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Pathogenesis-related gene expression in rice is correlated with developmentally controlled *Xa21*-mediated resistance against *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Disease resistance mediated by the resistance gene *Xa21* is developmentally controlled in rice. We examined the relationship between Pathogenesis Related (PR) defense gene expression and *Xa21*-mediated developmental disease resistance induced by *Xanthomonas oryzae* pv. *oryzae* (Xoo). *OsPR1a*, *OsPR1b*, and *OsPR1c* genes were cloned and their induction was analyzed, in addition to the *OsPR10a* gene, at the juvenile and adult stages in response to a wildtype Xoo strain that induces a resistance response (incompatible interaction) and an isogenic mutant Xoo strain that does not (compatible interaction). We found that the adult stage leaves are more competent to express these *OsPR1* genes and that the *Xa21* locus is required for the highest levels of induction.

Keywords: *Oryza sativa* var. indica; *Xanthomonas oryzae* pv. *oryzae*; Pathogenesis-related proteins; Developmental disease resistance; Adult plant resistance; Quantitative real time PCR

1. Introduction

Resistance to disease in many host plants depends on the developmental stage at which a plant is exposed to a pathogen. Host–pathogen interactions involving both monocots and dicots and various bacterial, fungal, and viral pathogens exhibit developmentally controlled resistance. For example, in various rice lines, resistance to *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal agent of bacterial leaf blight, varies with developmental stage [1–5]. Generally in rice, adult plant resistance exhibits race- and cultivar-specificity and is affected by environmental conditions. At later growth stages, it is thought that resistance against all isolates increases in a race-nonspecific manner [3]. Studies of the bacterial blight resistance response conditioned by the resistance gene *Xa21* by us and others indicate that *Xa21*-mediated resistance to Xoo is not fully expressed in early stages of development but, as the plant matures further, resistance increases [6,7]. The Xoo-*Xa21* interaction provides a model system to study the molecular basis of developmental control of disease resistance because the *Xa21* gene has been cloned [8] and functionally characterized [9,10]. Several genes from Xoo have been cloned that are essential for AvrXa21 activity [11–15]. In this host–pathogen system, near-isogenic lines of rice and Xoo strains allow for precisely controlled comparisons. Our previous studies showed that expression of the *Xa21* gene is independent of plant developmental stage, infection with Xoo, or wounding [7]. Expression of the *Xa21* gene in leaves is not correlated with expression of *Xa21*-mediated disease resistance. Recently, it has been proposed that levels of the XA21

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protein are developmentally controlled by proteolytic activity [10]. It may be that levels of the XA21 protein control levels of resistance.

The connection of disease resistance and plant development for any plant pathogen-host combination is far from understood [16]. In some plant pathogen interactions, defense gene expression is positively correlated with developmentally controlled resistance [17,18]. In a study of the interaction between tobacco and *Phytophthora parasitica*, increased resistance was shown to coordinate with the developmental transition from vegetative to flowering phase [19]. The apoplastic accumulation of PR1 (pathogenesis-related 1) protein directly correlated with the developmental transition to resistance. In addition to PR1 accumulation, differentially secreted proteins involved in stress response, cell wall modification and defense were identified and associated with resistance to this oomycete at the late developmental stage of tobacco [20]. Results from two studies in Arabidopsis conflict. One study showed that leaves of mature Arabidopsis plants were more resistant than leaves of young plants to *Pseudomonas syringae* pv. *tomato* [21], whereas another study did not detect differences [22]. Unlike the case in tobacco, *PR1* gene expression did not appear to be involved [21]. These studies on two different host pathogen systems, illustrate the diversity of defense mechanisms operating in the developmental control of resistance.

Expression of PR proteins is generally pathogen- and host–specific pointing to the need to examine the relationship in each host pathogen interaction [23,24]. In rice, it has been shown that infection with *Magnaporthe grisea* highly induced the transient expression of *PR1a*, *PR1b* and *PR10* in the incompatible interaction compared to the compatible interaction [25,26]. Rice lesion mimic mutants with the highest levels of *PR1b* expression showed enhanced resistance in a race non-specific manner to *M. grisea*. One of these mutants (*spl*11) showed enhanced race non-specific resistance to Xoo [27]. Another study showed that constitutive expression of PR1b and PR10a was correlated with enhanced resistance to *M. grisea* and *Burkholderia glumae* [28]. Expression of *PR* genes may contribute to non-race specific, broad-spectrum resistance against bacterial and fungal pathogens. Overexpression experiments have illustrated that there is a certain level of specificity in the antimicrobial activity by PR proteins [29].

