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ORIGINAL ARTICLE

Molecular cloning, functional analysis and localization of a novel gene encoding polygalacturonase-inhibiting protein in *Chorispora bungeana*

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Abstract Polygalacturonase-inhibiting proteins (PGIPs) are plant defense proteins. To date, no spatial distribution of PGIPs and interaction between *PGIPs* and nitric oxide (NO) in plant were described. Here, we first reported the full-length cDNA sequence of *PGIP* of *Chorispora bungeana* (*CbPGIP1*). Notably, immunofluorescence localization showed that the CbPGIP was evenly distributed in leaves but it was mainly localized in epidermis and vascular bundle in stems and roots. Further studies indicated that CbPGIP had higher abundance in roots than in stems and leaves. Conversely, the bulk PGIP of *C. bungeana* showed a higher activity in leaves than in stems and roots. In addition, quantitative real-time polymerase chain reaction demonstrated that *CbPGIP1* expression was induced by *Stemphylium solani*, salicylic acid (SA), 4, −4°C and NO. This is a first report attempting to predict if NO can induce the *PGIP* expression. Taken together, these findings showed that the gene was spatially regulated and NO and SA might take part in *CbPGIP1* expression induced by biotic and abiotic stresses. This study highlighted the potential importance of *CbPGIP1* and NO in plant resistance.

Keywords *Chorispora* · Cold stress · Immunofluorescence · Nitric oxide · Polygalacturonase · Inhibiting protein

Abbreviations

BSA	Bovine serum albumin
JA	Jasmonate
LRR	Leucine-rich repeat
NO	Nitric oxide
PGA	Polygalacturonic acid
PGIPs	Polygalacturonase-inhibiting proteins
PGs	Polygalacturonases
SA	Salicylic acid

Introduction

During pathogenesis, the cell wall is the first barrier that plant cells use to oppose the attack of pathogens. Plant fungal pathogens have evolved a battery of hydrolytic enzymes to hydrolyze the plant cell wall during the early stages of pathogenesis (Bezier et al. 2002). Polygalacturonases (PGs) are the first enzymes to be secreted by pathogens when they encounter plant cell wall (Jones and Jones 1997). PGs degrade plant cell wall, which make other hydrolyzation enzymes to degrade cell wall more easily (Karr and Albersheim 1970). Poylgalacturonase-inhibiting proteins (PGIPs) are cell wall proteins that inhibit the activity of fungal PGs and retard the invasion of plant tissues by pathogens (Ferrari et al. 2006; Spadoni et al. 2006; Joubert et al. 2007; Cheng et al. 2008; Hegedus et al. 2008; Janni et al. 2008; Protsenko et al. 2008).

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PGIPs are leucine-rich repeat (LRR) proteins, which are associated with numerous dicotyledonous plants as well as in the pectin-rich monocotyledonous plants (Di Matteo et al. 2006; Cheng et al. 2008). PGIPs and the products of many plant resistance genes share a LRR structure, and the plants use the LRR motif for their "immune" function and recognition of non-self-molecules (Jones 2001; De Lorenzo and Ferrari 2002; Di Matteo et al. 2003). In addition, signal transduction in plants toward activating defense genes is mainly accomplished by LRR regions in resistance genes (Shanmugam 2005). Several reports show that PGIP effectively and specifically bind to and inhibit PGs of fungi and insects, which are important fungal virulence factors (D'Ovidio et al. 2004). Although the high induction of the PGIP after inoculation with *Botrytis cinerea* (*B. cinerea*) indicates that PGIP has a role in disease resistance (Mehli et al. 2005; Schaart et al. 2005), the direct role of PGIP in plant defense has been demonstrated by transgenic plants over-expressing PGIP gene (Di et al. 2006; Richter et al. 2006; Janni et al. 2008). Thus, in transgenic tomato plants expressing the pear fruit PGIP, the growth of *B. cinerea* was reduced, and tissue breakdown was diminished by as much as 15% (Powell et al. 2000). In addition, Ferrari et al. (2003) showed that both AtPGIP1 and AtPGIP2 encode functional inhibitors of PG from *B. cinerea* and their over-expression in *Arabidopsis* significantly reduces the disease symptoms. Janni et al. (2008) also showed that wheat-expressing bean PGIP increased the disease. Now it is apparent that PGIP does play a crucial role in the resistance to infection by fungal pathogens.

Abiotic stresses, such as SA, low temperature, jasmonate (JA), and oligogalacturonides, can also induce the PGIP expression (Li et al. 2003; Di et al. 2006; Hegedus et al. 2008). For example, in *Arabidopsis*, cold induces the expression of *AtPGIP1* but not *AtPGIP2* (Ferrari et al. 2003). In peach, *PGIP* expression can be induced by exogenous SA (Liang et al. 2005), which is also an important component of the susceptible disease response (O'Donnell et al. 2001). In addition, nitric oxide (NO) is a signal molecule mediating responses to biotic and abiotic stresses in the plant. This molecule has also been involved in the response to drought, heat, UV and salt stress and in disease resistance (Lamotte et al. 2005; Xue et al. 2005; Mur et al. 2006). Although these studies have established that NO does play a role in resistance, the relationship between NO and the PGIP gene expression was not established.

