

Molecular cloning and characterization of a pathogenesis-related protein SmPR10-1 from *Salvia miltiorrhiza*

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Abstract A full-length cDNA, *SmPR10-1*, encoding a pathogenesis-related class 10 protein, was isolated from *Salvia miltiorrhiza*, a well-known Chinese herbal plant. From the coding region of nucleotide sequence, the amino acid sequence can be predicted which contained conserved domain (K–A–X–E–X–Y) in the C-terminal helix found in most members of the PR10 protein family. Phylogenetic tree analysis indicated that the SmPR10-1 protein showed a sequence similarity which was much higher to dicot proteins than to other PR10 proteins. Prokaryotic expression of SmPR10-1 protein in fusion with a His-tag produced a 22 kDa protein in *E. coli* BL21 (DE3), which exhibited ribonuclease activity in vitro. The purified protein was used to assay antifungal activity and the results showed SmPR10-1 only inhibited the growth of *Phytophthora infestans*. The RT-PCR results showed that the *SmPR10-1* was expressed in high transcript level in leaves, stalks and low levels in roots. This protein seems to involve in the

plant active defense response through activation of the methyl jasmonate signaling pathway rather than salicylic acid or abscisic acid pathway.

Keywords *Salvia miltiorrhiza* · Pathogenesis-related protein 10 · Prokaryotic expression · Ribonuclease activity · Antifungal activity

Introduction

Plants cannot move to escape environmental challenges and are consistently exposed to various pathogenic microorganisms such as bacteria, fungi, viruses, together with insects (Dangl and Jones 2001). In this case, both active and passive mechanisms, resisting pathogens, are involved in plant defenses. Pathogen access and infection to plant cells can be prevented by physical and chemical responses which constitute the passive defense mechanisms (Gómez-Gómez et al. 2011). The active defense requires synthesis of defense-related proteins and is regulated through complex signaling network pathways, such as jasmonates (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene signaling compounds. If these mechanisms are activated, the molecular, physiological and morphological changes can be produced (Bari and Jones 2009).

Most of these defense-related proteins correspond to pathogenesis-related (PR) proteins. The production of PR proteins is a major process of active plant defense induced by pathogen attacks or exposure to other stress conditions. According to primary structures, biological activities and serological relationships, PR proteins are currently classified into 17 different families (Sels et al. 2008; van Loon et al. 2006). The majority of PR proteins localize either extracellularly or intracellularly in the vacuole. On the

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contrary, the PR10 proteins are the only intracellular and cytoplasmic family (Chadha and Das 2006). Most genes of PR10 family encode proteins with molecular masses of 15–18 kDa (Liu and Ekramoddoullah 2006). PR10 ORF is generally interrupted by an intron of 76–359 bp at a position highly conserved in seed plants (Hoffmann-Sommergruber et al. 1997). However, one subfamily of the *Malus domestica* PR 10 gene is intronless (Gao et al. 2005). The PR10 family proteins, first identified as a major pollen allergen (Bet v1) from white birch (Breiteneder et al. 1989), then the PR10 proteins have been identified in a number of species throughout the plant kingdom, including dicot and monocot species, such as pea (Barratt and Clark 1991), bean (Walter et al. 1996), soybean (Crowell et al. 1992), potato (Matton and Brisson 1989), asparagus (Warner et al. 1992), rice (Midoh and Iwata 1996), maize (Xie et al. 2010), and saffron (Gómez-Gómez et al. 2011). It has been reported that these proteins manifest various functions ranging from allergenic, antifungal, ribonuclease activities, enzymatic activities in plant secondary metabolisms, and roles in abiotic stresses (Lee and Facchini 2010; Liu et al. 2003; Xie et al. 2010). Therefore, PR10 proteins can play significant parts in plant defense against pathogen attack. Similar to other PR proteins, transcription of PR10 is induced by various phytohormones, such as methyl jasmonate (MeJA), gibberellins (GA), abscisic acid (ABA) and ethylene (Adie et al. 2007; Bari and Jones 2009; Gómez-Gómez et al. 2011).

Salvia miltiorrhiza Bunge is a well-known traditional Chinese herbal plant. As the first Chinese medical material that enters the international market, it has great economic and medical value, and its root, called “Danshen” in Chinese, has been extensively employed in traditional Chinese medicine to treat cardiovascular diseases, menstrual disorders and to prevent inflammation (Cheng 2006; Ma et al. 2012). Like most other plants, *S. miltiorrhiza* is susceptible to a wide variety of pathogens that attack the root system, and many of these are caused by fungal infections. The yield and quality of the medicinally significant herb *S. miltiorrhiza* are severely affected by fungal infections which cause root rot diseases. The root rot diseases result in the low quality of *S. miltiorrhiza* which cannot reach the criteria of clinical medication. Chemical pesticide can hardly control the root rot disease and the abuse of pesticide will cause environmental contamination, seeking an effective measure of biological control is, therefore, in desperate need. Recent studies regarding *S. miltiorrhiza* were mainly focused on identification and characterization of novel genes involved in terpenoid biosynthesis (Gao et al. 2009; Guo et al. 2013; Ma et al. 2012), however, little research on resistance genes has been reported. So we analyzed the previous transcriptome data of *S. miltiorrhiza* (Wenping et al. 2011) and obtained a

number of plant pathogenesis-related genes including plant pathogenesis-related protein 10 gene. The results of this study provided not only the first and fundamental information about SmPR10-1 protein properties but also a candidate gene for future genetic engineering of Chinese medical herbs, *S. miltiorrhiza*, against pathogen attack.

