M) in MS media and wounding for 12-, 24-, and 48-h. The treated plant materials were immediately frozen in liquid nitrogen and stored at -70°C until required. Frozen samples were ground to a fine powder using liquid nitrogen. Sample extracts were made with a buffer containing 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl at 4°C. Extracts were heated at 80°C for 10 min and soluble proteins were recovered by centrifugation. Supernatants were transferred into tubes, and protein concentrations were determined using a Bradford protein assay kit (BioRad, USA) with bovine serum albumin as a standard. One hundred

micrograms of total protein in 0.2 mL of reaction solution (0.25 M sodium phosphate buffer, 2.5 mM EDTA, and 25 mM  $\beta$ -mercaptoethanol, pH 6.0) was incubated with 7.5  $\mu$ g of papain in 0.1 mL of solution (25 mM sodium phosphate buffer, pH 6.0) at 37°C for 5 min. The reaction was started by the addition of 0.2 mL of 1 mM *N*-benzoyl-L-arginine-2-naphthylamide (BANA), and stopped by the addition of 2% HCl/ethanol. The color was developed by the addition of 1 ml of 0.06% *p*-dimethylaminocinnamaldehyde/ethanol. Absorbance of the solution was measured at 540 nm. The velocity of the reaction was expressed as  $\Delta A_{540} \text{ h}^{-1} \text{ mL}^{-1}$  (Abe et al. 1994).

## Results

### Isolation and molecular analysis of *PgCPI*

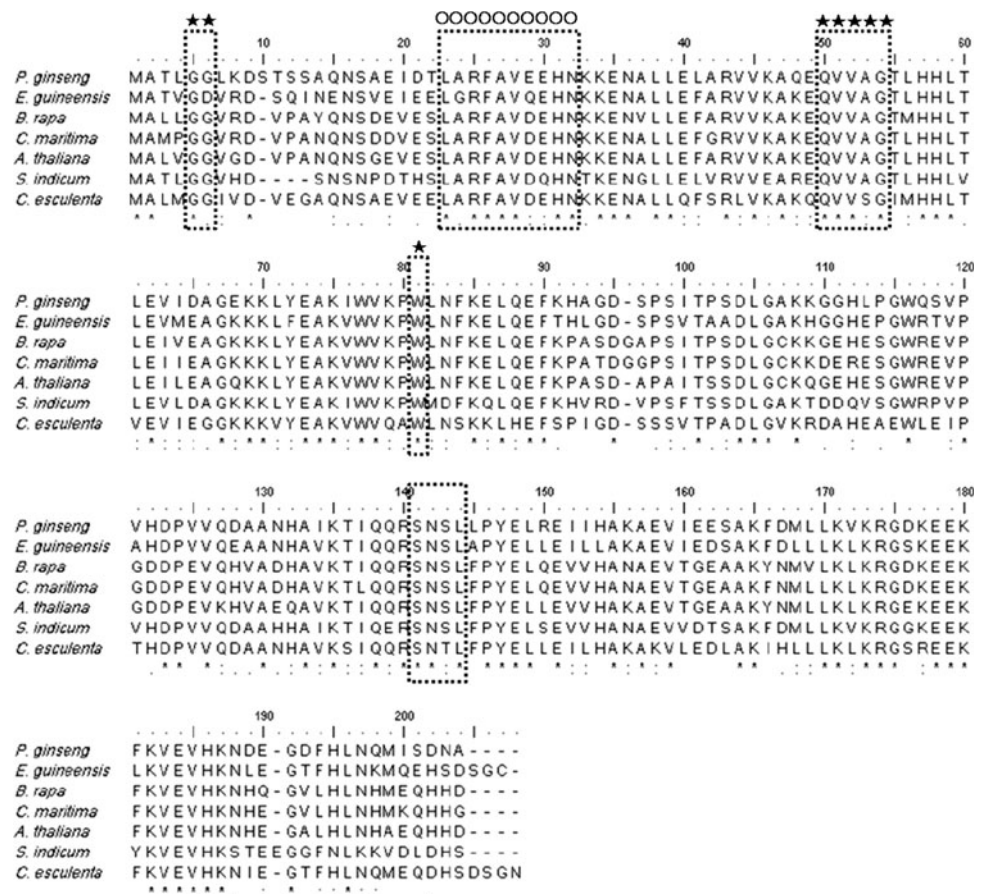
As part of a genomic project to identify genes in medicinal plant *P. ginseng*, the cDNA library consisting about 20,000 cDNAs were previously constructed (Kim et al. 2006). Using EST (expressed sequence tags) clones prepared from the 14-year-old root of *P. ginseng*, we identified a cDNA clone showing sequence similarity with previously reported

