Overexpression of *OsPGIP1* Enhances Rice Resistance to Sheath Blight

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Abstract

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Rice sheath blight (SB), caused by necrotrophic pathogen *Rhizoctonia solani*, is one of the most destructive rice diseases, and no major resistance genes are available. Polygalacturonase-inhibiting proteins (PGIP) are extracellular leucine-rich repeat proteins and play important roles in plant defense against different pathogenic fungi by counteracting secreted fungal polygalacturonases (PG). However, the role of PGIP in conferring resistance to rice SB remains to be thoroughly investigated. Here, we showed that *OsPGIP1* is capable of inhibiting PG derived from *R. solani*. Our real-time reverse-transcription polymerase chain reaction results indicated that resistant rice ‘YSBR1’ and ‘Jasmine 85’ express significantly higher levels of *OsPGIP1* than susceptible ‘Lemont’. Our results also show

that *OsPGIP1* is most highly expressed at the late tillering stage in the sheath of YSBR1, coinciding with the critical stage of SB development in field. More importantly, the *OsPGIP1* level is highly elevated by inoculation with *R. solani* in resistant cultivars but not in susceptible Lemont. Overexpression of *OsPGIP1* significantly increased rice resistance to SB and inhibited tissue degradation caused by *R. solani*-secreted PG. Furthermore, *OsPGIP1* overexpression did not affect rice agronomic traits or yield components. Together, our results not only demonstrate the important role of *OsPGIP1* in combatting the rice SB disease but also provide a new avenue to the improvement of rice SB resistance by manipulating an endogenous gene.

Rice is one of the most important staple foods in the world (Kumar et al. 2009). As with many other crops, the production of rice is constantly challenged by the invasion of many phytopathogenic fungi, bacteria, viruses, and nematodes. Sheath blight (SB), caused by *Rhizoctonia solani*, is one of the most devastating diseases in many areas of the world where rice is cultivated (Deepti and Thrimurthy 2006; Juge 2006; Lee and Rush 1983; Sarkar et al. 2003). The conventional approach to controlling fungal pathogens is mainly dependent on the use of chemical fungicides, which have serious undesirable effects such as being harmful to the ecological system, producing residual poisons to humans and animals, and high costs. The use of resistant cultivars has been considered the most environmentally friendly approach to controlling diseases (McKenzie et al. 1986; Xie et al. 1992). Due to the fact that rice resistance to SB disease is controlled by multiple loci and is quantitatively inherited, there are no cultivars that carry high resistance or complete immunity to rice SB in the field, although different degrees of resistance have been reported (Li et al. 1995; Meng et al. 2001; Yin et al. 2009).

More and more endogenous or exogenous genes have been identified and applied in breeding for disease resistance in recent years. Among them, the genes encoding polygalacturonase-inhibiting proteins (PGIP) have proven effective in inhibiting fungal disease development (Akhgari et al. 2012; Dang et al. 2012; Liu et al. 2014;

Mohammadzadeh et al. 2012). PGIP are plant extracellular leucine-rich repeat (LRR) proteins that locate to plant cell walls, where they counteract the activity of fungal polygalacturonase (PG) and prevent plant cell walls from degradation and release of nutrients that feed the fungus; and, thus, hinder the fungal invasion process (Protzenko et al. 2008; Zhang et al. 2014). At the same time, PGIP may cause the accumulation of oligogalacturonides, an active elicitor which induces host defense response (Cervone et al. 1989; De Lorenzo et al. 2001; Federici et al. 2006; Hegedus et al. 2008).

The use of PGIP to counter fungal infection should be relatively environmentally friendly and safe in transgenic breeding procedures because PGIP are employed endogenously in plants for defense purpose. However, not all PGIP are effective in inhibiting the PG secreted by the target pathogenic fungi. Not only do PGIP from different plants differ in their inhibitory activity but also PGIP from one plant sometimes show very different activities on inhibiting PG from different fungi or different PG from the same fungus (De Lorenzo et al. 2001). Most PGIP from different plants are very similar in sequence but can be very different in activity, which is presumably the outcome of coevolution between plants and pathogenic fungi (Kalunke et al. 2014).

