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Simultaneous Expression of the Protease Inhibitors in a Rice Blast-Resistant Mutant

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We have previously identified genes for four different protease inhibitors (PIs) that were induced upon rice blast infection in a rice blast resistant mutant SHM-11. Our expression analysis of the PIs indicated that induction of the PIs was the highest 24 hr after rice blast inoculation in the rice mutant SHM-11. Three PIs in the group of serine PIs were highly expressed while a cystein PI was weakly expressed upon rice blast inoculation. Four PIs were weakly induced 48 hr after pathogen inoculation in rice blast susceptible wild type rice plant. The simultaneous expression of three serine PIs was apparent from SHM-11 and two of them were induced in rice blast resistant Taebaegbyeo. One of them was induced in rice blast resistant Hwayeongbyeo while none of them were expressed in rice blast susceptible Nagdongbyeo and rice blast resistant Dongjinbyeo. Our results suggest that the expression of PI gene is rice cultivar specific and may be linked with the rice blast resistance in a specific rice mutant by the simultaneous expression of the PI genes.

Keywords **:** defense, expression, protease inhibitors (PIs), rice blast-resistance

Plants defend themselves against pathogen by a variety of mechanisms including activation of complex structural and biochemical changes in plant cells (Hutcheson, 1998). Production and accumulation of pathogenesis-related (PR) proteins in pathogen-challenged cells is one of the defense responses (Dixon and Lamb 1990; Dixon et al., 1994; Song and Goodman, 2001). The PR proteins are rapidly induced in plant tissues in response to incompatible pathogen infection and confer disease resistance on the challenged plant. It has been suggested that PR proteins are effective to inhibit pathogen growth and to restrict systemic pathogen spreading in plant (van Loon, 1997). PR proteins are classified into 14 groups based on their homologies (van Loon and van Strien, 1999). A number of rice PR genes have been isolated encoding products such as PR-1 proteins, chitinase, and β-glucanase (Agrawal et al., 2000; Kim et al., 2000; Kim et al., 2003; Simmons et al., 1992; Xiong et al., 2001; Xu et al., 1996).

Rice blast, caused by the fungal plant pathogen *Magnaporthe grisea*, is one of the most devastating diseases in rice worldwide, causing severe losses (Baker et al., 1997). Both the rice plant and rice blast fungus are good model systems for studying plant-pathogen interactions. Rice is recognized as a model cereal crop because of its small genome and the availability of a transformation system and various pathosystems (Baker et al., 1997; Izawa and Shimamoto, 1996; Valent, 1990).

Previously, we have identified a number of rice genes involved in rice defense response against rice blast using the suppression subtractive hybridization method (Han et al., 2004). The rice genes involved in defense response were identified from rice blast resistant mutant SHM-11. The SHM-11 is a gamma-ray irradiated rice mutant showing rice blast resistance against several strains of *M. grisea*, while its wild type Sanghaehyanghyella (SAH) is susceptible to the corresponding fungal pathogen (Han et al., 2004). The identified defense response genes include several PR proteins. Among the identified PR proteins, four different protease inhibitors (PIs) were highly expressed during rice blast infection in the rice mutant SHM-11. PIs are in fact PR6 and known to be involved in defense against insect and nematode attack and are highly expressed during wounding and insect attack (Koiwa et al., 1997). There has been a report about antifungal activity of trypsin and chymotrypsin inhibitors from cabbage leaves against plant pathogenic fungus (Lorito et al., 1994). A family of serine PIs was isolated in rice and over-expression of one of these conferred resistance to blast infection (Qu et al., 2003). Two types of PI, serine protease inhibitors and cysteine protease inhibitors identified from our previous study differed from

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the rice PIs previously reported by Qu et al. (2003).

In this study, we describe the simultaneous expression of four different PIs in a rice blast resistant mutant and cultivar specific expression of individual PIs in different rice cultivars.

Materials and Methods

Plant materials. The rice (*Oryza sativa*) plant used in this study is a *japonica*-type cultivar Sanghaehyanghyella (SAH), i.e. black and sticky rice. SAH is highly susceptible to rice blast, although it provides good food additives because of its content of anthocyanin, iron, and zinc. The rice mutant SHM-11 was previously reported as a rice blast-resistant mutant (Han et al., 2004). Other rice cultivars Hwayeongbyeo, Dongjinbyeo, Nagdongbyeo, and Taebaegbyeo were also used for PI gene expression study. All rice cultivars and mutant were grown in a temperature-controlled glasshouse with natural lighting before extracting RNA and evaluating disease resistance.

