

Molecular cloning, expression, purification and functional characterization of an antifungal cyclophilin protein from *Panax ginseng*

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Abstract. Cyclophilins (CyPs), a member of peptidyl-prolyl *cis-trans* isomerases (PPIases), are ubiquitously distributed in organisms such as bacteria, yeast, plants and animals. CyPs have diverse biological functions, with some exhibiting anti-fungal and antiviral activities. In this study, *Panax ginseng* cyclophilin (*pgCyP*), a novel gene encoding an antifungal protein from *Panax ginseng*, was cloned, and its protein product was expressed in *Escherichia coli*, and then fractionated by affinity chromatography. The open reading frame of the *pgCyP* full-length coding sequence was found to encode a single-domain CyP-like protein of 174 amino residues with a calculated molecular weight of 18.7 kDa. The pGEX system was used to express *pgCyP* fused to glutathione S-transferase. After affinity purification, the protein showed a strong fungal resistance effect on *Phytophthora cactorum*. In addition, *pgCyP* showed high PPIase activity. To the best of our knowledge, the present study is the first successful effort to clone and characterize a CyP-like protein gene from *Panax ginseng*.

Introduction

Pathogenic fungi cause a wide range of damage in organisms, including in plants, humans and other animals. To protect

themselves against fungal pathogens, living organisms produce a myriad molecules. Different classes of antifungal proteins isolated from various plants include chitinases, cyclophilins (CyPs), defensins, lectins and lipid transfer proteins (1-7), all of which kill or suppress the infection of pathogenic microorganisms. In addition, the introduction of genes encoding these proteins into crop species has been found to confer enhanced resistance on the resulting transgenic lines (8).

CyPs, also known as immunophilins, peptidyl-prolyl *cis-trans* isomerases (PPIases) and cyclosporine A-binding proteins, are expressed in a variety of organisms (plants, yeast, fruit flies, parasites, rats and humans) (9) and exhibit high homology to one another. In plants, CyPs were first reported in 1990 with the isolation of CyP cDNA sequences from tomato, maize and oilseed rape (10). CyPs have endogenous PPIase activity that isomerizes the *cis-trans* conformation of imide linkage in substrates (11). Multiple CyP members in plants such as rice and *Arabidopsis* are associated with diverse functions and regulatory pathways related to their foldase, chaperoning, scaffolding and other (unknown) activities (12-15). Antifungal and antiviral activities of CyPs can relieve the multiple stresses exerted by fungi and viruses (16). CyP-like antifungal proteins have been isolated from black-eyed pea, mung bean, Chinese cabbage and chickpea (4,17,18). The CyP of Chinese cabbage has been shown to have pronounced effects on a variety of fungal pathogens (17).

Plant CyPs have two isoforms that differ according to the number of domains. The first isoform possesses only a single PPIase domain, whereas the second type is composed of a catalytic PPIase domain plus either a leucine zipper domain at the amino end, a tetratricopeptide repeat domain at carboxyl end or another domain related to sub-cellular localization (19). Members of the subfamily comprising divergent CyPs have another loop containing the consensus sequence XXGKXLH, a conserved Glu and two invariable Cys residues (20). It was reported that the divergent loop can mediate protein-to-protein interactions or may be part of a P-loop or ATP-binding site formed by residues 42-GEKICIGKS-49 and 163-VVIAD-167 (21).

Panax ginseng is a Chinese traditional herb that is believed to have medicinal restorative properties (22). During growth,

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Abbreviations: CyP, cyclophilin; PPIase, peptidyl-prolyl *cis-trans* isomerase; GST, glutathione S-transferase; PDA, potato dextrose agar; RT-PCR, reverse transcription-polymerase chain reaction; IPTG, isopropyl β -D-1-thiogalactopyranoside

Key words: antifungal activity, cyclophilin, expression and purification, *Panax ginseng*, peptidyl-prolyl *cis-trans* isomerase

ginseng is exposed to various soil-borne pathogenic microorganisms, including fungi, bacteria and nematodes. However, the manner in which ginseng resists fungi, especially through its protein contents remains to be investigated. From ginseng transcriptome databases previously established (23), a *Panax ginseng* cyclophilin (pgCyP) of interest was identified since it was highly induced during the period in which plant is highly blight-prone. This observation suggested that pgCyP is involved in the anti-microorganism process.

