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Note

Ectopic Expression of Wild Rice *OgGRP* Gene Encoding a Glycine Rich Cell Wall Protein Confers Resistance to *Botrytis cinerea* Pathogen on *Arabidopsis*

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A full-length cDNA of *OgGRP* gene encoding a glycine-rich cell wall protein was isolated from wild rice (*Oryza grandiglumis*). Deduced amino acid sequences of *OgGRP* are composed of 148 amino acids (16.3 kDa), and show 85.9% homology with *Osgrp-2* (*Oryza sativa*). RT-PCR analysis showed that RNA expression of *OgGRP* was regulated by defense-related signaling chemicals, such as cantharidin, endothall, jasmonic acid, wounding, or yeast extract treatment. In relation to pathogen stress, the function of *OgGRP* was analyzed in *OgGRP* over-expressing *Arabidopsis thaliana*. Overexpression of *OgGRP* in *Arabidopsis* contributed to moderate resistance against fungal pathogen, *Botrytis cinerea*, by lowering disease rate and necrosis size. In the analysis of the transgenic *Arabidopsis* lines to check the change of gene expression profile, induction of *PR1*, *PR5* and *PDF1.2* was confirmed. The induction seemed to be caused by the interaction of ectopic expression of *OgGRP* with SA- and JA-dependent signaling pathways.

Keywords : *Botrytis cinerea*, glycine-rich cell wall protein, *Oryza grandiglumis*, resistance, wild rice

The plant cell wall composes the first defence structure against external invasion. It is consisted of structural proteins, cellulose, hemicellulose, lignin, peptic and poly-saccharides (Keller et al., 1989). The major structural proteins of plant cell wall are glycine-rich proteins (GRPs), hydroxyproline-rich glyco-proteins (HRGPs) and proline-rich proteins (PRP) (Keller, 1993; Showalter, 1993). Plant GRPs contain numerous glycine residues and characteristic repetitive and regular structures such as the (Gly-X)n motif. In the (Gly-X)n motif, any amino acid including glycine can located in X position. A number of GRPs also contain hydrophobic domain in N-terminal region. In general, GRPs are assumed to be located in the cell wall or interface between cell wall and plasma membrane. Thus, the N-terminal region in GRPs may be responsible for the association of GRPs to the cell wall or plasma membrane (Cassab, 1998; Showalter, 1993) or be supposed to interact with extra- or inter-cellular proteins (Wyatt and Carpita, 1993). To date, a number of GRPs have been identified and characterized from various plants including petunia, bean, tobacco, *Arabidopsis*, tomato, carrot, rice, maize (Fang et al., 1991; Keller, 1993; Luo et al., 1991; Showalter, 1993). GRPs have been shown to be transcriptionally regulated by various stimuli, including wounding, cold, light (Kaldenhoff and Richter, 1989), water stress (de Oliveira et al., 1990), circadian clock hormone such as auxins (Reddy and Poovaiah, 1987), gibberellins (Nicolas et al., 1997), methyl jasmonate, ethylene (Molina et al., 1997), abscisic acid (Sachetto-Martins et al., 2000), salicylic acid (Hooft et al., 1986), and pathogen infection (Fang et al., 1991; Molina et al., 1997; Pawlowski et al., 1997). In *Arabidopsis thaliana*, *AtGRP3* plays a role in cell wall as a ligand, which interacts with the extracellular domain of cell wall-associated kinase, *Wak1* and resulted in induction of *PR1* and *Wak1* (Park et al., 2001).

Oryza grandiglumis (Döll) Prodoehl (CCDD) has been used as one of the materials for massive screening of disease resistance genes because of its potential for resistance to blast and bacterial blight (Vaughan, 1994). Suppression subtractive hybridization (SSH) and cDNA microarray technique have been employed for rapid screening of defense-related alleles from *O. grandiglumis* (Kim et al., 2005). Among defense-related genes, a glycine-rich cell wall protein, *OgGRP*, was isolated and characterized in this study. In addition to gene expression by various stimuli and antifungal activity, change of gene expression profile in transgenic plant was also investigated.

