**SHORT COMMUNICATION**

# INDUCTION OF PATHOGENESIS-RELATED PROTEINS IN RICE BACTERIAL BLIGHT RESISTANT GENE XA21-MEDIATED INTERACTIONS WITH *XANTHOMONAS ORYZAE* pv. *ORYZAE*

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**SUMMARY**

In this study, we investigated pathogenesis-related (PR) protein expression in interactions between rice plants with bacterial blight resistant gene *Xa21* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial blight. Three PR genes, *OsPR1a*, *OsPR1b*, and *OsPR10a*, were cloned and the fusion proteins were expressed in *E. coli* to generate polyclonal antibodies. The proteins isolated at 0, 12, 24, 36, 48, 60, 72, and 144 h post-inoculation (hpi) from rice leaves inoculated with incompatible *Xoo* strains were analyzed using Western blotting. Significant inductions were observed for all three PR proteins in the late stages of inoculation. We also compared the expression of PR proteins among four different modalities: (i) incompatible interactions (R); (ii) compatible interactions in the absence of the R-gene ($S_R^-$); (iii) compatible interactions in the absence of the avirulence factor in *Xoo* ($S_{Avr}^-$); (iv) mock inoculated control treatments (M). Results showed that the three PR proteins are enhanced in the R, $S_R^-$, and $S_{Avr}^-$ interactions more than in the control treatments. The induction of PR proteins in the R interaction is the highest, suggesting that these proteins are involved in the defense mechanism.

*Key words*: rice, bacterial blight, pathogenesis-related proteins, Western blotting.

Rice (*Oryza sativa*), the staple food for nearly half the world's population, is heavily affected by bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Nearly 30 genes resistant to *Xoo* have been identified in recent years (Chu *et al*., 2006; Gu *et al*., 2005; Iyer and McCouch, 2004; Jiang *et al*., 2006; Song *et al*., 1995; Sun *et al*., 2004; Yoshimura *et al*., 1998). Among them, *Xa21* encodes a receptor-like kinase (Song *et al*., 1995) and belongs to the non-RD kinase family (Dardick and Ronald, 2006). Its transcription occurs regardless of the host developmental stage, infection with *Xoo*, or wounding (Century *et al*., 1999). Both the *in vitro*-expressed kinase domain and *in vivo*-purified XA21 protein are capable of autophosphorylation. Three phosphorylated residues in the juxtamembrane domain are implicated in maintaining XA21 stability (Liu *et al*., 2002; Xu *et al*., 2006). The functions have been determined of XB3, XB15, and OsWRKY62, the three XA21-binding proteins identified with yeast two-hybrid system (Park *et al*., 2008; Peng *et al*., 2008; Wang *et al*., 2006). Bip3 was identified recently through immunoprecipitation of the XA21 complex (Park *et al*., 2010). The identification of components involved in the *Xa21*-mediated resistance pathway will eventually unravel the mechanism of disease resistance.

PR proteins are defined as plant proteins that are induced in pathological or related situations (Kitajima and Sato, 1999). These inducible defense mechanisms have been identified in many plant species. A high mRNA level of *Oschib1*, a class III chitinase-encoding gene, was detected after inoculation of rice with *Magnaporthe grisea* or *Xoo*. Expression of *Oschib1* was induced more rapidly when an avirulent strain of *M. grisea* was inoculated (incompatible interaction) than when a virulent strain was used (compatible interaction) (Park *et al*., 2004). *RPR10a* transcripts were induced within the first 12 h following inoculation with *M. grisea* and by 48 h had returned to a lower level, while transcripts of *RPR10b* were also enhanced, but were not strongly visible until 48 h after inoculation (McGee *et al*., 2001). The accumulation of *PBZ1* mRNA was not induced by wounding, but it was induced by inoculation with the rice blast fungus (Midoh and Iwata, 1996). Subtractive hybridization and differential screening of a cDNA library led to the isolation of many induced genes from *Xoo*-infected rice, including a pathogenesis-related protein 1 (*OsPR1*) cDNA (Cho *et al*., 2004). Os*PR1b* and *PBZ1* were induced in rice plants inoculated with *Rhizoctonia solani*, the causal agent of rice sheath blight (Zhao *et al*., 2008).