Like many other plant PGIPs, the rice PGIP family consists of at least seven members (Lu et al. 2012). Among them, *OsPGIP1* can inhibit several PG from different fungal pathogens and is the mostly effective protein against the PG of *Sclerotinia sclerotiorum* (Janni et al. 2006). *OsPGIP2* shares common *cis* elements with *OsPGIP1* in its promoter with regard to meristem expression and ABA (Absciscic acid) responsiveness (Kumar et al. 2009). Interestingly, not only does the *OsFOR1* gene play an important role in the formation or maintenance of floral organ primordia but its gene product also shows PGIP activity when tested with *Aspergillus niger* PG (Jang et al. 2003). These studies indicated that *OsPGIP* genes might contribute to rice resistance to pathogenic fungi. However, it remains unknown how an individual *OsPGIP* gene is expressed and how each of them contributes to rice SB resistance. In this study, we investigated the expression pattern of *OsPGIP1* in detail and show that its

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overexpression confers significant resistance to rice SB. Importantly, its overexpression has no detrimental effects on rice development or yield-associated components, making it a good candidate for rice breeding against SB.

Materials and Methods

Plant materials and growth conditions. Rice cultivars with different resistance levels to SB were used in the present study. The resistance levels of these cultivars are ordered as follows: 'YSBR1' (resistant) > 'Jasmine 85' (moderately resistant) > 'Lemont' and 'Xudao3' (both are high susceptible). Seed were surface sterilized before germination by incubating in sodium hypochlorite (0.5% [vol/vol]) for 10 min followed by rinsing thoroughly with sterile water. The seedlings were grown in a greenhouse with a 14-h photoperiod daily at 25°C until maturity for analyzing the expression pattern of *OsPGIP1*.

Stress treatment and tissue collection. To analyze the expression profile of *OsPGIP1*, five plants with uniform morphological appearance at each developmental stage were selected and sectioned into roots, sheaths, and leaves, in addition to spikes at the heading stage. We also analyzed the *OsPGIP1* expression in sheaths in etiolated condition by growing the plants in darkness at 25°C in a biochemical incubator from sowing to four-leaf stage, when the sheath tissues were collected. For cold treatment, the plants were grown in normal conditions to four-leaf stage before being transferred to a growth chamber with light at 4°C and grown for 72 h, where sheath tissues were collected for experimentation. For wounding treatment, healthy sheaths of plants at the tillering stage were scratched and penetrated with a hemostat, and the damaged sheaths were collected 24 h post-treatment. Rice plants at the tillering stage were artificially inoculated with the *R. solani* YN-7 isolate using the method reported by Zuo et al. (2013), and the sheaths were harvested at 24 h postinoculation. All the samples collected above were frozen in liquid nitrogen and stored at -80°C till RNA extraction.

RNA extraction and real-time reverse-transcription polymerase chain reaction analyses. Total RNA was isolated from the treated seedlings or different parts of the plant using RNAiso Plus reagent (TaKaRa). Total RNA was treated with DNase I and used for the first-strand cDNA synthesis using the PrimeScript II reverse transcription (TaKaRa), according to the manufacturer's instructions. The gene-specific primers were designed using Primer Premier 5.0 (<http://www.tucows.com/preview/205452>) with default parameters. The synthesized cDNA was then used as template in real-time polymerase chain reaction (PCR) on ABI Prism 7300 real-time PCR system (Applied Biosystems).

The quantitative reverse-transcription (RT)-PCR primers used in real-time PCR reaction were *Osp1*-RT-F (5'-GCTGCCACCACGAAGAAGA-3') and *Osp1*-RT-R (5'-GTTGGCGTCCTGGAAGACGGA-3'), which resulted in a fragment of 192 bp. The *OsACTIN1* gene (X16280, GenBank) was used as the internal control to determine the loading quantity of each cDNA. The PCR primers specific to the *OsACTIN1* gene were (*Osa-q-F*) 5'-GAAGTGGTAGGTCAAGGCTG-3' and (*Osa-q-R*) 5'-ACACGGAGCTCGTTGTAGAAG-3'. Each reaction was repeated three times. Two biological replicates for each sample were included.