In this research, the *SmPR10-1* gene was isolated, and the SmPR10-1 protein was expressed in *Escherichia coli* as a fusion recombinant protein with His-tag, then the potentially antifungal activity of SmPR10-1 protein was investigated against phytopathogenic fungi which was isolated from *S. miltiorrhiza* root. The ribonuclease (RNase) activity of this protein was analyzed and the expression patterns in *S. miltiorrhiza* root under different treatments were also examined.

Materials and methods

Plant materials, treatment of plants with elicitors and phytohormone

Seeds of *S. miltiorrhiza* were obtained from the National Resource Center for Chinese Materia Medica, Chinese Academy of Chinese Medical Sciences, Beijing, China. The seeds were germinated under controlled environment at 25 °C with 16/8 h light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/dark photoperiod. After 2 weeks, the seedlings were transplanted to a container full of Hoagland solution (Hoagland and Arnon 1950) and grew for 2 more weeks. The solution was changed every 2 days. Plant tissues were collected and frozen in liquid nitrogen until they are used. The elicitor yeast extract (YE) and silver ion Ag^+ were prepared as described by Ge and Wu (Ge and Wu 2005). The seedlings were treated with elicitor 30 μM Ag^+ , 100 $\mu\text{g ml}^{-1}$ YE and phytohormone 50 μM ABA, 100 μM MeJA, 100 μM salicylic acid (SA), respectively, or with sterile water as control.

RNA isolation and cloning of SmPR10-1 gene

Previously, to investigate the inducible nature of tanshinone biosynthesis, a cDNA library was constructed from *S. miltiorrhiza* root tissue and a microarray chip was produced from ~8700 random cDNA inserts ranging in size from 0.5 to 2.5 kb (Gao et al. 2009). Total RNA was isolated from fresh roots using the TRIzol reagent (Life technologies, USA) according to the manufacturer's protocol. Reverse transcription was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) according to the manufacturer's instructions. The produced cDNA was used as template to amplify *SmPR10-1* coding region by using specific primer pairs SmPR10-F (5'-

ATGGGTGTGATCACTTACGA-3') and SmPR10-R (5'-TCAGTAGTCGGGGTTGGCGG-3') according to the sequence information of cDNA library. The PCR products were cloned into pGEM-T-Easy vector (Promega, USA), and transformed into *E. coli* DH5 α competent cells. The correct recombinant plasmid pGEM-T-SmPR10-1 was verified by DNA sequencing analysis.

Expression vector construction

Prokaryotic expression vector pET28a-SmPR10-1 was constructed by PCR with the forward primer SmPR-10-M1 (5'-CGGAATTCATGGGTGTGATCA-3', *EcoRI* site underlined) and the reverse primer SmPR-10-M2 (5'-CCGCTCGAGTGTAGTCGGGGTT-3', *XhoI* site underlined) using recombinant plasmid pGEM-T-SmPR10-1 as template. The PCR products were purified, digested with the restriction endonucleases *EcoRI* and *XhoI*, and cloned into the expression vector pET-28a(+) (Novagen, Germany), which was digested with the same restriction enzymes to generate the prokaryotic expression vector. Based on the manufacturer's instructions, PCR products and digested expression vector pET-28a(+) were purified by applying the GeneJET PCR Purification Kit (Thermo, USA). *E. coli* BL21 (DE3) cells were transformed with the plasmid pET28a-SmPR10-1 and used for prokaryotic expression. The correct recombinant plasmid pET28a-SmPR10-1 was verified by DNA sequencing.

Expression and purification of SmPR10-1 proteins

The *E. coli* BL21 (DE3) cells with the plasmid pET28a-SmPR10-1 grew in LB liquid medium (containing kanamycin 50 $\mu\text{g ml}^{-1}$) at 37 °C overnight. Details of expression and purification are described in Supplementary materials and methods.

Dialysis and concentration of SmPR10-1 protein

According to the instructions of the manufacturer, dialysis tubing cellulose membrane (MWCO 10,000 Da) (Sangon Biotech Co., Ltd. China) was applied. Details of dialysis and concentration are described in Supplementary materials and methods.

RNase activity assay

For RNA degradation assay, the purified SmPR10-1 protein was mixed with leaf total RNA from *S. miltiorrhiza* based on the method described by Xie et al. (2010). RNase activity of purified SmPR10-1 protein was determined according to Zhou et al. (2002). The effect of chemicals usually used in the process of protein extractions (such as

DTT and EDTA) and metal ions (Zn^{2+} , Mg^{2+} , and Ca^{2+}) on RNase activity was examined as well. Details of RNase activity assay are described in Supplementary materials and methods.

Antifungal activity assay

The tested fungal species were *Fusarium oxysporum*, *Verticillium dahliae* and *Phytophthora infestans*, all of which were isolated from the roots of *S. miltiorrhiza*. Details of antifungal activity assay are described in Supplementary materials and methods.