In addition to the induction of PR gene transcription, the expression of PR proteins has also been report-

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ed. Western blotting analysis revealed that OsPR-10 was induced earlier and to a greater extent in incompatible reactions (Kim *et al*., 2003). Rice JIOsPR10 protein was also examined using Western blotting, and found to be expressed in developmental tissues, including flowers and roots. The protein was also expressed under abiotic stress, such as senescence and wounding (Kim *et al*., 2008).

The interaction between *Xa21*-containing rice plants and *Xoo* Philippine Race 6 (PR6) strain is incompatible, while compatible interactions will occur in the absence of R or the avirulence gene. Transcription of *OsPR1a*, *OsPR1b,* and *OsPR10a* in rice plants containing *Xa21,* when inoculated with *Xoo* PR6 strain were up-regulated, indicating that the induction of these *OsPR* genes are dependent of host-pathogen interactions and that the *Xa21* locus is required for the highest levels of induction (Ponciano *et al*., 2006). However, the expression of PR proteins was not reported. We have now generated OsPR1A, OsPR1B, and OsPR10A-specific antibodies and used Western blotting to detect the expression of PR proteins at different time points following inoculation with *Xoo.*

TP309 is a rice variety (*Oryza sativa japonica*) whose transgenic line 4021-3, containing the homozygous *Xa21* gene, was generated in Dr. P.C. Ronald laboratory at the University of California, Davis. The interaction between 4021-3 and *Xoo* PR6 is incompatible (R). PR6-infected TP309 and Philippine race 10 (PR10)-infected 4021-3, both showing susceptible interactions, are designated as $S_R^-$ and $S_{Avr}^-$, respectively (Xu *et al*., 2006). The mock control treatments (M) were inoculated using distilled water. The inoculation with *Xoo* was carried out using the leaf-clipping method (Kauffman *et al*., 1973). Samples from rice leaves, inoculated with *Xoo* were collected at 0, 12, 24, 36, 48, 60, 72, and 144 h post-inoculation (hpi).

We designed primers based on cDNA sequences of *OsPR1a* (LOC_Os07g03710.1), *OsPR1b* (LOC_Os01g 28450.1), and *OsPR10a* (LOC_Os12g36880.1) from the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu) using PrimerCE software (Cao *et al*., 2010). OsPR1aF (5'GGAATTCATGGCGAGTTCG TCGAGCAGGTT3') and OsPR1aR (5'CCCAAGCTT TCAGTAGGGAGATTGGCCGACG3') primers were used for PCR amplification of *OsPR1a.* OsPR1bF (5'GGAATTCATGGAGGTATCCAAGCTGG3') and OsPR1bR (5'CCCAAGCTTTTAGTAAGGCCTCTGT CCG3') primers were used for PCR amplification of *OsPR1b.* OsPR10aF (5'GAAGATCTGATGGCTCCGGC-CTGCGTCT3') and OsPR10aR (5'GGAATTCTTAG-GCGTATTCGGCAGGGTG3') primers were used for PCR amplification of *OsPR10a.* The underlined letters are the restriction enzyme recognition sites for *Eco*R I, *Hind* III, and *Bgl* II, respectively. PCR was performed with the following temperature regime: 94°C for 5 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, then followed by 72°C for 10 min. The PCR product was purified with a Fragment Purification Kit (TaKaRa, Japan) and analyzed in 1% agarose gel.

