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Biochemical changes of rice genotypes against blast (*Magnaporthe oryzae*) disease and SSR marker validation for resistance genes

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ABSTRACT

Rice blast caused by *Magnoporthe oryzae* is a major devastating fungal disease and represents a potential threat to world rice productions. However, information about the genetic and biochemical basis of disease tolerance is still limited. In this study, we tested the presence and diversity of resistant R genes using SSR markers, and the antioxidant enzymes catalase (CAT), ascorbate peroxidise (APX) and guaicol peroxidise (POD), activity and also the concentration of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in resistant (BAUdhan 3) and susceptible (BRRIdhan 28) genotype. Molecular marker analysis reveals the presence of all ten studied resistant genes in BAUdhan 3. Among the markers studied, three markers namely RM224, RM72 and RM206 produce distinct band only in resistant genotype BAUdhan 3, which might be used to screen resistant genotypes. The enzymatic activity of APX, CAT and POD increased in the inoculated plant for both cultivars but the increase was more prominent for BAUdhan 3. The *M. oryzae* infections significantly increased the H_2O_2 content in BRRIdhan 28 and not much changed in BAUdhan 3. The MDA concentration was higher in the leaves of inoculated plants of BRRIdhan 28. The higher activities of APX and POD in the leaves of the inoculated plants of BAUdhan 3 resulted in lower H_2O_2 accumulation which can minimize the cellular damages possibly caused by reactive oxygen species. The result shows that the presence of more resistance genes and an effective antioxidative system in BAUdhan 3, which limits the damage caused due to fungal infection and thus contributes to greater resistance.

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INTRODUCTION

Rice (*Oryza sativa* L.) is an important food crop for around 65% of the world's population (Kumar *et al.*, 2010). Among the different diseases, rice blast is the most devastating diseases, caused by the fungal pathogen *Magnaporthe oryzae*, which seriously hampers the total production of rice in the world. Rice blast is considered the most dangerous fungal disease due to its pathogenic complexity directly linked to the host, pathogen and also weather conditions (Lee, 1994; Li *et al.*, 2007; Kwon & Lee, 2002). There are a number of pathogenic races of blast fungus present in the field, which are highly variable in most of the rice growing regions (Ou, 1979; Xia *et al.*, 1993; Valent & Chumley, 1994). Blast resistance genes screening using virulence analysis is time consuming and also challenging, as it needs to provide strict environmental control. Molecular markers based on polymerase chain reaction (PCR) can minimize this limitation and have an advantage to increase the precise identification of blast resistant genes and their incorporation into appropriate breeding schemes (Jia *et al.*, 2003; Wang *et al.*, 2007; Liu *et al.*, 2013). Markers that are closely linked to the resistance *R* gene provide resistance to a specific pathogenic race and can be easily and effectively applied in any Marker Assisted breeding (MAS) scheme. Due to the availability of a large number of DNA markers, it has now become a common tool for marker assisted selection. Selection of suitable SSRs can be done based on the banding pattern of the amplified fragment on regular agarose gels. To date several reports have been made for the identification of different blast resistance genes (Chen *et al.*, 2002; Berruyer *et al.*, 2003; Ballini *et al.*, 2008). Among them, twenty two namely *Pib*, *Pita*, *Pik-h*, *Pi9*, *Pi2*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pik-m*, *Pit*, *Pi5*, *Pid3*, *pi21*, *Pb1*, *Pish*, *Pik*, *Pik-p*, *Pia*, *NLS1*, *Pi25* and *Pi54rh* being cloned and characterized (Imam *et al.*, 2014). The identified blast resistance genes are almost host specific (Deng *et al.*, 2006) and located throughout all the chromosomes of rice except chromosome number three

(Liu *et al.*, 2014). To effectively control this devastating blast fungal infestation, the identification of genes showing a broad spectrum of resistance is imperative (Wang *et al.*, 2010). The effectiveness of resistant genes is often overcome through the emergence of a strong virulent pathogenic race (Wang *et al.*, 2010), resulting in the resistance of existing cultivars being lost within a few years in the field condition (Han *et al.*, 2001). Therefore, there is an immense need to detect resistant gene sources for the development of blast resistant rice varieties. Plants tend to overcome the challenges against pathogen attack by activating different defense response pathways (Staskawicz *et al.*, 1995). The activation of different defense signaling pathways varies depending on plant pathogen interactions.

