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Isolation and characterization of a pathogenesis-related protein 10 gene (*GmPR10*) with induced expression in soybean (*Glycine max*) during infection with *Phytophthora sojae*

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Abstract In previous study, a cDNA library enriched for mRNAs encoding ESTs that increased in abundance during infection with *Phytophthora sojae* was constructed by suppression subtractive hybridization from leaf tissues of a high resistant soybean, and an EST homologous to the class 10 of pathogenesis-related (PR) proteins was identified to be up-regulated by microarray and real-time PCR. Here, the full-length cDNA (termed *GmPR10*, GenBank accession number FJ960440; ADC31789.1) of the EST was isolated by rapid amplification of cDNA ends, and contains an open reading frame of 474 bp. The GmPR10 protein included a "P-loop'' motif. The constitutive transcript abundance of *GmPR10* in soybean was the highest in leaves, followed by roots and stems. Further analysis showed that *GmPR10* mRNA abundance was increased during infection with *P. sojae* following leaf treatments with gibberellin (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA), and abscisic acid (ABA). The dialytically renatured GmPR10 protein significantly inhibited *P. sojae* hyphal growth and exhibited RNase activity. Transgenic tobacco and soybean plants overexpressing *GmPR10* showed increased resistance to *P. nicotianae* Breda and *P. sojae*, respectively. These results suggest that the GmPR10 protein plays an important role in host defense against *P. sojae* infection. To the best of our knowledge, this is the first report on the functional characterization of a PR10 protein from soybean in defense against *P. sojae*.

Keywords Pathogenesis-related (PR) proteins · Soybean (*Glycine max*) · *Phytophthora sojae* · *PR10* gene

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Introduction

The production and accumulation of pathogenesis-related (PR) proteins in plants in response to invading pathogens or associated abiotic stress is one of the crucial components in the inducible repertoire of self-defense mechanisms in plants [26]. PR proteins do not typically accumulate in healthy plants, but are induced by pathogen infection or related abiotic stresses to improve the defense mechanisms in plants under these conditions [60]. Currently, PR proteins have been classified into 17 families based on structural differences, serological relationships, and/or biological activity [51, 59]. Six groups of PR proteins, including PR1 to PR4, PR9, and PR11, have been characterized as β-1,3-glucanases (EC 3.2.1.39), chitinases (EC 3.2.1.14), and peroxidases (EC 1.1 1.1.7), respectively. Other PR proteins, including PR6, PR12, PR13, PR14, and PR15, have been characterized as proteinase-inhibitors, defensins, thionins, lipid-transfer proteins, and oxalate oxidases (EC 1.2.3.4), respectively [16, 67]. However, the biological and biochemical functions of a few PR proteins, namely PR5, PR10, PR16, and PR17, remain unclear and their roles in the plant defense response have not been elucidated [16, 41, 62].

Among the 17 identified PR protein groups in plants, the class 10 PR proteins (PR10) represent one of the most

important families in response to fungal invasion [64]. Genes encoding these PR proteins have been isolated and characterized in many angiosperm species and a few gymnosperm species. Although most PR proteins are extracellular, the first intracellular PR protein, designated PR10, was isolated from cultured parsley cells after treatment with an elicitor [53]. The induction of PR10 expression has been demonstrated in a wide variety of plant species following infection with pathogens, including *Phytophthora megasperma* f. sp. *glycinea* on parsley [54], *Phytophthora infestans* on potato [36], tomato mosaic virus on *Capsicum annuum* [42], *Cronartium ribicola* on *Pinus monticola* [33], *Pseudomonas syringae* pv. *pisi* on *Vitis vinifera* [48], *Magnaporthe grisea* and *Acidovorax avenae* on rice [37], and *P. syringae* pv. *pisi* on alfalfa [6]. In addition, PR10 genes have also been induced under several abiotic conditions, such as drought [13], salinity and cold stress [24, 28, 55], extreme temperature [3, 56], ultraviolet radiation [47], heavy metals [34, 47], and herbicides [9]. The expression of some PR10 genes is also upregulated following treatments with plant hormones and signaling molecules, such as jasmonic acid (JA) [6, 37, 47], salicylic acid (SA) [37], ethylene (ET) [44], abscisic acid (ABA) [6], and kinetin (KT) [47].

PR10 proteins are typically described as small, acidic, intracellular proteins of 15–18 kDa [34], which are homologous to ribonucleases, such as birch Bet v1, pepper CaPR10, lupin LaPR10, jıcama SPE16, peanut AhPR10, pea PR10.1, and maize ZmPR10 [10, 42, 55, 63, 64]. The ribonuclease activity was demonstrated in major birch pollen allergen, Betv 1, which is homologous to PR10 proteins [7]. The RNase activity of PR10 proteins suggests a potential role in defenses against pathogenic infections. For example, PR10, isolated from hot pepper, functions as a ribonuclease in the antiviral pathway [42]. Several other studies have also reported that PR10 proteins displayed antimicrobial activities and in vitro ribonuclease activities [31, 66, 69].

Soybean (*Glycine max* (L.) Merrill.) is one of the most important food crops, which also serves as an oil and protein source for both human consumption and animal feed [29]. Phytophthora root and stem rot, caused by the biotrophic pathogen *Phytophthora sojae*, is a destructive disease of soybean worldwide [17, 58], which can reduce soybean yield of 10–40 % [1], and severe infection can result in a total yield loss [68]. In the interaction of *P. sojae* and soybean, the host responds to infection by increasing the expression of a number of genes associated with disease resistance, including pterocarpan phytoalexins, such as the glyceollins [2, 5, 15, 21, 22], and several PR proteins [23, 39, 40]. The accumulation of the glyceollins was extensively studied and thoroughly characterized [22]. Although there are some reports of individual PR proteins or PR protein-coding genes in soybean, the role of these proteins in defense against *P. sojae* has not been comprehensively studied.