On the basis of that finding, the *pgCyP* gene was cloned in the present study and expressed in a bacterial host. We tagged pgCyP with 6xHis to its end to facilitate chromatography. We also enhanced its expression amount by applying the pGEX vector with glutathione S-transferase (GST). The recombinant pgCyP exhibited strong antifungal activity against *Phytophthora cactorum* and also possessed PPIase activity.

Materials and methods

Biological material. Five-year-old plants of ginseng (*Panax ginseng* C.A. Meyer) were harvested from Fusong County (Jinlin, China). The freshly collected material was prepared for gene cloning.

Phytopathogenic fungal species used in this study were *Rhizoctonia solani* and *Cylindrocarpon destructans* (Hyphomycetes); *Phytophthora cactorum* (Oomycetes); *Fusarium solani*, *Alternaria panax* and *Botrytis cinerea* (Fungi imperfecti); and *Sclerotinia sp.* (Discomycetes). All the fungal species were obtained from Jilin Agricultural University (Changchun, China).

Plasmid constructs. Total RNA was separated from ginseng leaves. The *pgCyP* gene was obtained by carrying out polymerase chain reaction (PCR) amplification from leaf cDNA with synthetic nucleotide primers. The PCR product was inserted into pMD18-T (Takara, Dalian, China). After digestion with *Bam*HI and *Not*I, the generated DNA fragment was cloned in a pGEX-6pl vector.

Expression of recombinant pgCyP. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) to express *pgCyP* His₆. Transformed cells were cultured in Luria-Bertani medium supplemented with 50 µg/ml ampicillin at 37°C on a rotary shaker at 200 rpm. When the OD₆₀₀ value of the cell culture reached 0.6–0.8, protein overexpression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.5 mM, and the culture was grown for a further 5 h. Cells were harvested by centrifugation at 10,000 x g for 2 min. Protein expression levels were analyzed by SDS-PAGE and visualized with Coomassie Blue staining (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Isolation and purification of recombinant pgCyP. The transformed cell pellets were resuspended in wash buffer [20 mM Tris (pH 8.0), 100 mM NaCl and 3 M urea]. After centrifugation, the cells were lysed in lysis buffer [50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl (pH 8.0), 0.13 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml lysozyme and 1.33 mg/ml sodium deoxycholate] and sonicated at 4°C for 30 min. DNase I was

then added to a concentration of 2,000 U/ml and the solution was incubated at 37°C with shaking at 200 rpm for 1 h. The lysate was then centrifuged at 10,000 x g for 20 min at 4°C. The resulting inclusion bodies were washed twice with a solution consisting of 50 mM Tris, 100 mM NaCl, 2 M urea, 0.5% Triton-X and 10 mM EDTA at pH 8.0. The washed inclusion bodies were dissolved by stirring for 1 h in extraction buffer [1.5% sarkosyl, 25 mM triethanolamine amine and 1 mM EDTA (pH 8.0)] at 4°C. The solubilized inclusion bodies were centrifuged at 10,000 x g for 10 min at 4°C and then refolded by dialysis in binding buffer [10 mM NaH₂PO₄·2H₂O, 10 mM Na₂HPO₄·12H₂O (pH 7.8), 150 mM NaCl and 10 mM imidazole]. The refolded protein was purified by Ni-chelating Sepharose Fast Flow chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The adsorbed proteins were eluted with elution buffer containing 100 mM imidazole. Eluates were pooled together and the samples were renatured on a Sephadex G-25 column. Protein concentrations were determined by the Bradford Protein Assay kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China).