Pathogen inoculation. We used a strain of the fungal pathogen *M. grisea* KJ-201 to investigate blast resistance of the rice plants. The fungi were grown on potato dextrose agar (PDA) at 25°C. Preparation and inoculation of the *M. grisea* inocula followed the procedure of Kim et al. (2001). Approximately, fungal spore suspension of 5×10^5 spores/ml were sprayed on the 4 week old rice plants and maintained in the humidity chamber for 24 hr at 25°C. Resistance to rice blast was scored by the absence of lesions and the appearance of hypersensitive response spots on the sprayed leaves 5 to 7 days after inoculation, while the development of disease was indicated by lesion formation and leaf tissue collapse. The percentage of leaf area infected was also determined to evaluate the resistance of the rice cultivars to rice blast.

RNA extraction and Northern blot analysis. Total RNA was isolated from SAH, the blast resistant mutant SHM-11, and other rice cultivars as described (Prescott and Martin, 1987) one day after inoculation with *M. grisea* KJ-201. Total RNAs (20 μg per lane) were fractionated on 1.2% denaturing agarose gels in the presence of formaldehyde (Sambrook et al., 1989), and blotted onto positively charged nylon membranes (Roche, Germany). Radiolabeled probes were prepared with [α-^{32}P] dCTP (Amersham Pharmacia Biotech, USA) using the Prime-a-Gene labeling system (Promega, USA). The nylon membrane was hybridized with a ^{32}P-labeled PCR-amplified full-length cDNA probe of four different PIs. Hybridization was carried out as previously described and membrane washing was also carried out as previously described (Church and Gilbert, 1984). The membrane was finally exposed to an X-ray film (Kodak) for 24 to 48 hr at −80°C.

Results

Responses of rice cultivars to rice blast pathogen. We screened SAH, SHM-11, and other rice cultivars for disease responses to *M. grisea* KJ-201 strain. While the wild type SAH was susceptible to the KJ-201 strain of *M. grisea*, its mutant SHM-11 appeared to have gained resistance to the tested *M. grisea* strain, KJ-201. Among tested rice cultivars in this study, Taebaegbyeo, Dongjinbyeo, and Hwayeongbyeo were resistant to the fungal disease. Nagdongbyeo was highly susceptible to the tested fungal pathogen. Numerous blast lesions and severe tissue collapse was observed from the susceptible rice plants 5 and 7 days after blast inoculation, respectively. Neither of these symptoms appeared in the rice blast resistant rice cultivars except for the appearance of tiny local spots, probably indicative of hypersensitive cell death (data not shown).

Protease inhibitor (PI) genes are highly induced in the blast resistant mutant. Four genes encoding PIs were obtained from our previous study (Han et al., 2004). One (RBR21) was a cysteine PI, oryzacystatin, which has been reported to be expressed during rice seed ripening (Abe et al., 1987), and three of them (RBR1, RBR13, RBR27) resembled other cereal serine PIs. The four different PIs were subjected to time-course expression analysis by Northern blot after *M. grisea* KJ-201 inoculation in the rice blast resistant SHM-11 plant and its wild type SAH. Three serine PIs and a cysteine PI were highly induced 24 hr after pathogen inoculation and the accumulation of mRNA were

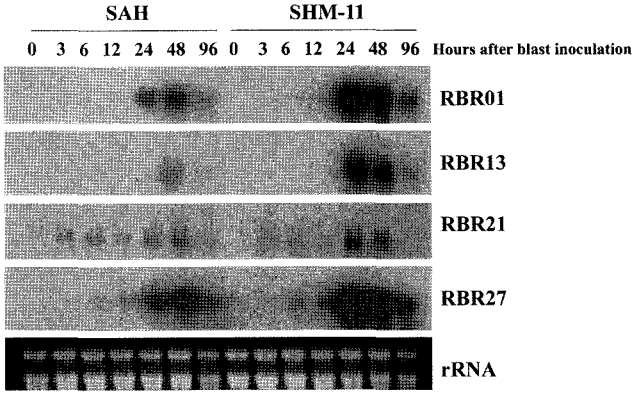

Fig. 1. Expression analysis of the PI cDNAs after inoculation with rice blast fungi. Four-week-old wild type (SAH) and mutant (SHM-11) rice plant were inoculated with *M. grisea* KJ-201 and total RNA sample were prepared over time from the rice plants. Total RNA 20 ug per lane was blotted on a nylon membrane and hybridized with the indicated ^{32}P-labeled probes.