Bioinformatics tools

The predicted secondary structure was performed with the PredictProtein server (Rost et al. 2004). SWISS-MODEL was employed to predict the three-dimensional (3D) model of the SmPR10-1 protein through homology modeling (<http://swissmodel.expasy.org/workspace/>) (Arnold et al. 2006; Schwede et al. 2003). The nuclear magnetic resonance (NMR) structure of the major cherry allergen Pru av 1 (PDB ID: 1e09) was used as the template. MEGA4 was applied to construct the phylogenetic tree with the neighbor-joining methods (Tamura et al. 2007). The sequences used in a phylogenetic context were downloaded from GenBank and were aligned in MEGA4 using ClustalW with neighbor-joining algorithm. SignalP 4.1 server was used to predict the signal peptide sequence of SmPR10-1 protein (<http://www.cbs.dtu.dk/services/SignalP/>) (Emanuelsson et al. 2007).

Results and discussions

Isolation and sequence analysis of SmPR10-1 cDNA

The *SmPR10-1* cDNA was isolated from cDNA library, which was constructed from *S. miltiorrhiza* root tissue. The full length of *SmPR10-1* gene (GenBank Accession No. KF877034) was 603 bp, in which there was a complete open reading frame (ORF) of 477 bp, encoding a protein of 158 amino acid residues. The predicted molecular mass of the protein was 17.3 kDa and the isoelectric point 4.87. All these data are in common with the majority of PR10 family proteins (Gómez-Gómez et al. 2011; Liu et al. 2006; Xie et al. 2010).

Figure 1a shows the conserved sequence (K-A-X-E-X-Y), which was found in most members of the PR10 family, existed in the C-terminal helix of SmPR10-1 protein (Jwa et al. 2001). In birch pollen Bet v1, the conserved P-loop sequence (G-X-G-G-X-G-X-X-K) was located, which may be an RNA binding site, and the two residues

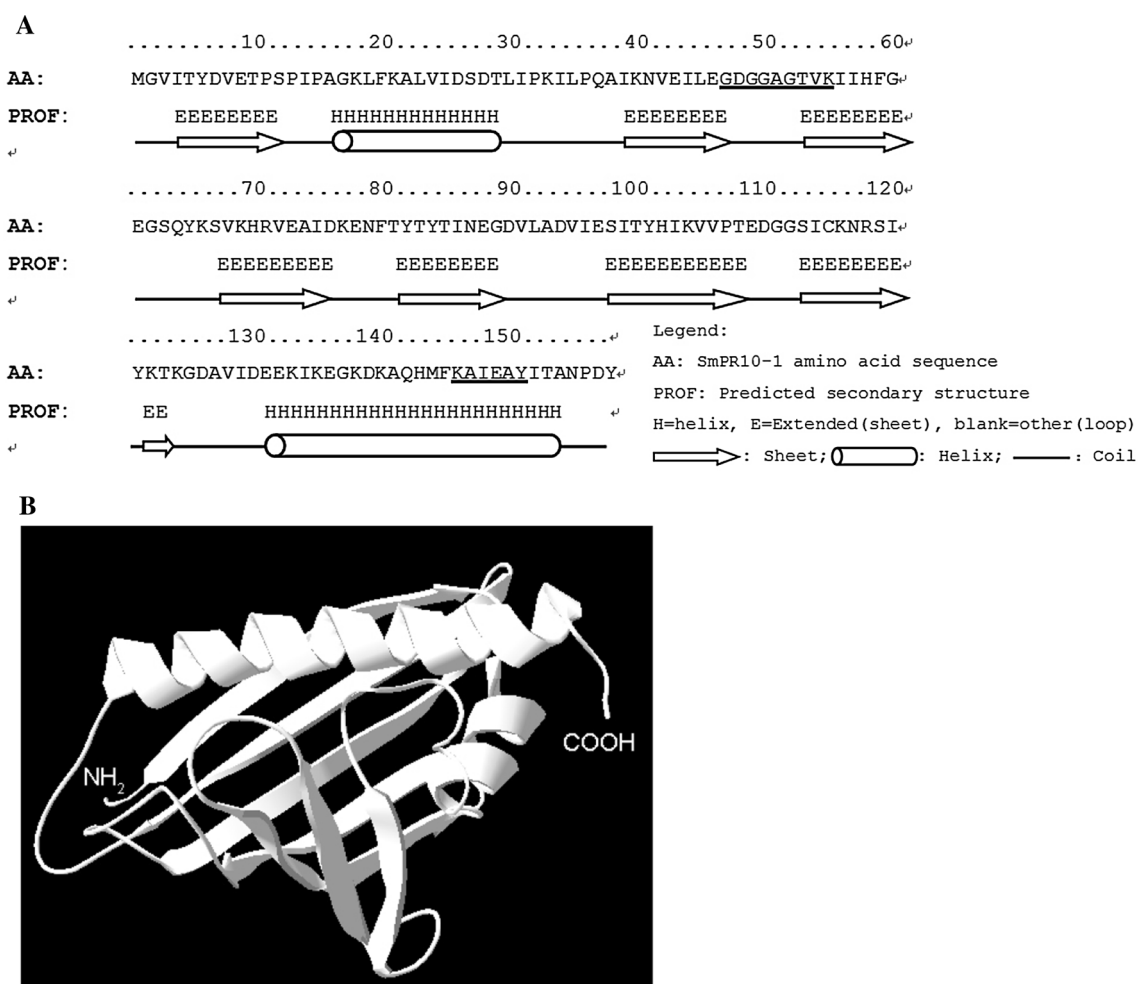


Fig. 1 Secondary structure prediction for the SmPR10-1 protein (**a**) and 3D model of SmPR10-1 protein (**b**). **a** The prediction was performed with the PredictProtein server. Conserved sequences (G-X-G-G-X-G-X-X-K) and (K-A-X-E-X-Y) are underlined. **b** 3D