PPIase activity assay. A mixture of the following components was incubated on ice for 10 min: 930 µl Assay Buffer [50 mM HEPES and 100 mM NaCl (pH 7.8)], 30 µl of 200 µM α-chymotrypsin (Sigma-Aldrich) and either 10 µl of GST-pgCyP-His₆ (1 µM) or the GST (1 µM) negative control. Each sample was placed in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), which was pre-cooled to 8°C. After the addition of 30 µl of 7.8 mM Suc-Ala-Ala-Pro-Phe-NA (Sigma-Aldrich), absorbance at 390 nm was immediately recorded every second for 5 min at 8°C.

pgCyP antifungal activity. GST-pgCyP-His₆ and GST protein were tested for possession of antifungal activity. Fungi were grown in potato dextrose agar (PDA) for 48 h at 28°C. The fungi were then spread onto PDA plates. Sterilized blank paper disks were placed on the plates and dotted with an aliquot of protein at different concentrations. The plates were incubated and monitored for up to 3 days. To determine the IC₅₀ of proteins against various fungal pathogens, the fungal spores were collected and placed in 96-well microtiter plates. Recombinant pgCyP (20 µl) or the negative control was then added to each well. After 12–36 h of incubation at 28°C, fungal growth was evaluated microscopically. The turbidity of each well was also measured by recording absorbance at 595 nm using a microtiter reader (Emax, Molecular Devices, Sunnyvale, CA, USA).

Sequence accession number. The nucleotide sequence of pgCyP in the present study has an accession no. KX034081 in GenBank.

Results

Cloning and sequence analysis of pgCyP in ginseng. As shown in Fig. 1A (lane 3), pgCyP cDNA was successfully generated by reverse transcription-polymerase chain reaction (RT-PCR) amplification from cDNA of ginseng leaves. The corresponding nucleotide sequence, consisting of 525 bp, was predicted to be 174 a.a. in length, as well as a theoretical isoelectric point