PCR products and expression vectors were digested with two restriction enzymes. The *OsPR1a* PCR product was ligated with pET30a-GST, a GST tag-containing version of pET30a (Novagen, Germany), created in our laboratory whose detailed sequence can be found in the PrimerCE vector database (Cao *et al*., 2010). *OsPR1b* and *OsPR10a* PCR products were integrated into the pET30a vector. The induction and purification of fusion proteins were carried out as described previously (Chen *et al*., 2009).

Rice protein extraction and Western blotting were performed as described previously with modifications (Wang *et al*., 2006). Briefly, the polyclonal antibodies were generated by BPI (Beijing Protein Innovation, China). For Western blotting, the membrane was incubated with a horseradish peroxidase-conjugated goat-anti rabbit antibody (Zhongshan Goldenbridge Biotechnology, China) for 1 h at room temperature. The blot was developed using a SuperECL Plus kit (Applygen, China), and the signal was exposed using X-ray film. The equal loading of proteins was confirmed by Ponceau S staining the membrane.

*OsPR1a, OsPR1b,* and *OsPR10a* were PCR-amplified using cDNA plasmids as templates and separated with a 1% agarose gel (Fig. 1A). The actual sizes of PCR products were consistent with the predicted 507, 495, and 477 bp, respectively. PCR-amplified products were cloned into expression vectors and verified by sequencing. Fusion proteins were induced and separated with SDS-PAGE. Proteins OsPR1A and OsPR1B accumulated highly in the pellet, while protein OsPR10A was found in both pellet and supernatant. The apparent molecular weight of the three recombinant fusion proteins shown in SDS-PAGE are consistent with the respective predicted sizes of 44.3, 22.9, and 22.0 kDa (Fig. 1B).

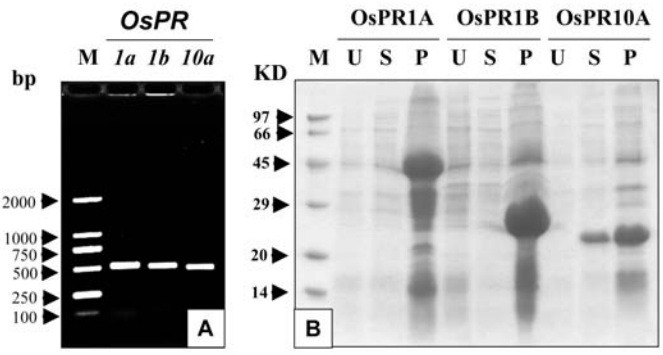


**Fig. 1.** A. PCR amplification of *OsPR1a, OsPR1b*, and *OsPR10a.* B. Expression of OsPR1A, OsPR1B, and OsPR10A fusion proteins in *E. coli.* U, not induced; S, Supernatant; P, Pellet.

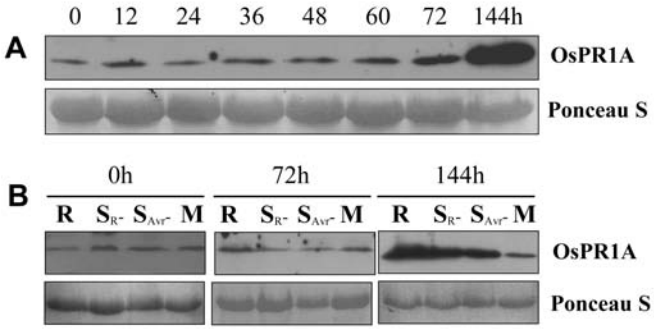


**Fig. 2.** Western blotting of OsPR1A protein expression in rice. A. Upper panel, OsPR1A protein expression in incompatible interactions at the time points indicated. B. Upper panel, OsPR1A protein expression at 0, 72 h, and 144 h in the R, $S_{R^-}$, $S_{Avr^-}$, and control interaction, respectively. Lower panel of A and B, Ponceau S stained membrane.

Purified OsPR1A, OsPR1B, and OsPR10A proteins were used as antigens to immunize rabbits. Since the amino acid sequence of protein OsPR1A and OsPR1B are quite close (64% of their amino acids are identical) (Ponciano *et al*., 2006), the antibody specificity for recombinant proteins was evaluated by Western blotting, which showed that recognition was specific (not shown).