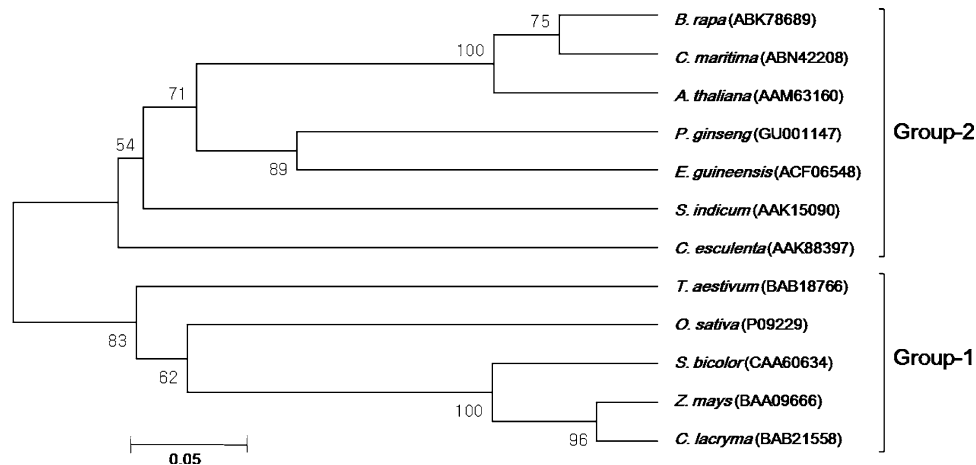
cystein proteinase inhibitor, designated *PgCPI*. The *PgCPI* (*P. ginseng* cystein proteinase inhibitor; Gene-Bank accession No. GU001147) encodes 609 base pairs (bp) in length with a 116-bp upstream sequence and a 196-bp downstream sequence. The predicted molecular weight of *PgCPI* is 22.5 kDa and isoelectric point is 5.93 using ProtParam (Wilkins et al. 2005).

Alignment of the phytocystatins indicates the highly conserved amino-acid residues (Fig. 1): (a) the active site motif QxVxG; (b) a G near N-terminus; and (c) the second loop near the C-terminal region that may contain a tryptophan residue (W). These three conserved regions have been demonstrated to directly interact with the active site cleft of cysteine proteinases belonging to the papain family (Turk et al. 1997). In addition, plant cystatins contain particular consensus motif [L-A-R-[FY]-A-[VI]-X(3)-N] sequence at the N-terminal end of an  $\alpha$ -helix segment (Margis et al. 1998). *PgCPI* also contains conserved motifs and catalytic active motifs, which supports its similar function with previously reported CPI (Fig. 1).

Phylogenetic tree of *PgCPI* with other homologous genes based on the a.a sequence is constructed (Fig. 2). It shows that *PgCPI* is more closely related to those in group 2. A GenBank BlastX search revealed that the

**Fig. 1** Multiple alignment of *PgCPI* with other homologous cystatins using *ClustalX* program. Cystatins shown are as follows with GenBank accession numbers in parenthesis: *Elaeis guineensis* (ACF06548), *Brassica rapa* (ABK78689), *Cakile maritima* (ABN42208), *Arabidopsis thaliana* (AAM63160), *Sesamum indicum* (AAK15090), *Colocasia esculenta* (AAM88397). Asterisk indicates identical residue, and strong and weak similarity is marked with colon and period, respectively. The strictly conserved amino acid residues, including glycines (G) near the N-terminus, a QxVxG in the central motif, and a tryptophan (W) near the C-terminus, are enclosed in dotted boxes and marked by stars. The boxed region with empty circles indicates the unique plant cystatins sequence (L-A-R-[FY]-A-[VI]-X(3)-N). Carboxy terminal extension with an amino acid motif (SNSL) is boxed by dotted-line