Chorispora bungeana (*C. bungeana*; family Brassicaceae) grows in the freeze–thaw tundra in border of glaciers where almost all the other flowering plants have great difficulty to grow. The perennial herb is mainly distributed in an ice-free cirque (with a 3,800–3,900 m height above sea level) beside the Glacier No. 1 in the source area of Urumqi River in Tianshan Mountains, Xinjiang province, China. It is a representative alpine subnival plant which can survive under frequent temperature fluctuations, nearly 4 to −10°C during favorable growth season from June to September (Zhang et al. 2006). Previous research showed that *C. bungeana* did not possess special morphological characteristics that helped to survive under freezing environment. Recently, some work had been done to explore its special cold-hardiness mechanism (Zhang et al. 2006). Molecular and physiological mechanisms were assumed to further account for its adaptation to the freezing environment.

However, there is little known about the PGIP's spatial distribution and its relationship with NO in plants. So, the aims of this project are sought to clone the full-length of *CbPGIP1* cDNA, determine the spatial distribution of CbPGIP and characterize its role in the defense of *C. bungeana* against *Stemphylium solani* (*S. solani*) and cold. The results obtained in this study demonstrated that CbPGIP1 may play a prominent role in plant biotic and abiotic resistance. These results will provide useful insight for understanding the diverse role of CbPGIP1 and its signal transduction in plant.

Materials and methods

Plant material

The wild species of *C. bungeana* (*Chorispora bungeana* Fisch. and C.A. Mey) were collected from an ice-free cirque (43°05′N, 86°49′E, with an altitude of 3,800–3,900 m) near the No. 1 glacier in the source area of Urumqi River in Tianshan Mountains, Xinjiang, China. The leaves of *C. bungeana* without serrate margins were selected for calli induction. The sterile leaves were cut into 10 mm segments, with margins being finely cut off, and cultured on MS basal medium containing 4 mg l^{-1} gibberellic acid, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg l^{-1} 1-naphthaleneacetic acid (NAA) with a 12-h long light period (4,000 lx) under 20°C. The medium was refreshed every 2 weeks. Cultured cells of *C. bungeana* were cultivated in MS medium containing 0.2 mg l^{-1} 2,4-D, 0.2 mg l^{-1} NAA, 0.2 mg l^{-1} 6-benzylaminopurine and 0.2 mg l^{-1} kinetin. After five or more subcultures, the synchronous steady cultures were collected and used in the following experiments.

Stress treatment

For cold treatment, calli of *C. bungeana* were taken at 4 or −4°C, respectively. For disease treatment, the calli were inoculated with 5×10^4 conidial spores of *S. solani* strain

SS31 per milliliter. In addition, 0.1 mM salicylic acid (SA; Sigma, St. Louis, MO, USA) and 0.2 mM sodium nitroprusside (SNP, a NO-releasing substance; Sigma) solution were applied to the calli. As controls, the calli were mock-inoculated in the same way with sterile water. The calli treated were harvested at 0, 6, 12, 24 and 48 h, respectively, immediately frozen in liquid nitrogen and stored at −80°C for further analysis.

RACE and the full-length cDNA cloning of *CbPGIP1*

Two primers, P1 (5′-ACCCTCCTCAAGATCAAGAAA-3′) and P2 (5′-CCACACAGTYTGTTRTAGCTAAC-3′) (where Y is A and T; R is A and G), which correspond to sequences of the conserved regions of PGIP genes were amplified by polymerase chain reaction (PCR). Total RNA samples were isolated from *C. bungeana* calli using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription-PCR product of 803 bp was cloned into pMD18-T vector (Takara, Kyoto, Japan) and sequenced. RACE was carried out using the GeneRacer kit (Clontech, Palo Alto, CA, USA). PCR amplifications were conducted using the GeneRacer 5′ or 3′ primer and the 5′ or 3′ gene-specific primers (gsp). Nested PCR amplifications were carried out using the GeneRacer 5′or 3′ nested primer and the 5′or 3′ nested gsp. The amplification products were gel-purified, cloned and sequenced. The gsp used in the RACE were designed from the 803 bp fragment sequence of *C. bungeana*, including 5′-RACE first gsp (5′-TCGGGAGGGATC TGAGCGGA-3′), 5′-RACE second gsp (5′-AGGTCCAG GAGCAGCAGTCTTCT-3′), 3′-RACE first gsp (5′-CATG TTCCAGTTCGATATCTCAGAGGT-3′), 3′-RACE second gsp (5′-GACTTGAACCACAATGGGATCACTG-3′). By comparing and aligning the sequences of 3′-RACE, 5′-RACE and the middle region products, the full-length cDNA sequence of *CbPGIP1* was obtained, which was amplified and sequenced. The PCR amplification for the full-length *CbPGIP1* was repeated three times. The gene was subsequently analyzed for molecular characterization such as the presentation of conserved motifs, sequence similarity, and secondary structure.

The sequence analysis was aligned using Clustal X1.83 (Thompson et al. 1994). All parameters were default values of the software. Neighbor-joining (NJ) and minimum evolution method analysis were carried out using MEGA3.1 with the following settings: two-parameter method was used to calculate the genetic distance matrix and construct the neighbor-joining tree and minimum evolution analysis. Gaps and missing data were completely deleted. Confidence limits for branches of the trees were performed by bootstrap analysis with 1,000 replications. The LRR motif of CbPGIP1 was predicted by the Motif Scan Server (http://scansite.mit.edu/motifscan_seq.phtml).