PR1a, *PR1b* and *PR10* gene expression in rice has been examined in relationship to the specific response to jasmonic acid (JA). Both *PR1a* and *PR1b* genes were specifically induced by JA in a light- and dose-dependent manner [26,30,31] and a JA inhibitor prevented accumulation of PR1A-like proteins [26]. However, the exogenous application of JA did not confer resistance against pathogens [26]. In related studies, JA treatment produced tissue- and developmental stage-specific induction of *PR1a* and *PR10* [32,33]. *PR10* was also induced by treatment with SA, H_2O_2, UV light and $CaCl_2$, suggesting that *PR10* responds to both biotic and abiotic stresses [25,33]. All of these responses may be governed by the activity of the stress-responsive OsMAPK5 [28].

The objective of our study is to assess whether activation of *PR* gene expression parallels developmental expression of the *Xa21*-mediated resistance response [7]. Our working hypothesis is that full resistance at the adult stage correlates with activation of defense responses upon pathogen infection and accordingly, the lack of full resistance at the juvenile stage correlates with the lack of activation of defense responses. To study the activation of defense genes in resistant ($Xa21^+$) and susceptible ($Xa21^-$) rice lines, we cloned the *OsPR1a*, *OsPR1b*, and *OsPR1c* genes and analyzed their induction. At juvenile and adult stages in resistant and susceptible rice lines, we assessed the expression pattern of these *PR1* genes and the *OsPR10a* gene in response to a wild type Xoo strain that induces a resistance response in $Xa21^+$ rice and an isogenic Xoo strain that does not due to a mutation in the *raxQ* gene that disrupts AvrXa21 activity [11]. We found that the developmental stage of rice and the *Xa21* locus are important in governing the level of expression of *PR1* defense genes.

2. Materials and methods

2.1. Plant material and bacterial inoculations

Growth, maintenance and bacterial inoculations of *O. sativa* var. indica IRBB21 and IR24 plants were conducted as described in Century et al. [7]. Xoo race 6 induces resistance [7,8] and was used in inoculation studies (called race 6). The isogenic raxQ- mutant Xoo strain (PR6raxQ::*Tn5*) lacking *avrXa21* activity based on lesion length analysis [11] was also included in inoculation studies (called raxQ-). Two developmental stages, juvenile leaf 2 and adult leaf 6 at just full expansion, were tested. About 30 1-cm^2 leaf tip sections were harvested at the leaf 2 stage and 15–20 sections at the leaf 6 stage. Untreated tissue (for real time reverse transcriptase (RT)-PCR analysis) was collected at the time of inoculation. Collected tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA was isolated.

2.2. Cloning of OsPR1a, OsPR1b, and OsPR1c and probe preparation

To clone the *OsPR1a* cDNA, RT-PCR was used. Total RNA (244 ng) from several mock-inoculated leaf 6 at 3 days post-inoculation (DPI) was used as the template in OneStep RT-PCR (Qiagen, Valencia, CA, USA). Primers (3U25, 5′-CACGAGTCGATCTCCATCATCTCTT-3′ and 778L18, 5′-GCAAATACGGCTGACAGT-3′) were designed from the sequence of rice *PR1a* (GenBank accession no. AJ278436). RT-PCR was performed according to the OneStep instructions with the following temperature regime: 50 °C for 30 min; 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 56.6 °C for 30 s, and 72 °C for

1 min; followed by 72 °C for 10 min. PCR products were electrophoresed on agarose gels and DNA from gel sections was purified with the QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. After cloning and transformation with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to instructions, plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen) and sequenced (Davis Sequencing, LLC, Davis, CA).

To clone *OsPR1b*, PCR was used on genomic DNA (200 pg). Primers were designed (655U19, 5′-CAAGTCCTGCGTACAAATC-3′ and 1908L18, 5′-TAGAGAAGTGCGGCGATG-3′) from the DNA sequence of rice *PR1* (GenBank accession no. U89895). PCR was performed (0.5 μM each primer, 200 μM dNTPs, 1 U of Taq DNA polymerase, and 1 X PCR buffer) with the following temperature regime: 95 °C for 1 min; 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min; and 72 °C for 10 min. The PCR product was purified, cloned and sequenced as described above.

OsPR1c was cloned using PCR and a high-fidelity thermostable DNA polymerase on genomic DNA as above. Two different sets of primers (1264U15, 5′-GCGGTCGTGGGTGGA-3′ and 2114L17, 5′- GGGGTTGGGTGGGTGTG-3′; and 844U18, 5′-CGCCTCGCCAACATTTCC-3′ and 1524L17, 5′-GTAACGAGCGAGGGACG-3′) were designed from the sequences of the putative rice PR protein precursor (GenBank accession no. AP003853, region complement 32520…33023) and genomic clone OSJNBa0091E23 that contains the same sequence. Two primer combinations (1264U15 and 2114L17, and 844U18 and 1524L17) were used in PCRs using 2.5 U of *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA, USA), 1 X cloned *Pfu* DNA polymerase reaction buffer, 250 μM dNTPs, and 300 μM each primer. PCR was performed with the following temperature regime: 94 °C for 1 min; 30 cycles of 94 °C for 1 min, 63 °C for 1264U15/2114L17 or 60 °C for 844U18/1524L17 for 1 min, and 72 °C for 1 min; and 72 °C for 10 min. The PCR product was purified and cloned with the ZERO Blunt TOPO cloning kit (Invitrogen).