To investigate OsPR1A protein expression in the incompatible interaction between rice and *Xoo*, the PR6 strain was used to inoculate rice 4021-3 plants. Inoculated leaves (about 1 cm) were harvested at 0, 12, 24, 36, 48, 60, 72, and 144 hpi. Western blotting analysis detected a band of 17.6 kD (Fig. 2A), which is consistent with the molecular weight of OsPR1A. The intensity of the band remained constant from 0 to 60 hpi, showed an enhancement at 72 hpi and a significant increase at 144 hpi, indicating that the induction of this protein takes place in the late stage of infection.

To compare the response between the R, $S_R^-$, $S_{Avr}^-$, and the control treatment, Western blotting was conducted at 0, 72, and 144 hpi. The results showed that at 0 hpi the four interactions produced low signals. At 72 hpi, the signal produced by the R interaction was higher than that produced by the $S_R^-$, $S_{Avr}^-$, and the control interaction. At 144 hpi, the signals produced by the R, $S_R^-$, and $S_{Avr}^-$ interactions were significantly higher than that produced by the control treatment. However, there were no significant difference among the $S_R^-$, and $S_{Avr}^-$ interactions (Fig. 2B).

When Western blotting was performed to examine OsPR1B protein expression in incompatible interactions, it was found that OsPR1B was barely detectable in the early stages of infection (0-48 hpi) showing a basal level expression, but the induction was much higher in the late stages (Fig. 3A). The size of the detected band is 17.5 kD, which is consistent with the molecular weight of protein OsPR1B. Comparison was carried out among R, $S_R^-$, $S_{Avr}^-$, and the control treatment at three time points (0, 72, and 144 hpi), disclosing a difference only at 72 and 144 hpi. At 72 hpi, the expression of the OsPR1B protein in the R interaction was higher than that in the $S_R^-$, $S_{Avr}^-$, and the control treatment. At 144 hpi, the expression of OsPR1B in the R interaction was the highest, followed by that in the $S_R^-$, $S_{Avr}^-$, and the control treatment (Fig. 3B).

When the expression in the incompatible interaction of protein OsPR10A was investigated, the signal was almost undetectable in the early stages, but increased significantly in the later stages (48, 60, 72, and 144 hpi) (Fig. 4A). At 48 hpi, the signal produced by the R interaction was higher than that in the $S_R^-$, $S_{Avr}^-$, and the control treatment. Moreover, the signal produced by the $S_R^-$ treatment was higher than that in the $S_{Avr}^-$ and the control treatment. At 144 hpi, the signal produced by the R interaction was higher than that in the $S_R^-$, $S_{Avr}^-$, and the control treatment, while there was no significant difference between the signals produced by the $S_{Avr}^-$ and the control treatment (Fig 4B).

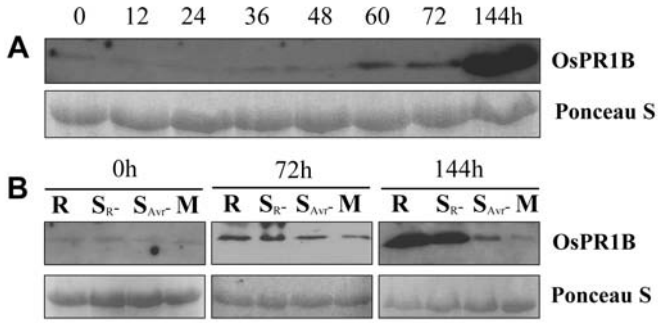


**Fig. 3.** Western blotting of OsPR1B protein expression in rice. A. Upper panel, OsPR1B protein expression in incompatible interactions at the time points indicated. B. Upper panel, OsPR1A protein expression at 0, 72 h, and 144 h in R, $S_{R^-}$, $S_{Avr^-}$, and the control interaction, respectively. Lower panel of A and B, Ponceau S stained membrane.