Fluorescence microscopy

Chorispora bungeana young leaves, stems and roots were collected and cut into pieces, then immediately fixed in 4% formaldehyde solution and then embedded in paraffin. After fixation and embedding, tissue sections (10 μM) were cut with a microtome (Leica, Wetzlar, Germany), placed onto poly-lysine at 37°C. Samples were deparaffinized by washing in Xylene series and rehydrated in an ethanol series, washed in PBS, and blocked with a 5% bovine serum albumin (BSA) solution in PBS. Then, sections were incubated with undiluted primary antibody overnight at 4°C, using a coverslip and a humid chamber. Rabbit polyclonal antibodies raised against a 108-residue peptide of AtPGIP1 were used as primary antibody. After several rinses in PBS, samples were washed in a solution of 0.1% BSA, 0.05% Tween-20 in PBS, and incubated for 2 h at room temperature with FITC-conjugated goat anti-rabbit IgG antibody used as secondary antibody (Molecular Probes, Eugene, OR, USA) diluted 1:3,000 in PBS (0.1% BSA in PBS). To eliminate background, slides were rinsed several times in PBS, and washed moderately in 0.1% BSA, 0.05% Tween-20 in PBS three times for 10 min at room temperature. Samples were stored at 4°C until observation. Immunofluorescence analysis was performed on 10 μm semi-thin section of leaves, stems and roots of *C. bungeana*. For detection, samples were examined with a Leica TCS-NT confocal laser microscope. In the confocal system, Alexa fluorescence was excited at 488 nm and emission read in the 515–545 nm interval with a green filter (BP530/30). Controls were made by replacing primary antibody with PBS. Besides, we have used Image J for fluorescent signal analysis as well.

Anti-AtPGIP1 rabbit polyclonal antibody was made against the N-terminal peptide containing 108 amino acids of AtPGIP1. In this study, the histidine-tagged fusion protein was induced by IPTG and identified by Western-blot analysis (not shown). Even though the signals that we detect belong to CbPGIP1, we need to take into account that we might be detecting other members from the same protein family as well. However, blast analysis showed no further PGIPs in *C. bungeana* matching the antigen amino acid sequence. Till now it is unknown which other family members, apart of CbPGIP1, exist in the plant. Furthermore, amino acid sequence of CbtPGIP1 is 93.52% identical with the antigen of AtPGIP1 (Fig. S3). The antibody is specific for the CbPGIP1, so it is reasonable to use the Anti-AtPGIP1 rabbit polyclonal antibody to detect the CbPGIP1 in immunofluorescence and Western-blot analysis. Additionally, other CbPGIPs might cross-react with the antibody, so we used the antibody to detect CbPGIP distribution and activity.

Western-blot analysis

Total proteins were prepared by homogenizing tissues (2 ml g^{-1} tissue) in 2–5 volumes of 1 M NaCl, 0.1 M sodium acetate, pH 6, 1% polyvinylpyrrolidone-40, 0.2% NaBisulfite, and homogenates were incubated for 1 h at 4°C and centrifuged for 15 min at 15,000*g*. The supernatant was precipitated with two volumes of cold acetone. Protein concentration was determined in parallel by the method of Bradford (1976). Approximately 50 μg of total proteins was analyzed by 15% SDS–PAGE for Western-blot analysis and activity assay.

The supernatant mentioned above was examined by SDS–PAGE and protein in the gel was blotted electrically to nitrocellulose membranes. Membrane was blocked with PBS (25 mM Tris–HCl, pH 7.5 and 137 mM NaCl) containing 5% non-fat dry milk for at least 2 h and incubated with affinity-purified polyclonal AtPGIP antibody (1:1,000) for 2 h in PBS containing 1% non-fat dried milk. After multiple washes with PBS containing Tween-20 in PBS, bound antibodies were detected with peroxidase-conjugated secondary antibodies.

CbPGIP activity assay

The *S. solani* PG was prepared according to Berger et al. (2000). CbPGIP1 activity in *C. bungeana* was analyzed by the radial diffusion assay (Taylor and Secor 1988). An agarose sheet was prepared by pouring 40 ml melted 1% agarose, 0.1 g l^{-1} polygalacturonic acid (PGA; Sigma), 3.65 g l^{-1} EDTA, and 0.2 g l^{-1} Na-azide in 0.1 M sodium acetate buffer (pH 5.0) into a 10 cm × 8 cm Plexiglasframe. When the agar was solidified, 4-mm diameter wells were cut in the agar sheet using cup-plate. Proteins were extracted as mentioned above. Sample mixtures of 100 μl total volume (40 μl bulk PGIP or boiled PGIP) mixed with 60 μl of PG from *Aspergillus niger* (*A. niger*; Sigma) or *S. solani* were loaded into wells. The plate was incubated for 24 h at 37°C and dyed with Ruthenium red (Ru-red; Sigma). After 1-h staining period, the plate was rinsed with distilled water several times over 1 h to remove excess (i.e., unbound) dye. PGIP can inhibit the PG activity, so the activity of PGIP can be analyzed by the clear halo consequence of PGA degradation by PG activity.

Quantitative real-time PCR

RNA extraction and cDNA synthesis from *C. bungeana* material were performed as described in the kit of Quantitative real-time PCR (Q-PCR; Takara, Otsu, Shiga, Japan). Q-PCR was performed on an iCycler iQ™ Real-Time PCR system, using SYBR green method (Takara). For detection of *CbPGIP1*, forward 5′-GATGCTTCGATGTTGTTTG GT-3′ and reverse 5′-ATTCCCAGTGATCCCATTGTG-3′ *CbPGIP1* primers were designed (product size 144 bp). For detection of a housekeeping actin gene (GenBank Accession No.AY825362), forward 5′-atacgctcttccacacgctattc-3′ and reverse 5′-tcacgatttcacgctctgct-3′ primers were designed (product size 125 bp). Each PCR reaction (20 μl) contained 200 nm primer and 0.5 μl cDNA. The thermal cycling conditions were 95°C for 10 s followed by 95°C for 5 s, 60°C for 20 s for 40 cycles, followed by melt cycle from 50 to 96°C. *CbPGIP1* was monitored by Q-PCR at 0, 6, 12, 24 and 48 h. Melting point analysis of the *CbPGIP1* RT–PCR and actin gene products resulted in a single peak in all samples, indicating the presence of a single PCR product. Data are represented as mean ± SD of the values obtained from triplicate experiments.