Generation of plant overexpression construct and rice transformation. For generation of the *OsPGIP1* overexpression construct, the full-length cDNA of *OsPGIP1* was amplified by primer pair *Osp*-POE-F (5'-cgGGATCCAATTGGAGTGGAGGCTACTACC-3')/*Osp1*-PE-R (5'-cggCTCGAGTGGGAGC TTAATTGCAGGA-3'). The product was subcloned into the protein expression vector pET-22b(+) and digested using *Bam*HI and *Xho*I to generate His-tagged fusion protein expression construct pET22-*OsPGIP1*. The pET22-*OsPGIP1* was introduced into *Escherichia coli* strain BL21 to express the fusion protein under induction with isopropyl β-D-1-thiogalactopyranoside. The bacterial cells were collected and broken by the ultrasonic method. Refolding of the recombinant protein derived from protein inclusion bodies was performed according to the procedure reported by De Bernardez (1998). The His-tagged fusion protein (His-*OsPGIP1*) was enriched using His-Bind Resin (Novagen) according to the manufacturer's protocol. After elution, the protein concentration was adjusted with 10% glycerol solution and stored at -70°C until use.

described previously (Chern et al. 2005), the overexpression construct of *OsPGIP1* was introduced into Xudao3 rice. The positive transgenic plants were confirmed by PCR using primers HygRT-F (5'-ATTTGTGTACGCCCCGACAGT-3') and HygRT-R (5'-GGATATGTCCTGCGGGTAAA-3') specific to the hygromycin phosphotransferase gene (*HPT*).

Expression of *OsPGIP1* protein in vitro. A full-length cDNA fragment of *OsPGIP1* was amplified by using primer pair *Osp*-POE-F (5'-cgGGATCCAATTGGAGTGGAGGCTACTACC-3')/*Osp1*-PE-R (5'-cggCTCGAGTGGGAGC TTAATTGCAGGA-3'). The product was subcloned into the protein expression vector pET-22b(+) and digested using *Bam*HI and *Xho*I to generate His-tagged fusion protein expression construct pET22-*OsPGIP1*. The pET22-*OsPGIP1* was introduced into *Escherichia coli* strain BL21 to express the fusion protein under induction with isopropyl β-D-1-thiogalactopyranoside. The bacterial cells were collected and broken by the ultrasonic method. Refolding of the recombinant protein derived from protein inclusion bodies was performed according to the procedure reported by De Bernardez (1998). The His-tagged fusion protein (His-*OsPGIP1*) was enriched using His-Bind Resin (Novagen) according to the manufacturer's protocol. After elution, the protein concentration was adjusted with 10% glycerol solution and stored at -70°C until use.

Assay of RsPG and *OsPGIP1* proteins. Purified fusion protein (1 ml at 0.2 g/ml) was plated on the surface of Czapek-Dox culture medium in a sterile hood. After drying in air, SB isolate YN-7 on a 6-mm-diameter disc was inoculated in the center of the plate. The plate was incubated at 28°C. The colony diameter was measured after 36 h of culture.

Extraction and assay of PG from *R. solani* were performed according to the procedure reported by Chen et al. (2010). A mixture containing 0.5 ml of RsPG at 0.2 g/ml, 0.5 ml of 0.25% polygalacturonic acid (Sigma) in 50 mM sodium citrate buffer at pH 5.0, and 1 ml of redistilled water was incubated at 37°C for 60 min. After addition of 2.5 ml of dinitrosalicylate solution, the mixture was incubated in boiling water for 10 min and then cooled down. One enzyme unit was defined as the amount of enzyme required to release 1 μg of reducing sugar at 37°C in 1 h. The same mixture was used to assess the inhibitory activity of *OsPGIP1* on RsPG, where 2.0 ml of *OsPGIP1* at 0.1 g/ml was used in each reaction. This method was named the reduced sugar assay. The diffusion assay was the second method that was employed to quantify the PG activity of RsPG in the present study, which was performed according to the procedure reported by Taylor and Secor (1998). The medium contained 0.8% type II agarose and 0.5% PCA (Polygalacturonic acid) in 0.1 M NaAC buffer (pH 4.7). After addition of RsPG or *OsPGIP1*, the petri-dish plate was incubated at 37°C for 17 h, and then the gel was stained with ruthenium red (0.05% [wt/vol] in water) for 2 h. The gel was rinsed thoroughly with water after staining. The RsPG enzyme activity and the *OsPGIP1* inhibitory activity were represented by the diameter of the agarose diffusion ring. The size reduction represents the inhibitory activity of *OsPGIP1* on the RsPG enzyme activity.

