Prokaryotic Expression of Rice Ospgip1 Gene and Bioinformatic Analysis of Encoded Product

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Abstract: Using the reference sequences of pgip genes in GenBank, a fragment of 930 bp covering the open reading frame (ORF) of rice Ospgip1 (Oryza sativa polygalacturonase-inhibiting protein 1) was amplified. The prokaryotic expression product of the gene inhibited the growth of Rhizoctonia solani, the causal agent of rice sheath blight, and reduced its polygalacturonase activity. Bioinformatic analysis showed that OsPGIP1 is a hydrophobic protein with a molecular weight of 32.8 kDa and an isoelectric point (pI) of 7.26. The protein is mainly located in the cell wall of rice, and its signal peptide cleavage site is located between the 17th and 18th amino acids. There are four cysteines in both the N- and C-termini of the deduced protein, which can form three disulfide bonds (between the 56th and 63rd, the 278th and 298th, and the 300th and 308th amino acids). The protein has a typical leucine-rich repeat (LRR) domain, and its secondary structure comprises α-helices, β-sheets and irregular coils. Compared with polygalacturonase-inhibiting proteins (PGIPs) from other plants, the 7th LRR is absent in OsPGIP1. The nine LRRs could form a cleft that might associate with proteins from pathogenic fungi, such as polygalacturonase.

Key words: Ospgip1 gene; polygalacturonase-inhibiting protein; prokaryotic expression; bioinformatic analysis; rice; Rhizoctonia solani

Plant pathogenic fungi produce an array of extracellular degrading enzymes to breach the plant cell wall, the first barrier to come into contact with the invading organisms, to gain access to plant tissues and degrade wall polymers (Walton, 1994). Among these enzymes, polygalacturonases (PGs) play an important role in cleaving the linkages between D-galacturonic acid residues in nonmethylated homogalacturonan, which is the major component of pectin. Polygalacturonase-inhibiting proteins (PGIPs) are important components of the plant cell wall. They can specifically combine with PGs to form a highly stable complex, which reduces the activity of PGs and favors the accumulation of elicitor-active oligogalacturonides (OGAs), thus eliciting the plant defense response. As a result, PGIPs play an important role in enhancing the host disease resistance (Roco et al, 1993; De Lorenzo et al, 2001; Federici et al, 2006).

In previous studies, PGs have not been considered as the major pathogenic factor of pathogens in monocots, especially in gramineous plants, because of the low proportion of pectin in their cell walls compared with that in dicotyledonous plants (30%). Therefore, the PGIPs of gramineous plants were not believed to be major factors in regulating plant disease resistance. Most of previous studies focused on PGIPs from dicotyledinous, such as bean (Spinelli et al, 2009), soybean (D’Ovidio et al, 2006), tomato (Stutz et al, 1994), cotton (James and Dubery, 2001), and rape (Li et al, 2003), whereas only a few studied monocotyledonous plants (Favaron, 2001). Recent research has revealed that PGs from monocot pathogens are also important pathogenic factors. Mutants of Claviceps purpurea, lacking both polygalacturonase genes cppg1 and cppg2, did not show any differences in vegetative properties, but lost almost all their pathogenicity towards rye (Oeser et al, 2002). Our previous research also showed that PGs from Rhizoctonia solani play an important role in its pathogenesis. We observed significant differences in
PG production among the isolates of this fungus, and the PG production of different isolates was positively correlated with their pathogenicity (Chen et al, 2006). Consequently, some researchers tried to use PGIPs in resistance breeding in gramineous plants. Transgenic wheat expressing a bean PGIP (PvPGIP2) presented a wide spectrum of specificities against fungal PGs and showed increased resistance to digestion by the PG of *Fusarium moniliforme* (Janni et al, 2008). When interacting with the fungal pathogen *Bipolaris sorokiniana*, the transgenic wheat lines showed a significant reduction in symptom progression (46% to 50%) compared with the control (Janni et al, 2008). *Ospgip1* was over-expressed in *Nicotiana benthamiana*, and the protein extract could completely inhibit different PGs, except that from *F. moniliforme* (Janni et al, 2006). In the present study, the inhibitory effects of the expression product of *Ospgip1* gene on PG activity and hyphal growth of *R. solani* were investigated. Additionally, bioinformatic analysis of OsPGIP1 was also conducted. The results would lay the foundation for further application of the *Ospgip* gene in rice resistance breeding to sheath blight.