model of SmPR10-1 protein was predicted with SWISS-MODEL software (the α -helix represented by the *spiral ribbon*, the β -sheet shown with an *arrow*, and the irregular coil shown as a *line*)

after the P-loop was conserved Lys⁵⁵, which might have a similar function (Gajhede et al. 1996). In SmPR10-1 protein, this motif was changed to GDGGAGTVK (Fig. 1a), which may have the similar function. The highly conserved PR10 protein family seems to be embodied in almost all plant species. According to predicted three-dimensional structure based on homology with other PR proteins, PR 10 protein structure contains seven anti-parallel β -sheets that wraps around a 25 residue-long C-terminal α -helix, and two short α -helices between β 1 and β 2 sheets (Liu and Ekramoddoullah 2006). In the β -sheets II and III of Bet v1 existed a glycine-rich loop called P-loop motif, which located in many nucleotide binding proteins (Markovic-Housley et al. 2003). As shown in Fig. 1B, the three-dimensional structure of SmPR10-1 protein is organized by two α -helices and seven β -sheets, which is not entirely novel. The novel PR10 protein is constituted by a highly curved, conserved, seven anti-parallel β -sheets which

embrace a long C-terminal α helix (α 3) in a grip like a palm and another two continuous short helices (α 1, α 2) complete the scaffold forming a hydrophobic cavity (Fernandes et al. 2013). Though the three-dimensional structure of SmPR10-1 protein is not entirely novel, the sequence and structure of SmPR10-1 are still similar to the most of other PR 10 proteins, which indicates that SmPR10-1 protein may have the similar functions, such as ribonuclease activity, antifungal activity, and roles in abiotic stresses (Lee and Facchini 2010; Liu et al. 2003; Xie et al. 2010).

On the basis of SiganLP prediction (Emanuelsson et al. 2007), the SmPR10-1 protein was possibly located in cytoplasm without the detection of any signal peptide sequence. The results of sequence alignment through BLASTP (NCBI) indicated that SmPR10-1 protein showed the highest homology, 69 % identity, with PR10 protein from *Vitis vinifera*. 20 members of PR10 family from

different species of GenBank database were selected to construct a phylogenetic tree and SmPR10-1 protein was placed in the phylogenetic context (Fig. 2). *Hordeum vulgare*, *Crocus sativus*, *Triticum aestivum*, *Zea mays* and *Oryza sativa* are monocots, and the rest of species are dicots, including *V. vinifera* and *S. miltiorrhiza*. Unsurprisingly the SmPR10-1 protein showed a sequence similarity which was much higher to dicot proteins than to other PR10 proteins (Fig. 2). The difference between monocots and dicots may be reflected by the divergences in the similarity of sequence between SmPR10-1, dicots and other PR proteins.

Expression and purification of SmPR 10-1 protein

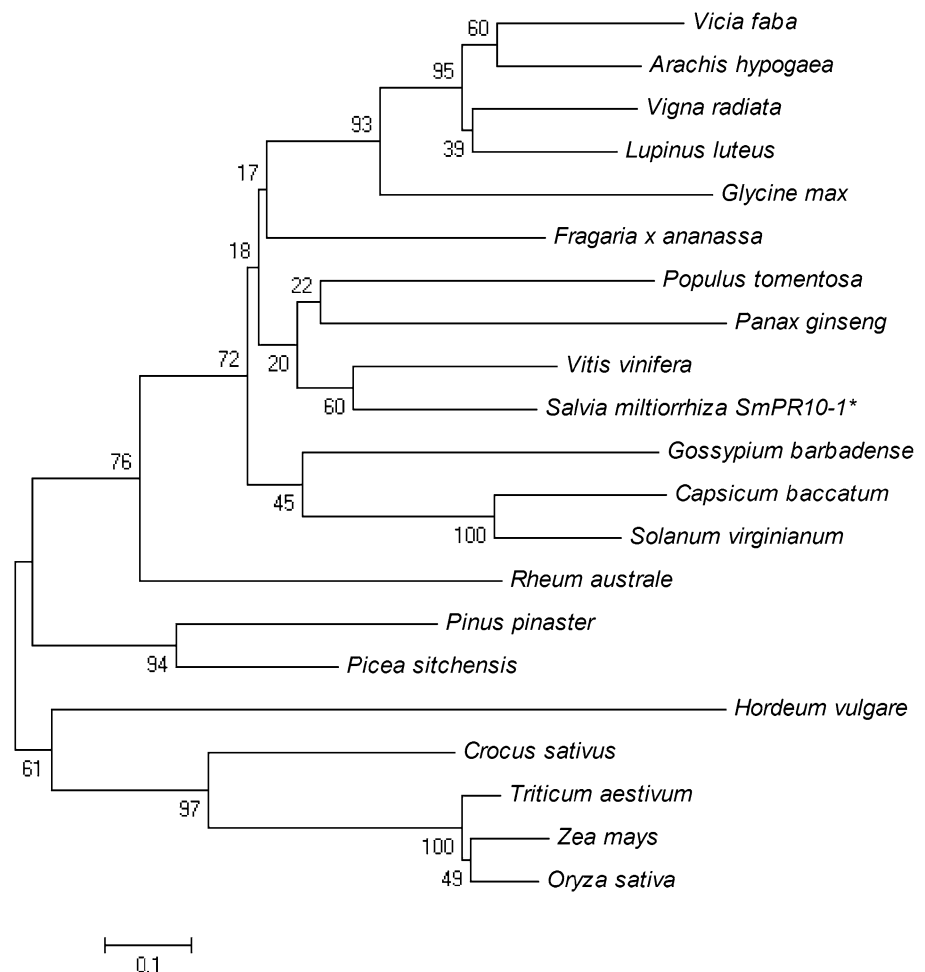
The recombinant protein contains 6 N-terminal histidine residues and other C-terminal amino acids of the vector pET-28a(+) and the calculated molecular weight is 22.5 kDa, similar to the value that is estimated. The SDS-PAGE analysis indicated that a specific 22.5 kDa protein band emerged in the soluble fraction after being induced by