In a previous study, a cDNA library enriched for mRNAs encoding ESTs that increased in abundance during infection with *P. sojae* was constructed by suppression subtractive hybridization (SSH) from leaf tissues of highly resistant soybean cultivar 'Suinong 10', and an EST homologous to the class 10 of PR proteins was identified to be up-regulated by microarray and real-time PCR [65]. However, no further studies have been conducted to examine the expression and biochemical activities of the protein. To better understand the relevance of this gene in plant defense mechanisms, the present research was undertaken to examine the expression patterns under biotic and abiotic stresses and the mode of PR10 antimicrobial activities in plants were presented in this paper.

The objectives of this study were to conduct a functional analysis of GmPR10, a novel PR protein isolated from 'Suinong 10' soybean after infection with *P. sojae*. To the best of our knowledge, this study is the first report on the biological activity of PR10 protein from soybean in defense against *P. sojae*. The enhanced abundance of host resistance in the transgenic plants and the antimicrobial activities of GmPR10 demonstrated in the present study might implicate *GmPR10* as a new tool for managing Phytophthora root and stem rot in soybean.

Materials and methods

Plant materials and pathogen inoculation

'Suinong 10', a popular soybean cultivar with gene-for-gene resistance against the predominant race 1 of *P. sojae* in Heilongjiang, China [68], was used in this study. Seeds of 'Suinong 10' were disinfected by soaking in 0.5 % (v/v) NaClO for 30 s, rinsed twice in sterile distilled water, and subsequently planted in pots filled with sterile vermiculite as the growth medium under a 14-h photoperiod at a light intensity of 350 μmol m^{-2} s^{-1} at 25 °C and 10-h darkness at 18 °C in a growth chamber. 7 days after planting, seedlings at the first-node stage (V1) were used for various treatments [18].

Inoculum was prepared for an isolate of *P. sojae* race 1, PSR01, which was isolated from infected soybean plants in Heilongjiang in 2007 [68], at 25 °C for 7 days on V8 juice agar in a polystyrene dish. The soybean plants were infected with *P. sojae* race 1 using the hypocotyl wound infestation technique described by Kaufmann and Gerdemann [27]. All the seedlings were incubated in a mist chamber at 25 °C, with 100 % relative humidity and a 14-h photoperiod at a light intensity of 350 μmol m^{-2} s^{-1}. The

unifoliolate leaves of infected 'Suinong 10' plants were harvested 3, 6, 12, 24, 36, and 72 h after inoculation, respectively, frozen in liquid nitrogen, and stored at −80 °C until used for RNA extraction and cDNA analysis. 'Dongnong 50' soybean, which was susceptible to *P. sojae* race 1, and 'Havana 425' tobacco, which was susceptible to *P. nicotianae* Breda, obtained from the Key Laboratory of Soybean Biology in Chinese Ministry of Education, Harbin, were used for gene transformation experiments. Professor WX Shan of College of Plant Protection, Northwest Agriculture and Forestry University, China kindly provided the *P. nicotianae* isolate.

RNA extraction and cDNA analysis

Total RNA was extracted from the leaves of the *P. sojae*-infected soybean plants using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The quality and concentration of RNA samples was examined through agarose gel electrophoresis and analyzed with a Lambda 35 UV/Vis Spectrometer (Perkin Elmer, USA). Total RNA was converted into cDNA using a random oligo dT primer and M-MLV reverse transcriptase according to manufacturer's instructions.

Cloning of a novel PR10 gene

The first-strand reaction product from the cDNA obtained above was used to clone full-length PR10 (termed *GmPR10*). The rapid amplification of cDNA ends (RACE) was conducted for soybean leaf mRNA using the CLONTECH SMART RACE cDNA Amplification Kit (Clontech, USA). Primers for *GmPR10* (5′RACE; 5′-TTGTGGATAG CATCCACAG-3′; 3′RACE; 5′-AGCCATGACATTAGAT TTCCATCATTG-3′) were synthesized based on the sequence of the fragment. The primers for *GmPR10* were used for PCR under the following conditions; one cycle (94 °C, 1 min), followed by 5 cycles (94 °C, 30 s; 57 °C, 30 s; 72 °C, 3 min), 5 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 3 min), 30 cycles (94 °C, 30 s; 53 °C, 30 s; 72 °C, 10 min). The PCR products were analyzed through electrophoresis, purified from an agarose gel, and then ligated into pGEM-T Easy vector. Clones containing about 600 and 250 bp fragments were identified through DNA sequencing. The sequence alignment was conducted using DNAMAN 5.2.2 software. A clone containing a fragment of 831 bp was obtained and confirmed through DNA sequencing.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was conducted to examine the transcript abundance of *GmPR10* under abiotic (phytohormone and chemical) and biotic (*P. sojae*) stress in 'Suinong 10' soybean.

For phytohormone and chemical treatments, 'Suinong 10' soybean leaves, sprayed with 0.5 mM SA, 50 mM ABA, 250 mg L^{-1} GA_3 or 200 mM H_2O_2, were harvested for RNA isolation 3, 6, 12, 24, 36, and 72 h after treatment, and subjected to semi-quantitative RT-PCR analysis.

The leaves of 'Suinong 10' soybean seedlings inoculated with *P. sojae* race 1 using the hypocotyl wound method [27] were also harvested 3, 6, 12, 24, 36, and 72 h after the treatment for semi-quantitative RT-PCR analysis.

Specifically, total RNAs were isolated from the leaves of 'Suinong 10' soybean seedlings treated as mentioned above and converted into cDNAs using M-MLV reverse transcriptase. Specific primer sequences were designed based on the full-length *GmPR10* cDNA and used to perform semi-quantitative RT-PCR reactions (GmPR10-F; 5′-TGGTCCTGGCACCATCAAGAAGAT-3′; GmPR10-R; 5′-GCTTCCACCAATTGGCTCTCGAAT-3′). Two primers, Actin F (5′-CTTGGAGGATCATGTTCGGTT-3′) and Actin R (5′-GCATCACAGTGCAATCTAGCT-3′), were used in the semi-quantitative RT-PCR as controls. All RNA templates were digested with *DNase*I. A total of 10 μL of PCR products was loaded on an agarose gel. The quantification of the band strength was accomplished through scanning the gels with the ultraviolet, and subsequently, the samples were subjected to RT-PCR analysis.

