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Modification of antioxidant enzymes activation and membrane lipid peroxidation in mutant rice resistant to *Magnaportheoryzae*

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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. Enzyme activities of catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO) and malondialdehyde (MDA) levels were evaluated at 1, 2, 3, 4 and 5 days after inoculation with M. oryzae and the seedlings treated with sterile water was used as control. While enzyme activities of CAT, POX and PPO in the mutant* variety *Pooya were significantly more than Mosatarom cultivar. Analysis of MDA levels also revealed distinct differences between two genotypes. According to our results, resistance to rice blast in mutant* variety *Pooya might be associated with modification of antioxidant enzymes activation and membrane lipid peroxidation.*

Keywords: Defense enzymes, Membrane lipid peroxidation, 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]. Upon pathogen invasion, an oxidative burst is one of the most rapid defense reactions elicited in the plant, which in turn leads to the transient production of high levels of reactive oxygen species (ROS) that include superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) [3]. ROS produced in the oxidative burst has been demonstrated not only to protect against invading pathogens but to function as signaling molecules to activate plant defense responses in many plant-pathogen interactions [4]. However, in order to avoid plethora ROS, the plant has evolved to efficiently scavenge these damaging effects by triggering. Such a system involves both enzymatic and non-enzymatic antioxidants, where the enzymatic protective mechanism operates by sequential and simultaneously activating a number of elevation/induction antioxidant enzymes (catalase, CAT; peroxidase, POX; polyphenol oxidase, PPO) [5].Active oxygen radicals may induce the chain-like peroxidation of unsaturated fatty acids in the membranes, leading to the formation of lipid peroxidation products such as malondialdehyde (MDA)[6]. Generally, the levels of ROS and the extent of oxidative damage depend largely upon the level of coordination among the ROS-scavenging enzymes [7].

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 [8].In the present study we analyzed the mechanisms antioxidant defense systems in response to rice blast disease. In addition, we evaluated the activities of some antioxidant enzymes and MDA levels 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. Mosataromis susceptible to rice blast, although it provides good food additives because of aromatic and volatile compounds. The Pooyamutant variety derived from Mosatarom was produced by gamma irradiation approach and registered as a blast-resistant mutant[9].The plantswere grown under natural light in a greenhouse (20-30 °C)for inoculation experiments.

Pathogen inoculation and sample collection

The *M. oryzae*isolateIA-89was cultured at 26 ℃ 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.

Determination of Antioxidant Enzymes Activity

For estimation of enzyme activity, plant material (leaves) was homogenized at 4 °C with a mortar and pestle in 0.1 M Tris-HCl buffer (pH 8.9) containing 10 mM mercaptoethanol and 4 % (m/v) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 13000 *g* for 30 min at 4 °C and resulting supernatants were kept at -70 °C and used for enzyme assays. A high-speed centrifuge (J2-21M, Beckman, Palo Alto, USA) and UV-visible recording spectrophotometer (UV-160, Shimadzu, and Tokyo, Japan) were used.

CAT (EC 1.11.1.6) activity was assayed from the rate ofH_2O_2 decomposition as measured by the decrease in absorbance at 240 nm. The reaction mixture comprised50 mM potassium phosphate buffer (pH 7.0), 15 mMH_2O_2, and 20 µl of protein extract. Activity was expressed as units (µmol of H_2O_2 decomposed per min per mg protein[U mg^{-1}(protein)] [10]. Proteins were determined using serum albumin as a standard [11].

For estimation of POX (EC 1.11.1.7),the reaction mixture comprised 4 mL of 0.2 M acetate buffer (pH 4.8),0.4 mL of H_2O_2 (3 %), 0.2 mL of 20 mM benzidine, and0.05 mL of enzyme extract. The increase in absorbance was recorded at 530 nm. The POX activity was defined as l µM of benzidine oxidized per min per mg protein [U mg^{-1}(protein)] [12].

PPO (EC 1.14.18.1) activity was estimated at 40 °C. The reaction mixture contained 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 mL of 20 mM pyrogallol and 0.1 mL of enzymes extract. The increase in absorbance was recorded at 430 nm. The PPO activity was defined as 1 µM of pyrogallol oxidized per min per mg protein [U mg^{-1}(protein)] [13].