**MATERIALS AND METHODS**

**Materials**

Three rice varieties, Lemont, Jasmine 85 and YSBR1 were used, which were conserved by our cultivar resource center.

Isolate YN-7 of *R. solani* was isolated from rice pathogenic plants in Jiangsu Province, China. *E. coli* isolates DH5α and BL21 were bought from TaKaRa Biological Ltd.

**DNA manipulation**

Genomic DNA was extracted from 0.5 g of green rice materials using the CTAB method (Khanuja et al, 1999). PCR amplification of the *Ospgip1* gene was carried out in a reaction volume of 40 μL with 2 μL of genomic DNA (25 ng/μL), 4 μL of 10×buffer (with Mg²⁺), 2 μL of each primer (10 μmol/μL), 2 μL of dNTP Mixture (2.5 mmol/L), 1 μL of Taq polymerase (5 U/μL), and 25 μL of ddH₂O. PCR conditions were: 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and a final step at 72 °C for 7 min. Primers were synthesized based on the nucleotide sequences available at GenBank and related references, with the following sequences: *OspgipF*, 5'-CGGGATCCATTTGAATGGAGGCTACTACC-3', and *OspgipR*, 5'-CCGCTCTGAGTGCGGAGCTTAGAATT GCAGGGA-3' (The underlined regions were the restriction enzyme sites of *BamH* I and *Xho* I).

Amplified products were separated on 1.2% agarose gels, recovered by the TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa Biological Ltd.), and cloned into pET-22b(+) Easy vector or directly subjected to nucleotide sequencing. Sequencing reactions were performed by TaKaRa Biological Ltd.

**Prokaryotic expression of OsPGIP1 gene**

*E. coli* cells were transformed with the recombinant vector using standard methods. A single positive clone was picked with a sterilized toothpick and transferred into 10 mL of LB/Amp (50 mg/L) culture liquid. The culture was put in a shaker at 37 °C with a constant speed of 200 r/min. When the OD₆₀₀ value of the culture reached 0.5, IPTG was added with the final concentration of 4 mmol/L. After the addition of IPTG, the culture was divided into several aliquots and placed at 26 °C in a shaker at 200 r/min continually for 0, 4, 6, 8, 12 and 14 h, respectively, or at 25, 28 and 30 °C, respectively, with a shaking speed of 200 r/min for 12 h. Soluble and inclusion body proteins were extracted from 1 mL of culture liquid for each treatment and the expression levels were analyzed by SDS-PAGE (Clark, 1998; Zhang et al, 2008).

**Inhibitory effect of OsPGIP1 on growth and PG activity of R. solani**

The antifungal activity of the expression product was studied on the basis of the inhibitory effect on mycelial growth and PG activity of *R. solani*. One milliliter of each inactivate inclusion body protein, soluble protein and inclusion body protein were spread into a thin layer on Czapek-Dox culture medium (Sucrose 30 g, NaNO₃ 3 g, MgSO₄·7H₂O 0.5 g, KCl 0.5 g, FeSO₄·7H₂O 0.01 g, K₂HPO₄ 1 g, Agar 13 g, distilled water 1000 mL, pH 7.2), in which pectin was used to replace sucrose as the carbon source. Sterile distilled water served as a control. Fungal discs (6 mm)
were punched from 48 h culture of *R. solani* and placed in the middle of the Petri plate. The plates were incubated at 28 °C and the inhibitory effect on mycelial growth was observed after 36 h of incubation. Determination of the activity of *R. solani* PG (RsPG) was performed according to Chen et al (2010), except that 2 mL of each inactivated inclusion body protein, soluble protein and inclusion body protein were added in the reaction tubes. The inhibition effect of OsPGIP was calculated based on the variation in RsPG activity.

**Bioinformatic analysis of OsPGIP**

Amino acid sequence deduction and conserved domain analysis were performed by ExPASy (http://us.expasy.org/tools/dna.html). Protein secondary and tertiary structure prediction used the online software at the following web sites [http://scratch.proteomics.ics.uci.edu (Cheng et al, 2005), http://www.ch.embnet.org/software/COILS_form.html (Fong et al, 2004), and http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/ (Lambert et al, 2002)].