**Fig. 2** Phylogenetic tree of PgCPI with closely related cystatins. Cystatins in both groups are as follows: *Brassica rapa* (ABK78689), *Cakile maritima* (ABN42208), *Arabidopsis thaliana* (AAM63160), *Elaeis guineensis* (ACF06548), *Sesamum indicum* (AAK15090), *Colocasia esculenta* (AAM88397), *Triticum aestivum* (BAB18766),

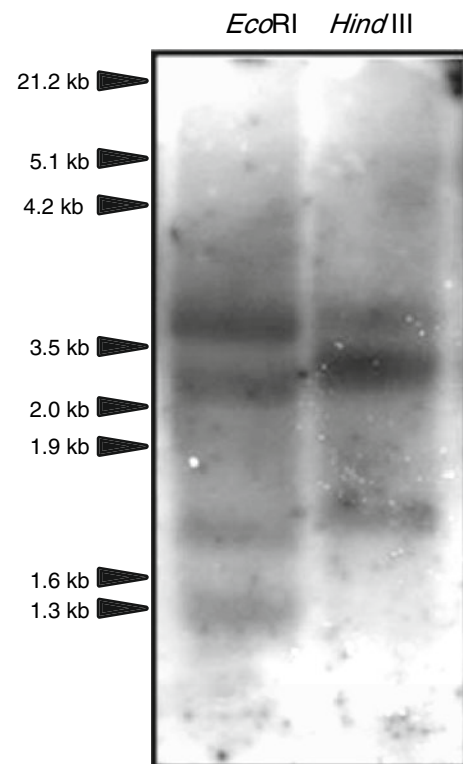
*Oriza sativa* (P09229), *Sorghum bicolor* (CAA60634), *Zea mays* (BAA09666), and *Coix lacryma* (BAB21558). The tree was constructed using the Clustal X method (Neighbor-joining method). The bar represents 0.05 substitutions per amino acid position

deduced amino acid sequence of PgCPI shares a high-degree homology with cystein proteinase inhibitor of *Elaeis guineensis* (ACF06548; 77% identity, 89% similarity), *Brassica rapa* (ABK78689; 71% identity, 85% similarity), *Arabidopsis thaliana* (AAM63160; 71% identity, 85% similarity) *Cakile maritima* (ABN42208; 71% identity, 83% similarity), *Sesamum indicum* (AAK15090; 68% identity, 83% similarity), and *Colocasia esculenta* (AAM88397; 64% identity, 84% similarity). In contrast, PgCPI shares low identity with the CPI proteins from animals (not included in this phylogenetic tree).

Southern hybridization was performed to determine the genomic organization of the PgCPI gene in *P. ginseng*. Genomic DNA was completely digested with *EcoRI* and *HindIII* and labeled with DIG-labeled PgCPI probe. The hybridization result shows four bands by *EcoRI* restriction digested lanes and three bands by *HindIII* restriction digested lanes (Fig. 3), respectively. This observation is in agreement with the DNA-DNA hybridization results from a chinese cabbage (Lim et al. 1996) and a potato (Gruden et al. 1997), where cystatin is encoded as multi-gene family. In contrast, a single copy of CPI gene was detected in *Amaranthus hypochondriacus* (Valdés-Rodríguez et al. 2007) and chestnut (Pernas et al. 2000). Member of multi-copy family genes implies that PgCPI may have functional redundancy.