For DNA and RNA blots, specific probes were designed using non-conserved sequences of *OsPR1a*, *OsPR1b*, and *OsPR1c* based on a nucleotide alignment. Probes were produced with PCR using *OsPR1a*-specific primers (656U18, 5′-TATGCTACGTGTTTATGC-3′ and 776L17, 5′-AATACGGCTGACAGTAC-3′), *OsPR1b*-specific primers (65U17, 5′-GTTATTTATACACACGG-3′ and 221L18, 5′-ATAACCTGAAACAGAAAC-3′), and *OsPR1c*-specific primers (690U17, 5′-CGTCCCTCGCTCGTTAC-3′ and 858L19, 5′-GAAAAGAGCAAGACGCATC-3′). Plasmids containing the cloned genes (100 ng) were used as templates in PCRs (0.25 μM each primer, 50 μM dNTPs, 1 U of DNA Taq polymerase, and 1 X PCR buffer) that were performed with the following temperature regime: 95 °C for 1 min; and 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. PCR products were purified with the QIAquick PCR Purification kit (Qiagen) according to instructions. Purified PCR products were used as probes. The 800 bp insert from a *OsPR10a* clone (kindly provided by Hamer) was released by *Eco*RI and *Xba*I digestion, and purified after electrophoresis with the QIAquick Gel Extraction kit (Qiagen) according to instructions and used as a probe. Twenty-five ng of each probe was labeled with P^{32}-dCTP with the Strip-EZ™ DNA kit (Ambion, Austin, TX, USA) according to manufacturer's instructions.

2.3. DNA and RNA blot analysis

Genomic DNA was isolated from leaves, digested with restriction enzymes and fractionated on a 0.7% agarose gel and blotted onto Hybond-N+ (Amersham Life Science, Piscataway, NJ) following standard techniques. DNA was crosslinked to the membrane with UV light.

To isolate RNA, ground, frozen leaf samples were homogenized in TRIzol reagent (Invitrogen). After a chloroform extraction and isopropanol precipitation, RNA was purified using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions for RNA clean-up. Total RNA (5 μg) was fractionated on a pre-run 1% agarose denaturing gel (19.7 mM MOPS, pH 7.0; 6.2% formaldehyde) at 120 V. The RNA was blotted onto a nylon membrane and UV-crosslinked.

All membranes were pre-hybridized with ULTRAhyb™ solution (Ambion) at 42 °C for up to 75 min and hybridized with P^{32}-labeled probes overnight at 42 °C. After hybridization, membranes were briefly rinsed twice with Wash solution I (0.2X SSC and 0.1% SDS), washed three times for 10 min each with Wash solution II (0.1X SSC and 0.1% SDS) at 42 °C, and rinsed with Wash solution I. Phosphor screens were exposed to the membrane for up to 4 days, and scanned with the Storm® Gel and Blot Imaging System (Molecular Dynamics, Sunnyvale, CA, USA). For analysis of RNA blots, the intensity of each hybridized band was quantified with ImageQuant® software (Molecular Dynamics). To allow relative comparisons, intensities were normalized to 25S rRNA at each time point.

2.4. Quantitative real time RT-PCR analysis

Total RNA (4 μg) was used as template to obtain a cDNA yield of 200 ng/μl using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real time PCR on cDNAs of *OsPR1a*, *OsPR1b*, *OsPR10a* and *OsP1E6* (rice endogenous constitutively expressed control gene; J. Leach, personal communication; sequence complementary to region 34882…35025 of Indica AAAA02026344, Ctg026344 from *O. sativa* ssp. indica WGS contigs database) was carried out using Applied Biosystems 7300 Real Time PCR System and the TaqMan® chemistry (Applied Biosystems, Foster City, CA, USA).

To calculate mean relative expression levels of each *OsPR* gene, cDNAs from three independent RNA isolations

were analyzed in triplicate in the same 96-well micro chamber plate. Samples to be directly and statistically compared were analyzed in the same plate. The relative expression ratio was calculated according to Pfaffl [34]. Efficiencies were calculated [34] as 1.90 for *PR1a*, 2.06 for *PR1b*, 1.98 for *PR10a*, and 1.91 for *P1E6*. Expression of each target *OsPR* gene was normalized to expression of the constitutively expressed, endogenous reference gene *OsP1E6* and to its expression in untreated, matched control tissue. Means of the three independent experiments were statistically compared.