Cloning, purifying and renaturing of the GmPR10 protein

Two specific primers, *GmPR10*-F (5′-CGCCATATGATG GGTGTTGTTACTCAGATT-3′, *Nde*I underlined) and *GmPR10*-R (5′-ACGAGCTCACATAATCAGGATTTGC CAAAAG-3′, *Sac*I site underlined), were used to amplify the coding region of *GmPR10* gene. The following PCR cycling parameters were used; 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and a final cycle at 72 °C for 8 min. The pGEM-T Easy vector containing the *GmPR10* gene was used as the template. The PCR products were digested with *Nde*I and *Sac*I. The recombinant plasmid pET29b-*GmPR10* was generated from the ligation of the digested PCR products and the digested plasmid pET-29b. *E. coli* BL21 (DE3) cells were transformed with plasmid pET29b-*GmPR10* for protein expression and grown in LB medium containing 50 mg mL^{-1} kanamycin at 37 °C to an absorbance of 0.5 at 600 nm. The cultures were induced using 1 mM IPTG. After 5 h of induction, the cells were harvested through centrifugation at 4,000 rpm for 10 min at 4 °C. The cells were resuspended in 10 mL ice-cold 1× binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9), and sonicated on ice for 10 min until the sample was no

longer viscous. Following centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant was decanted. Some of the pellets were used to determine the recombinant GmPR10 protein solubility. Other pellets were dissolved in solution containing 1× binding buffer with 6 M urea. The supernatant was harvested through centrifugation at 12,000 rpm for 15 min at 4 °C and loaded onto a His-bind Resin column (Novagen, USA). The eluates, including the recombinant GmPR10 protein, were renatured through dialysis. The eluates and all the samples were analyzed through SDS-PAGE (4 % (v/v) and 15 % (v/v) polyacrylamide in the stacking and resolving gels).

RNase activities assays of recombinant protein

To elucidate the ribonuclease activity of the *GmPR10* gene product, recombinant GmPR10 protein expressed in *E. coli* was purified. The RNase activity assay of purified recombinant GmPR10 protein was assayed by culturing for 2–4 h at 37 °C according to the method of Bantignies et al. [4], with some modifications. The RNA degradation assay was used to examine whether dialytically renatured GmPR10 protein had RNase activity. Briefly, 50 μL reaction mixtures contained 10 μg total RNA extracted from 'Suinong 10' soybean. RNA was visualized under UV light. The proteins were removed from the reaction mixtures and water containing only dissolved RNA was used as a negative control.

In vitro antimicrobial activity of recombinant GmPR10 protein

The inhibition of pathogen growth by GmPR10 was assayed using *P. sojae* according to Schlumbaum et al. [52]. The PSR01 isolate of *P. sojae* race 1 was grown on carrot agar plates at 28 °C for 4 days. When the colony diameters were 3–4 cm, sterile filter paper discs (30 mm in diameter) were placed on the plate surface at 1 cm from the hyphal tips of the culture and treated with 25 or 15 μg of the re-natured recombinant GmPR10 protein, eluation buffer (without recombinant GmPR10 protein) or 25 μg of protein extract from *E. coli* transformed with pET-29b. After incubation at 28 °C for 24 h, the growth zones of the pathogen were measured.

Vector construction and tobacco, soybean transformation

Two specific primers, *GmPR10*-F (5′-GCTCTAGAATGG GTGTTGTTACTCAGAT-3′, *Xba*I site underlined) and *GmPR10*-R (5′-TCGAGCTCTCAATAATCAGGATTTGC C-3′, *Sac*I site underlined), were designed to amplify the coding region of the *GmPR10* gene for recombinant vector construction and transformation. The following PCR cycling parameters were used; 94 °C for 5 min, 35 cycles of 94 °Cfor 30 s, 54 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min. The PCR products were cloned into pMD^{TM}18-T vector, followed by transformation into *E. coli* DH5α cells (Shanghai Biotech Inc, Shanghai, China) and sequenced. The PCR products and pCAMBIA3301 (www.cambia.org) with the bar gene as the selective marker and GUS as the reporter gene, were digested with *Xba*I and *Sac*I. The recombinant plasmid pCAMBIA3301-*GmPR10* was generated from the digested PCR products and the digested plasmid pCAMBIA3301. *E. coli* DH5α cells were transformed with plasmid pCAMBIA3301-*GmPR10*. The plant expression vector was introduced into *Agrobacterium tumefaciens* LBA4404 using the freezing and thawing method. 'Dongnong 50' soybean and 'Havana 425' tobacco were used for the gene transformation experiments. The soybean cotyledonary node was used as explants for the transformation using the *Agrobacterium*-mediated transformation method [43], and tobacco leaf discs were transformed according to the methods of Horsch et al. [25]. T_1 seeds were collected, dried at 25 °C, grown in soil to test the viability of the transgenic tobacco and soybean plants. T_2 seeds were set using the same method for the T_1 seeds.

PCR analysis of transgenic plants

To confirm transgene insertion in the transgenic tobacco and soybean plants, genomic DNA was extracted from the transformants using the CTAB method and PCR analysis was conducted. Two primers, BarF (5′-ATATCCGAGCG CCTCGTGCAT-3′), and BarR (5′-GGTCTGCACCATCG TCAACCACT-3′), were designed to target the regions of the Bar reporter gene. Using genomic DNA as template, PCR was performed with a initial denaturation condition of 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s and a final cycle at 72 °C for 8 min.