the 0.5 mM IPTG (Fig. 3a). Based on the manufacturer's instructions, the Ni Sepharose 6 Fast Flow Purification System (GE Healthcare) was used to purify the fusion SmPR10-1 protein. After dialysis and concentration, about 20 mg purified protein was obtained from 1L of *E. coli* culture. A single band, about 22 kDa, was observed (Fig. 3a) after SDS-PAGE analysis which was consistent with calculated molecular weight of the recombinant protein. Purified protein was confirmed by MALDI-TOF/TOF-MS analysis with standard methods in Ultraflex II MALDI-TOF/TOF Mass Spectrometer (Bruker Daltonic Inc., Germany) (data not shown).

RNase activity

Previous reports indicated that some PR10 proteins from major birch pollen allergen Bet v1 (Bufe et al. 1996), cotton (Zhou et al. 2002), maize (Xie et al. 2010) and saffron (Gómez-Gómez et al. 2011) have shown plant ribonucleases activity. The SmPR10-1 was over-expressed in *E. coli* to determine the possible RNase activity similar

Fig. 2 The phylogenetic tree was constructed by the neighbor-joining algorithm using MEGA4 with bootstrap of 1000 replicates. The accession numbers of proteins used in this analysis are: *Vicia faba* (AFD29283.1), *Arachis hypogaea* (CAJ43118.1), *Vigna radiata* (AAX19889.1), *Lupinus luteus* (2QIM_A), *Glycine max* (ADC31789.1), *Fragaria × ananassa* AET44159.1), *Populus tomentosa* (ADP69174.1), *Panax ginseng* (ACY36943.1), *Vitis vinifera* (CAC16166.1), *Gossypium barbadense* (AAP76504.1), *Capsicum baccatum* (ABC74797.1), *Solanum virginianum* (AAU00066.1), *Rheum australe* (ACH63224.1), *Pinus pinaster* (ADJ53040.1), *Picea sitchensis* (ABK23161.1), *Hordeum vulgare* (AAP04429.1), *Crocus sativus* (ADL09408.1), *Triticum aestivum* (ACG68733.1), *Zea mays* (ADA68331.1) and *Oryza sativa* (NP_001049857.1). The scale bar corresponds to 0.1 amino acid substitutions per site



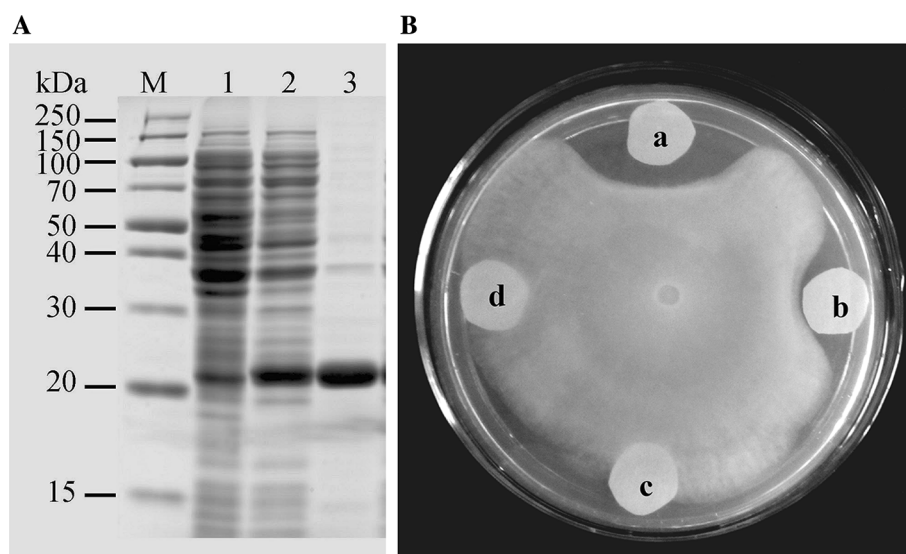


Fig. 3 **a** SDS-PAGE (15 %) analysis of SmPR10-1 protein expression in *E. coli*. Cells. Lane M, molecular weight markers; lane 1, soluble fraction of *E. coli* with the pET28a-SmPR10-1 plasmid before IPTG induction; lane 2, soluble fraction of *E. coli* with the pET28a-SmPR10-1 plasmid after IPTG induction; lane 3, purified SmPR10-1