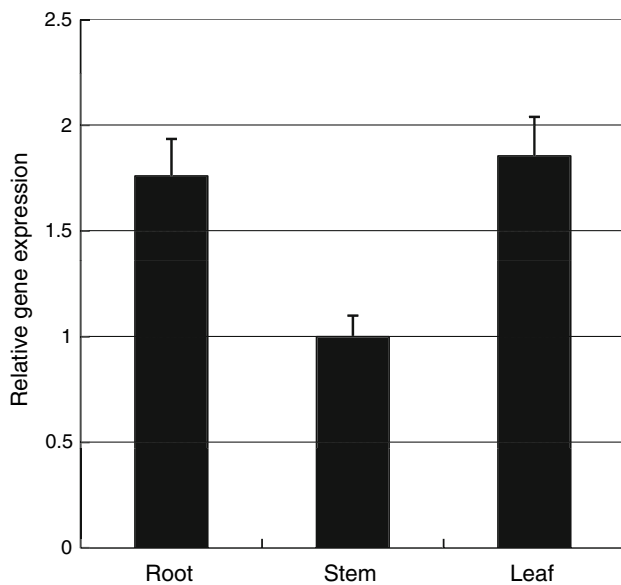
#### Temporal expression patterns of PgCPI

The expression patterns of PgCPI in different *P. ginseng* organs were examined in 5-week-old seedlings using quantitative RT-PCR (qRT-PCR) analysis. It is clear that PgCPI is constitutively expressed in root, leaf, and shoot



**Fig. 3** Southern blot analysis of PgCPI from *P. ginseng*. Equal amounts (10  $\mu$ g) of genomic DNA were digested with the restriction enzymes (*EcoRI* or *HindIII*), separated by electrophoresis, and transferred onto a Zeta-probe GT membrane. The blot was hybridized with the DIG-labeled PgCPI-specific DNA fragment. The size is marked in the left side

(Fig. 4). Among tested organs, relatively higher levels of PgCPI transcripts (average: 2.04-fold) were observed in leaf. In *Arabidopsis*, one of cystatins, *AtCYSa* was also



**Fig. 4** Relative expression of *PgCPI* in different organs of *Panax ginseng*. Total RNAs from root, stem, and leaf were extracted and used for cDNA synthesis. Same amount of cDNA from different organs was used for the qRT-PCR. All PCR reactions were performed in triplicate. Data represent means  $\pm$  SE for three independent replicates

stronger in leaf than in root (Zhang et al. 2008). In chestnut, high level of cystatin transcripts was evident in leaf compared to that in root and stem (Pernas et al. 2000).

#### Differential expressions of *PgCPI* against abiotic stresses

The expression patterns of *PgCPI* under various stresses, such as stress-related chemicals; NaCl (100 mM), CuSO<sub>4</sub> (500  $\mu$ M), ABA (100 mM), jasmonic acid (0.2 mM), and MeJA (45.4  $\mu$ M), and chilling (4°C), high light (500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>), UV (1.35  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>), and wounding stress were investigated by qRT-PCR using plantlets. Under salt stress, the transcripts of *PgCPI* were gradually increased up to 4.36-fold at 24-h post treatment and then relatively decreased after 48-h treatment (2.65-fold) compared with that at 24-h time point (Fig. 5a). Four- and eight-hour chilling stress increased the mRNA expression level to approximately double, and its level gradually down-regulated up to the half of the control after 48-h treatment (Fig. 5b). Under the copper-treated condition, the transcription level of *PgCPI* gene was the highest at 4-h post-treatment (Fig. 5c). High light exposure showed zig-zag pattern; however, the *PgCPI* expression reached maximum 5.4-fold at 12-h post-treatment and then declined to the control level at 48-h exposure time point (Fig. 5d). UV irradiation mostly increased the expression level of