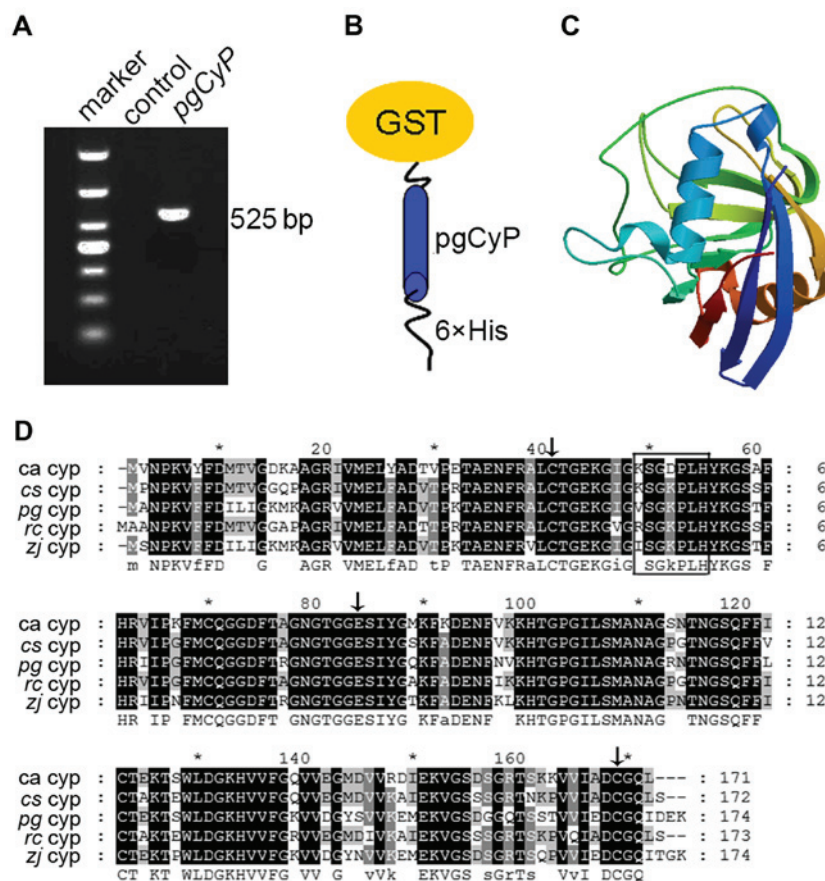


Figure 1. Detection of the *pgCyP* gene transcript and sequence analysis. (A) PCR amplification of *pgCyP* by RT-PCR from total mRNA of ginseng leaves. Marker refers to the DNA molecular marker, while control is the mock-DNA negative control. (B) Schematic representation of constructs of GST-*pgCyP*-His₆. (C) Predicted 3D structure model of *pgCyP*. The 3D structure model was predicted using the SWISS-MODEL server. (D) Alignment of the amino acid sequences of CyPs of *Panax ginseng* and other species. Residues comprising the divergent loop are shown in a black box, whereas the conserved Cys residues (Cys-40 and Cys-168) and Glu (Glu-83) are indicated with black arrows. *pgCyP*, *Panax ginseng* cyclophilin; PCR, polymerase chain reaction; GST, glutathione S-transferase; RT-PCR, reverse transcription-polymerase chain reaction; CyP, cyclophilin; ca, Chinese cabbage; cs, *Citrus sinensis*; zj, *Ziziphus jujuba*; rc, *Ricinus communis*.

of 7.67 and a molecular weight of 18.7 kDa. As it lacked a transit peptide, this predicted protein was probably localized in the cytosol (24). The *pgCyP* protein contained a PPIase domain and a divergent loop (48-VSGKPLH-54). Two CyPs (positioned at 40 and 168) as well as a Glu (positioned at 83) were conservatively kept similar to the other members in the CyPs family. The predicted secondary structure of *pgCyP* comprised 6.32% α helices, 21.26% extended strands and 72.42% random coils. On the basis of protein sequence alignment, *pgCyP* was closely associated with *Ziziphus jujuba* CyP, *Citrus sinensis* CyP19-3 and *Ricinus communis* CyP proteins. *pgCyP* shares 90% identity with its homolog in *Ziziphus jujuba* (Fig. 1D).

In the phylogenetic tree of CyP-like proteins shown in Fig. 2, *pgCyP* may be classified, as expected, into the clade of CyP proteins from dicot species, where it is most closely related to *Ziziphus jujuba* belonging to the same family. A 3D *pgCyP* model was created through primary protein sequence (25) using the SWISS-MODEL server (Fig. 1C).

Construction, expression and purification of GST-*pgCyP*-His₆. cDNA of the *pgCyP* isolated from this study, which differs from the *pgCyP* previously reported (23), and possibly belongs to a different ginseng CyP subfamily, was cloned into vector pMD18-T. The resulting plasmid was then digested with

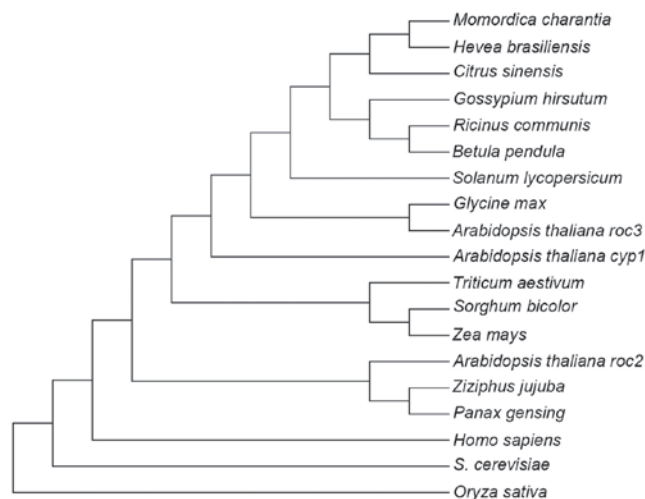


Figure 2. Phylogenetic tree of cyclophilin proteins from plants, humans and yeast. The software program MEGA5 was used for alignment of amino acid sequences and to generate a phylogenetic tree based on the neighbor-joining method.