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Materials and Methods

Plant materials and treatments. *O. grandiglumis* (CCDD, 2n=4X=48) seeds were sown and grown in the National Crop Experiment Station (NCES), Rural Development Administration in Korea. Two-month-old leaf tissues were treated with 20% yeast extracts, wounding and chemicals, all which were prepared by dissolving appropriate solvent or water (Rakwal et al., 2001), and fresh water was used as control treatment. Tissues were incubated under 16 h of light and 8 h of darkness at 24°C. Samples were harvested at the indicated times, and used immediately or stored at −80°C.

Isolation of the *OgGRP* and sequence alignment. The full-length DNA of *OgGRP* was cloned using Genome-Walker™ Universal kit (Clontech, USA) according to manufacturer's instruction. To obtain the full-length cDNA, RT-PCR was performed using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, USA) by manufacturer's guide. PCR reaction was as follows: cDNA synthesis and pre-denaturation at 48°C for 30 min and at 94°C for 2 min, followed by 28 cycles of denaturing at 94°C for 15 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Gene-specific primers used for amplification were *OgGRP*-5 (5'-TTA GTG GTG TCC GTT GCC TCC GCC-3') and *OgGRP*-3 (5'-ATG GCT ACT ACT AAG CAT TTG GCT CTT-3'). Multiple sequence alignment was conducted by CLUSTALX (Thompson et al., 1997), and annotated using GeneDoc. Hydropathy plot was analyzed using ProtScale program in ExPASy (Kyte and Doolittle., 1982) and transmembrane helices domain was analyzed using TMHMM program in ExPASy (http://www.cbs.dtu.dk/services/TMHMM).

RNA extraction and semi-quantitative RT-PCR analysis. Total RNA was extracted from treated leaves using Concert™ Plant RNA Reagent (Invitrogen, USA) according to manufacturer's guide, and 30 ng of total RNA of *O. grandiglumis* was used for semi-quantitative RT-PCR. RT-PCR was performed using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, USA) according to manufacturer's instruction. PCR condition was as follows: cDNA synthesis and pre-denaturation at 48°C for 30 min and at 94°C for 2 min, followed by 28 cycles of denaturing at 94°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min. For RT-PCR, gene specific primers, *OgGRP*-F (5'-ACC CGC GCC ATA GCC AGA GCC TTG CCC AGA-3') and *OgGRP*-R (5'-ACA CTA CTC AGC TAA CTG AAG TAG CAA AGC-3'), were used. As a control, Rice 18S rRNA primers were used for wild rice 18S rRNA; 18S rRNA-F (5'-ATG ATA ACT CGA CGG ATC GC-3') and 18S rRNA-R (5'-CTT GGA TGT GGT AGC CGT TT-3') (Kim et al., 2003). PCR products were separated on 1% agarose gels, and stained with ethidium bromide. The numerical value intensity of the bands obtained from the RT-PCR product was estimated with a Scion Image Instrument (Scion Corp., MD) using the software LabWorks 4.6 and calculated according to control band assigned to a value of 100.

For RT-PCR of *OgGRP* overexpressing *Arabidopsis* transgenic plants, gene specific primers, *OgGRP*-F and *OgGRP*-R, *AtPR1* (F-5'-ATG AAT TTT ACT GGC TAT TCT CGA-3' and R-5'-'TTA GTA TGG CTT CTC GTT CAC ATA-3'), *AtPR5* (F-5'-ATG GCA AAT ATC TCC AGT ATT CAC-3' and R-5'-CGA CAT TGT TCT GAT CCA TGA AAT-3') and *AtPDF1.2* (F-5'-ATG GCA AAG TTT GCT TCC ATC ATC-3' and R-5'-TTA ACA TGG GAC GTA ACA GAT ACA-3') were used. As a control, *Arabidopsis* 18S rRNA primers from *Arabidopsis* were used; 18S rRNA-F (5'-ACG CGC GCT ACA CTG ATG TA-3') and 18S rRNA-R (5'-TGA TGA CTC GCG CTT ACT AGG A-3'). The numerical value intensity of the bands obtained from the RT-PCR product was estimated with a Scion Image Instrument (Scion Corp., MD) using the software LabWorks 4.6 and calculated according to control band assigned to a value of 1.