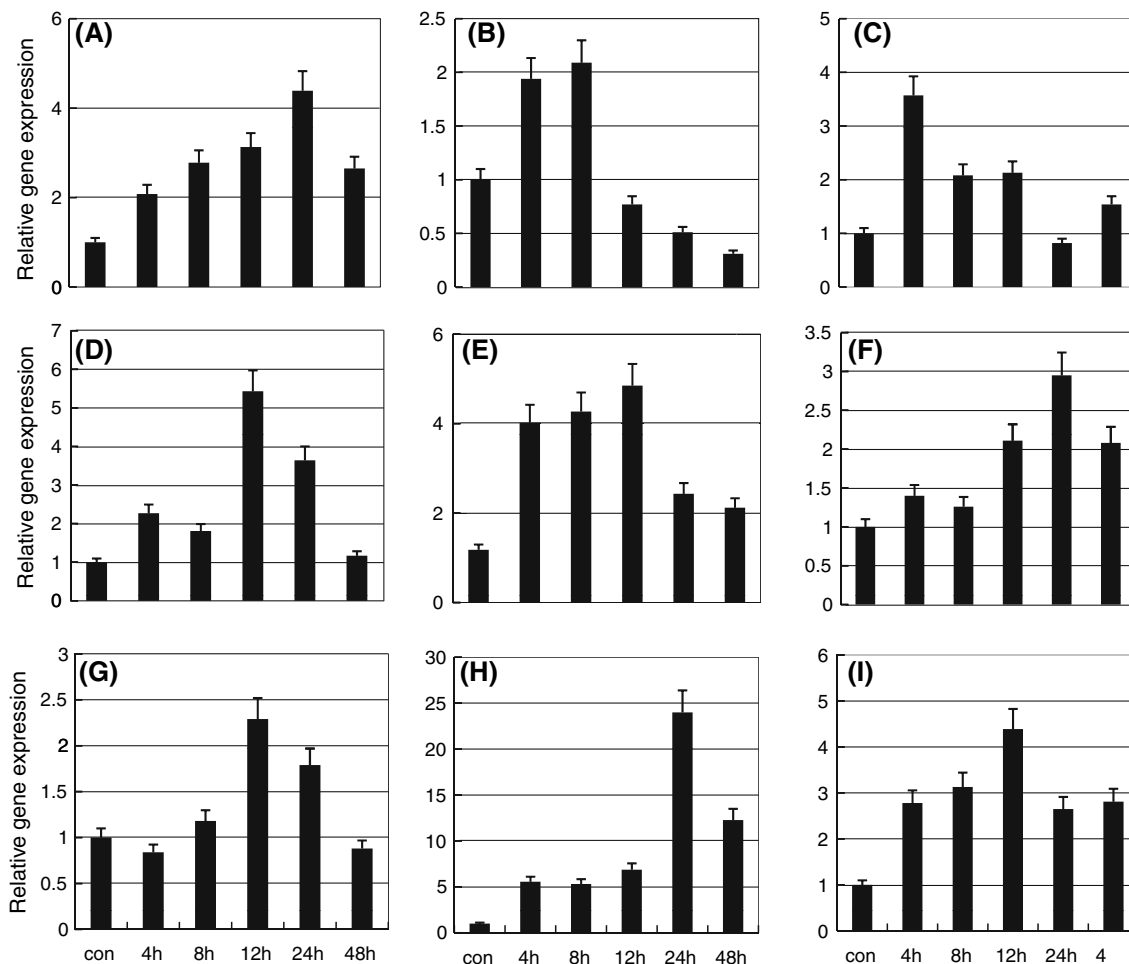
*PgCPI* from 4-h to 12-h irradiation with 4-h interval up to fourfold, following rather decreased but still higher than control level (Fig. 5e). ABA treatment resulted in up-regulation of *PgCPI* expression in all treated conditions with highest at 24-h point (2.95-fold) (Fig. 5f). ABA treatment followed similar expression patterns just like salt treatment. Jasmonic-acid treatment resulted in up-regulation of *PgCPI* expression at 8-h (1.18-fold) and 12-h (2.29-fold) time point, and the initial 4-h and latest 24-h time point gave similar expression levels to the control level (Fig. 5g). Wounding resulted in overall the highest up-regulation of *PgCPI* expression compared with the other tested stress conditions. Its expression reached about 5.5-fold at 4-, 8-, 12-h treatment and reached the highest point at 24-h (24-fold), and slightly decreased at 48-h (12.27-fold) treatment (Fig. 5h). MeJA also resulted in up-regulation of *PgCPI* expression in all tested conditions; at 4-h (2.73-fold), 12-h (4.39-fold), and 48-h (2.81-fold) (Fig. 5i). Overall, the transcripts level of *PgCPI* showed highest at 12-h time point if it responded relatively slow to the selected abiotic stresses. Relative quick response was also observed even after 4-h time point in case of chilling, CuSO<sub>4</sub>, UV, wounding, and MeJA treatments.