In response to different biotic and abiotic stresses, plants tend to develop antioxidant defense pathways to overcome the deleterious effect caused due to overproduction of reactive oxygen species (ROS) (Panda, 2007). Among the different ROS molecules generated during pathogen infection, hydrogen peroxide (H_2O_2) is a key component that directly induced oxidative stress, causing cellular damage involving cell membrane, nucleic acid and protein by the production of malondialdehyde (MDA) (Apel & Hirt, 2004). The balance between ROS accumulation and their degradation was largely depending on ROS scavenging enzymes synthesized inside the cell (Liang *et al.*, 2003). To remove the generated excess ROS during plant pathogen interaction, plants tend to induce the activity of different ROS scavenging enzymes such as catalase (CAT), ascrobate peroxidase (APX), guaicol peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) (Hiraga *et al.*, 2001; Apel & Hirt, 2004; Dixon *et al.*, 2010). Some studies have been reported about the induction of antioxidative system due to pathogen infection (Agrawal *et al.*, 2002a, 2002b; Díaz-Vivancos *et al.*, 2008), but there was no clear evidence about the direct relationship between ROS accumulation and cellular damage, and induction of antioxidant enzyme activity in rice during fungal infection. Thus, the present study aimed to determine the content of H_2O_2, damage to the cell membrane (MDA), and the activity of major enzymes CAT, POD and APX in two contrasting blast resistance rice genotypes.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Rice seeds of BAUdhan 3, BRRIdhan 28 and BRRIdhan 29 were collected from the experimental farm, dept. of Genetics and Plant Breeding, BAU, Mymensingh. Seeds were surface sterilized using 10% NaOCl solution for 15 minutes, rinsed in sterilized water, germinated on moist filer papers for 2 days and then planted on plastic pots filled with soil and put at 26 °C temperature for 2-4 weeks.

Genomic DNA Extraction and Quantification

Genomic DNA was extracted using the CTAB method (Doyle & Doyle, 1987) with slight modification. Briefly, about 0.1 g of young leaves was collected from each genotype grown in plant growth room and ground using mortar and pestle. After that CTAB buffer was added and incubated for 10 minutes at 65 °C. Then chloroform was added, vortexed and centrifuged for another 15 minutes. Finally, ethanol was used to elute and wash the precipitate. The extracted DNA samples were quantified spectrophotometrically by measuring A260/A280 and quality was checked by electrophoresis using 1.0% agarose gel.

Markers Specific for Rice Blast R Genes and Marker Analysis

Ten previously reported SSR markers (Singh *et al.*, 2015) were used to identify the presence of blast resistance genes. The details of primers are shown in Table 1.

The reaction mixture or PCR cocktail was prepared separately for each genotype and each SSR marker. About 10 µL of the reaction mixture was prepared in individual PCR tubes, each of which contained 50 ng genomic DNA, 1 µL forward primer, 1 µL reverse primer, 5 µL PCR master mix and double distilled water. PCR Addbio® Taq Master Mix was used to prepare the PCR cocktail. The PCR was performed following a thermal cycling program involving an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 2 °C below Tm of respective primers for 30 sec, primer extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 8 min. The amplified PCR products were separated using 2.5% agarose gel prepared in TAE buffer and visualized using ethidium bromide in a gel documentation system. All PCR reactions were repeated at least two to confirm the results.

Inocula Preparation, Inoculation and Sample Collection

Rice blast isolates were isolated from neck infected rice samples were cultured on readymade oatmeal agar at 26 °C for 17 days. The surface of the culture media was then gently rubbed with a sterile paint brush to remove aerial mycelia and then exposed for 3 days to fluorescent light at 26 °C to induce sporulation. Conidia from sporulated culture media were scraped and suspended in water containing Tween 20 (0.01%), then filtered and adjusted the concentration of conidia to 1×10^5 µL water. The conidial suspension was sprayed on 21 days old rice seedlings, and incubated at 25 °C temperature in a humid

Table 1: List of SSR Markers, Resistance Genes and their Details

Sl. No.	Gene Name	Chromosome Locus	Marker	Product Size
01	Pi-9	6	RM 541	158
02	Pi-1	11	RM 224	157
03	Pi5-(t)	11	RM 21	157
04	Piz-5	6	RM 527	233
05	Pi-b	2	RM 208	173
06	Pi-ta	12	RM 247	131
07	Pi33	8	RM 72	166
08	Pi-27(t)	1	RM 259	162
09	Pitp (t)	1	RM 246	116
10	Pi-kh	11	RM 206	147

chamber, and leaf samples were collected after 48, 72 and 96 h of inoculation.