Results

Cloning and analysis of CbPGIP1

The cloned full-length *CbPGIP1* cDNA from *C. bungeana* was 1,203 bp with a poly A tail of 22 bp (Fig. 1). A *CbPGIP1* contained a 999-bp ORF, which codes for 332 residues of a deduced amino acid sequence with a calculated molecular weight of about 36.9 kD and with an isoelectric point (p*I*) of 6.93 (Figs. 1a, S1). The protein exhibited closest sequence similarity to a subgroup of AtPGIP1 and we thus referred to it as CbPGIP1 (GeneBank accession number: EU441203). Based on Signal P-HMM (Hidden Markov Models) and Signal P–N, N (neural networks) prediction to N-terminal peptides, CbPGIP1 has an N-terminal signal peptide of 23 amino acids (Fig. 1a).

The deduced amino acid sequence of CbPGIP1 gene revealed a high degree of similarity with previously isolated PGIPs and contains features characteristic of PGIPs in other plants, including the presence of nine highly conserved cysteine residues, four potential *N*-glycosylation sites (N108, N132, N240 and N293) and nine potential phosphorylation sites (Fig. S1). Prediction of structural analysis showed that the full-length cDNA of *CbPGIP1* contains one N-terminal LRR repeat and a set of eight LRR repeats (Figs. 1a, S2). The LRR domain is often flanked by cysteine rich domains (Fig. S1). The secondary structure of the *CbPGIP1* showed that α-helix and random coil constituted interlaced domination of the main part of the secondary structure (Fig. 1b). Furthermore, the prediction structure of CbPGIP1 showed typical curved and elongated shape (Fig. 1b), which is a typical PGIP shape. As shown in Fig. 1, the central LRR domain was folded in a right-handed superhelix (residues 29–310), consisting by one N-terminal LRR repeat and a set of eight LRR repeats. The