with a specific protein band at ~22 kDa. **b** Antifungal activity of SmPR10-1 against *P. infestans*. **a** Nystatin (20 units); **b** 20 µg SmPR10-1; **c** 20 µg heat-inactivated SmPR10-1 (boiled for 15 min); **d** 20 µl 20 mM PBS buffer (pH 7.4)

to some other PR10 proteins. A test was carried out to examine whether the purified SmPR10-1 protein possessed RNase activity with the total RNA isolated from *S. miltiorrhiza* leaf as the substrate.

The significant RNase activity was observed when total RNA incubated along with native recombinant SmPR10-1 protein (Supplementary Fig. 1, lanes 3 and 4). The RNase A (Takara, Japan) was employed as positive control, while the other negative control incubated with heat-inactivated recombinant protein did not show evident RNase activity (Supplementary Fig. 1, lanes 5 and 6).

The effect of chemicals usually used in the process of protein extractions (such as DTT and EDTA) and metal ions (Zn^{2+} , Mg^{2+} , and Ca^{2+}) on RNase activity was examined. Zn^{2+} , Mg^{2+} , Ca^{2+} and EDTA slightly affected the RNase activity of SmPR10-1 (93.4, 92.5, 90.7 and 93.6 %) (Supplementary Table 1). However, DTT significantly inhibited the RNase activity of SmPR10-1 (12.3 %) (Supplementary Table 1). All these features are belonging to the plant ribonucleases II based on traditional biochemical classification (Wilson 1975).

In vitro antifungal activity of SmPR10-1 protein

Some PR10 proteins have been reported to show in vitro antifungal activity in different plants, such as *Oxalis tuberosa* Mol. (Flores et al. 2002), *Solanum surattense* (Liu et al. 2006), *Capsicum annuum* (Park et al. 2004) and *Crocus sativus* (Gómez-Gómez et al. 2011). This research investigated the possible antifungal activity of

SmPR10-1 protein in which a test was conducted to investigate whether this protein possessed the antifungal activity against *F. oxysporum*, *V. dahliae* and *P. infestans* isolated from roots of *S. miltiorrhiza* cultivated in the fields. The results indicated that SmPR10-1 inhibits mycelial growth of *P. infestans* (Fig. 3b) rather than *V. dahliae* or *F. oxysporum* (data not shown). As shown in Fig. 3b, after 72 h incubation, paper filter discs with the SmPR10-1 protein (20 µg) and positive control nystatin (20 units) had produced crescents of inhibition around the discs. The control paper filter discs with boiled SmPR10-1 protein and PBS buffer did not show the inhibition effect.

SmPR10-1 protein revealed the selectivity of inhibition, which is similar to AhPR10 and CsPR10. It was reported that AhPR10 protein showed antifungal activity against *F. oxysporum* and *Rhizoctonia solani* instead of *Sclerotium rolfsii*, *Aspergillus xavus*, *A. niger* and *P. infestans* (Chadha and Das 2006). Through the substitution mutant, the mutant protein AhPR10-K54 N where lys54 is mutated to asn54 loses its RNase activity and AhPR10-K54 N protein exhibited the non antifungal activity against *F. oxysporum* and *R. solani*. The finding shows that the RNase activity of AhPR10 is indispensable for its antifungal activity (Chadha and Das 2006). CsPR10 was described as showing antifungal activity against *F. oxysporum* instead of *Penicillium* sp. and *V. dahliae* (Gómez-Gómez et al. 2011). The SmPR10-1 protein displayed the selective antifungal activity and the ribonuclease activity, which suggests that it exerts its antifungal activity after internalizing inside the

mycelium of sensitive fungi through its ribonuclease activity (Chadha and Das 2006).

Detection of the expression pattern of *SmPR10-1*

This study investigated the expression pattern of *SmPR10-1* in different tissues. A semi-quantitative RT-PCR analysis was conducted using RNA extracted from different tissues of *S. miltiorrhiza* (Fig. 4a). As can be seen from Fig. 4a, *SmPR10-1* was expressed in roots, stalks and leaves of *S. miltiorrhiza*. It was highly expressed in leaves, then in stalks and weakly expressed in roots. The results indicated that *SmPR10-1* is expressed constitutively in all examined tissues. These results are inconsistent with other PR10 genes expression patterns, which perform highest expression in roots than in leaves (Hashimoto et al. 2004; Kim et al. 2008; Xie et al. 2010). This may indicate that *SmPR10-1* belongs to the pre-infection members of the *S. miltiorrhiza* defense mechanism. It has been reported that in anthers and tepals of saffron, *CsPR10* shows the highest transcriptional level, which may indicate its significance in the defense of these tissues and it might protect the saffron flower from being attacked by pathogen throughout the short florescence (Gómez-Gómez et al. 2011). *SmPR10-1* may also have the similar function like *CsPR10* in saffron flower to protect leaves against pathogen attack.