Effects of RsPG on rice cell membrane. The healthy sheaths of plants at the later tillering stage were collected and cut into small discs with a diameter of 5 mm. The discs were transferred into dactylethraes with 5 ml of RsPG (0.1 g/ml) and were fully immersed in the solution with vacuum applied. After 1 h, the discs were removed and rinsed with Milli-Q water and transferred into new dactylethraes with 5 ml of Milli-Q water. Vacuum pressure was applied to ensure that the discs were immersed in the water for 1 h. Electrical conductivity (EC) was measured for the water mixed with the sheath lexivium by using a DDS-307 conductivity meter. The discs were removed and put into 5 ml of boiling Milli-Q water and incubated for 15 min. The boiling EC (BEC) of the water mixed with the sheath lexivium was measured. In the negative control, all the components in solution were same as above, except for the exclusion of RsPG (Chen et al. 2006). The damaged cell membrane rate (DCMR) for each treatment was calculated using the formula DCMR (%) = [(tEC - cEC)/(BEC - cEC)] × 100, where t and c represent treatment and control samples, respectively.

Evaluation of agronomic traits and SB resistance of transgenic lines. The homozygous transgenic lines and wild-type Xudao3 were planted in the field in a completely randomized design with two replications for evaluation of their agronomic traits. All tested lines were sown in the seedling nursery on 7 May and transplanted on 8 to 10 June. Each plot contained two rows, 35 cm apart. Each row contained 12 plants with 20 cm between plants. Heading date, plant height, flag leaf length, flag leaf width, tillering number, the number of productive panicle, panicle length, the number of the first branch, seed number per panicle, and 1,000-grain weight (1,000GW) were measured for all rice lines tested. All traits were measured according to the description of Zuo et al. (2007).

The YN-7 strain (originally named RH-9), with moderate pathogenicity, isolated from the rice plants of Jiangsu Province and used in our laboratory for about 10 years (Chen et al. 2009), was used to inoculate transgenic lines. Autoclaved, truncated thin matchsticks with a length of 0.8 to 1.0 cm were incubated with the YN-7 strain on potato dextrose broth medium for 3 to 5 days in darkness at room temperature (25°C), and were then used as the inoculum (Zuo et al. 2013). Inoculation was performed at the late tillering stage of rice, as described by Zuo et al. (2013). Each line was grown in four replicate plots with three rows per plot in field. The three largest tillers of each of the middle 10 plants in the central row of each plot were inoculated with the SB fungus. Disease severity was rated approximately 30 days after heading. The modified 0-to-9 disease scoring system reported by Zuo et al. (2013) was used in this study, where 0 was defined as no disease and 9 indicates that the plant was dead or collapsed. Each tiller was individually scored. The disease severity of each line in each replicate was obtained by averaging the disease scores of at least 30 tillers. Statistical analysis was subsequently performed.

Statistic analyses. Data of SB disease severity and agronomic traits were analyzed using the one-factor analysis of variance procedure in the SPSS12.05 program. Mean comparisons (least significant difference) were used to reveal the difference among rice lines tested for the SB disease severity, agronomic traits, and yield-related traits,

where $P = 0.05$ and $P = 0.01$ were used to measure whether the differences were statistically significant or highly significant, respectively.

Results

OsPGIP1 was able to inhibit the activity of RsPG and the growth of *R. solani*. Because PGIP were believed to be able to inhibit the activity of PG from fungi, we tested the effects of the OsPGIP1 protein on inhibiting the activity of RsPG by both the reduced sugar assay and diffusion assay. We found that the activity of RsPG in control was up to 320 U/mg and the activity showed no changes after addition of either the *E. coli*-expressed, soluble protein (SP) fraction or inactive fusion protein (IFP) treated by boiling. In contrast, significant differences ($P = 0.0001$) were found when purified fusion protein (PFP) from the refolded inclusion body was added, which resulted in a 50% reduction in PG activity to approximately 160 U/mg (Fig. 1A). These results indicated that OsPGIP1 was insoluble in our *E. coli* protein expression system but, after refolding treatment, it clearly gained biological activity represented by the strong inhibition on RsPG activity. In the diffusion assay, we found that the diameter of the diffusion ring of PFP plus PG was apparently shorter than that of the control of water plus PG as well as that of SP plus PG and of IFP plus PG (Fig. 1B). Further, we found that the purified OsPGIP1 fusion protein from three independent *E. coli* clones carrying the construct all showed strong inhibition on the RsPG activity ($P = 0.0001$; Fig. 1C and D). Then, we tested whether the purified OsPGIP1 fusion protein can inhibit the growth of *R. solani*. We found no significant differences between the negative control and treatment with the SP or IFP sample. In contrast, significant differences in colony size were found between the control and the treatment with the PFP sample ($P = 0.001$; Fig. 1E and 1F). At 48 h after treatment, the addition of PFP was found to reduce the size of *R. solani* by 1.1 cm in diameter, equivalent to an inhibition rate of 15.7%. Taken together, we conclude that the *E. coli*-expressed OsPGIP1 fusion protein is able to inhibit the RsPG enzyme activity as well as the growth of *R. solani*.

