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Expression patterns of pathogenesis-related genes in a mutant rice resistant to blast disease

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ABSTRACT

Rice blast, caused by Magnaporthe oryzae, is the most devastating fungal disease in the rice-growing world. Between 10% and 30% of the annual rice harvest is lost due to infection by rice blast. Mutant plants with altered response to pathogens, either gain or loss of resistance, are useful for dissecting defense mechanisms. For this purpose two rice genotypes including mutant variety *Pooya (resistant) and its wild-type cultivar Mosatarom (susceptible) were used in greenhouse tests. Expression patterns of PR1a and PR10a genes were analyzed at 1, 2, 3, 4 and 5 days after inoculation with M. oryzae and the seedlings treated with sterile water was used as control. The PR1a and PR10a genes responded to M. oryzae differently expression levels in Pooya compared with the Mosatarom. The induced expression levels of PR1a and PR10a were higher in the Pooya than in the Mosatarom, and the difference was greater for PR1a. According to our results, rapid induction and high level expression of PR1a and PR10a genes might be involved in resistance of mutant* variety *Pooya to blast.*

Keywords: Pathogenesis-related proteins, Mutant Rice, Blast disease, Resistance

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INTRODUCTION

Rice blast, caused by *Magnaporthe oryzae*, is the most devastating fungal disease in the rice-growing world [1]. Between 10% and 30% of the annual rice harvest is lost due to infection by rice blast [2]. Plants defend themselves against pathogen challenges by the activation of defense response pathways [3]. The recognition between plant *R* gene products and pathogen a virulence gene products leads to the rapid, coordinated expression of defense genes, whose products participate in fighting back against pathogen infection [4]. The known defense genes in rice mainly encode Pathogenesis Related (PR) proteins such as *PR1a* [5] and *PR10a* [6]. The rapid induction of defence gene expression is required for fighting back against pathogens in plants. The activation speeds and expression levels of defence genes vary in different plant-pathogen interactions.

Mutants are widely used in plant research, such as plant physiology, genetic, and plant breeding studies. Mutant plants with altered response to pathogens, either gain or loss of resistance, are useful for dissecting defense mechanisms [7]. In the present study we analyzed the mechanisms controlling defense genes in response to rice blast

disease. In addition, we evaluated the expression of *PR1a* and *PR10a* in a rice blast-resistant mutant at the control conditions and after inoculation with *M. oryzae*.

MATERIALS AND METHODS

Plant material and growing conditions

Two rice (*Oryza sativa*) genotypes consist of mutant variety Pooya and its wild-type cultivar Mosatarom was used as plant materials. Mosatarom is susceptible to rice blast, although it provides good food additives because of aromatic and volatile compounds. The Pooya mutant variety derived from Mosatarom was produced by gamma irradiation approach and registered as a blast-resistant mutant [8]. The plants were grown under natural light in a greenhouse (20-30 °C) for inoculation experiments.

Pathogen inoculation and sample collection

The *M. oryzae* isolate IC-25 was cultured at 26 °C on prune-agar and, harvested spore were suspended in 0.01% Tween-20 solution. For leaf inoculations, rice seedlings at the 4-leaf stage were used. The seedlings were sprayed with a spore suspension of 1×10^5/mL. The inoculated seedlings were maintained in a growth chamber at 26°C in darkness for 24 h, followed by a light/dark cycle of 14/10 h with 95% humidity. The leaves were collected at 1, 2, 3, 4, and 5 days post-inoculation (DPI), frozen in liquid nitrogen and then stored at -80 °C. Seedlings treated with sterile water for 1, 2, 3, 4, and 5 d were used as controls.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from control and treated rice leaves using the pBiozol reagent according to the manufacturer's protocol. The quality and concentration of RNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis. Total RNA was treated with DNaseI (Fermentase, Germany) to remove DNA contamination before cDNA synthesis according to the manufacturer's instructions. Three micrograms of DNase-treated RNA was reverse transcribed into complementary DNA (cDNA) using Revert Aid^{TM} Reverse Transcriptase (Fermentas, Germany), oligo dT18 and random hexamer primers (MWG, Germany) in a total volume of 20 µl reaction mixture, according to the manufacturer's instructions. The mRNA expression levels of *PR1a* and *PR10a* were measured by quantitative real-time PCR using the primers listed in table 1 [9]. The relative expression level was quantified in comparison with the house keeping gene *actin* as an internal control. Quantitative real-time PCR was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem/MDS SCIEX, Foster City, CA, USA), with 10 ng cDNA, 10 µl of SYBR Green I master mix (Takara, Shiga, Japan), and 200 nM of forward and reverse primers up to final reaction volumes of 20 µl, according to the manufacturer's instructions. The PCR was performed through the following instruction: an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 30 s, and extension at 72 °C for 10 s. The PCR reactions were performed in triplicate. Melt curves were run immediately after the last PCR cycle to examine if the measurements were influenced by primer-dimer pairs.