Determination of Catalase (CAT), Ascorbic Peroxidase and Guaicol Peroxidase (POD) Activity

Activities of Catalase (CAT) (Aebi, 1984), Ascorbate peroxidase (APX) and Guaicol Peroxidase (POD) (Nakano & Asada, 1981) were measured in inoculated and non-inoculated plants. To measure CAT activity 0.1 mL of enzyme extract was added to 50 mM potassium phosphate buffer (pH 8.0), 0.1 mL of EDTA and 0.1 mL of H_2O_2 mixture and changes in absorbance were recorded immediately at 240 nm at 30 seconds intervals. 0.1 mL of enzyme extract was added to 0.6 mL of 50 mM potassium phosphate buffer (pH 8.0), 0.1 mL of EDTA, 0.1 mL of H_2O_2 and 0.1 mL of ascorbate mixture and changes in absorbance were recorded immediately at 290 nm at 30 seconds interval to measure APX activity. To measure POD activity, 50 mM potassium phosphate buffer (pH 8.0), EDTA, H_2O_2 and 0.1 mL of Guaiacol were added and mixed well and changes in absorbance were recorded immediately at 470 nm at 30 seconds intervals.

Determination of Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) Content

The activity of Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) content were measured according to the following protocol (Heath & Packer, 1968; Velikova *et al.*, 2000). Briefly, 0.1 g leaf (with similar age, and young expanded leaf) was ground into powder, and then the powder was put into a tube containing 1 mL 0.1% (w/v) TCA and mixed. Homogenized samples were centrifuged and then the supernatants were transferred to a new tube. After centrifugation, the supernatants were kept in the dark for 1 h after mixing with phosphate buffer (10 mM, pH 7.0) and potassium iodide (1 M) (in the ratio of 0.5 mL: 0.5 mL: 1 mL). The absorbance of the resulting solution was recorded at 390 nm. For MDA content measurement, 20% TCA containing 0.5% TBA was added and mixed well. The mixture was boiled at 95 °C for 15 min and quickly cooled on ice. The content of MDA can be calculated by measuring absorbance at 532 nm.

RESULTS

Molecular Screening of Blast Resistant Genes

Molecular screening for ten major blast resistance genes (*Pi-9*, *Pi-1*, *Pi-5(t)*, *Piz-5*, *Pi-b*, *Pi-ta*, *Pi-33*, *Pi27(t)*, *Pitp(t) and Pi-k^h*) in one resistant BAUdhan 3, and two susceptible BRRIdhan 28 and BRRIdhan 29 genotypes were performed using ten SSR markers namely RM541, RM224, RM21, RM527, RM208, RM247, RM72, RM259, RM246 and RM206. BAUdhan 3 showed a positive band associated with all ten resistance genes. Susceptible genotypes BRRIdhan 28 and BRRIdhan 29 showed around six positive bands indicating the presence of *Pi-9*, *Pi-5(t)*, *Piz-5*, *Pi-b*, *Pi-ta and Pi27(t)* resistant genes (Figure 1). PCR results were confirmed for the *Pi-1* and *Pi-33* genes through visualization of DNA fragments about 157 and 166 bp using the primer RM224 and RM72 on chromosome numbers 11 and 8, respectively. *Pi-1* and *Pi-33* genes were only scored in tolerant genotype BAUdhan 3. The Presence of *Pitp(t) and Pi-k^h* were determined by visualization of the amplicon of 120 and 300 bp using the marker RM246 and RM206. Resistant genotype BAUdhan 3 showed a distinct positive band for both genes and no amplicon was observed in BRRIdhan 28 and BRRIdhan 29 (Figure 1).

Content of H_2O_2 (Hydrogen peroxide) and MDA (Malondialdehyde)

The concentration of H_2O_2 ranged from 90-140 µM/gm FW in BRRIdhan 28 and 130-160 µM/gm FW in BAUdhan 3. The trend of H_2O_2 accumulation was almost the same in BRRIdhan 28 and BAUdhan 3 at 48 and 72 hai, only a significant difference was found between the inoculated and non-inoculated plants for BRRIdhan 28 at 72 hai and 96 hai with the higher values occurring for the non-inoculated and inoculated plants, respectively (Figure 2). After pathogen inoculation the MDA