Fig. 1. Inhibitory effects of OsPGIP1 on RsPG enzyme activity and fungal growth. **A**, Inhibitory effects of different protein fractions of *Escherichia coli* expressing OsPGIP1 on the polygalacturonase (PG) activities of *Rhizoctonia solani* by the reduced sugar assay. The protein fractions include soluble protein (SP), inactive fusion protein (IFP), and purified fusion protein (PFP). Water (W) was used as the negative control. **B**, Inhibitory effects of different protein fractions of *E. coli* expressing OsPGIP1 on the PG activities of *R. solani* by the diffusion assay. **C**, PFP proteins purified from different *E. coli* transformants (PT-03, PT-12, and PT-17) were tested for inhibitory activities to PG from *R. solani* by the reduced sugar assay. EV = expression product of empty vector; PT-03, PT-12, and PT-17 were expression products of different *E. coli* expressing OsPGIP1. **D**, PFP proteins purified from different *E. coli* transformants (PT-03, PT-12, and PT-17) were tested for inhibitory activities to PG from *R. solani* by the diffusion assay. **E** and **F**, Inhibitory effects of different protein fractions of *E. coli* expressing OsPGIP1, including SP, IFP, and PFP, on the growth of *R. solani*. Different lowercase letters on the bar show significant statistical difference between treatment and control at 5% significance level. Each bar represents the average and standard deviation of at least three samples. Pictures were taken 48 h after inoculation.

OsPGIP1 showed a high expression level and was strongly induced by SB fungus in resistant cultivars. To determine the expression pattern of *OsPGIP1*, we assessed its transcript levels by real-time RT-PCR in three cultivars (YSBR1, Jasmine85, and Lemont) that carry high, medium, and low levels of SB resistance, respectively. We found that *OsPGIP1* was expressed at higher levels in leaf and sheath tissues of YSBR1 and Jasmine 85 compared with those of susceptible Lemont across all three tested developmental stages. No or little expression was observed in roots or spikes. *OsPGIP1* is expressed higher at the seedling and tillering stages than at the heading stage (Fig. 2A).

Because *R. solani* mainly infects rice sheath, we then compared the expression levels of *OsPGIP1* in sheaths of different cultivars. We found that the *OsPGIP1* expression levels in highly susceptible Lemont were much lower than those in resistant YSBR1 and moderately resistant Jasmine 85 (Fig. 2B). Severe SB disease is generally developed at around the tillering stage (Zuo et al. 2010). At the tillering stage, we found the highest *OsPGIP1* levels in YSBR1, followed by Jasmine 85, then Lemont. These results are consistent with their SB resistance levels in the field (Fig. 2B) and suggest that *OsPGIP1* may be involved in the defense against *R. solani*.

Then, using real-time RT-PCR, we tested whether the *OsPGIP1* expression is induced by the infection of *R. solani* (Fig. 2C). We found that the *OsPGIP1* level was most strongly induced in YSBR1 and induced to a lesser extent in Jasmine 85 but not induced at all in Lemont at 24 h post-SB inoculation. After all results were taken together, we concluded that *OsPGIP1* is highly expressed in rice sheath and leaves and is strongly induced by *R. solani* in the resistant cultivars but not in the susceptible cultivar.

Expression profiles of *OsPGIP1* under abiotic stresses. PGIP are ubiquitous plant cell wall proteins and the expression of PGIP has been found to be induced by various stress stimuli (Ferrari et al. 2003). Therefore, we tested *OsPGIP1* expression levels under three different abiotic stress conditions: darkness, low temperature, and wounding (Fig. 3). As expected, the *OsPGIP1* expression level was significantly induced in all cultivars by darkness and cold treatments (Fig. 3A and B); in contrast, the wounding treatment only significantly induced expression in YSBR1 and Jasmine 85 but not in Lemont (Fig. 3C). Among the three cultivars, YSBR1 showed the strongest induction on *OsPGIP1* expression by wounding. These results suggest that *OsPGIP1* plays roles in tolerance to diverse abiotic and biotic stresses.