The following combinations of forward/reverse primers and probes were used for the real time PCR reactions: for *OsPR1a*, 5′-CGTCTTCATCACCTGCAACTACTC-3′, 5′-CATGCATAAACACGTAGCATAGCA-3′, and probe 5′-6FAMCCGGGCAACTTCGTCGGCCTAMRA-3′; for *OsPR1b*, 5′-AGCTGGCCATTGCTTTGG-3′, 5′-CGTTGTGGAGCCTCACGTAGT-3′, and probe 5′-6FAM TACGTGAGGCTCCACAACTAMRA-3′; for *OsPR10a*, 5′-CGCCGCAAGTCATGTCCTA-3′, 5′-GCTTCGTCTCCGTCGAGTGT-3′, and probe 5′-6FAMTCGGATGTGCTCGAGGCAGAAAGCTAMRA-3′; and for *OsP1E6*, 5′-TTGCACCTAGGAGCGTGGAT-3′, 5′-AACTGCACACAACAGTTTGCTCTT-3′, and probe 5′-6FAM TGTG GAAGGTGCCATTGGTTGGAGATAMRA-3′. AmpliTaq Gold enzyme (1.25 U) in 1X Gold buffer (Applied Biosystems) was used in each real time PCR reaction in combination with 3 mM $MgCl_2$, 400 μM dNTPs, 900 nM each primer and 250 nM TaqMan® probe with the following temperature regime: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 62 °C for 30 s; and 60 °C for 1 min. The data were analyzed using the 7300 Sequence Detection Software (Applied Biosystems) with manually set baseline and threshold.

3. Results

3.1. Cloning and analysis of PR1 genes and predicted gene products from rice var. indica IRBB21

Three *O. sativa PR1* genes (*OsPR1a* [GI:117655416], *OsPR1b* [GI:117655418], and *OsPRc1* [GI:117655420]) were cloned from rice line IRBB21 carrying *Xa21*. The copy number of each *OsPR1* gene was assessed using *OsPR1*-specific probes designed from non-conserved sequences in the untranslated regions of each of the three genes. Using the unique probes, we found that *OsPR1a*, *OsPR1b* and *OsPR1c* are present as single copy number genes (data not shown) within this multi-gene family.

The *OsPR1a* ORF is predicted to encode an acidic protein of 17.6 kDa (168 amino acids) with a pI of 4.4, *OsPR1b* ORF is predicted to encode a basic protein of 17.5 kDa (164 amino acids) with a pI of 9.0, and *OsPR1c* ORF is predicted to encode a basic protein of molecular weight of 18.1 KDa (167 amino acids) with a pI of 9.5. The *OsPR1c* ORF is highly similar (83–86%) to several monocot PR protein genes including wheat PR-1.1 (AJ007348) and PR1 (AF384143); barley PR proteins pbr1-2 (Z26320), PR1 (Z21494), PR protein (Z26333), and pbr1-3 (Z26321); and maize PRm gene (X54325).

We studied the relationships among *OsPR1* genes in IRBB21 and found that the ORFs are about 70% identical to each other. When compared to the genome sequences of indica line CO39 and japonica line Nipponbare, we found that the nucleotide sequence within each ORF was more highly conserved than that in the untranslated regions outside the ORF. At the amino acid level, the OsPR1 proteins are 64–48% identical to each other (Fig. 1). All three protein sequences have the conserved signal sequence cleavage site at the N-terminus and the six conserved cysteine residues in the body of the protein that are found in PR1 family members in plants [35]. In addition, all three proteins have the eight amino acid domain (GHYTQVVW) that is also conserved in vertebrate sequences of cysteine-rich secretory proteins (CRISPs) ([35]; Fig. 1).

3.2. Expression of OsPR genes in response to inoculation with Xoo race 6 and raxQ

To study the interaction of expression of *OsPR* genes in response to Xoo and developmental disease resistance in the *Xa21*-line IRBB21 as described [7], RNA gel blot analysis was performed. RNA from juvenile leaf 2 and adult leaf 6 inoculated with race 6 and raxQ- during a time course post-inoculation was probed with *OsPR1a*, *OsPR1b*, *OsPR1c* and *OsPR10a*. Transcript levels were normalized to those of 25S rRNA (Fig. 2). Less rRNA was observed after 4 DPI in leaves inoculated with the raxQ- strain because leaf tips began to die as the disease progressed. No effect on rRNA in race 6 inoculated samples was observed.

From the expression profiles of all four genes, we found general overall patterns of transcript accumulation (Fig. 2). First, levels of transcript at the adult leaf 6 stage are higher than those at the juvenile leaf 2 stage for both treatments. Second, transcripts accumulated to a greater extent in leaves inoculated with race 6 than in those inoculated with the raxQ- strain. Third, expression of these genes in response to Xoo is maximum at 4 DPI. Fourth, *OsPR1c* is only weakly expressed under both conditions.