Southern hybridization analysis

Southern hybridization analysis of PCR-positive soybean plants (T_2) was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Cat., Germany) according to the manufacturer's instructions. Specifically, 20 μg of genomic DNA was digested with *Hin*dIII and electrophoresed on a 1.0 % (w/v) agarose gel. The DNA was transferred to nylon membranes using the alkaline transfer protocol and UV cross-linked [49]. PCR products of bar genes and target gene from plasmid were used as the probe. The probe was labeled using digoxigenin (DIG)-11-dUTP with DIG High Prime DNA Labeling reagents (Roche, Mannheim, Germany). Hybridization was

performed at 42–45 °C. Washing, blocking, and detection were performed according to the manufacturer's instructions. The Southern hybridizations were exposed to X-ray film (Kodak, Japan) using two intensifying screens at room temperature for 20 min and subsequently developed.

Identification of resistant transgenic tobacco and soybean lines

To investigate whether the *GmPR10*-transformed plants resist pathogen infection, artificial inoculation procedures were modified from the methods of Dou et al. [14]. Leaves of transgenic tobacco plants were infected with inoculum of *P. nicotianae* Breda, and those of soybean plants that have been tested through Southern hybridization were treated with *P. sojae* race 1 inoculum. The leaves were incubated in a mist chamber at 25 °C, with 90 % relative humidity under a 14-h photoperiod at the light intensity of 350 μmol m^{-2} s^{-1} for 72 h. Non-transformed leaves were used as controls. Disease symptoms on each leaf were observed and photographed using Canon IXUS 860IS camera at 96 h after inoculation.

Results

GmPR10 cDNA cloning and sequence analysis

The full-length cDNA sequence of *GmPR10* was assembled from the PCR-amplified 5′ and 3′ RACE products (using primers GmPR10-F and GmPR10-R, respectively), and confirmed through sequencing of a PCR product amplified from genomic DNA. Sequence data from this article have been deposited in GenBank under accession number FJ960440; ADC31789.1.

The full-length cDNA of *GmPR10* was 831 bp and contained a single ORF spanning 474 bp, flanked by 134 and 223-bp stretches at the 5′ and 3′-untranslated regions, respectively. The ORF encoded a 157-amino-acid polypeptide with a predicted molecular mass of 17 kDa and an isoelectric point of 5.22. The sequence of the *GmPR10* genomic DNA was also amplified and showed that encompassed ORF was the same as the full length of the *GmPR10* mRNA, indicating that there were no introns in the PR10 gene (data not shown). There is a striking conserved sequence motif GXGGXG at amino acid residues 46-51(Fig. 1, underlined in bold) of *GmR10*, known as the ''P-loop''(phosphate-binding loop), which is frequently observed in protein kinases and nucleotide-binding proteins [50] and presumed to function as a binding site for nucleotides involved in the RNase activity for some PR10 proteins [34]. To identify residues important for the biological function of the *GmPR10* gene product, NetPhos, a

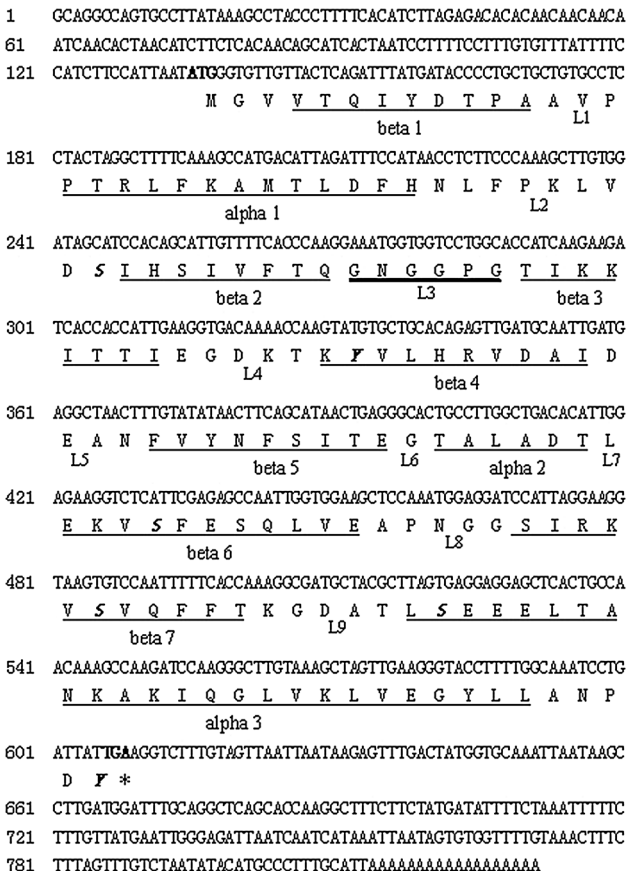

Fig. 1 Nucleotide and amino acid sequence of *GmPR10* cDNA. P-loop motif is *underlined in bold* and putative phosphorylation sites are marked *italically in bold*

web-based software (http://www.cbs.dtu.dk/services/NetPhos/), predicted four serine (Ser 37, 99, 117, and 129, in bold italic) and two tyrosine (Tyr 66 and 157, in bold italic) residues as potential phosphorylation sites (Fig. 1).

Sequence comparison showed that the putative soybean GmPR10 protein was homologous to PR10 proteins from other plants. Its deduced amino acid sequence displayed 89, 76, 72, 70, 70, 69, and 68 % similarity to *Betula platyphylla* (EU526262), *Cicer arietinum* (AJ4046414), *Retama raetam* (AF439272), *Lupinus luteus* (AF322226), *P. sativum* (X13383), *Pachyrhizus erosus* (AY433943), and *Medicago sativa* (AJ311050) (Fig. 2).