Determination of Malondialdehyde (MDA)

The extent of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) The leaves (0.5 g) were homogenizedin 5 mL of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10000*g* for 20 min. To 1 mL aliquot of the supernatant, 4 mL of 0.5 % thiobarbituric acid(TBA) in 20 % TCA was added. The resulting mixture washeated at 95 °C for 30 min and then was quickly cooled inan ice bath. The absorbance was determined at 532 and600 nm, after centrifugation at 10000*g* for 15 min. The value for non-specific absorption at 600 nm was then subtracted from that of 532 nm. The concentration of MDA was calculated using absorption coefficient of155 mM^{-1} cm^{-1} [14].

Data analysis
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 [15].

RESULTS AND DISCUSSION

Activities of antioxidant enzymes and MDA levels in the Pooya and Mosatarom at the control conditions
The activities of three antioxidant enzymes and the MDA levels all exhibited significant differences between Pooya and Mosatarom at the control conditions (Table 1). SOD, CAT and PPO activities were more in Pooya compared with those in Mosatarom. Only MDA levels were lower in the Pooya than in the Mosatarom. Significant differences between these two genotypes for oxidative indexes (CAT, POX, PPO and MDA) at the control conditions reflect the impact of gamma radiation on genetic diversity of these traits.

Table1.Activities of antioxidant enzymes and MDA levels of Pooya (mutant variety) and Mosatarom (wild-type) at the control conditions

Genotypes	CAT [U $mg^{-}1$(protein)]	POX [U $mg^{-}1$(protein)]	PPO [U $mg^{-}1$(protein)]	MDA (µmol/g. F.W.)
Mosatarom(wild-type)	8.34±0.59	22.59±2.06	20.36±0.71	4.81±0.88[a]
Pooya (mutant)	15.47±1.04[a]	37.84±3.86[a]	25.58±1.32[a]	2.72±0.56

Values represent the mean from three independent experiments ± standard deviation.
[a] *Values represent a significant difference between the Pooya (mutant* variety*) and wild-type(Mosatarom) according to the Student's t-test with P< 0.05*

Activities of antioxidant enzymes and MDA levels in the Pooya and Mosatarom after inoculation with *M. oryzae*
The three antioxidant enzymes and MDA levels all responded to *M. oryzae* differently in Pooya compared with the Mosatarom. Although the CAT activities increased stably in both genotypes, the relative CAT activities were all significantly higher in the Pooya than in the Mosatarom. The highest catalase activity was observed in Pooya at 5 DPI (Fig 1A).This increase in catalase activity may provide its protection from oxidative damage by rapid removal of H_2O_2. These results were in agreement with those recorded that, the activity of antioxidant enzymes in leaves under *Fusarium oxysporum* infection increased and might be affective in scavenging mechanism to remove H_2O_2 and O^{2-}produced in leaves [16].

The peroxidase and polyphenol oxidase activity was similar in the Pooya, as the highest was observed on 2 and 3 DPI and decreased with increasing duration of disease at the 4 and 5 DPI. Also, the activities of peroxidase and polyphenol oxidase in Pooya at 2 and 3 DPI were significantly higher than Mosatarom (Fig 1B, C). These findings indicate to a positive relationship between resistance and peroxidase activity. Peroxidase also produces free radicals and hydrogen peroxide which are toxic to many microorganisms [17]. Also, an increase in peroxidase activity is considered as a preliminary indicator for resistance of broad beans to chocolate spot disease [18]. These compounds act as barriers against pathogen invasion. POXs are usually associated with induced resistance [19] and they are also implicated in several plant defense mechanisms such as lignin synthesis, oxidative cross linking of different plant cell wall components or generation of reactive oxygen species [20].

PPO is the major enzyme responsible for oxidation of phenolic compounds [21].The higher activity of PPO in resistant cultivar must have resulted in more oxidation of phenolic substances to form more toxic quinones and the reversed disproportionation of quinones to semiquinone radicals that may lead to generation of ROS. These oxidative products are toxic substances for the extra-cellular enzymes produced by the pathogen [22]. Therefore, it is likely to govern the same biochemical mechanism for resistance in the present study.