Based on our Northern blot results, we used real-time RT-PCR to refine our analyses of *OsPR1a*, *OsPR1b* and *OsPR10a* expression at the time of maximum expression, 4 DPI, in both *Xa21*-line IRBB21 and its near-isogenic parent IR24 that does not have *Xa21*. We directly compared expression levels at two developmental stages and found that the *Xa21*-line IRBB21 responds with significantly greater levels of expression of *OsPR1a* and *OsPR1b* when inoculated with race 6 at the adult stage than at the juvenile stage (Fig. 3). On the other hand, expression of *OsPR1a* and *OsPR1b* in IR24 and of *OsPR10a* in both lines does not appear to be developmentally regulated in a statistically significant manner (Fig. 3).

```
PR1a     1 MASSSSRL-SCCLLVLAAAAMA----ATAQNSAQDFVDPHNAARADVGVGPVSWDDTVAA
PR1b     1 MEVSKLAI-A--LAMVAAMALP----SQAQNSPQDYVRLHNAARAAVGVGPVTWDTSVQA
PR1c     1 MEASKLAICS--LFVLAVAAATVVHCSDAQNSPQDYLSPQNAARSAVGVGPMSWSTKLQG
                                     ^                          *

PR1a    56 YAESYAAQRQGDCKLEHSDSGGKYGENIFWGSAGGDWTAASAVSAWVSEKQWYDHGSNSC
PR1b    50 FAENYASQRSGDCSLIHSSNHNNLGENLFWGSAGGDWTAASAVQSWVGEKSDYDYASNSC
PR1c    53 FAESYARQRKGDCRLQ--HSGGPYGENIFWGSAGADWTAADAVRSWVDEKKYYNYASNSC
                    *          * *                    *             *

PR1a   116 SAPEGSSCGHYTQVVWRDSTAIGCARVVCDGDLGVFITCNYSPPGNFVGQSPY
PR1b   107 A--QGKVCGHYTQVVWRASTSIGCARVVCSNGRGVFITCNYKPAGNFVGQRPY
PR1c   106 A--AGKVCGHYTQVVWRDSTNVGCARVRCDANRGVFIICNYEPRGNIVGRRPY
                 *=========          *    *         *
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Fig. 1. Multiple alignment of three members of the *OsPR1* protein family from *Xa21*-line IRBB21. Alignment was made with Clustal. ^, indicates conserved cleavage site for signal sequence; column of amino acids with * below indicates residues conserved with other *PR1* protein sequences; = underlining, indicates residues that are conserved with distantly related family members including vertebrates [35].

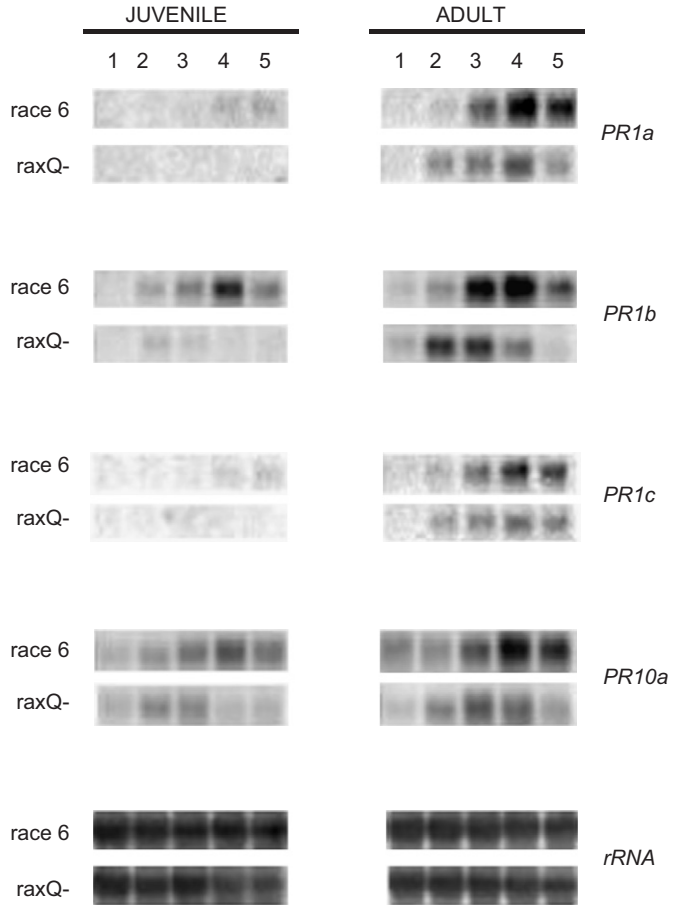

Fig. 2. Time course analysis of *OsPR1a*, *OsPR1b*, *OsPR1c* and *OsPR10a* gene expression. RNA for gel blots was harvested from juvenile stage leaf 2 and adult stage leaf 6 at 1, 2, 3, 4 and 5 days post-inoculation with *Xanthomonas oryzae* pv. *oryzae* race 6 and raxQ- mutant. RNA gel blots were probed with the corresponding *OsPR* gene and rRNA gene probes.

These results suggest that *Xa21* in concert with development play a role in controlling expression of *OsPR1a* and *OsPR1b* genes. To further examine the role of *Xa21* in *OsPR* gene responses, we directly compared expression levels in IRBB21 and IR24 adult stages to race 6 inoculation. IRBB21 responds to race 6 with greater levels of expression of *OsPR1a*, *OsPR1b* and *OsPR10a* than IR24 (Fig. 4). Similarly, IRBB21 responds to raxQ- inoculation with higher levels of *OsPR1a* and *OsPR1b* expression than IR24 (Fig. 4). However, *OsPR10a* is equally expressed in response to raxQ- in both lines. These results demonstrate that *Xa21* allows greater *OsPR1* gene responses to Xoo strains whether or not they have *avrXa21* activity. Direct comparisons of responses to race 6 and raxQ- show that although the mean expression levels induced by race 6 in IRBB21 are consistently higher than the mean levels induced by raxQ-, particularly *OsPR1a*, the differences are not statistically significant (Fig. 5).

4. Discussion