Transcript abundance patterns of *GmPR10* as induced by various stimuli

The tissue-specific transcript abundance of *GmPR10* was also determined using semi-quantitative PCR. Total RNA was isolated from different organs, including the leaves, roots and stems of 'Suinong 10' soybean seedlings. The results showed that *GmPR10* is constitutively and highly expressed in the leaves, followed by the roots and stems

Fig. 2 Alignments of amino acid sequences of GmPR10 with other plant PR10 proteins

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Cicer arietinum   MGVFTFEQETASTVPPAKLYKAMVKDADVIIPKAVDAIKTVETVEGNGGPGTIKKLTFVE  60
Glycine max       MGVVTQIYDTPAAVPPTRLFKAMTLDFHNLFPKLVDSIHSIVFTQGNGGPGTIKKITTIE  60
Lupinus luteus    MGIFTFEDESTTTVAPARLYKALVKDADTIIPKAVEAIQSVEIVEGNGGPGTIKKLTLIE  60
Medicago sativa   MGVINFEEETTSIVAPATLHKAFVTDADNLIPKVVHVIKSIDIVEGNGGSGTIKKLTFVE  60
M.truncatula      MGVFNFEDETTSIVAPARLYKALVTDSDNLIPKVIDAIQSIEIVEGNGGAGTIKKLTFVE  60
P.erosus          MGVFVFRDETSSSVAPAKLYKALTKDSDTIAQKIDGPIQSIELVEGNGGVGTIKKITANE  60
P.sativum         MGVFNVEDEITSVVAPAILYKALVTDADTLTPKVIDAIKSIEIVEGNGGAGTIKKLTFVE  60
Retama raetam     MGVFTFKEENVSPVAPAKLPKAFVKDSDTIIPKVVEPIQSIEIVEGNGGPGTVKKLTVVE  60

Cicer arietinum   GGQTLYVLHKIEAIDEANLGYNYSIVGGAGLSETVEKYHFEAKLCEGPNGGSIGKVSVKY  120
Glycine max       GDKTKYVLHRVDAIDEANFVYNFSITEGTALADTLEKVSFESQLVEAPNGGSIRKVSVQF  120
Lupinus luteus    GGETKYVLHKIEAIDEANFGYNYSIVGGIGLPDTIEKISFETKLFEGANGGSIGKVTIKI  120
Medicago sativa   GGETKYDLHKVDLVDDANWAYNYSIVGGDSLPDTVEKISFEAKLSAGPNGGSIAKLSVKY  120
M.truncatula      GGETKYDLHKVDIVDDVNFAYNYSIVGGGGLPDTVEKISFESKLSAGPDGGSIAKLTVKY  120
P.erosus          GDKTSFVLQKVDAIDEANLGYDYSIVGGTGLPESLEKLSFETKVVAGSGGGSISKVTLKF  120
P.sativum         DGETKHVLHKVELVDVANLAYNYSIVGGVGFPDTVEKISFEAKLSAGPNGGSIAKLSVKY  120
Retama raetam     GGKTSYVLHKIDAIDEANFGYNYSIIGGTGLEEILEKVTFETKLLPGPNGGSVGEVTVTY  120

Cicer arietinum   QTKGD.AKPNEKEVQEG.KAKGDALFKAIEGYVLANPNY                       157
Glycine max       FTKGD.ATLSEEELTAN.KAKIQGLVKLVEGYILANPDY                       157
Lupinus luteus    ETKGD.AQPNEEEGKAA.KARGDAFFKAIENYIIAHPEY                       157
Medicago sativa   FTKGD.ATPSEEELKSG.KAKGDGLFKALEGYCLANPDY                       157
M.truncatula      FTKGD.AAPSEEEIKGG.KARGDGLFKALEGYVLANPDY                       157
P.erosus          HTKGD..APLSDAVRDDALAKGAGFFKAIETYL                             151
P.sativum         YTKGDAAAPTEEQLKSD.KAKGDGLFKALERYCLAHPDY                       158
Retama raetam     HTKGD..APLSDEVREGSKAKGTALFKAVEDFVLANPDY                       157
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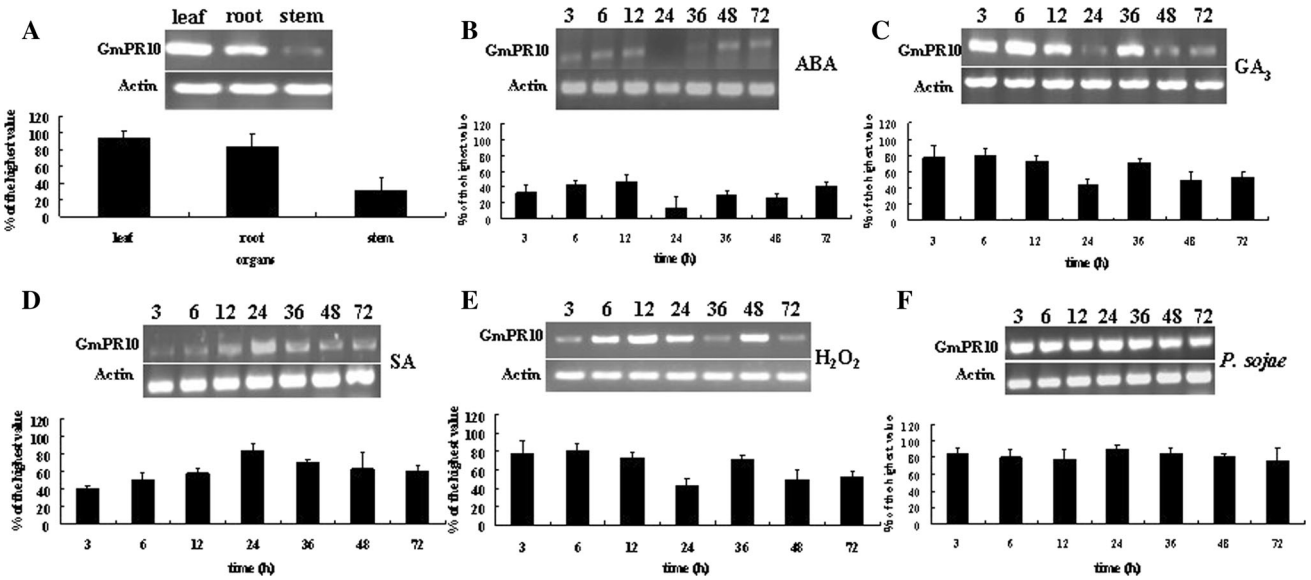