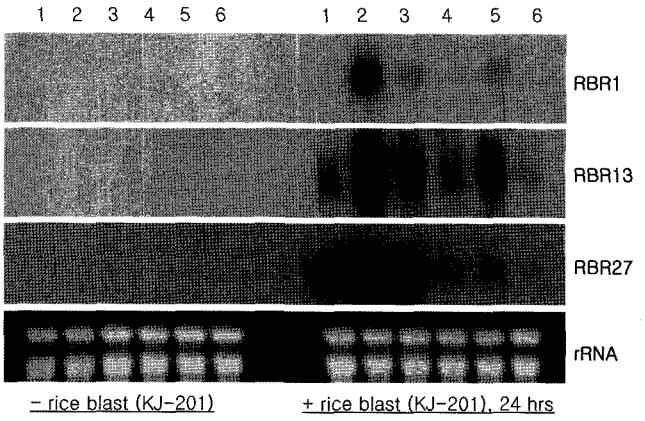

Fig. 2. Expression of PIs from different rice cultivars before and 24 hr after rice blast race KJ-201 infection. Taebaegbyeo, SHM-11, Dongjinbyeo and Hwayeongbyeo are KJ-201 resistant cultivars and Nagdongbyeo and Sanghaehyanghyella (SAH) are KJ-201 susceptible cultivars. 1, SAH; 2, SHM-11; 3, Taebaegbyeo; 4, Nagdongbyeo; 5, Hwayeongbyeo; 6, Dongjinbyeo

reduced after 24 hr in SHM-11 (Fig. 1). The weak expression of the PIs in SAH was observed 48 hr after pathogen inoculation. Expression of cysteine PI (RBR21) was relatively low both in SAH and in SHM-11 compared to expression of other PIs, although its expression in SHM-11 was slightly higher than that in SAH. The time-course Northern blot analysis indicated that the PIs were all simultaneously induced upon pathogen infection from rice blast resistant rice mutant. Expression of PIs in rice blast susceptible wild type SAH was much weaker and slower than that in SHM-11.

Rice cultivar specific expression of PIs. In order to investigate if the simultaneous expression of four PIs in the rice mutant is unique phenomena compared to other rice cultivar, Northern blot analysis was performed with three serine PIs probe using RNAs from different rice cultivars with or without *M. grisea* KJ-201 inoculation. Expression of serine PIs were only observed from pathogen infection irrespective of rice cultivars (Fig. 2). The simultaneous expression of all three serine PIs (RBR1, RBR13, RBR27) were only observed from SHM-11. Two of them (RBR13, RBR27) were expressed in Taebaegbyeo, which is highly resistant to several *M. grisea* races. However, the RBR1 was not expressed in Taebaegbyeo. The PI, RBR13, was also expressed from rice blast resistant Hwayeongbyeo. In contrast, no significant expression of three PIs was detected from rice blast resistant Dongjinbyeo and blast susceptible Nagdongbyeo and SAH. Therefore, culvar-specific expression of three different PIs was recognized from this analysis.

Discussion

PR proteins are classes of proteins induced and accumulated during incompatible interaction between pathogen and plant. Induction of many PR proteins has been previously described in rice treated with either elicitors or an incompatible pathogen (Kim et al., 2000; Xiong et al., 2001). The genes studied in this study include ones encoding PIs. PIs are well known for their role on defense response against insect and nematode attack and they are highly expressed during wounding and pest attack (Koiwa et al., 1997). Previously, we identified two types of PI, serine PIs and a cysteine PI. Expression analysis of four PIs in this study indicated that they are highly induced in a rice blast-resistant mutant compared to its wild type. Our result revealed that the PI genes were also expressed to some extent in the blast susceptible wild type plant 48 hr after blast inoculation. Therefore, it is likely that the blast resistance phenotype is determined at an early stage of the plant pathogen interaction, as previously defined (Morris et al., 1998). Although three serine PIs (with 53-65% identity) and one cysteine PI (oryzacystatin) were different in their amino acid sequences, they were simultaneously induced in the blast resistant mutant. To our knowledge, this is the first report of the simultaneous expression of three different PIs in a rice plant upon rice blast infection. It is not clear why the two types of genes are highly expressed in the mutant, but their expression may be inhibited by the same signaling pathway in the wild type. PIs may be involved in the inhibition of fungal enzymes essential for pathogenicity. Possible site of action of the PIs is on the exogenous fungal proteinase involved in pathogenicity. So far, there is no evidence that the PI inhibition influence fungal growth or pathogenicity.

In this study, we report the cultivar specific expression of different PIs in rice blast resistant cultivars, such as Hwayeongbyeo and Taebaegbyeo. No expression of PIs in KJ-201 resistant Dongjinbyeo suggested that the Dongjinbyeo might have different PR proteins involved in rice blast resistance. Lorito et al. (1994) previously reported that the cabbage serine PIs have no effect on the host-specific pathogen, suggesting that there is specificity between the PIs and the fungal pathogen. So far, the antifungal specificity of PIs or cultivar specific expression of PIs was not extensively characterized.