Table 1. The primer sequences used in quantitative RT-PCR

Gene	Accession	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product (bp)
PR1a	AJ278436	TGGGTGTCGGAGAAGCAGTG	GGTGATGAAGACGCCGAGG	159
PR10a	AF274850	ACACTCGACGGAGACGAAGC	CAGGGTGAGCGACGAGGTA	176
actin	X15865	GAGCTACGAGCTTCCTGATGGA	CCTCAGGGCAGCGGAAA	65

Data analysis

Induction of defense gene expression was determined using the delta-delta method [10]. Firstly, the threshold cycles (C_T) of the PCR results for each gene were averaged and used for quantification of the transcripts. Secondly, the ΔC_T value was determined by subtracting the average C_T value of the endogenous control genes *actin* from the average C_T value of the defense gene, respectively. Lastly, the $\Delta\Delta C_T$ value was determined by subtracting the ΔC_T of the control (sample treated with sterile water) from the ΔC_T of the inoculated sample. The $2^{-\Delta\Delta CT}$ value was given to estimate the fold change of gene expression [11, 12]. Each value was the mean of three independent biological experiments that contained eight leaves in each experiment, and standard deviations were given. The results were analyzed using the Student's *t*-test [11].

RESULTS AND DISCUSSION

Expression levels of *PR1a* and *PR10a* in the Pooya and Mosatarom at the control conditions

Both *PR1a* and *PR10a* genes exhibited significant expression between the Pooya and Mosatarom at the control conditions (Fig. 1). Expression levels of *PR1a* and *PR10a* was more in Pooya compared with those in Mosatarom. According to the same genetic background between Pooya (mutant variety) and Mosatarom (wild-type), differences in expression of *PR1a* and *PR10a* genes at the control conditions represents the impact of gamma irradiation.

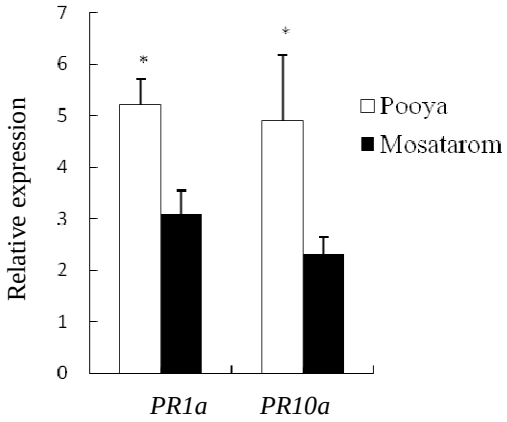

Fig. 1. Expression levels of *PR1a* and *PR10a* in the Pooya (mutant variety) and Mosatarom (wild-type) at the control conditions. The transcript levels were calculated relative to the *actin* gene. Error bars represent the standard deviation of the mean from three independent experiments. Asterisks indicate a significant difference ($P < 0.05$, *t*-test) between the Pooya and Mosatarom within the same gene

Expression levels of *PR1a* and *PR10a* in the Pooya and Mosatarom after inoculation with *M. oryzae*

The *PR1a* and *PR10a* genes responded to *M. oryzae* differently expression levels in Pooya compared with the Mosatarom.

PR1a and *PR10a* were up-regulated in both genotypes throughout most of the experimental period (*PR10a* level was below that of control at 1 DPI in Pooya). Notably, the induced expression levels of *PR1a* and *PR10a* were higher in the Pooya than in the Mosatarom, and the difference was greater for *PR1a*.

The plant PR proteins can be classified into 17 independent families (PR-1-PR-17) basing on their amino acid sequences, serological relationships, and enzymatic activities [13]. PR-1 proteins were first found to be expressed in tobacco in response to tobacco mosaic virus (TMV) infection having 14 to 17 kDa molecular weights [14]. Later, homologues of tobacco PR-1 proteins have been identified in barley, tomato, maize, rice, and so forth [15]. These widely distributed proteins of plant kingdom have antifungal activity at the micromolar level against a number of plant pathogenic fungi [16], but their mechanism of action is not known. OsPR1a, a rice acidic PR class 1 protein, is highly responsive to pathogen attack, wounding, and salicylic acid. The blast fungus infection can induce *PR1a* transcript accumulation in both compatible and incompatible interactions [5].