#### Differential expressions of *PgCPI* under biotic stresses

To have insight into how *PgCPI* responds to the biotic stresses, fungus-infected ginseng plants and nematode-infected ginseng knots were analyzed. The expression of *PgCPI* drastically increased by *Botrytis cinerea* fungus infection compared with that of uninfected control ginseng (Fig. 6a). It shows the relative expressions of *PgCPI* transcripts in infected bud (B), rhizome (Rh), lateral root (LR), and sub-root (SR) are increased to 18.4-, 70.2-, 73.3- and 60.13-fold, respectively, compared with the control. Nematode-infected root knots were also investigated for the differential expression of *PgCPI*. All nematode-infected different parts of root knot samples displayed more *PgCPI* transcripts than the control (Fig. 6b).

#### Papain inhibitory activity by *PgCPI*

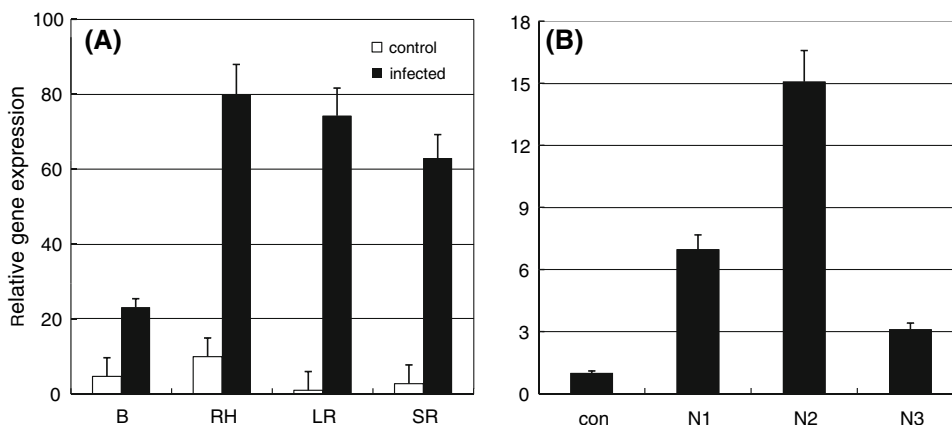
Papain is a cysteine protease enzyme present in papaya (*Carica papaya*) and mountain papaya (*Vasconcellea cundinamarcensis*), and is a generally used protein to prove for the cysteine proteinase inhibitor function. We determined the papain inhibitory activity using crude protein extracts from *P. ginseng* plant using *N*-benzoyl-L-arginine-2-naphthylamide (BANA) as a substrate (Abe et al. 1994). Crude protein extract itself showed inhibitory activity (Fig 7). As wounding and MeJA treatments were shown to



**Fig. 5** Relative expression patterns of *PgCPI* against several abiotic stresses at various time points (h). **a** NaCl (100 mM), **b** chilling (4°C), **c** CuSO<sub>4</sub> (500 μM), **d** high light (500 μmol m<sup>-2</sup> s<sup>-1</sup>), **e** UV (1.35 μE m<sup>-2</sup> s<sup>-1</sup>), **f** ABA (100 mM), **g** Jasmonic acid (0.2 mM),

**h** wounding, **i** MeJA (45.5 μM). Plantlets were grown in MS media containing appropriate chemicals or under stresses for the given time points. Data represent means ± SE for three independent replicates

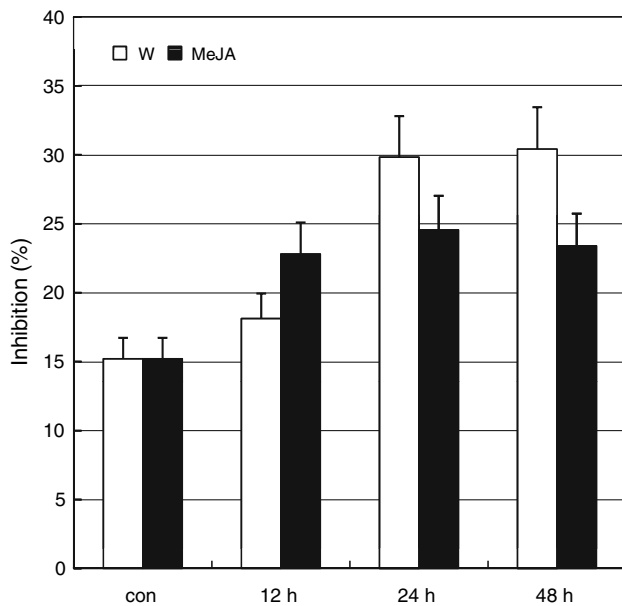
**Fig. 6** Relative expression of *PgCPI* under biotic stresses. **a** *PgCPI* expression in different parts of fungus (*Botrytis cinerea*) infected roots compared with the uninfected control (*B* bud, *Rh* rhizome, *SR* sub root, and *LR* lateral roots). **b** The level of *PgCPI* transcripts in nematode (*Meloidogyne* sp.) infected root knots (*con* not-infected control); *N* nematode-infected root samples (N1–N3). Data represent means ± SE for three independent replicates