*Bam*HI and *Not*I. After target fragments were cloned into pGEX-6p1, the protein were carrying a tandem His tag and GST at both ends (Fig. 1B).

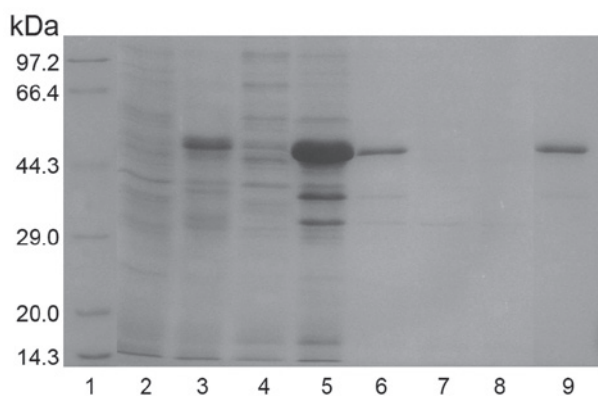


Figure 3. Expression, purification and refolding of GST-pgCyP-His₆. Samples collected after purification of GST-pgCyP-His₆ were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Lane 1, marker; lane 2, sample before IPTG induction; lane 3, sample subjected to IPTG induction; lane 4, soluble-protein fraction; lane 5, insoluble-protein fraction; lane 6, solubilized denatured insoluble-protein fraction; lane 7, purification flow-through fraction; lane 8, purification wash fraction; lane 9, purification elution fraction with 100 mM imidazole. GST, glutathione S-transferase; pgCyP, *Panax ginseng* cyclophilin; IPTG, isopropyl β-D-1-thiogalactopyranoside.

Escherichia coli BL21 (DE3) competent cells were transformed with the pGEX-6p1/pgCyP-His₆ plasmid. Overexpression of recombinant protein was induced at different IPTG concentrations for different lengths of time. The optimal IPTG concentration and induction time was 0.5 mM and 5 h, respectively. The induced protein was resolved in SDS-PAGE. As expected, a protein band of 46 kDa was evident (Fig. 3). Moreover, the analysis indicated GST-pgCyP-His₆ present as inclusion bodies (Fig. 3).

After solubilizing the inclusion bodies in extraction buffer, pgCyP was purified using Ni-NTA affinity chromatography as described in Materials and methods. The target protein with His tag was fractionated with 100 mM imidazole. GST-pgCyP-His₆ was confirmed in SDS-Page (lane 9, Fig. 3).

In vitro antifungal activity of pgCyP. After purification and renaturation, the recombinant protein was tested for its ability to inhibit fungal growth *in vitro*. Pathogenic fungus included *Sclerotinia* sp., *R. solani*, *P. cactorum*, *F. solani*, *C. destructans*, *A. panax* and *B. cinerea*. The fusion protein at concentrations of 1.28 and 2.14 μM had pronounced effects on the growth of *P. cactorum* (Fig. 4A), but had no effect on the growth of the other fungi. The IC₅₀ of pgCyP against *P. cactorum*, was 2.55 μM (Fig. 4B). GST, as a negative control, was inactive. Hereby we demonstrate *in vitro* resistance activity against fungi of pgCyP.

PPIase activity of recombinant pgCyP. CyPs are PPIases. In the present study, we showed pgCyP has PPIase activity. PgCyP is capable of accelerating isomerization of imide between the Ala and Pro peptide bonds in contrast to spontaneous inter-conversion in the negative control. GST protein, the control, had no isomerize activity.