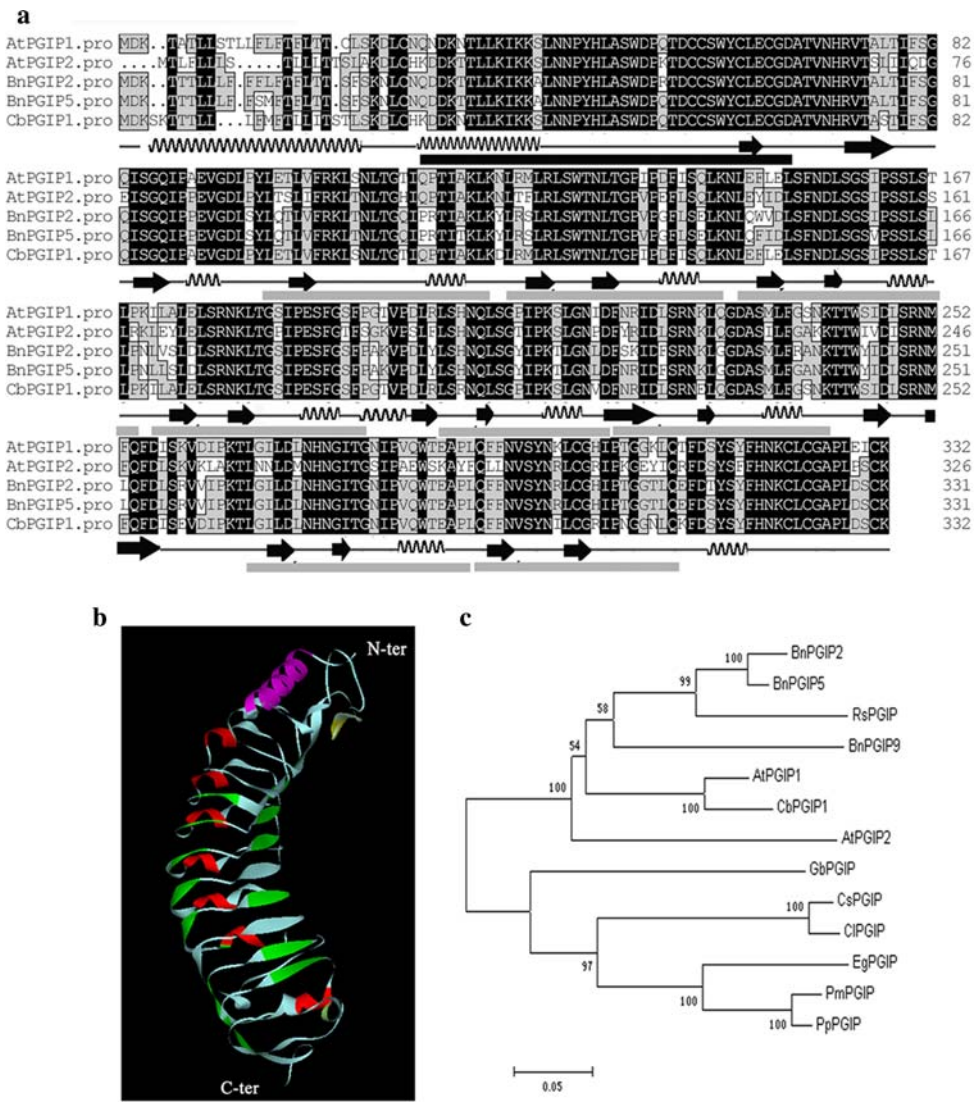

Fig. 1 Multiple alignments, analysis of amino acid sequence, predicted structural model and phylogenetic tree of CbPGIP1. **a** Multiple alignment and analysis of amino acid sequence of CbPGIP1. *Black* and *gray boxes* indicate highly conserved and partially conserved amino acid, respectively. The secondary structure of the CbPGIP1 was predicted by Predict Protein Server (http://bubic.bioc.columbia.edu/pp/), and the amino acid sequence alignment was performed by DNAMAN software (prediction accuracy = 87.52%). A *black bar*, a *gray bar*, *broad arrows* and revolving lines under the sequences denote the one N-terminal LRR repeat, eight LRR repeats, β-sheets and α-helices, respectively. **b** Ribbon representation of predicted structure and secondary structure organization of the LRR motif. Predicted structural model of the CbPGIP1 (residues 25–331) are produced by the Swiss-Model program (http://www.expasy.ch/) and then colored with DS Visualizer software (version 1.5; Accelrys). β-sheets in LRR are colored *green*, and α-helices are colored *red* (*light red* for the N-terminal α-helix and *red* for α-helices in the LRR). **c** Phylogenetic tree showing the close relationship of the different PGIP sequences and CbPGIP1 sequence. A phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal X1.83 using MEGA3.1

residues of the LRR motif contributing to form the secondary structure elements are colored green (β-sheets) and red (α-helices) (Fig. 1b). Comparison of the residues, which can form sheets and helices in LRR motif, revealed that the residues of sheets were more conserved than the residues of helices. LRR domain can form β-sheet/β-turn motif, and is critical for its affinity and specificity for the PG ligands (Leckie et al. 1999). Compared to AtPGIP1, there was one amino acid substitution in CbPGIP1 which caused the 1-LRR repeat lost (L75 → S77) (Fig. 1a).

In addition, the sequences were aligned using Clustal X1.83 (Thompson et al. 1994). Comparison of the predicted protein sequences of the CbPGIP1 with other plant PGIPs shows that CbPGIP1 is the most similar to the AtPGIP1 (92%), BnPGIP5 (81.1%), BnPGIP2 (79.6%) and AtPGIP2 (75.7%) (Fig. 1c).

Localization analysis of CbPGIP in leaves, stems and roots by immunofluorescence

The localization of CbPGIP in leaves, stems and roots was assessed by the indirect immunofluorescence. CbPGIP is evenly distributed in leaves, but it is mainly localized in epidermis and vascular bundles in stems and roots (Fig. 2d–f). The fluorescence signal of control was nearly absent in stem, but not in leaf and root (Fig. 2a–c). The autofluorescence might result from other substances in the mature tissues. We used the Image J software to analyze the fluorescence of these tissues. The fluorescence in leaf was 1.3 times and fluorescence in root was 1.5 times higher than control (Fig. S4). The background of stem was nearly absent and the fluorescence was 1.8 stronger than control (Fig. S4). Moreover, compared with control fluorescence, the vascular bundles fluorescence was almost 1.6 times and epidermis fluorescence was approximate 2.5 times higher (data not shown).

C. bungeana PGIP expression in leaves, stems and roots

To explore the constitutive activity of PGIP in *C. bungeana*, the PGIP expression was determined by radial diffusion assay and Western-blot analysis. The ability of PGIP to interact with the fungal PG-S (PG from *S. solani*) and PG-A (PG from *A. niger*) was initially verified by cup-plate assays. The clear halo as a consequence of PGA degradation by PG-A is greater than the PG-S (Fig. 3). When bulk PGIP protein from leaves, stems or roots were added, it was observed that the clear halo as the result of PGA degradation by PG-A and bulk PGIP protein was much smaller than after the use of PG-A alone (Fig. 3a) and also smaller after the use of PG-S alone (Fig. 3b). Based on the appearance of the clear halo as a consequence of PGA degradation by PG, it is concluded that PGIP of *C. bungeana* in leaves, stems and roots can inhibit the activity, but the activity of PGIP in roots is higher than in stems and leaves.

It has been previously demonstrated that the bulk activity extracted from plant tissues correspond with the activity of one PGIP (De Lorenzo et al. 2001). Since there is no evidence for only one CbPGIP1 in the plant, we performed Western-blot analysis using specific antibody in order to analyze the constitutive expression level of CbPGIP in leaves, stems and roots. Results indicated that CbPGIP is expressed in all tissues, including leaves, stems and roots (Fig. 3c), whereas CbPGIP showed higher abundance in leaves than stems and roots (Fig. 3c).

CbPGIP1 expression is induced by *S. solani*, 0.1 mM SA, cold stress and 0.2 mM NO

In order to verify whether the CbPGIP1 gene undergone a different regulation following pathogen infection and 0.1 mM SA, cold stress and 0.2 mM SNP application, we performed Q-PCR analysis using primers specific for the gene. Transcript analysis was performed on cDNA synthesized from RNA isolated from calli treated by different stresses at different time. Conditions were optimized to show induction relative to basal levels (time 0). Here, Q-PCR assays demonstrated that *CbPGIP1* was expressed in untreated calli and was induced in treated calli (Figs. 4, 5).