Fig. 3 Transcript abundance patterns of *GmPR10* under various conditions. Transcript abundance of *GmPR10* in **a** organs of sterile seedlings, **b** soybean seedlings treated with 50 mM ABA, **c** soybean seedlings treated with 250 mg L^{-1} GA_3, **d** soybean seedlings treated with 0.5 mM SA, **e** soybean seedlings treated with 200 mM H_2O_2, and **f** soybean seedlings infected with *P. sojae* race 1. Experiments were performed at least three times for each treatment and only representative results were presented

(Fig. 3a). The responses of *GmPR10* transcript abundance to phytohormone stresses depended on the treatments. Treatment with ABA (50 mM) on the leaves resulted in the gradual increase of *GmPR10* transcript abundance after 12 h, but the transcript abundance was reduced to its lowest at 24 h and subsequently increased from 24 to 72 h (Fig. 3b). The GA_3 treatment resulted in similar *GmPR10* transcript abundance patterns as the ABA treatment (Fig. 3b, c). In soybean leaves sprayed with 0.5 mM SA, the transcript abundance of *GmPR10* increased within 24 h and subsequently decreased between 24 and 72 h (Fig. 3d). The transcript abundance of *GmPR10* in response to H_2O_2 showed a steady increase within 12 h and decreased between 12 and 72 h, but the transcript abundance had a moderately quick increase after 48 h (Fig. 3e). The transcript abundance of *GmPR10* was also investigated after treatment with *P. sojae*. A significant induction of *GmPR10* expression was detected in the leaves 3 h after

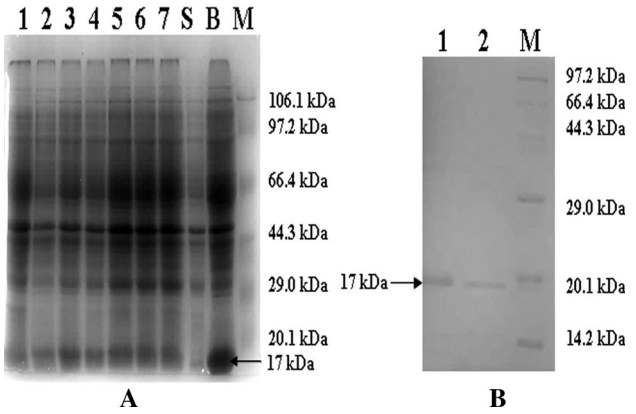

Fig. 4 Expression and purification of *GmPR10* from *E.coli* BL21 (DE3). **a** *Lanes 1–7*, *E. coli* BL21 containing vector pET-29b harboring *GmPR10* gene induced by IPTG from 1 to 7 h. *Lane S*, supernatant. *Lane B*, precipitation including inclusions. **b** Purification of recombinant GmPR10 protein from *E. coli* BL21 transformed with vector pET-29b containing the GmPR10. *Lanes 1* and *2*, purified recombinant GmPR10 protein. *Lane M*, protein marker

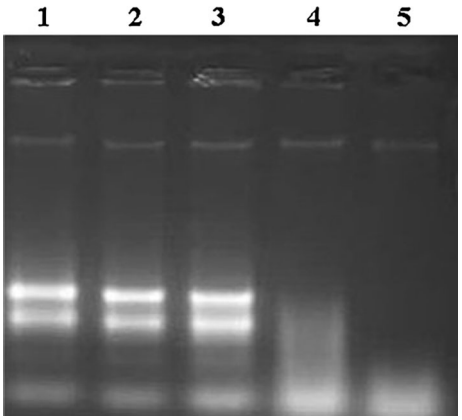

Fig. 5 Ribonuclease activity of the purified recombinant GmPR10 using 10 μg of total RNA from leaves of 'Suinong' 10 soybean. Gel electrophoresis was performed to separate hydrolyzed RNAs on a 1.0 % (w/v) agarose gel. Each reaction mixture containing recombinant GmPR10 protein (10 μg) and total RNA (10 μg) from soybean was incubated for 2–4 h at 37 °C. *Lanes 1–2*, RNA + elution buffer was investigated at 2 and 4 h, respectively. *Lane 3*, RNA + boiled dialytically renatured GmPR10 protein was investigated at 4 h. *Lanes 4–5*, RNA + dialytically renatured GmPR10 protein was investigated at 2 and 4 h, respectively

treatment, and the transcript abundance remained high up to 72 h (Fig. 3f).

Expression of *GmPR10* in *E. coli* and properties of GmPR10 protein

No expression of *GmPR10* was detected without induction using 1 mM IPTG, in all *E. coli* extracts with or without the pET-29b vector (data not shown). However, GmPR10 protein expression was remarkably enhanced from 1 to 7 h after IPTG induction (Fig. 4a, lanes 1–7), reaching maximum expression at 5 h (Fig. 4a, lane 5). The recombinant GmPR10 protein was expressed in the form of insoluble inclusion bodies (Fig. 4a, lane S, B). The purified recombinant protein migrated at 17 kDa in SDS-PAGE (Fig. 4b). The value was consistent with the predicted molecular weight calculated from the amino acid sequence.