Overexpression of *OsPGIP1* improved rice resistance to SB.

To evaluate the effect of *OsPGIP1* on resistance to rice SB, we overexpressed the gene in SB-susceptible Xudao3, which is widely planted in the Jiangsu province of China. We obtained 21 independent transgenic plants (named T251 to T271) through *Agrobacterium*-mediated transformation and confirmed the presence of the transgene by PCR using primers specific to the *HPT* gene (Supplementary Fig. S1A). We identified five lines with single copy of the transgene by analyzing the segregation ratios of T₁ plants from these T₀ lines for those that segregated at a 3:1 ratio (Supplementary Table S1). We confirmed the elevated expression levels of *OsPGIP1* in these five transgenic lines using real-time RT-PCR (Supplementary Fig. S1B). We selected three (T262, T263, and T266) of the five lines that showed normal plant heights and seed set rates to develop homozygous lines (Supplementary Fig. S1C). The strong expression of *OsPGIP1* in these three independent homozygous lines were further confirmed and shown in Figure 4A.

Fig. 2. Real-time reverse-transcription polymerase chain reaction analyses of *OsPGIP1* expression in different rice tissues. **A**, Expression analysis of *OsPGIP1* in various rice varieties and tissues at different development stages. Total RNA was extracted from same amount rice tissues. The β -actin gene was used as internal control. R, S, L, and P represent rice root, sheath, leaf, and panicle, respectively. **B**, Expression levels of *OsPGIP1* in sheaths of three cultivars at different developing stages. **C**, Induction of *OsPGIP1* expression in sheath by *Rhizoctonia solani* infection. Plants were inoculated with YN-7 at the later tillering stage and the sheath tissues were harvested before inoculation and at 24 h postinoculation. Different lowercase letters on the column indicate statistical difference at the 5% significant level revealed by *t* test. Each bar represents the average and standard deviation of at least three replicates.

These homozygous transgenic lines and the wild-type control were inoculated with the YN-7 SB isolate in the field. We found that all three transgenic lines displayed significantly ($P = 0.034$) lower SB disease severity than the wild type (Fig. 4B). Compared with the wild type, overexpression of *OsPGIP1* reduced the SB disease severity score by 0.9 to 1.2, equivalent to a reduction in lesion length by around 10 cm measured on 30 days after heading in the *japonica* 'XD 3' genetic background. Further, we tested whether the transgenic lines increase tolerance to tissue degradation caused by the RsPG enzyme. We treated detached sheaths of the transgenic rice lines with RsPG and found that the antidecomposing abilities of the transgenic lines were significantly enhanced compared with the wild type (Fig. 4C). Taking these results together, we conclude that overexpression of *OsPGIP1* is able to improve resistance to rice SB by countering the PG activity of RsPG and reducing tissue degradation caused by RsPG.

Overexpression of *OsPGIP1* carries no detrimental effects on rice development. To determine the breeding potential of *OsPGIP1* overexpression in rice, we measured a few agronomic traits in the three transgenic lines T262-3, T263-4, and T266-2. We found no differences between these transgenic lines and the wild-type control in agronomic traits, including heading date, plant height, flag leaf length, flag leaf width, tillering number, panicle length, and the

number of the first branch (Table 1). We also found that all three transgenic lines had almost the same values on yield-associated components, including the number of productive panicle, seed number per panicle, and 1,000GW, as the wild-type control (Fig. 5; Table 2).

Discussion

PGIP are a class of plant immune proteins induced during early fungal infection that inhibit PG function, prevent cell wall degradation, and limit fungal growth and colonization (Di Matteo et al. 2006; Federici et al. 2006; Juge 2006). Like plant resistance genes, *PGIP* genes belong to complex multigene families, whose members show nearly identical biochemical characteristics but present distinctive recognition specificities to different PG from pathogens (Desiderio et al. 1997). For instance, *Phaseolus vulgaris* PGIP1 is able to interact with

Fig. 3. Analysis of *OsPGIP1* expression under different abiotic stresses by real-time reverse-transcription polymerase chain reaction. **A**, Expression of *OsPGIP1* in etiolated seedlings grown in darkness for 3 weeks and in control seedlings grown under natural environment. **B**, Expression of *OsPGIP1* in cold environment. Seedlings were placed in 4°C chamber for 72 h compared with plants in a 25°C chamber with the same light conditions. **C**, Expression of *OsPGIP1* after wounding treatment. Sheaths were harvested at 24 h after wounding with a hemostat. Different lowercase letters on the bar show significant differences between treatments and controls at 5% significance level. Each bar represents the average and standard deviation of at least three samples.