PR10 expression in cotton was induced after treatment with JA and elicitor, but not with SA (Zhou et al. 2002). In rice, SA, JA and H₂O₂ induced *PR10* mRNA expression, while ethylene and ABA did not (Jwa et al. 2001). The induced transcriptional level of *SmPR10-1* gene was investigated in root tissues of *S. miltiorrhiza* after phytohormone and elicitor treatment. Ag⁺, YE and Ag⁺ + YE were performed as elicitors to induce the production of

tanshinone in *S. miltiorrhiza* roots (Ge and Wu 2005; Yan et al. 2006). The *SmPR10-1* transcripts increased after application of elicitor and MeJA treatment, but the *SmPR10-1* transcripts were hardly detected after ABA and SA treatment (Fig. 4b). After elicitor treatment, much higher level of expression was detected, indicating the possible involvement of *SmPR10-1* in active defenses of plants against pathogens. The signaling molecules MeJA and SA are regarded to involve in plants disease resistance and ABA involves in plant response to abiotic stress. The results showed that in root tissues of *S. miltiorrhiza*, the *SmPR10-1* was induced by activating the MeJA signaling pathway instead of the ABA or SA signaling pathway. Furthermore, the expression of *SmPR10-1* gene was inhibited after ABA and SA treatment.

In conclusion, *SmPR10-1* gene has been isolated from the root of *S. miltiorrhiza*, which is confirmed as one of PR10 family. *SmPR10-1* shows the highest expression in stalks and leaves. It may involve in the active defenses of plants against pathogens through activation of the MeJA signaling pathway to inhibit the mycelial growth of fungi, for it shows selective antifungal activity and ribonuclease function. These results of this study provided not only the first and fundamental information about *SmPR10-1* protein properties but also a candidate gene for future genetic engineering of Chinese medical herbs, *S. miltiorrhiza*, against pathogen attack.

Author contribution statement Lu-qi Huang and Ye Shen as the corresponding authors designed the study and led this project. Zhi-shuang Wang prepared the plant materials and isolated gene. Li-gang Ma carried out bioinformatics analysis. Mei-lan Chen provided fungal species and technical supports about fungus. Le Zhao performed protein expression and purification, assay anti-fungal activity, and drafted the manuscript.

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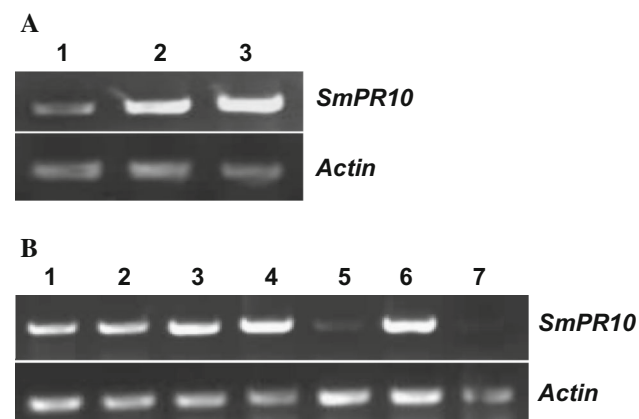


Fig. 4 **a** Expression patterns of *SmPR10-1* in different tissues. 1 Roots, 2 stalks, 3 leaves. **b** Expression patterns of *SmPR10-1* in root tissue under different induction treatments and various elicitors and phytohormone treatments. 1 Control, 2 Ag⁺, 3 yeast extract (YE), 4 Ag⁺ + YE, 5 ABA, 6 MeJA, 7 SA

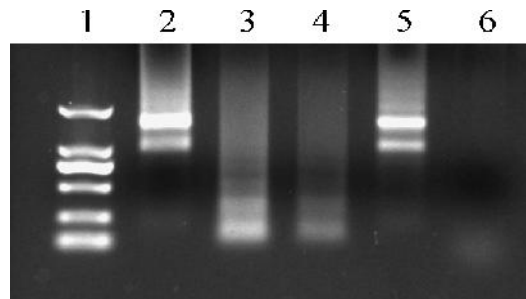
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Supplementary Fig. 1 RNase activity of purified recombinant SmPR10-1 protein.

RNase activity was examined with the 10 μ g total RNA isolated from *S. miltiorrhiza* leaf as the substrate. Lane 1, DNA DL2000 marker (Takara, Japan); Lane 2, RNA of leaf tissues; Lane 3, RNA + native SmPR10-1 protein (10 μ g); Lane 4, RNA + native SmPR10-1 protein (20 μ g); Lane 5, RNA + heat-inactivated SmPR10-1 protein (10 μ g); Lane 6, RNA + RNase A (10 μ g).