Construction of *OgGRP* for *Arabidopsis* transformation. The *OgGRP* cDNA fragment was cloned into *Sma*I and *Sac*I enzyme sites of the pCAMBIA-IVS 1301 vector yielding pCAMBIA-*OgGRP*. The plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404. Transgenic *Arabidopsis* was generated by floral dipping method (Clough and Bent, 1998). Transgenic lines were selected on MS medium containing Hygromycin (50 μg/mL) and RT-PCR analysis was carried out as described above.

Pathogen Growth Assay. *B. cinerea* was grown on potato dextrose agar (Difco, France) at 25°C for 10 days. Four-week-old transgenic plants were inoculated with *B. cinerea* spore suspension (3×10^5 conidia/mL) by 5 μL of spore solution dropping on the surface of leaf. The inoculated plants were incubated in high humidity chamber at 22°C. The susceptibility to *B. cinerea* was evaluated by necrosis size and disease index at 3 or 6 days after inoculation (DAI) (Zimmerli et al., 2001). Disease index was scored by the symptom of inoculated leaves as follows; 0, no necrosis; 1, mild necrosis; 2, half necrosis; 3, severe necrosis symptom.

Results and Discussion

Pathogenesis-related transcripts were isolated and screened

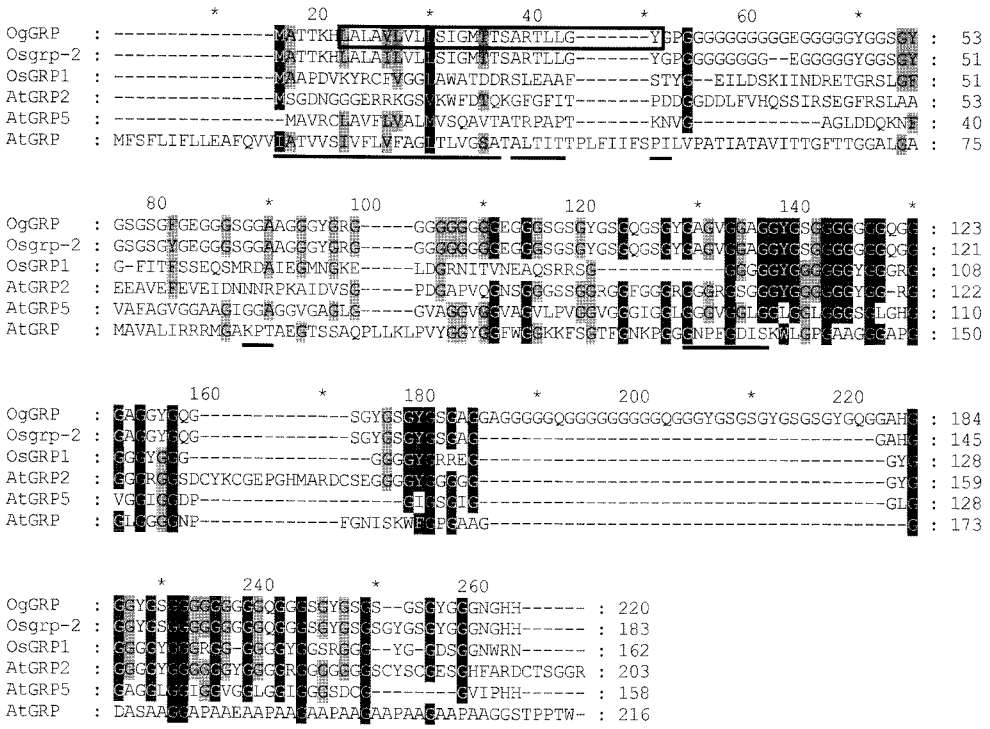

Fig. 1. Multiple deduced amino acids alignment of *OgGRP* (*O. grandiglumis*), *Osgrp-2* (*O. sativa L.*, GenBank Acc No. U40708), *OsGRP* (*O. sativa L.*, AF010579), *AtGRP* (*A. thaliana*, AK175635), *AtGRP2* (*A. thaliana*, AF360202) and *AtGRP5* (*A. thaliana*, AY096677). Identical amino acid residues are in black boxes, 80% similar in dark gray boxes, 60% similar in light gray boxes. Amino acid residues are numbered at the end of lines. Boxed amino acids indicate a putative transmembrane helices domain. Hydrophobic regions are underlined.