Disease resistance mediated by the resistance gene *Xa21* is developmentally controlled in rice [6,7]. Recently, it was discovered that the developmental control point may be a proteolytic cleavage of the XA21 protein [10]. In this study we examined defense events downstream of XA21 by assessing the relationship between *OsPR* defense gene expression and *Xa21*-mediated developmental disease resistance. Three *PR1* genes (*OsPR1a*, *OsPR1b* and *OsPR1c*) that were expressed in leaves were cloned from rice line IRBB21. The sequences of the predicted protein products of acidic OsPR1A and the basic OsPR1B and OsPR1C proteins are more than 64% identical (Fig. 1). A recent phylogenetic analysis of the PR1 protein family in rice predicted the presence of 32 members in the rice genome [24]. Although the precise function of any PR1 protein is not known, it is obvious that secretion, the secondary structure mediated by disulfide bonds, and a conserved eight amino acid domain must play essential roles.

To understand the role of genes other than *Xa21* in developmental control of *Xa21*-mediated disease resistance, we studied expression of *OsPR1a*, *OsPR1b*, *OsPR1c* and *OsPR10a* genes in response to inoculation with Xoo race 6 with and without *avrXa21* activity in near-isogenic

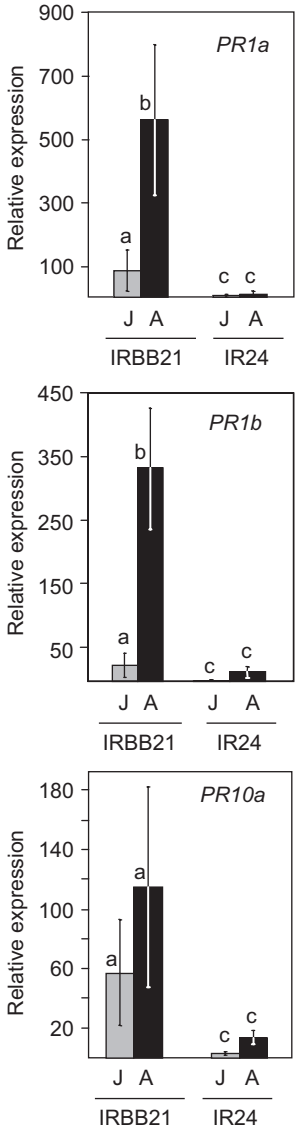

Fig. 3. Relative expression of *OsPR1a*, *OsPR1b*, and *OsPR10a* in IRBB21 (*Xa21*$^{+}$) and IR24 (*Xa21*$^{-}$) leaves. Gray bars represent gene induction by Xoo race 6 in juvenile (J) and black bars in adult (A) stage leaves. Error bars represent the standard deviation of the mean from three independent experiments. Different lower case letters indicate significant differences (*t*-test, $P \leqslant 0.05$) of each pair-wise comparison of juvenile vs. adult. Relative *PR* gene expression ratios were calculated according to [34].

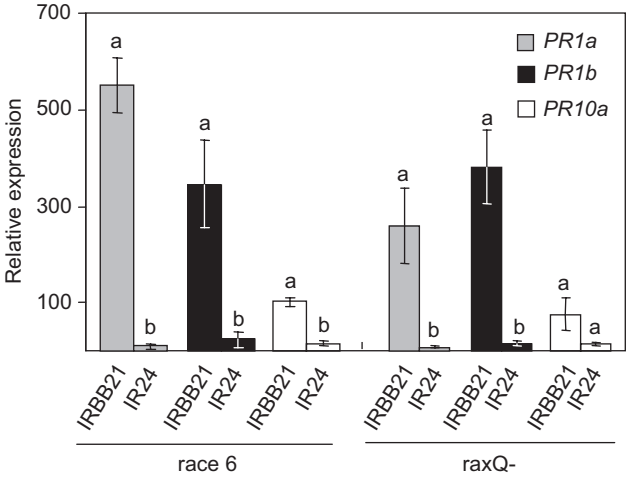

Fig. 4. Relative expression of *OsPR1a*, *OsPR1b*, and *OsPR10a* induced by Xoo race 6 and raxQ- strains in adult stage rice leaves of the *Xa21*$^{+}$ line (IRBB21) compared to *Xa21*$^{-}$ line (IR24). Error bars represent the standard deviation of the mean of three independent experiments. Different lower case letters indicate significant differences (*t*-test, $P \leqslant 0.05$) of each pair-wise comparison of IRBB21 vs. IR24.

rice lines with and without *Xa21*. From the *PR1* expression profiles in the *Xa21*$^{+}$ line, we consistently found that levels of expression in the adult leaf 6 stage are higher at 4 DPI than those at the juvenile leaf 2 stage (Figs. 2 and 3). These results indicate that *PR1* gene induction in rice is developmentally regulated and importantly suggests that enhanced resistance seen at the adult stage may in part be due to enhanced *PR1* gene expression. It is well known that expression of various defense genes is developmentally controlled [16,22] but the role of that control in effective resistance is not well studied. Previous studies addressing the role of PR1 proteins in developmental resistance to *Phytophthora* in tobacco showed that the transition from susceptibility to resistance correlates with the transition from vegetative to flowering phase and levels of PR1 protein in the apoplast were directly correlated with the developmental increase in resistance [19,20]. Another study analyzed developmental expression of *PR1a* in transgenic tobacco containing a fusion between the *PR1a* promoter and the GUS reporter gene [36]. It was