Ribonuclease activity of recombinant PR10 proteins

To examine whether the GmPR10 protein possessed RNase activity, a RNA degradation assay was performed according to the method of Bantignies et al. [4], with some modifications. The total RNA isolated from leaves of 'Suinong 10' soybean was incubated with or without recombinant GmPR10 (Fig. 5). The elution buffer did degrade the total RNA (Fig. 5, lane 1, 2). The control sample, which was incubated with the boiled dialytically renatured *GmPR10* protein, did not show significant RNase activity against soybean RNA (Fig. 5, lane 3). However, when incubated with the dialytically renatured GmPR10 protein, significant RNase activity was clearly visible through the migration of the degradation products in the agarose gel (Fig. 5, lane 4, 5).

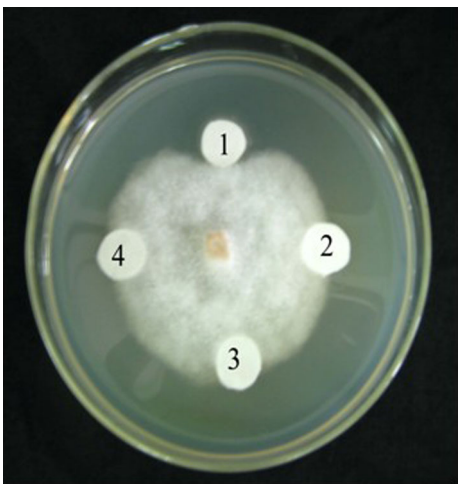

Fig. 6 Inhibition of *Phytophthora sojae* growth by recombinant GmPR10 protein. 1, 25 μg renatured recombinant GmPR10 protein. 2, 15 μg renatured recombinant GmPR10 protein. 3, 15 μL elution buffer. 4, 25 μg protein of *E. coli* transformed with pET-29b

In vitro antimicrobial activity of recombinant GmPR10 protein

To examine the antimicrobial activity effect of the recombinant GmPR10 protein on growth of *P. sojae*, paper filter discs containing recombinant GmPR10 proteins (15 or 25 μg) were placed near the tip of growing hyphae of *P.*

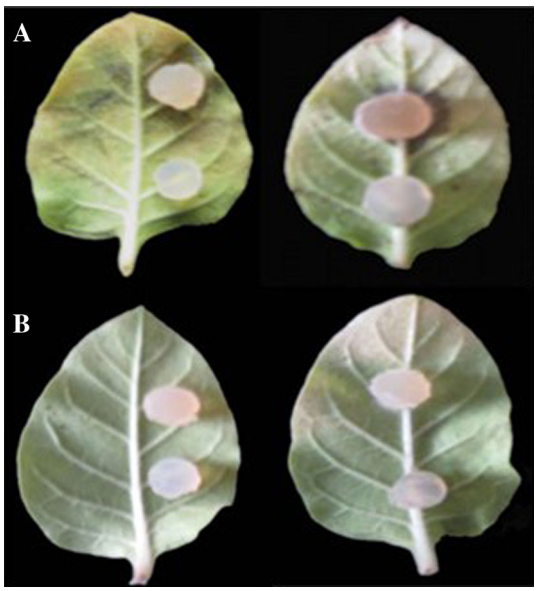

Fig. 7 Over-expression of *GmPR10* gene in tobacco leaves enhanced the resistance to *Phytophthora nicotianae* Breda. *Row A*, the leaves of non-transgenic tobacco at 72 h after infestation. The upper disc is culture medium with *P. nicotianae* and the lower disc is only the culture medium. *Row B*, the leaves of transgenic tobacco at 72 h after infestation. The upper disc is culture medium with *P. sojae* and the lower disc is the culture medium

sojae. After incubation, a 2–3 mm zone with inhibited hyphal growth was detected when 25 μg of recombinant protein was applied to the paper filter discs containing the recombinant GmPR10 protein (Fig. 6). A total of 15 μg of recombinant protein and a control paper filter disc containing elution buffer or the protein from *E. coli* transformed with pET-29b did not show the inhibition effect.

Enhanced resistance to *P. nicotianae* and *P. sojae* in transgenic plants

GmPR10 was over-expressed in tobacco and soybean to evaluate the antimicrobial activity of this protein. A total of 46 T_2 transgenic tobacco plants, confirmed through PCR, and 16 T_2 transgenic soybean plants, confirmed through PCR and Southern hybridization, were obtained. Of these plants, 10 transgenic tobacco plants and 10 transgenic soybean plants were selected at random to investigate the susceptibility or resistance to *P. nicotianae* or *P. sojae*, respectively. After 72 h incubation with *P. nicotianae* or *P. sojae*, a remarkable difference in the development of disease symptoms was observed between the transgenic and the non-transgenic tobacco and soybean plants, respectively. After 72 h incubation with *P. nicotianae*, severe symptoms (necrosis and chlorosis) around the infection areas were observed in non-transgenic tobacco plants (Fig. 7, row A), but the transgenic *GmPR10* tobacco plants showed almost no visible lesions (Fig. 7, row B). After

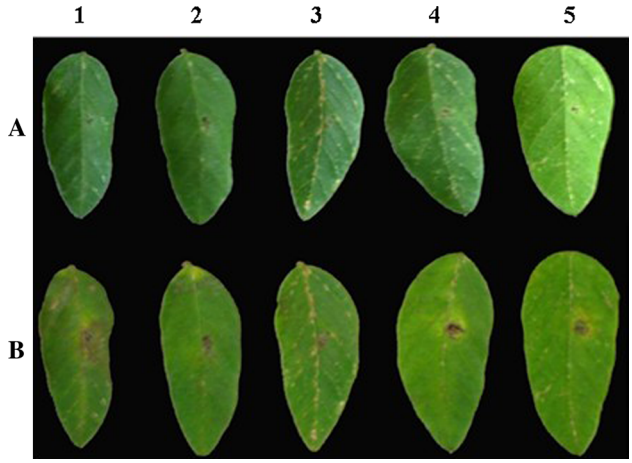

Fig. 8 Over-expression of the *GmPR10* gene in soybean leaves enhanced the resistance to *Phytophthora sojae*. *Row A*, soybean leaves 24 h after inoculation. *Row B*, soybean leaves 72 h after inoculation. *Lane 1*, leaves of non-transgenic soybean. *Lanes 2–5*, leaves of transgenic soybean

72 h incubation with *P. sojae,* the leaves of the non-transgenic soybean plants exhibited clear and large lesions compared with those of the transgenic plants (Fig. 8). These results indicate that the over-expression of *GmPR10* gene in tobacco and soybean plants improved resistance to *P. nicotianae* and *P. sojae*, respectively.