from wounding and elicitor-treated wild rice by combination of PCR-based subtraction and microarray analysis (Kim et al., 2005; Jeon et al., 2008). One of the induced genes by the stresses was *OgGRP* (*Oryza grandiglumis* Glycine-Rich cell wall Protein, Genbank accession No. EU360956). The full length cDNA of *OgGRP* obtained by 5' and 3' RACE was 663 bp long. *OgGRP* polypeptide is composed of 148 amino acids with a predicted molecular mass of 16.3 kDa and an isoelectric point (pI) of 6.89. Deduced amino acid sequences of *OgGRP* shows 85.9% homology with those of rice *Osgrp-2* (Genbank accession No. U40708).

In N-terminal region of *OgGRP*, amino acids from 7 to 29 predict transmembrane helices domain and subsequent hydropathy plot analysis shows several hydrophobic domains in N-terminal region that is assumed to be associated with the cell wall or cell membrane (Fig. 1). The remaining sequences are characteristic of highly hydrophilic signature. Southern blot analysis was performed to determine the copy number of *OgGRP* in the genome of *O. grandiglumis*. According to the Southern blot analysis using full-length gene fragment as a probe, multiple bands were observed from the wild rice genomic DNAs (data not shown). This indicates that *OgGRP* belongs to a large gene family like other GRPs from many plant species (Lin et al., 2005; Sachetto-Martins et al., 2000). RT-PCR was carried out to analyze gene expression pattern of *OgGRP* by pathogen-related stresses in *O. grandiglumis* (Fig. 2). RT-PCR analysis showed that *OgGRP* transcripts were induced by cantharidin (CN), endothall (EN), SA or JA, which have been known as plant defense-inducing chemicals (Fig. 2). Especially, enhanced *OgGRP* expression by EN and JA treatment was observed; the increase of 4-fold at 24 h and 5-fold at 72 h than non-treatment plants, respectively. Yeast extracts and wounding treatment also induced gene expression of *OgGRP* in *O. grandiglumis*. CN and EN are protein phosphatase inhibitor and previously reported to be involved in the induction of isoflavonoid phytoalexins on cut surface of soybean cotyledons (MacKintosh et al., 1994). SA and JA are the endogenous plant hormones well-known for their important roles in pathogen infection. Their induced level by pathogen infection triggers separate sets of genes encoding antimicrobial proteins (Thomma et al., 1998).

This result implies that *OgGRP* expression is regulated by defense signaling. The basal expression of *OgGRP* detected from the control samples indicates that GRP may have the essential role as structural proteins in wild rice. There have been many reports on GRPs' involvement in

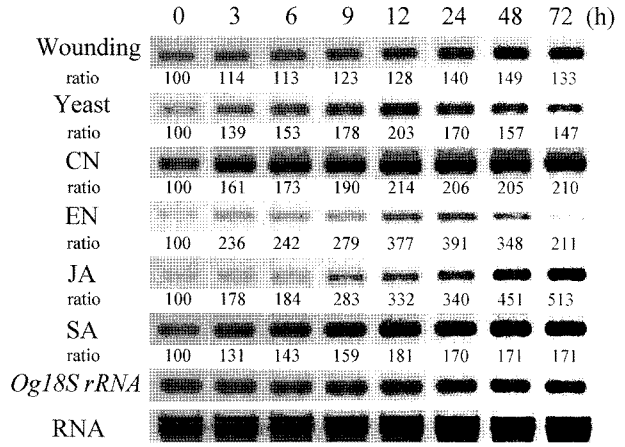

Fig. 2. RT-PCR analysis of *OgGRP* gene transcripts in *O. grandiglumis*. Leaves were treated with wounding, 20% yeast extract solution, 100 μM CN, 100 μM EN, 100 μM JA, or 100 μM SA. The relative expression of *OgGRP* gene was quantified using the software LabWorks 4.6 and calculated according to control band assigned to a value of 100. The relative expression ratios of *OgGRP* gene by each treatment were then calculated based on average of the results gathered from three times. Total RNAs were isolated from the treated samples at the indicated time. As a quantitative control, wild rice 18S rRNA was amplified.