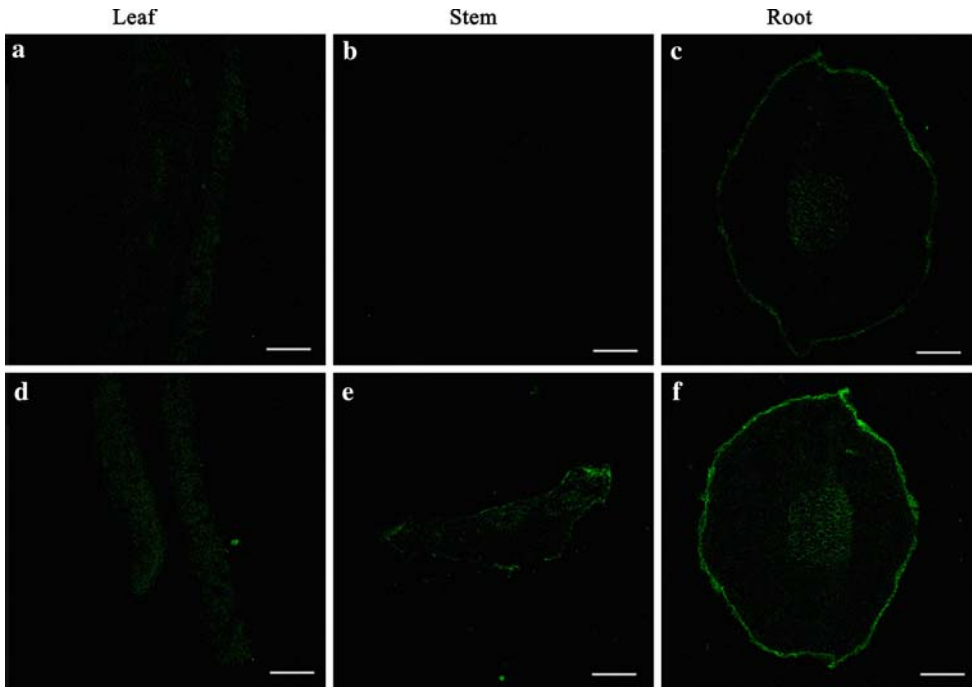

Fig. 2 Localization of CbPGIP in leaves, stems and roots. **a–c** Transverse sections of leaves, stems and roots are negative controls, in which anti-AtPGIP1 antiserum was omitted (*scale bars* 200 μm). **d–f** Immunofluorescence analysis reveals spatial distribution of CbPGIP1. Transverse sections of leaves, stems and roots were treated with anti-AtPGIP1 antiserum and visualized by fluorescent anti-rabbit IgG-FITC conjugate (*scale bars* 200 μm). **d** The fluorescence of the antibody-labeled cells is localized evenly. **e**, **f** The fluorescence of the antibody-labeled cells is mainly localized in epidermis and vascular bundles

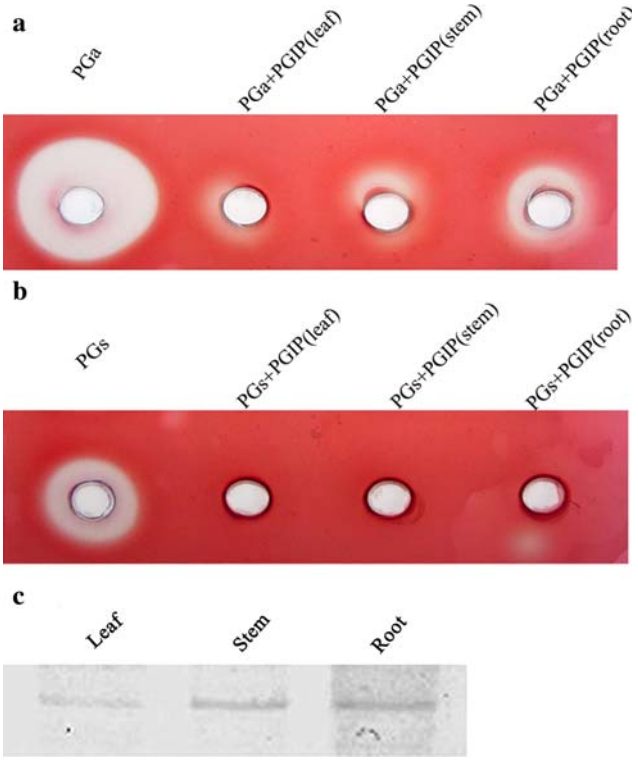

Fig. 3 Differential CbPGIP expression in leaves, stems and roots. 50 μg total proteins was loaded in each well for the activity assay and each lane for Western-blot analysis. **a**, **b** Bulk PGIP activity in different organs was analyzed by radial diffusion assay. The controls show fungal PG-A and fungal PG-S activity degrading the PGA and without any plant extracts. The CbPGIP activity in different organs was represented by PG-A and fungal PG-S activity degrading the PGA and with plant extracts. **c** Comparison of spatial distribution content of CbPGIP in different organs by Western-blot analysis. *Lane 1* leaves, *lane 2* stems, *lane 3* roots