Fig. 4. Resistance of homozygous transgenic lines to sheath blight in the field. **A**, Expression levels of *OsPGIP1* in the plants of three homozygous transgenic lines (T262-3, T264-3, and T266-2) and wild type (WT) Xuedao3. **B**, Sheath blight disease scores of the transgenic lines and WT at 30 days after heading or 45 days after inoculation. **C**, Reduction in damage caused by RsPG in the homozygous transgenic lines. Different lowercase letters on the bar show significant differences between treatments and controls at 5% significance level. Each bar represents the average and standard deviation.

PG from *Aspergillus niger* but not from *Fusarium moniliforme*; in contrast, PvPGIP2 interacts with both PG. In rice, of the seven OsPGIP, OsPGIP1 lacks an entire LRR and has been confirmed to inhibit several fungal PG, including RsPG (Jang et al. 2003; Janni et al. 2006; Lu et al. 2012). In this study, we purified an OsPGIP1 fusion protein and found that it could inhibit RsPG activity and *R. solani* growth in vitro (Fig. 1). These results suggest that OsPGIP1 recognizes RsPG.

R. solani is a necrotrophic pathogen and generally requires cell-wall-degrading enzymes to destroy host cells for its early infection (Chen et al. 2006). RsPG are a class of cell-wall-degrading enzymes and have their highest activity among the enzymes secreted by *R. solani*, which indicates its key role in SB infection. Upon *R. solani* infection, we found strong *OsPGIP1* induction in rice, especially in SB-resistant varieties (Fig. 2C), which is consistent with the report by Lu et al. (2012). *OsPGIP1* expression was also found significantly induced by wounding but only in SB-resistant varieties, whereas it was significantly induced in all varieties tested under cold and

darkness treatments. This implies that wounding may be more similar to SB infection than cold and darkness stresses, which is consistent with the fact that necrotrophic pathogens generally damage plant tissues during infection. These results further support the hypothesis that *OsPGIP1* is involved in defense against *R. solani* infection. For rice growing in the field, plants at the later tillering stage generally acquire maximum numbers of tillers and, therefore, create a favorable microclimate for *R. solani* infection (Zuo et al. 2010). Interestingly, we found that *OsPGIP1* is highly expressed in sheaths, especially at the later tillering stage (Fig. 2B), which could be the result of coevolution, allowing rice to use the OsPGIP1 protein to counter *R. solani* infection. All these observations support an important role played by *OsPGIP1* in conferring resistance to rice SB.

Janni et al. (2006) reported that *OsPGIP1* was constitutively expressed in rice roots, leaves, and spikes. However, in this study, we found diverse expression patterns of *OsPGIP1* in either cultivars, tissues, or development stages but not constitutive expression across

Table 1. Agronomic trait data from populations of the homozygous transgenic lines^a

Plant genotype	Heading date	Plant height	Tiller number	Flag leaf length	Flag leaf width	Panicle length	Number of the first branch
Wild plant	100.89 ± 1.85	95.87 ± 0.64	9.13 ± 0.51	20.98 ± 0.66	1.79 ± 0.078	19.28 ± 0.24	13.33 ± 0.33
T262-3	100.32 ± 3.21	96.29 ± 1.56	9.22 ± 0.84	20.68 ± 0.57	1.82 ± 0.041	18.12 ± 0.54	13.00 ± 0.58
T263-4	99.71 ± 2.41	96.73 ± 1.07	9.00 ± 0.33	20.94 ± 0.61	1.81 ± 0.092	18.75 ± 0.61	13.16 ± 0.48
T266-2	98.14 ± 1.63	96.52 ± 2.00	9.67 ± 0.34	21.27 ± 0.68	1.82 ± 0.063	18.08 ± 0.64	12.57 ± 0.57
<i>F</i> and <i>P</i>	<i>F</i> = 0.51, <i>P</i> = 0.68	<i>F</i> = 0.44, <i>P</i> = 0.71	<i>F</i> = 2.67, <i>P</i> = 0.095	<i>F</i> = 0.45, <i>P</i> = 0.72	<i>F</i> = 1.58, <i>P</i> = 0.25	<i>F</i> = 2.14, <i>P</i> = 0.13	<i>F</i> = 1.91, <i>P</i> = 0.16

^a *F* and *P* values are from analysis of variance. *P* values greater than 0.05 indicate no statistically significant differences. Numerical values in the table are average ± standard deviation.