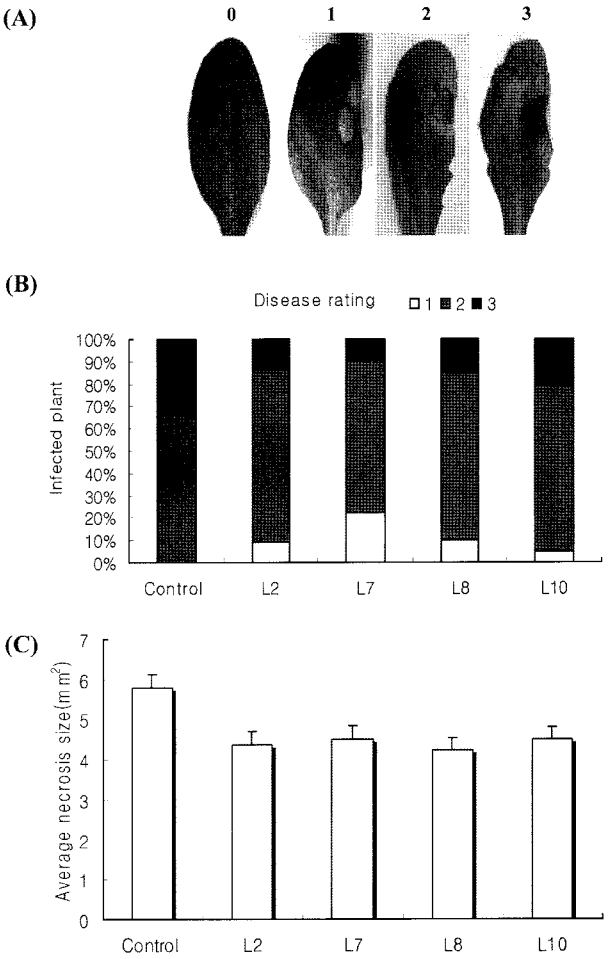

Fig. 3. Disease rating and average necrosis size of transgenic *Arabidopsis* seedlings overexpressing *OgGRP*. *Arabidopsis* plants were inoculated with *B. cinerea* (5 μL drop of a suspension of 3×10^5 conidia/mL), and observed for three days. (A) Disease index of the inoculated leaves. Healthy leaves were scored as '0', mild necrotic leaves as '1', noticeable necrotic leaves as '2', and severe necrotic leaves as '3'. (B) Susceptibility of *OgGRP* overexpressing transgenic plants against *B. cinerea*. It was evaluated from each transgenic line of L2, L7, L8, and L10. (C) Average necrosis size of four transgenic lines was smaller than that from control. Bars indicated the standard errors. All these experiments were carried out more than 3 times with 5 replications.