The remaining important question from this study is to define the roles of the identified PI genes in blast resistance. Analysis of direct antifungal activity of PIs against *M. grisea* and a transgenic study of the identified PI genes should also give us a clue on the function of the various PIs during disease resistance.

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References

Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S. 1987. Molecular cloning of a cysteine proteinase inhibitor of rice (oryzacystatin). Homology with animal cystatins and transient expression in the ripening process of rice seeds. *J. Biol. Chem.* 262:16793-16797.

Agrawal, G. K., Jwa, N.-S. and Rakwal, R. 2000. A novel rice (*Oryza sativa* L.) acidic *PR1* gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. *Biochem. Biophysic. Res. Commun.* 274:157-165.

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S. P. 1997. Signaling in plant-microbe interactions. *Science* 276: 726-733.

Church, G. M. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81:1991-1995.

Dixon, R. A. and Lamb, C. J. 1990. Molecular communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:339-367.

Dixon, R. A., Harrison, M. J. and Lamb, C. J. 1994. Early events in the activation of plant defence responses. *Annu. Rev. Phytopathol.* 32:479-501.

Han, C. U., Lee, C. H., Jang, K. S., Choi, G. J., Lim, H. K., Kim, J. C., Ahn, S. N., Choi, J. E., Cha, J. S., Kim, H. T., Cho, K. Y. and Lee, S. W. 2004. Identification of rice genes induced in a rice blast-resistant mutant. *Mol. Cells* 17:462-468.

Hutcheson, S. W. 1998. Current concepts of active defense in plants. *Annu. Rev. Phytopathol.* 36:59-99.

Izawa, T. and Shimamoto, K. 1996. Becoming a model plant: the importance of rice to plant science. *Trends Plant Sci.* 1:95-99.

Kim, C. Y., Lee, S.-H., Park, H. C., Bae, C. G., Cheong, Y. H., Choi, Y. J., Han, C.-D., Lee, S. Y., Lim, C. O. and Cho, M. J. 2000. Identification of rice blast fungal elicitor-responsive genes by differential display analysis. *Mol. Plant-Microbe Interact.* 13:470-474.

Kim, J.-C., Choi, G. J., Park, J.-H., Kim, H. T. and Cho, K. Y. 2001. Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica*. *Pest Manag. Sci.* 57:554-559.

Kim, S. T., Cho, K. S., Kim, S. G., Kang, S. Y. and Kang, K. Y. 2003. A rice isoflavone reductase-like gene, *OsIRL*, is induced by rice blast fungal elicitor. *Mol. Cells* 16:224-231.

Koiwa, H., Bressan, R. A. and Hasegawa, P. M. 1997. Regulation of protease inhibitors and plant defense. *Trends Plant Sci.* 2:379-384.

Lorito, M., Broadway, R. M., Hayes, C. K., Woo, S. L., Noviello, C., Williams, D. L. and Harman, G. E. 1994. Proteinase inhibitors from plants as a novel class of fungicides. *Mol. Plant-Microbe Interact.* 7:525-527.

Morris, S. W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C. C., Frederiksen, R. A., Bhandhufalck, A., Hulbert, S. and Uknes, S. 1998. Induced resistance responses in maize. *Mol. Plant-Microbe Interact.* 11:643-658.

Prescott, A. and Martin, C. 1987. A rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant. Mol. Biol. Rep.* 4:219-224.

Qu, L.-J., Chen, J., Liu, M., Pan, N., Okamoto, H., Lin, Z., Li, C., Li, D., Wang, J., Zhu, G., Zhao, X., Chen, X., Gu, H. and Chen, Z. 2003. Molecular cloning and functional analysis of a novel type of Bowman-Birk inhibitor gene family in rice. *Plant Physiol.* 133:560-570.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Mannual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Simmons, C. R., Litts, J. C., Huang, N. and Rodriguez, R. L. 1992. Structure of a rice beta-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elictors. *Plant Mol. Biol.* 18:33-45.

Song, F. and Goodman, R. M. 2001. Molecular biology of disease resistance in rice. *Physiol. Mol. Plant Pathol.* 59:1-11.

Valent, B. 1990. Rice blast as a model system for plant pathology. *Phytopathology* 80:33-36.

Van Loon, L. C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* 103:753-765.

Van Loon, L. C. and Van Strien, E. A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55:85-97.

Xiong, L., Lee, M.-W., Qi, M. and Yang, Y. 2001. Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. *Mol. Plant-Microbe Interact.* 14:685-692.

Xu, Y., Zhu, Q., Panbangred, W., Shirasu, K. and Lamb, C. 1996. Regulation, expression and function of a new basic chitinase gene in rice (*Oryza sativa* L.). *Plant Mol. Biol.* 30:387-401.