increase the highest expression of *PgCPI* (Fig. 5h, i), increased papain inhibitory activity was also resulted as expected (Fig. 7). Papain inhibitory activity indicates that *PgCPI* is functioning as one of cystatins.

**Discussion**

Phytocystatins have been characterized in several monocots and dicots including rice, maize, soybean, chestnut,



**Fig. 7** Papain-inhibitory activity by PgCPI. Papain-inhibitory activities of ginseng extract from untreated control (*con*), wounded (*W*) and MeJA-treated leaves for 12-, 24-, and 48-h. Relative inhibitory activities are expressed as percentages of inhibition of papain-proteolytic activity. Data represent means  $\pm$  SE for four independent replicates

potato, and tomato because of their regulatory and protective functions (Megdiche et al. 2009). However, the functional characterization of cystatins in medicinal plants has never been studied in detail. In this study, we report an ORF of *PgCPI* with deduced full a.a sequence from ginseng, *P. ginseng* C. A. Meyer, which shares high sequence similarity with conventional cystatins. Most of phytocystatins are small proteins with a molecular mass ranging from 12 to 16 kDa. However, the so-called multicystatins from potato and tomato show the molecular mass is  $\sim$ 80 kDa, whereas cystatins from soybean, cabbage, sesame, and strawberry are in the range of  $\sim$ 23 kDa (Wang et al. 2008). The predicted molecular weight of *PgCPI* is 22.5 kDa, which suggests its similar functional roles found in the latter group cystatins. Analysis of the deduced *PgCPI* amino acid sequence revealed the presence of cystatin-like domains. It includes the cystatin-specific motifs GG (residues 5–6), QVVAG (50–54) and PW (80–81), and the plant cystatin-specific sequence (LARFAVDEHN) at the N-terminal region (Fig. 1). Moreover, as is the case for most phytocystatins, the *PgCPI* protein contains an asparagine at position 36, which is required by some animal cystatins to inhibit animal and plant legumain. Conserved carboxy terminal extension with an amino acid motif (SNSL) also suggests its similar inhibitory functional roles (Martinez et al. 2007).

Phytocystatins are implicated in plant response to adverse environmental stresses. When ginseng plantlets