Discussion

Plant diseases are a major concern in the production of agricultural crops and medicinal herbs. Although an increasing

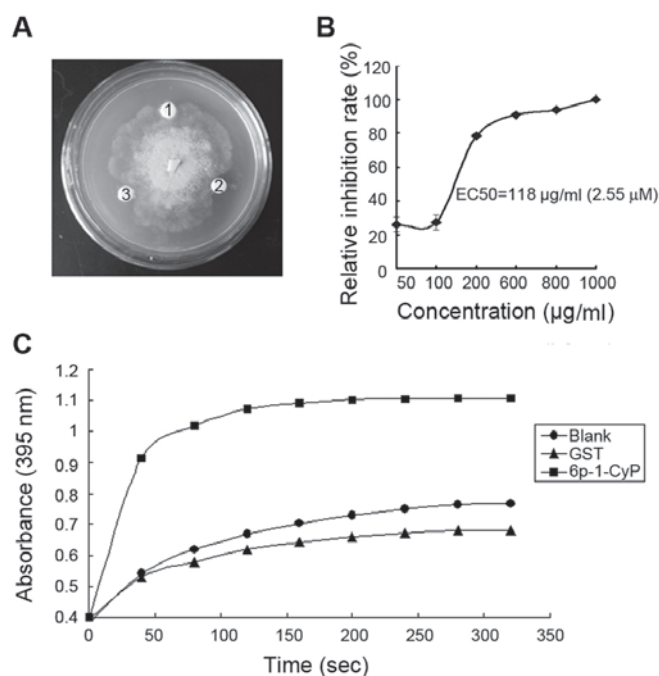


Figure 4. Antifungal activity and peptidyl-prolyl *cis-trans* isomerase activity of pgCyP. (A) Antifungal activity of purified pgCyP and GST protein tested against *Phytophthora cactorum* at concentrations of 2.14 μM (GST), 1.28 μM (pgCyP) and 2.14 μM (pgCyP). (B) Growth inhibition of fungal pathogen by purified pgCyP. (C) Spectrophotometric assay performed with pgCyP or GST in the absence of the two proteins. The graph is representative of at least three independent experiments. pgCyP, *Panax ginseng* cyclophilin; GST, glutathione S-transferase.

number of antimicrobial peptides have been isolated from plants, there is little research on anti-microorganism proteins of ginseng. In the present study, we cloned and isolated a ginseng CyP protein. PgCyP contains an ORF of 525 bp encoding 174 a.a. Ginseng blight is very prevalent during the rainy season (July to September), and our transcription expression level analysis revealed that CyP has a high expression in roots during the same period. This finding suggests a relationship between pgCyP expression and ginseng blight. In the antifungal test, pgCyP exhibited strong antifungal activity at micromolar concentrations against *P. cactorum*.

The expression vectors PET26b, PET28a and pRSET B were used; however, they failed to express pgCyP. GST, a chaperone for protein folding, is frequently selected to help isolation of soluble protein (26). However, use of GST-fusion system for insoluble GST-fusion protein isolation remains challenging (27). Ni-NTA affinity chromatography was effective, and the one-step method was used to isolate target proteins with His tag. Using this method, we were able to obtain pure GST-fused protein. We then attempted to hydrolyze the GST domain. Following protease treatment, the incised protein was unstable and rapidly degraded. Consequently, the GST tag was retained while performing the antifungal activity tests.

The functional properties of the generated fusion protein suggest pgCyP has PPIase activity. Taken together, our results provide evidence that pgCyP has antifungal activity. Previous findings have indicated that the CyP protein of Chinese cabbage has antifungal activity against *B. cinerea*, *T. harzianum*, *T. viride*, *R. solani*, *F. solani* and *F. oxysporum* (17). In the

present study, however, pgCyP affected *P. cactorum*, growth only, with no activity observed against *R. solani*, *F. solani* or *B. cinerea*. pgCyP shares 78% identity with its homolog in Chinese cabbage. Thus, the divergent pattern of antifungal activity observed between the two homologous proteins may be related to differences in amino acid sequences. We aim to investigate the antifungal spectrum of pgCyP in a future study.

In conclusion, in this study, we carried out the successful heterologous expression, purification and characterization of pgCyP and investigated its structure and function. While CyPs are reported to be involved in biotic stress response, their exact functions remain to be identified. To demonstrate the possible molecular functions executed, identification of its downstream substrates is needed. Transgenic plants expressing the *pgCyP* gene may facilitate revealing the physiological functions in the future.

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