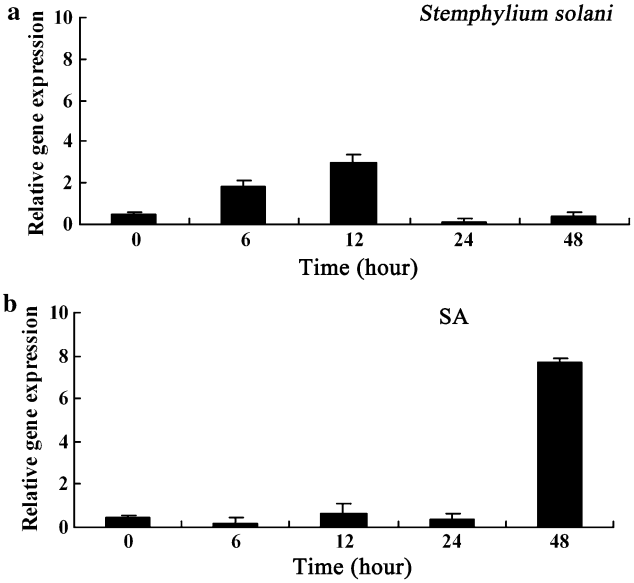

Fig. 4 Differential *CbPGIP1* expression in response to *S. solani* infection and 0.1 mM SA application. **a**, **b** *CbPGIP1* gene is induced by *S. solani* infection and 0.1 mM SA application by Q-PCR analysis at different times after stresses application, using gene-specific primers. The assays were performed on cDNA synthesized from RNA isolated from calli induced by *S. solani* and SA at different times. Conditions for Q-PCR were optimized to show induction relative to basal levels (time 0). The *CbPGIP1* gene expression level of 0 h is control. Data are represented as mean ± SD of the values obtained from triplicate experiments

Both the *S. solani* infection and 0.1 mM SA treatment caused a differential transcript accumulation of the *CbPGIP1*. The *CbPGIP1* gene transcript level rapidly increased and reached the maximum peak at 12 h following the pathogen infection (Fig. 4a). Then the *CbPGIP1* gene transcript quickly decreased and the level was back to the basal point. As shown in Fig. 4b, *CbPGIP1* gene transcript increased to maximum at 48 h following 0.1 mM SA treatment. Its accumulation was evident at a late stage of 0.1 mM SA treatment (48 h). Conversely, its accumulation was at an early stage of fungal infection (12 h).

Both the 4 and −4°C stresses caused a differential transcript accumulation of the *CbPGIP1*. The 4°C stress caused *CbPGIP1* transcript induction at 12 h following stress treatment, whereas the −4°C stress caused *CbPGIP1* transcript induction at 6 h following stress treatment (Fig. 5a, b). However, the maximum of *CbPGIP1* transcript level induced by 4°C was higher than the gene transcript level induced by −4°C.

In addition, NO is an important endogenous signal also mediating the transcriptional activation of CbPGIP1. In the study, 0.2 mM SNP application caused transcript accumulation of the *CbPGIP1*. The *CbPGIP1* gene transcript level rapidly increased and reached the maximum peak at 6 h following the NO treatment (Fig. 5c).

Discussion

We have isolated and characterized CbPGIP1 from *C. bungeana*. Its identity was confirmed by structural data, functional analysis and its close sequence similarity with other PGIPs. Here, the residues of *CbPGIP1* were analyzed. Compared to AtPGIP1, there was one amino acid substitution in CbPGIP1 which caused the 1-LRR repeat lost. Comparing to AtPGIP1 containing ten LRR repeats, CbPGIP1 contained one N-terminal LRR repeat and a set of eight LRR repeats, which would be involved in the function of *CbPGIP1* to biotic and some abiotic stresses. In plants, LRR acts as a prominent defense reactive molecule (Federici et al. 2006; Bonivento et al. 2008; Cheng et al. 2008) and plays a relevant role in both, resistance and development (Jang et al. 2003; Li et al. 2003). Furthermore, signal transduction in plants toward activating defense

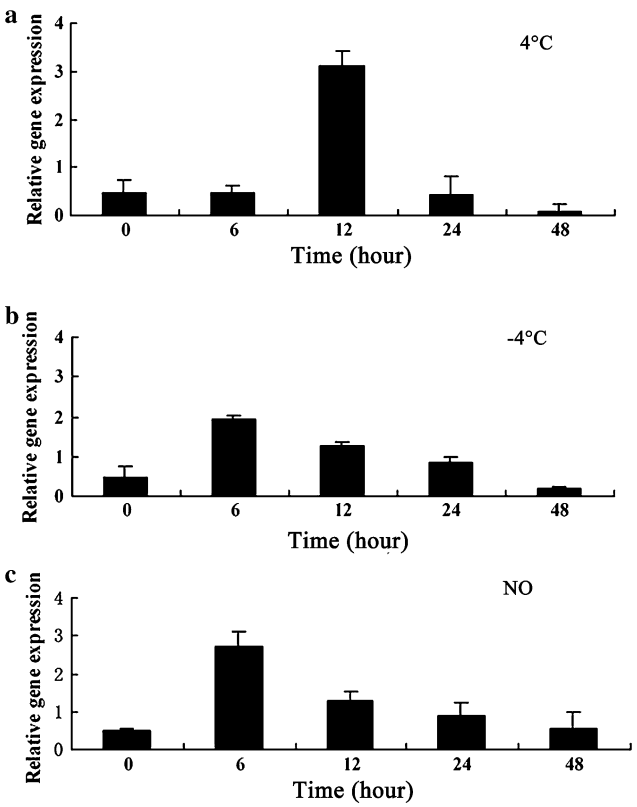

Fig. 5 *CbPGIP1* gene expression induced by cold and 0.2 mM SNP application. The assays were performed on cDNA synthesized from RNA isolated from calli induced by abiotic stresses. Different *CbPGIP1*expression was analyzed by Q-PCR analysis at different times. Conditions for Q-PCR were optimized to show induction relative to basal levels (time 0). **a** 4°C, **b** −4°C, **c** 0.2 mM SNP application. Data are represented as mean ± SD of the values obtained from triplicate experiments

genes is mainly accomplished by LRR regions in resistance genes (Shanmugam 2005). For example, CbPGIP1 with one N-terminal LRR repeat and a set of eight LRR repeats is induced by SA, but not in *AtPGIP1* with ten LRR repeats (Ferrari et al. 2003). A gene with a different LRR region is differentially regulated by a signal molecule.