Discussion

PR10 is one of the most important families of PR proteins that play important roles in plant defense against microbial attack [60, 64]. In previous study, a novel up-regulated cDNA encoding a PR10 protein was screened in highly resistant soybean cultivar 'Suinong 10' [65]. Here, the isolation and characterization of the novel PR10 (termed *GmPR10*) gene and corresponding gene products from soybean (*Glycine max*) were performed in order to obtain a better understanding of the function of this protein in defense against *P. sojae*.

The sequence analysis indicated that GmPR10 contained no signal peptide, suggesting that GmPR10 is an intracellular protein located in the cytosol, similar to other intracellular pathogenesis-related proteins (IPR) of the PR10 family. Most intracellular PR10 genes possess introns and exons, but *GmPR10* lacked introns, similar to a subclass of the *Malus* PR10 family [20]. GmPR10 shared several conserved features of known IPR PR10 proteins, such as a small molecular mass, acidic pI, and putative phosphorylation sites. The predication of the three dimensional (3D) structure of GmPR10, based on the data from http://swissmodel.expasy.org/, showed that this protein had a long C-terminal α-helix (α3) wrapped in a seven-stranded anti-

parallel β-sheet (from β1 to β7) and two N-terminal short α-helices (α1 and α2), with nine connecting loops (L1–L9) (Fig. 1, underlined). Although the conserved P-loop motif (deduced to GxGGxG), which is the only highly conserved region across the PR10 family, is typically localized in the L4 region between β2 and β3, that of *GmPR10* was localized in the L3 region (Fig. 1, underlined in bold). Most PR10 genes are clustered on the chromosomes [20, 30], and the analysis of homologs of *GmPR10*, based on data obtained from the web (http://www.phytozome.net/soybean), indicated that a total of eleven genes were clustered on 8 linkage groups, namely three on Gm 09, two on Gm 17, and one each on Gm 01, 06, 07, 15, 17, 18, 19.

The transcript abundance of PR10 genes has been widely investigated in response to biotic and abiotic stresses. Early studies have reported that PR10 expression changes in response to viruses [42, 45], bacteria [8, 48], and fungus [26, 46, 57]. The transcript abundance of PR10 is also regulated through plant hormones and defense-related signaling molecules, such as JA [33, 38], ABA [61], GA_3 [35], SA [37], and H_2O_2 [35]. In the present study, the transcript abundance of *GmPR10* was induced by *P. sojae*, and the mRNA was maintained at high abundance during most of the infection process (Fig. 3f). The effects of ABA, GA_3, SA, and H_2O_2 on *GmPR10* expression were also investigated, and the mRNA abundance of *GmPR10* were gradually up-regulated during most of the 12 h of treatment. The transcript abundance of *GmPR10* was relatively low when treated with ABA compared with other treatments. These data suggested that *P. sojae*, plant hormones and defense-related signal chemicals might be involved in the signal transduction pathway, leading to *GmPR10* activation.

The antimicrobial activity conferred through PR10 proteins has been verified in Ocatin from *Oxalis tuberosa* [19], CaPR10 from *Capsicum annuum* [42], and SsPR10 from *Solanum surattense* [35] through in vitro microbe inhibition experiments. Further studies showed that the over-expression of PR10 genes in transgenic plants enhanced the resistance of potato to early dying disease [11] and Arabidopsis to *P. syringae* [64]. However, no enhancement of resistance have been observed for STH-2, a member of the Ypr10 family, in potato [12] and PR-10-1 in pea [55]. These differences might reflect the selectivity of the inhibition through the PR10 proteins [10]. In the present study, the antimicrobial activities of GmPR10 were evaluated both in vitro using purified PR10 proteins against *P. sojae* and in vivo through the inoculation of of transgenic tobacco and soybean plants over-expressing *GmPR10*. GmPR10 significantly inhibited the hyphae growth of *P. sojae*, and transgenic tobacco and soybean plants over-expressing the *GmPR10* gene showed higher tolerance to *P. nicotianae* and *P. sojae*, respectively. These results suggest that the enhanced resistance to plants in the tobacco and soybean plants might be associated with the transcript abundance of *GmPR10*.

Although the antimicrobial activities of *GmPR10* have been verified through in vitro and in vivo experiments, the resistance mechanism remains unknown. PR10 proteins with RNase activity might protect plants during programmed cell death near infection sites or act directly on the pathogens [34]. The P-loop motif is considered as a potential RNA phosphate-binding site involved in RNase activity for some PR10 proteins [4, 7, 31, 42, 57, 69]. In the RNA degradation assay, the recombinant GmPR10 protein showed significant ribonucleolytic activity, where total RNA was nearly degraded within 4 h of incubation (Fig. 5), implying that ribonucleolytic activity might be one of the important roles of this protein in the plant defense response to pathogen attack.

In conclusion, expression of a novel *GmPR10* gene isolated from 'Suinong 10' soybean was induced to high transcript abundance in the plant leaves infected with *P. sojae* and was also induced by GA_3, H_2O_2, SA, and ABA. The recombinant GmPR10 proteins showed RNase and growth inhibitory activities against *P. sojae*. The over-expression of *GmPR10* in tobacco and soybean plants enhanced the resistance to *P. nicotianae* and *P. sojae*, respectively. These results suggest that the GmPR10 protein plays an important role in the host defense against *P. sojae* infection.

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