**Phylogenetic analysis**

Several amino acid sequences that showed more than 30% similarity to OsPGIP were selected from GenBank for cluster analysis. The dendrogram was created with Vector NTI Suite 8.0.

**RESULTS**

**Cloning of Ospgip1 gene**

A 930 bp open reading frame of *Ospgip1* gene was amplified from rice genome DNA using the primer pair of *OspgipF/OspgipR* and the vector pET-22(+) was cloned to form the recombinant vector pET-pgip1. The recombinant vector was transformed into *E. coli* BL21. Positive clones verified by restriction enzyme digestions using *Bam*H I and *Xho* I, were named *E. coli* BL21(DE3)/pET-pgip1 (Fig. 1).

**Optimal conditions for prokaryotic expression**

The amount of OsPGIP1 expressed after the addition of IPTG to the culture of isolate *E. coli* BL21(DE3)/pET-pgip1 varied according to the induction time (4, 6, 8, 12 and 14 h). Initially, the OsPGIP1 expression level increased with prolonged induction time, and then plateaued after 12 h (Fig. 2). The OsPGIP1 expression level was similar when the culture was incubated at 25, 28 or 30 °C (Fig. 3). Higher temperatures improve the formation of inclusion bodies, therefore, the optimum induction conditions for liquid culture were determined to be 25 °C for 12 h.

**Inhibitory effect of OsPGIP1 on R. solani growth and RsPG activity**

One milliliter inactivate inclusion body protein, soluble protein and inclusion body protein were spread in a thin layer on Czapek-Dox culture medium. After being inoculated with a fungal disc in the middle, the Petri dish was incubated at 28 °C for 36 h. The

![Fig. 1. Map of recombinant vector digested with restriction enzymes *BamH* I and *Xho* I.](image)

M, Marker; Lanes 1 to 6, Digesting product of recombinant vector.

![Fig. 2. Effect of the induction time on the expression quantity of recombinant protein.](image)

M, Marker; Lanes 1 to 6, Induction time of 0, 4, 6, 8, 12 and 14 h, respectively.
results showed that the diameter of the expanded colony on the plate with the inclusion body protein was smaller than that of the distilled water control (5.93 cm vs. 7.00 cm; Fig. 4). The diameters of the expanded fungal colonies on the inactivated inclusion body protein and soluble protein plates were similar to the control.

When the inclusion body protein was added to the PG reaction solution, the activity of RsPG was inhibited significantly (54.3% of the control; Fig. 5). This result indicated that the refolded inclusion body protein could strongly inhibit the activity of RsPG.

**Structure prediction of OsPGIP1**

OsPGIP1 consists of 309 amino acids, among which 22 amino acids are positively charged and 22 are negatively charged. The protein contains nine tandemly leucine-rich repeat (LRR) units. Compared with the PGIPs from other plants, the 7th LRR was absent in OsPGIP1. There were no coiled helixes in the secondary structure of OsPGIP1, which mainly consisted of $\alpha$-helixes, $\beta$-sheets and irregular coils.

A 3D-model of the tertiary structure of OsPGIP1, which is up to 67.4% identical with other plant PGIPs, was predicted using an automated homology modeling program. We observed that the LRR domain of OsPGIP1 had nine $\beta$-sheets, which formed a cleft that might form the active domain for inactivating with PGs of plant pathogens (Fig. 6).

**Other characteristics and functions of OsPGIP1**

The molecular formula, molecular weight, and pI of OsPGIP1 were determined as $C_{1468}H_{2320}N_{394}O_{428}S_{13}$, 32.8 kDa and 7.26, respectively. Results of solubility prediction showed that the insolubility ratio and instability coefficients were 86.3% and 31.82%, respectively, which indicated that the deduced protein was hydrophobic and stable. The primary structure of the deduced protein was mainly hydrophobic and its mean hydrophobic coefficient was 0.183. All these characteristics indicated that the prokaryotic expression product of Ospgip1 gene was insoluble and formed an inclusion body protein.

Transmembrane domain, subcellular localization
and signal peptide prediction showed that the deduced protein had no transmembrane domains and included a signal peptide, whose cleavage point was between the 17th and 18th amino acids (Fig. 7). The deduced protein was predicted to be located in the cell wall and the endoplasmic reticulum, but not in the mitochondria or the cell nucleus. Thus, these results indicated that PGIP is an important component of the plant cell wall.