were exposed to abiotic stresses, the *PgCPI* was differentially expressed with respect to different periods of exposure times. In *Arabidopsis thaliana*, wide range of stresses including treatment with NaCl and cold was associated with accumulation of *CPI* transcripts (Valdés-Rodríguez et al. 2007). Chilling stress was reported as a cause for cystatin increase in avocado (Dopico et al. 1993). Similarly, our result also shows that *PgCPI* is induced by NaCl and chilling (Fig. 5a, b) although longer treatment of chilling resulted in down-regulation of its expression. By  $\text{CuSO}_4$  treatment, transcripts of *PgCPI* increased until 12-h time point and slightly decreased compared to the untreated control (Fig. 5c). Similarly, the mRNA level of proteinase inhibitor II from *Nicotiana glutinosa* L. peaked at 6-h and then subsequently decreased (Choi et al. 2000). By high light and UV exposure, *PgCPI* transcripts were increased until 12 h (Fig. 5d, e). In barley, response of the *Icy* gene to light stresses was also investigated (Gaddour et al. 2001). High light and UV irradiation can generate oxidative stress through ROS (reactive oxygen species) in plants (Green and Fluhr 1995), which lead production of oxidative damage. Plant cysteine proteinases play an instrumental role in PCD (programmed cell death) triggered by oxidative stresses. PCD constitutes the main form of cell death in animals, plants, and other organisms. With ABA treatment, *PgCPI* transcript was increased until 24-h (2.95-fold) time point (Fig. 5f). It has been demonstrated that mRNA and protein of multicystatin from cowpea are accumulated in response to drought stress and also accumulated slightly in leaves by ABA treatment (Diop et al. 2004). Important role of ABA is the control of stomata in response to water stress (Mansfield and McAinsh 1995). When leaves are exposed to water stress, there is a rapid ABA synthesis and it moves to the guard cells. This movement resulted in losing of  $\text{K}^+$  from the guard cells within minutes, lowering turgor pressure and causing the stomata to close. Up-regulation of *PgCPI* against jasmonic acid (Fig. 5g) is also observed in chestnut cystatin (Pernas et al. 2000). Wounding caused the most dramatic up-regulation of *PgCPI* continuously, and MeJA treatment also increased its expression in all time points (Fig. 5h, i). Induced level of transcripts by wounding and MeJA corresponds to the results from cystatins of soybean and potato (Botella et al. 1996; Hildmann et al. 1992). Tomato cystatin also responds to MeJA (Zhao et al. 1996). MeJA is considered as an important signal between pathogen-infected and uninfected plants by promoting pathogen-resistance in uninfected plant (Megdiche et al. 2009). It is noteworthy that the extensively changed defense protein synthesis by wounding leads to localized resistance at the site of the lesion via the production of phytoalexin, enhanced lignification and suberization of the cell wall, and systemic induction of protease inhibitor (Ebel and Mithofer 1998).



Antifungal activity of phytocystatins from taro, strawberry, and chestnut has been reported previously (Yang and Yeh 2005). In addition, several cystatins are reported to enhance resistance against nematodes (Urwin et al. 1997). Both biotic stresses also increased *PgCPI* gene expression (Fig. 6), although it is not clear where the interaction between the cystatin and its putative targets takes place. A possible link between fungal toxicity and proteinase inhibition relies on the fact that a proteinase activity is required for the processing of the membrane-bound chitin synthase precursor (Machida and Saito 1993). Thus, the observed effects of a ginseng cystatin on fungal growth could be related to its indirect inhibition of fungal cell wall development. Measuring catalytic activity of papain, a molecular model protein of cysteine proteinase, is adapted for the functional test of cysteine proteinase inhibitor (Abe et al. 1994). Inhibition of papain activity by addition of crude protein extracts from ginseng was observed, and the inhibition rate increased when the ginseng plants were stressed in accordance with increased transcripts of *PgCPI* (Fig. 7). Taken together, these data support the hypothesis that the *PgCPI* from ginseng may also work as a genuine cystatin such as other previously reported cystatins in crop plants (Zhao et al. 1996).

Plant tolerance to abiotic stresses is a complex trait that involves multiple physiological and biochemical mechanisms, and function of numerous genes. Many genes respond to salt, drought, oxidative, and cold stresses, and the proteins encoded by these genes are thought to function in protecting cells from these stresses. A recent study, focusing on the physiological functions of plant proteinase inhibitors, revealed that plant proteinase inhibitors might actively participate in regulation of proteolytic process by inhibiting the activity of endogenous proteinases (Solomon et al. 1999). In addition to regulating proteolytic processes, plant proteinase inhibitors may act as storage proteins and serve in plant defense (Mosolov and Valueva 2005). The transcriptional accumulation of *PgCPI*, elicited by biotic and abiotic stresses, suggests that *PgCPI* is probably involved in a general response mechanism of ginseng plants which can be shared or at least in part by both stress types. Such a general responsive system probably mediated by ABA or jasmonic acid has been recently proposed for herbaceous plants (Moons et al. 1997). Genetically engineering ginseng plant by overexpressing *PgCPI* gene and further biochemical analysis using natively purified *PgCPI* will shed more light for its molecular function. It is ultimately necessary for the improvement of the tolerance against all stressed conditions during long-term cultivation time of perennial herb ginseng.

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