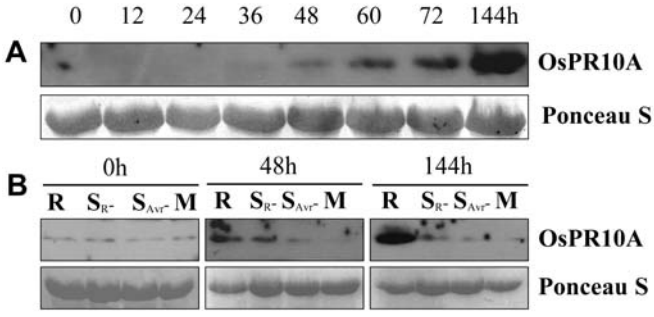


**Fig. 4.** Western blotting of OsPR10A protein expression in rice. A. Upper panel, OsPR10A protein expression in incompatible interactions at the time points indicated. B. Upper panel, OsPR10A protein expression at 0, 48 and 144 h in R, $S_{R^-}$, $S_{Avr^-}$, and the control interaction, respectively. Lower panel of A and B, Ponceau S stained membrane.

Previous report have shown that transcription of *OsPR1a*, *OsPR1b*, and *OsPR10a* genes is correlated with both *Xa21* and the *Avr* factor for the highest levels of induction (Ponciano *et al*., 2006). We have now found that the expression of the PR proteins in the incompatible interactions is significantly higher than that in the compatible interaction. In particular, the expression of OsPR1B and OsPR10A in $S_{R}^{-}$ interactions is higher than that in the $S_{Avr}$- interactions, suggesting that the *Avr* factor plays an important role in the induction of PR proteins. In the late stage of infection, the expression of OsPR1A, OsPR1B, and OsPR10A in the R, $S_{R}^{-}$, and $S_{Avr}$- interactions was significantly higher than that in control treatments, suggesting that either R or *Avr* factor may contribute to the accumulation of PR proteins.

Induction of OsPR1A, OsPR1B, and OsPR10A proteins occurred at 72, 60, and 48 hpi, respectively indicating that induction of OsPR10A is the earliest, while OsPR1A is induced at a later stage. The induction of PR genes detected by Northern blotting occurred at three days (72 hpi) (Ponciano *et al*., 2006). Rice *RPR10a* transcripts were induced as early as 12 hpi with *M. grisea* (McGee *et al*., 2001). In the compatible interactions between rice and *M. grisea* race KJ101, accumulation of OsPR-10 was first observed 48 hpi, while in the incompatible interaction (*M. grisea* race KJ401), it was detected 36 hpi (Kim *et al*., 2003). Compared with previous results, it seems that the induction of PR proteins occurred later in response to infection with *Xoo* than in infection with *M. grisea.* Based on the Western blotting results, the induction of PR proteins can last for several days, suggesting that they are not only involved in the initiation of the defense response, but also in maintaining the inhibition of bacterial multiplication. No significant difference in the mock-inoculated control samples at different time points were observed, indicating that the induction of the PR proteins is not affected by wounding. In contrast, the expression of the JIOsPR10 protein began to increase at 4 h post-wounding, reached a maximum level at 12 h, and was still detectable at 24 h (Kim *et al*., 2008).

In summary, our results indicate that both the R gene and the *Avr* factor can contribute independently to the induction of the OsPR1A, OsPR1B, and OsPR10A proteins. However, the highest level of PR protein expression occurred when both factors are present simultaneously.

Inductions take place during the late stage of inoculation with *Xoo*, suggesting that the three PR proteins are involved in the defense response. Up- or down-regulation of these PR proteins by means of over-expression or RNA interference may alter resistance to *Xoo* in rice. The data also suggest that PR proteins may play different roles in the interactions as indicated by the level and time of induction.

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