Previous studies have shown that PGIP expression is associated with various plant organs, suggesting that the spatial distribution plays a critical role in biotic resistance (De Lorenzo and Ferrari 2002). Some reports showed that PGIPs are expressed predominantly in fruit tissues of tomato, apple, raspberry and strawberry (Di et al. 2006). Moreover, some reports indicated that PGIPs of soybean are highly expressed in leaves, but reach the highest levels in cotyledons and the lowest levels in roots and hypocotyls (Favaron et al. 1994). Our results showed that the CbPGIP expression in leaves was evenly localized in all tissues but CbPGIP in stems and roots was mainly localized in the epidermis and vascular bundle. This spatial distribution might be related with its role in disease resistance. On the one hand, the epidermis is the first line of plant resistance. On the other hand, the pathogen can infect other parts of the plant through the vascular bundle. So the spatial distribution of CbPGIP would restrict the pathogen invasion and limit the disease infection area. Additionally, Western-blot analysis with a specific antibody has shown that CbPGIP has higher abundance in roots than stems and leaves.

To further illustrate the relationship between the spatial distribution of *C. bungeana* PGIP and disease resistance, we have analyzed the PGIP activity from different tissues by an agarose diffusion assay. The result indicated that bulk PGIP extracted from leaves, stems and roots all inhibited both purified *S. solani* and *A. niger* PG. The enzymatic activity was estimated from the clear halo consequence of PGA degradation by PG. The results of bulk PGIP activity of different tissues are not consistent with the Western-blot analysis. Bulk PGIP showed higher activity in leaves than in stems and roots. The differences found between the two assays might be due to several reasons. First, protein abundance is not directly correlated with protein activity, since protein activity is often determined by post-transcriptional modification such as phosphorylation. Second, the clear halo observed in the agarose assay might be due to the activity of other members from the same protein family. Third, the discrepancy between activity and intensity in the Western-blot analysis might also be due to the presence of CbPGIP that cross-reacted with the antibody but did not inhibit the two PGs used in the assay. At least, one PGIP of the PGIP family in *C. bungeana* can interact with PGs. In further work we will study the PGIP family in *C. bungeana* and the function of individual *PGIP* genes.

PGIPs may be essential to the general resistance to biotic stresses (Albersheim and Anderson 1971; Di et al. 2006), since PGIPs are induced by biotic stress in bean (Bergmann et al. 1994), apple (Yao et al. 1999), soybean (D'Ovidio et al. 2006), strawberry (Mehli et al. 2005; Schaart et al. 2005), *Arabidopsis* (Ferrari et al. 2003, 2006), peach (Liang et al. 2005) and *Brassica napus* (Li et al. 2003). In our study, the *CbPGIP1* gene transcript was notably induced by *S. solani*. High up-regulation of *CbPGIP1* might provide protection of disease. Interestingly, a carrot antifreeze protein showed a high degree of identity to PGIPs (Worrall et al. 1998). Ferrari considered that low temperature like other stresses can increase susceptibility to diseases, so cold induction of defensive proteins might provide protection from infections (Ferrari et al. 2003). *C. bungeana* is a representative alpine subnival plant, so it is an ideal plant to illuminate how cold regulates the *CbPGIP1* gene expression. In this work, the *CbPGIP1* gene was quickly induced in −4°C at 6 h, whereas in 4°C, induction of *CbPGIP1* was postponed, the maximum peak occurring at 12 h. Then the *CbPGIP1* gene declined and returned to basal line. Therefore, a dual role for *CbPGIP1* in protection against pathogens and

cold stress is conceivable. *CbPGIP1* might be vital for disease and cold resistance.

PGIP expression is mediated by signal molecules, such as SA and NO. SA plays an important role during incompatible plant–pathogen interactions in both local and systemic resistance, resulting in the stimulation of the initial oxidative burst and leading to defense gene expression (Loake and Grant 2007). In addition, NO has also been suggested to act as a signal molecule mediating responses to biotic and abiotic stresses in the plant, especially, in disease and cold resistance (Boccara et al. 2005; Lamotte et al. 2005; Mur et al. 2006). Bean, potato and peach *PGIP*s accumulated in response to SA treatment (Bergmann et al. 1994; D'Ovidio et al. 2004), whereas *AtPGIP1*, *BnPGIP1* and *BnPGIP2* gene expression was unaffected (Ferrari et al. 2003; Li et al. 2003). Till now, the relationship between NO and the *CbPGIP1* expression was not established. Interestingly, our report revealed that 0.1 mM SA was a strong inducer of *CbPGIP1* and 0.2 mM SNP did so but to a lesser extent. The induction of *CbPGIP1* in response to SA and NO suggests that CbPGIP1 may share at least part of a signal pathway.

Our results showed that SA and NO induced the *CbPGIP1* gene expression in the same manner as cold and pathogen infection did. Cross-talk between NO and SA signaling pathways is very common in plant response to abiotic and biotic factors (Tuteja and Sopory 2008). In conclusion, *CbPGIP1* gene expression induced by pathogen infection and cold stress may be regulated by the SA and NO signaling pathways. Our study highlights the potential of both *CbPGIP1* and NO in plant resistance. The interaction of CbPGIP1 with NO paved the way to intensely study the cross-talk between signal molecules and defense gene expression.

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