Fig. 5. Agronomic characteristics of the transgenic homozygous lines. **A**, Plants at late tillering stage; **B**, panicles; and **C**, seed.

samples tested. For instance, relatively low expression of *OsPGIP1* was detected in roots of Lemont and YSBR1 at seedling stage and of Jasmine 85 and YSBR1 at tillering stage; and almost no expression of *OsPGIP1* was observed in roots at heading stages and in panicles in all three varieties tested (Fig. 2A). These various results compared with those of Janni et al. (2006) should be attributed to the difference in varieties used. After different stress treatments, plant *PGIP* genes were also found to present diverse induction expression patterns (Di et al. 2012; Machinandiarena et al. 2001; Oliveira et al. 2010), indicating their roles against various stresses. In *Medicago truncatula*, the expression of *MtPGIP1* was induced at 6 h after infection with *Colletotrichum trifolii* and maintained the high expression levels until over 72 h (Song and Nam 2005). The expression levels of *PGIP* genes were increased in potato leaves in response to wounding and infection by incompatible *Phytophthora infestans* (Machinandiarena et al. 2001). In this study, we found that *OsPGIP1* was strongly induced by dark and cold treatments in all varieties tested, and by wounding only in SB-resistant varieties (Fig. 3). This indicates that *OsPGIP1* also may be involved in tolerance to stresses from darkness and cold in addition to resistance to *R. solani* infection.

We further overexpressed *OsPGIP1* in the commercially susceptible *japonica* XD 3, and found that it enhanced resistance to SB in the field and inhibited tissue degradation caused by RsPG in biochemical analysis (Fig. 4C). We believe that the reduction in tissue degradation can account for the increased SB resistance in these transgenic lines. We found that overexpression of *OsPGIP1* reduced SB disease scores by 0.9 to 1.2 in field studies (Fig. 4B).

The effect of *OsPGIP1* (0.9 to 1.2) is comparable with effects conferred by major SB resistance quantitative trait loci (QTL) identified thus far. For example, *qSB-9*, which is the only SB QTL stably detected in multiple environments and mapping populations, only reduces the SB disease score by approximately 1.0 under the same rating scale (Zuo et al. 2014). Moreover, except for two, all other SB QTL were only preliminarily mapped (Srinivasachary and Savary 2011; Zuo et al. 2010, 2014), which greatly reduces their potential for use to develop SB-resistant varieties due to the fact that most of them colocalize with genes or QTL conferring undesirable traits. Importantly, we found that the *OsPGIP1* overexpression plants maintained the same morphological and developmental traits and values in yield-associated components as the wild type in this study. These results demonstrate that overexpression of *OsPGIP1* results in no inferior effects on rice development while improving SB resistance. In fact, overexpression of *PGIP* genes has been reported in many other plants for improving plant disease resistance without apparent detrimental effects on plant development (Ferrari et al. 2012; HuangFu 2014; Hwang et al. 2010; Powell et al. 2000; Richter et al. 2006). Nguema-Ona et al. (2013) reported that overexpression of grapevine *PGIP* genes had no significant influence on plant morphology or normal growth processes although a few transgenic lines showed subtle changes in pectin and cellulose components in cell walls. Consequently, we conclude that overexpression of the rice endogenous *OsPGIP1* gene is a feasible approach to the improvement of rice SB resistance.

Table 2. Values of yield-associated components collected from populations of the homozygous transgenic lines^a

Plant genotype	Panicle number per plant	Seed number per panicle	1,000 seed-grain weight
Wild plant	8.47 ± 0.40	129.3 ± 2.33	26.83 ± 0.67
T262-3	8.78 ± 0.77	127.7 ± 1.25	26.56 ± 1.32
T263-4	8.55 ± 0.69	130.5 ± 0.87	27.84 ± 0.83
T266-2	8.89 ± 0.20	132.2 ± 3.19	27.31 ± 0.69
<i>F</i> and <i>P</i>	<i>F</i> = 1.95, <i>P</i> = 0.17	<i>F</i> = 3.10, <i>P</i> = 0.06	<i>F</i> = 1.14, <i>P</i> = 0.22

^a *F* and *P* values are from analysis of variance. *P* values greater than 0.05 indicate no statistically significant differences. Numerical values in the table are average ± standard deviation.

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