Supplementary Materials and Methods

Molecular cloning and characterization of a pathogenesis-related protein SmPR10-1 from *Salvia miltiorrhiza*

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Supplementary materials and methods

Expression and purification of SmPR10-1 proteins

The *E. coli* BL21 (DE3) cells containing the pET28a-SmPR10-1 plasmid grew in LB liquid medium (containing kanamycin 50 $\mu\text{g}\cdot\text{ml}^{-1}$) at 37 °C overnight, then 5ml cultured cells were inoculated in 500 ml of LB liquid medium (volume ratio 1:100) at 37 °C, 200 rpm for 2~3 h. When the OD₆₀₀ reached 0.6, isopropyl β -D- thiogalactopyranoside (IPTG) (Merck) was added to a final concentration of 0.5 mM to initiate overexpression for 6 more hours at 28 °C. The cells were collected through centrifuging at 6000 g, 4 °C for 10 min. The precipitated cells were resuspended in 10 ml lysis buffer (20 mM NaH₂PO₄, 500 mM NaCl, and 20 mM imidazole, pH 7.3) containing 1mM phenylmethylmethylsulfonyl fluoride (PMSF). Protein expression was analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide separation gel) with Mini-PROTEAN 3 Cell (Bio-Rad) based on Laemmli (Laemmli 1970), then detected by Coomassie Brilliant Blue R-250 staining. The resuspended cells were broken by 99 cycles of ultrasonication at 10 s/10 s (VCX 500 Watt Ultrasonic Processors, Sonics & Materials, Inc., USA) with 40% maximum intensity on ice with PMSF. The insoluble materials were removed through centrifuging at 10,000g 4°C for 20 min and the supernatants were transferred to new centrifuge tubes to be further purified. Based on the manufacturer's protocol, the Ni Sepharose 6 Fast Flow Purification System (GE Healthcare) was used to purify the recombinant fusion SmPR10-1 protein. The concentration of protein was determined with the

method of Bradford (Bradford 1976).

Dialysis and concentration of SmPR10-1 protein

According to the instructions of the manufacturer, dialysis tubing cellulose membrane (MWCO 10,000 Da) (Sangon Biotech Co., Ltd. China) was applied. The purified SmPR10-1 protein was dialyzed in 1.5 L dialysis buffer (1×PBS buffer, pH 7.4) at 4°C overnight. The protein was processed for further dialysis at 4°C for 2 d with fresh dialysis buffer which was replaced every 4 h. The dialyzed protein was concentrated by Amicon Ultra-15 device (MWCO 10,000 Da, Millipore). SDS-PAGE was used to analyze the SmPR10-1 protein so as to examine whether it is intact.

RNase activity Assay

RNase activity of purified SmPR10-1 protein was examined based on the methods described by Zhou. (Zhou et al. 2002). The assay was carried out by mixing 10µg purified protein and 10µg leaf total RNA, then the mixture was incubated at 50 °C for 30 min. In order to terminate the reaction, the 1 ml precooled 4 M LiCl was added to the mixture, which was centrifuged at 10,000g 4 °C for 15 min to remove the insoluble RNA. The absorbance of the supernatant was measured at 260 nm with a spectrophotometer (Nanodrop ND-1000, Thermo). The definition of one enzyme activity unit was the amount of protein that caused the absorbance to increase by 1.0 after incubation for 30 min (Zhou et al. 2002). The RNase A (Takara, Japan) was employed as positive control, while the heat-inactivated SmPR10-1 protein (100 °C heating for 15 min) was used as the negative control for RNase activity assay.

The effect of chemicals usually used in the process of protein extractions (such as DTT and EDTA) and metal ions (Zn^{2+} , Mg^{2+} , and Ca^{2+}) on SmPR10-1 RNase activity was examined as well. The purified 20 µg SmPR10-1 protein and 20 µg leaf total RNA were mixed together, then each reagent was added to the 1.0 mM final concentration and the mixture was incubated at 50 °C for 30 min. Then the RNase activity was examined in the same way (Xie et al. 2010).

Antifungal Activity Assay

The tested fungal species were *Fusarium oxysporum*, *Verticillium dahliae* and *Phytophthora*

infestans, all of which were isolated from the roots of *S. miltiorrhiza*. The assay was conducted by applying Petri dishes with 20 ml of potato dextrose agar (PDA). After the development of mycelium, 4 sterile blank filter paper discs (0.6 cm diameter) were placed and each of the disc was about 1 cm away from the edge of the mycelium. The 20 µg purified SmPR10-1 protein in 20 mM PBS buffer (pH 7.4) was loaded on a disc. The Petri dishes were incubated at 23 °C for 72 h, which made the growth of mycelium envelop peripheral discs with the control (PBS buffer) and produce crescents of inhibition around discs with samples showing antifungal activity (Ye and Ng 2009). The nystatin (20 units) was used as positive control, while the heat-inactivated SmPR10-1 protein (boiled for 15 min) and PBS buffer (20mM, pH 7.4) were employed as negative controls.

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Supplementary Table 1 Effect of reagents on the RNase activity of SmPR10-1

Reagents (1 mM)	SmPR10-1 (%) ^a
Control ^b	100 ± 4.6
CaCl ₂	93.4 ± 2.8
MgCl ₂	92.5 ± 4.7
ZnCl ₂	90.7 ± 1.5
EDTA	93.6 ± 3.7
DTT	12.3 ± 2.3

^aData shown are means of three replicates ± standard error.

^bControl without reagents.