cell wall reinforcement or in signal transduction of defense-related response (Lin et al., 2005; Lu et al., 2007; Molina et al., 1997). Members of GRPs are more ubiquitous than expected in their temporal and spatial expression. RNA induction of *OgGRP* in response to defense signaling prompted us to test the role of *OgGRP* against fungal infection (Fig. 3). For the ectopic expression of *OgGRP*, pCAMBIA-*OgGRP* construct was made (data not shown). Subsequently, transgenic *Arabidopsis* plants overexpressing *OgGRP* driven by the 35S CaMV constitutive promoter were generated. Four transgenic lines (L2, L7, L8, L10) showing high ectopic expression of *OgGRP* were tested for *B. cinerea* resistance compared to wild type *Arabidopsis* plants (Fig. 3). *B. cinerea* was inoculated to the homozygous transgenic plants as previously described (Zimmerli et al., 2001). *B. cinerea* spore suspension (3×10^5 conidia/mL) was drop-inoculated and the susceptibility of control and the transgenic lines were scored by disease index (0 to 3) (Fig. 3A). A third of control plants inoculated was scored as disease index 3 (highest susceptibility) and the rest of them as index 2 (Fig. 3B). In case of transgenic lines, 9~21% showed disease index 3, 68~77% for 2 and 5~23% for 1, respectively. The result demonstrated that transgenic lines showed moderate resistance against *B. cinerea* disease infection. In the test of necrosis size after fungal infection, *OgGRP* overexpressing transgenic lines displayed much smaller size of necrosis (4.2~4.5 mm^2) in all 4 lines than in control plants (5.78 mm^2) (Fig. 3C). Considering both results from disease index and necrosis size, ectopic expression of *OgGRP* confers moderate resistance against *B. cinerea* infection on *Arabidopsis*. Considering the fact that *B. cinerea* is known to attack more than 200 different plant species (Elad, 1997; Prins et al., 2000) and causes a problematic disease, probing of new gene resources like *OgGRP* from wild species is desirable and highly recommended effort.

In the test of *OgGRP* role against fungal infection using *OgGRP* overexpressing transgenic lines, we observed their mild resistance against *B. cinerea*. To further understand the basis of resistance in transgenic *Arabidopsis*, we carried out

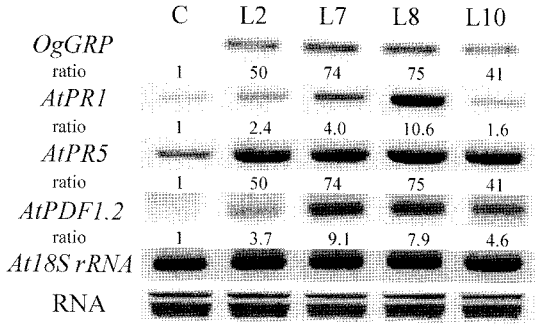

Fig. 4. Expression of *OgGRP*, *AtPR1*, *AtPR5* and *AtPDF1.2* in wild type (C) and *OgGRP* overexpressing transgenic *Arabidopsis* seedlings. Total RNAs were isolated from 4-week-old plants. The relative expression of each gene was quantified using the software LabWorks 4.6 and calculated according to control band assigned to a value of 1. As a quantitative control, *Arabidopsis* 18S rRNA was amplified.

RT-PCR analysis of defense-related endogenous genes that might confer the resistance against pathogen infection in *Arabidopsis*. Gene expression profiles of *AtPR1*, *AtPR5* and *AtPDF1.2* involved in each SA and JA signal pathway were investigated (Fig. 4). As previously confirmed, no expression of *OgGRP* was detected in non-transgenic line but all the transgenic lines had different levels of *OgGRP* expression. In control plants, *AtPR1* and *AtPR5* RNA transcripts were induced slightly but not for *AtPDF1.2*. All 3 genes, however, strongly induced or expressed in 4 transgenic plants. Especially, in case of *AtPR5* and *AtPDF1.2*, their expression pattern showed the increase of 75-fold at L8 and 9.1-fold at L7 than non-transgenic plants, respectively. It is well known that *PR1* and *PR5* are generally activated by SA signaling and plant defensin gene *PDF1.2*, along with a *PR3* and *PR4* gene, is induced by pathogens via JA-dependent pathway (Thomma et al., 1998). Considering the induced expression of *OgGRP* by JA and SA treatment (Fig. 2), overexpression of *OgGRP* in *Arabidopsis* interacts with those components of SA- and JA- dependent signaling pathways.

In conclusion, we cloned a novel *OgGRP* encoding a glycine-rich cell wall protein, and observed its induced expression by defense-related signaling. And ectopic expression of *OgGRP* in *Arabidopsis* conferred moderate resistance against *B. cinerea*. According to the gene profile analysis, the resistance was resulted from a certain interaction of *OgGRP* with components of SA- and JA- dependent signaling pathways.

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