shown that expression was greatest in older fully expanded leaves prior to senescence. It is clear in two of these studies that *PR1* gene expression levels are coordinated with the developmental stage of the plant. In contrast, Kus and coworkers [21] found that *PR1* expression in Arabidopsis did not correlate with the developmental increase in resistance observed in mature plants. *PR1* was not expressed in adult plants in response to either infection with virulent *Pseudomonas* or mock inoculation in spite of the fact that it was expressed at the juvenile stage in response to *Pseudomonas*. On the other hand, Chen and Chen [22] show that *PR1* is not expressed in Arabidopsis at young (3–4 weeks) or mature stages (5–6 weeks), and there was no corresponding difference in resistance to *Psuedomonas* at the two stages. The different results among these five studies may be due to natural variation in host responses to specific pathogens, inoculation conditions and in the roles of various PR1 proteins. However, direct comparison of results may not be appropriate because in these studies, senescence may be a complicating factor. By sampling responses of particular leaves when they have just fully expanded, our studies avoid the complications associated with ongoing senescence of the oldest leaves when either whole plants or young leaves on old plants are used.

Our data on the higher competence in adults to induce *OsPR1* gene expression in the presence of *Xa21* (Fig. 3),

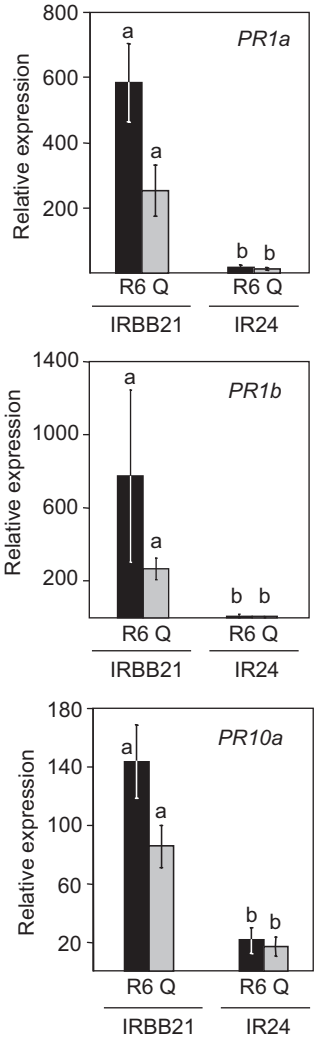

Fig. 5. Relative expression of *OsPR1a*, *OsPR1b*, and *OsPR10a* in IRBB21 (*Xa21*$^+$) and IR24 (*Xa21*$^-$) adult stage leaves. Black bars represent gene induction by Xoo race 6 (R6) and grey bars by Xoo raxQ- (Q). Error bars represent the standard deviation of the mean of three independent experiments. Different lower case letters indicate significant differences (*t*-test, $P \leqslant 0.05$) of each pair-wise comparison.

along with the higher expression levels in *Xa21*$^+$ lines compared to *Xa21*$^-$ lines overall (Fig. 4), clearly show that the *Xa21* locus potentiates *PR1* gene expression. Surprisingly, the loss of activity of *avrXa21* in raxQ- is not associated with a statistically significant loss of induction of *OsPR* genes in the *Xa21*$^+$ line (Fig. 5), suggesting either that *Xa21*-specific resistance in not directly involved or that the *avrXa21* activity is not completely eliminated by the raxQ- mutation. Taken together, our results indicate that *OsPR1a* and *OsPR1b* play a role in adult resistance and that the role of *avrXa21* will have to be further examined once the effector has been isolated. The timing of the increase in race 6-induced expression starting at 3 DPI (Fig. 2) correlates with the beginning of inhibition of race 6 growth in adult *Xa21*$^+$ leaves [8,11]. The coincidence of the timing of induction and magnitude of the response suggests that *OsPR1a* and *OsPR1b* expression may be important for mounting the resistance response to Xoo in adult leaves. *OsPR1a* and *OsPR1b* appear to be good markers for resistance in general in this interaction and for developmentally controlled resistance.