Analysis of MDA levels revealed that with increasing time of disease MDA levels increased in both genotypes. However, MDA levels in Pooya at all times except for the 2 DPI were significantly lower than Mosatarom (Fig 1D). Upon pathogen invasion, hosts trigger a defense mechanisms resulting in the generation of reactive oxygen species (ROS), superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) [3]. Increasing evidence reveals that accumulation of ROS severely affects bio-molecules such as proteins, lipids and nucleic acids, leading to oxidative damage at the cellular level [23]. Lipid peroxidation is an indicator of oxidative stress and is estimated as MDA, the principal product of poly-unsaturated fatty acid peroxidation. MDA levels were shown to increase in *Botrytis cinerea*-elicited maritime pine suspensions [3], whereas several studies have documented that lipid peroxide levels were unaffected by *B. cinerea*in *Capsicum annuum* fruits, and even decreased in elicited *Arabidopsis* plants

[24,25]. Our present results show that the MDA levels increase gradually upon *M. oryzae* invasion, suggesting that lipid peroxidation might have been induced due to the production of ˙OH generated in response to pathogen infection.

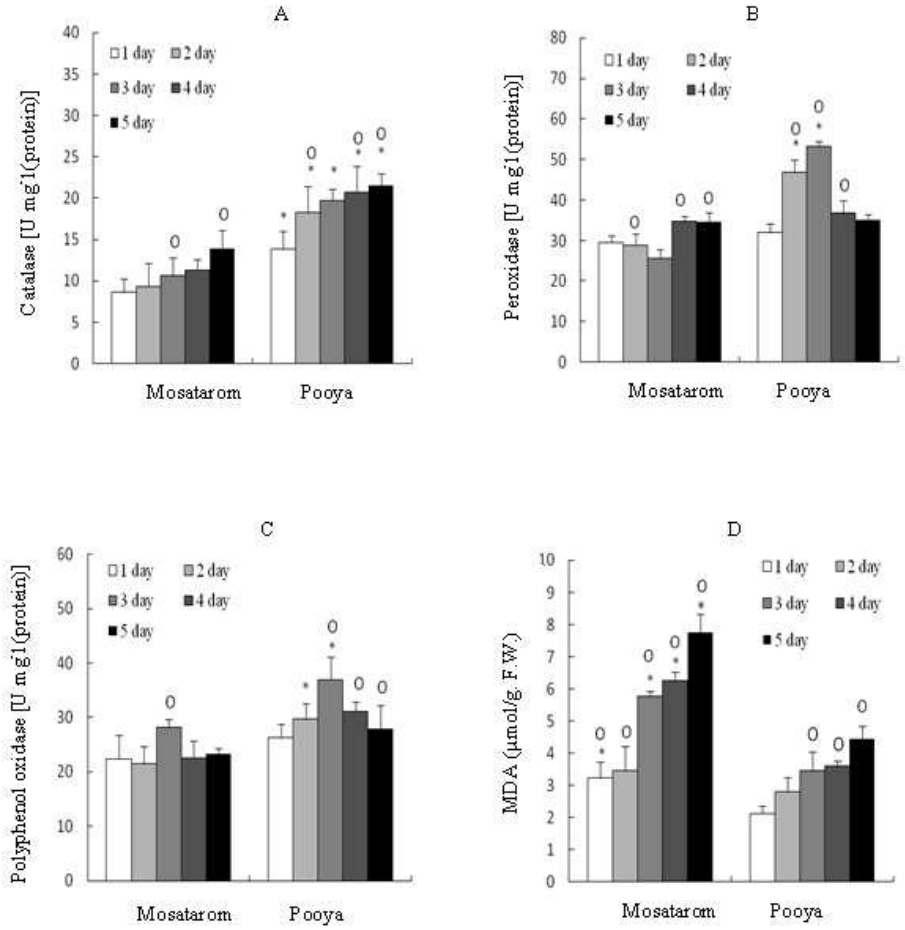

Fig 1. A) Catalase activity, B) Peroxidase activity, C) Polyphenol oxidase activity and D) MDA levelsin Pooya (mutant variety) and wild-type (Mosatarom)after inoculation with *Magnaportheoryzae*. Circles indicate a significant difference between the controls and the inoculated samples, and asterisks indicate a significant difference between the Pooya (mutant variety) and wild-type(Mosatarom) according to the Student's *t*-test with *P*< 0.05

CONCLUSION

Difference in antioxidant enzymes activity and MDA levels 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, resistance to rice blast in mutant variety Pooya might be associated with modification of antioxidant enzymes activation and membrane lipid peroxidation.

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