The PR-10 family proteins are intracellular proteins with unknown enzymatic function. Some proteins of PR-10 family are induced under various stress conditions and act as common allergens [17, 18]. However, few PR-10 proteins are also constitutively expressed, indicating a role of these proteins in plant development [19]. The members of this family have low molecular weight (around 15-16 kDa) and are slightly acidic, resistant to proteases, and mostly intracellular and cytosolic [20, 21]. PR-10 proteins are structurally not related to any other class of PR proteins. Apart from direct function in defense, these proteins are implicated in a general function during overall stress as well as during physiological changes in certain developmental stages [22]. Most plant PR-10 proteins, such as sorghum PR-10 [23], barley PR-10 [24], and asparagus PR-10 [25], are activated upon pathogen attack or after treatment with fungal elicitors. In other plants, PR-10 is induced by drought [26], salt stress [27], and

the plant hormones abscisic acid and methyl jasmonate [27]. *OsPR10a* is known to be induced by probenazole, and thus is also called a probenazole-inducible gene, *PBZ1* [6].

Our results indicate that *PR1a* and *PR10a* might be involved in resistance of mutant variety Pooya to blast. Although they were induced to higher relative levels in the Pooya, both *PR1a* and *PR10a* were also up-regulated throughout the whole experimental period in the Mosatarom. Rapid induction and high levels of defence gene expression are necessary for plants to fight back against pathogens. In most cases, the inducible levels of gene expression in compatible interactions are lower than those in incompatible interactions.

CONCLUSION

Differential expression patterns of *PR1a* and *PR10a* genes was observed in mutant variety Pooya (resistant) and its wild-type cultivar Mosatarom (susceptible) at the control conditions and under *M. oryzae* inoculation. According to our results, rapid induction and high level expression of *PR1a* and *PR10a* genes might be involved in resistance of mutant variety Pooya to blast.

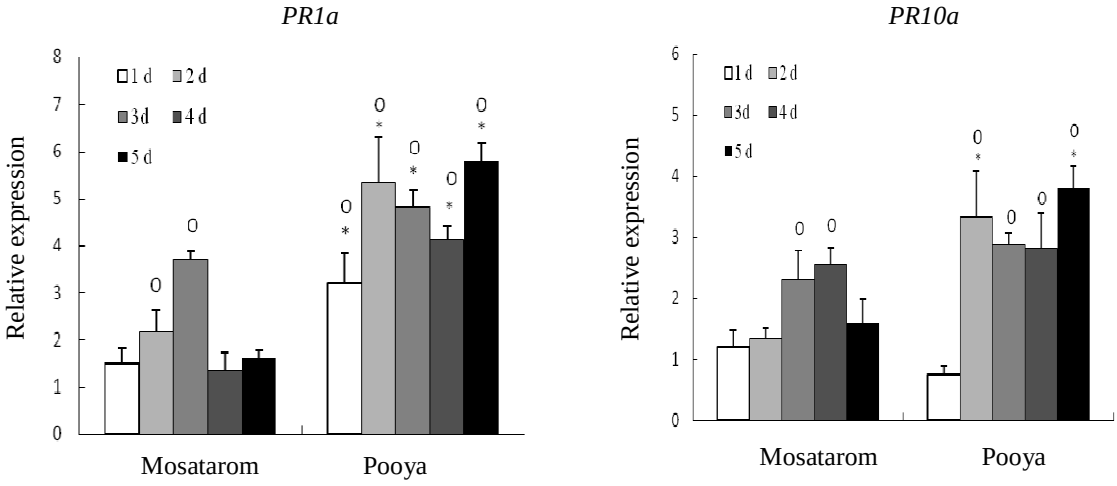

Fig 2. Expression levels of *PR1a* and *PR10a* in the Pooya (mutant variety) and Mosatarom (wild-type) after inoculation with *Magnaporthe oryzae*. The leaves were obtained at 1, 2, 3, 4 and 5 days post-inoculation. The transcript levels of *PR1a* and *PR10a* were normalized to those of the *actin* gene as house-keeping gene. Then, the expression levels of *PR1a* and *PR10a* genes in the inoculated samples were calculated relative to those of controls that were treated with sterile water at respective times. Error bars represent the standard deviation of the mean from three independent biological experiments. Circles indicate a significant difference ($P < 0.05$, *t*-test) between the controls and the inoculated samples. Asterisks indicate a significant difference ($P < 0.05$) between the Pooya and Mosatarom in the same treatment time

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