Based on our results, *OsPR1a* and *OsPR1b* appear to be good candidates for engineering enhanced resistance against Xoo in rice, despite the fact that their molecular function is still unknown. To assess their role in defense against Xoo, we are in the process of screening transgenic plants with over-expressed and silenced *OsPR1a* and *OsPR1b*. *OsPR10a* expression does not appear to be important in the defense response to Xoo, although it was found to be associated with resistance to *M. grisea* [27,37,38]. *OsPR1c* expression levels were the lowest of the *PR* genes analyzed (Fig. 2). There are genes that are similar to *OsPR1c* with over 78% identity in maize and barley that were induced upon challenge by the fungal pathogens, *Fusarium moniliforme* and *Erysiphe graminis* f. sp. *hordei*, respectively [39–41]. However, the induction was not specific to incompatible interactions suggesting that these *PR1c* homologues responded in general to pathogen infection.

OsPR1a, OsPR1b and *OsPR10a* were transiently expressed in response to race 6 inoculation (Fig. 2) in a manner similar to that shown previously in an incompatible interaction with *M. grisea* [42]. Assuming that *OsPR1a* and *OsPR1b* are involved in resistance, the transient nature of their expression suggests that they are not involved in maintaining the defense response unless their protein products are very stable. Instead it is possible that the early stages of a defense response and maintenance of it are two different functions, and these genes are involved only in initiating the inhibition of bacterial multiplication. Interestingly for understanding the significance of the timing of gene expression, in a study of an incompatible interaction in rice at the cellular level, nearly 65% of Xoo cells were found to be enveloped by fibrillar material (FM) within 3 DPI [43]. On the other hand, FM was not observed in the compatible interaction. The correlation among the timing of (1) inhibition of bacterial growth in the *Xa21* incompatible interaction [8], (2) expression of *OsPR1a*, and *OsPR1b*, and (3) reported FM envelopment suggests a relationship among the three events. If PR1A and PR1B proteins play a role in production of FM or act synergistically with FM to inhibit Xoo growth, it is possible that once Xoo are surrounded by FM, continuous expression of these genes is not necessary. To examine the relationship among these three factors, further studies are necessary.

There is a general correlation between *PR1* gene expression and SA levels in the phenomenon of systemic acquired resistance (SAR; [44]). The developmentally controlled resistance of tobacco to *Phytophthora* [19] and of Arabidopsis to *Pseudomonas* [21] is eliminated when SA levels were reduced by the *NahG* transgene. In Arabidopsis, two additional mutations in SA production, *sid1* and *sid2*, prevented adult plant resistance [21]. These results suggest

that SA is involved in developmental resistance. In rice, the role of SA in resistance is far from clear but several SAR components have been identified [37,45–49]. The role of SA in full resistance in adult rice leaves is yet to be determined. Finally, there appears to be an interaction between activity of the rice MAPK called OsMAPK5 and expression of both PR1b and PR10a and resistance to fungal and bacterial pathogens [28]. It would be worth examining this interaction in a developmental context.

In conclusion, we have shown that the developmental stage of rice and the presence of *Xa21* are important in governing the level of expression of *PR1* defense genes. We found that adult stage leaves are more competent to express defense genes in *Xa21* rice lines.

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