There were four cysteines in both the C- and N-terminal regions, which could form three disulfide bridges. One bridge was located in the N-terminal region (56th–63rd) and the other two were in the C-terminal region (278th–298th and 300th–308th). Function site analysis showed that OsPGIP1 was predicted to have two protein kinase K phosphorylation sites, six casein type II phosphorylation sites, five N-nutmeg acylation sites, and four asparagine N-glycosylation sites. Both the phosphorylation sites and glycosylation sites contribute to the interaction with PGs of plant pathogens.

**Phylogenetic development of OsPGIP1**

Fourteen PGIP sequences from dicotyledons (*Arabidopsis* and apple) and monocotyledons (wheat, corn and sorghum) were used for phylogenetic analysis. The results showed that the sequences of PGIPs were significantly different between dicotyledons and monocotyledons, and even among the monocotyledons. OsPGIP1 is phylogenetically most closely related to those of corn and sorghum (Fig. 8).

**DISCUSSION**

Sheath blight is one of the most important diseases in rice. With the wide application of multi-tillering, semi-dwarf rice cultivars, and the increase of nitrogen fertilization, this disease is becoming increasingly serious. In southern China, sheath blight disease has become the most serious disease in rice (Liao et al, 1997). To date, no completely
resistant or immune variety has been found, and the genes conferring disease resistance were all identified as quantitative trait loci (QTLs) (Zuo et al., 2006). Exploration QTLs with large effects and pyramiding them by marker assisted selection to develop resistant varieties would be the most economical and effective method for controlling the disease.

In recent years, application of plant pgips genes has become a focus of resistance breeding. In the plant genome, the pgip genes were clustered into a small gene family, and their encoded proteins were found to be able to reduce PG activity from fungi. Interestingly, different PGIPs from different plants, or even from the same plant, have various effects on reducing the fungal PG activity. Similarly, the inhibitory activity of PGIPs on PG also varied with different fungi PGs and even with different PGs from the same fungus (D’Ovidio et al., 2006). RsPG, a cell wall degrading enzyme that is secreted by R. solani during early infection, is considered as one of the most important pathogenetic factors of R. solani (Chen et al., 2006). Therefore, the use of pgip genes in rice breeding practice may offer another strategy for increasing rice resistance to R. solani. In this study, we found that the prokaryotic expression product of the Ospgip1 gene could inhibit the activity of RsPG, which provides important evidence for the utility of the gene in rice breeding against sheath blight.

Plant PGIPs, like many resistance proteins, belong to the LRR protein family. PGIPs can recognize PGs from pathogens through its LRR domains. The PvPGIP2 of Phaseolus vulgaris can recognize PGs from different pathogens, which is mainly attributed to the fact that its LRR domain (LxxLxxLxx) changed easily (Sicilia et al., 2005). In P. vulgaris, only eight different amino acids were found between PvPGIP1 and PvPGIP2, and among them, five are located in the LRR domain and two in the region neighboring the LRR. Generally, it is PvPGIP2, but not PvPGIP1, that interacts with PGs from F. moniliforme. However, when the 253th amino acid (a lysine in the LRR region) of PvPGIP1 was substituted by glutamine that is specific to the LRR region of PvPGIP2, this single mutation could confer the ability of PvPGIP1 to interact with PGs. The reverse replacement in PvPGIP2 resulted in the loss of its inhibitory effect on PGs from F. moniliforme by up to 70% (Leckie et al., 1999). By mutagenesis analysis, Fong et al. (2004) found that 9 out of 313 amino acids of PvPGIP2 were required for its functional interaction with PGs, and seven of them are located in the LRR domain. These limited numbers of ‘hot spots’ could be responsible for the specificity of PGIPs and could be subjected to positive selection upon contact with diverse pathogens.

Bioinformatic analysis indicated that OsPGIP1 contains a typical LRR domain but lacks the 7th LRR, when compared with PGIPS from other plants. The LRRs in OsPGIP1 could form a cleft that might play an important role in the interaction with PGs and reducing the activity of PGs. However, the amino acids that are responsible for the interaction of PGIPs with PGs have not yet been determined and require further study.

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