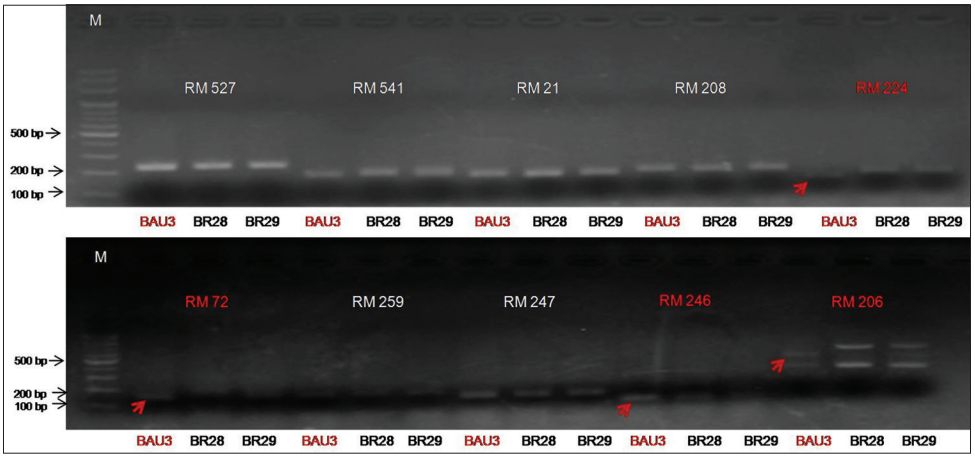

Figure 1: Agarose gel electrophoresis pattern using ten gene specific SSR markers. Genomic DNA was extracted, quantified and equal amount used for PCR amplification. The lane M indicates 100 bp DNA ladder. BAU3: BAUdhan 3, BR28: BRRIdhan 28 and BR29: BRRIdhan 29

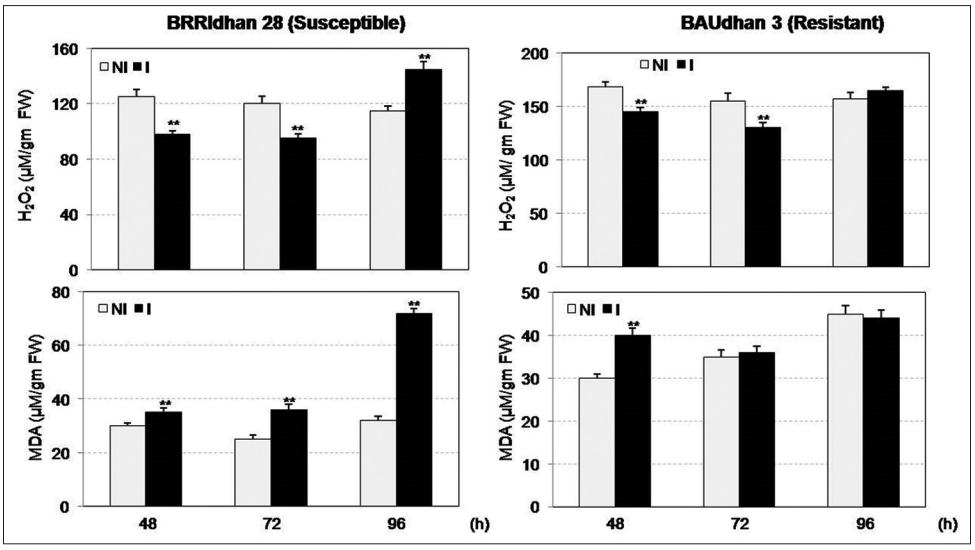

Figure 2: Concentration of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaves of rice plants of BRRIdhan 28 and BAUdhan 3 non-inoculated (NI) and inoculated plants (i) at 48, 72 and 96 hours after inoculation (hai). The vertical bar indicates concentration of H2O2 and MDA form non-inoculated and inoculated leaf with *M. oryzae*.The values indicate means ± SE (n=5). Asterisk indicates significant difference between inoculated and non-inoculated plants. *P<0.05, **P<0.01

content ranged between 35-70 µM/gm FW in BRRIdhan 28 and 30-43 µM/gm FW in BAUdhan 3. Significant differences in MDA concentration were observed between inoculated and non-inoculated plants of BRRIdhan 28 at 48, 72 and 96 hai, but in BAUdhan 3 only after 48 h. The content of MDA is always higher in inoculated plants compared to non-inoculated plants except in BAUdhan 3 at 96 hai indicating less cellular damage and resistance (Figure 2).

Ascorbate Peroxidase Activity (APX)

Ascorbate peroxidase (APX) activity ranged between 6-8 µM/min/mg in BRRIdhan 28 and 6-14 µM/min/mg in BAUdhan 3 (Figure 3) considering both inoculated and non-inoculated leaves. Significant increase and differences were observed for APX activity in inoculated and non-inoculated plants for both BAUdhan 3 and BRRIdhan 28 at 48, 72 and 96 hai. The increase of APX activity was much higher (two times) in BAUdhan 3 compared to BRRIdhan 28 at both 72 and 96 hai (Figure 3).

Catalase (CAT) Activity

The catalase (CAT) activity ranged between 0.5-0.9 µM/min/mg in both tolerant and susceptible genotypes. The catalase (CAT) activity was initially increased at 48 hai and then decreased in inoculated plants of susceptible genotypes BRRIdhan 28 at 72 and 96 hai. Catalase activity was significantly increased in resistant genotype BAUdhan 3 at 48 and 96 hai. The activity of CAT is comparatively lower in BRRIdhan 28 than BAUdhan 3 at 72 and 96 hai (Figure 3).

Guaicol Peroxidase Activity (POD)

The peroxidase (POD) activity profile ranged from 80-120 µM/min/mg in BRRIdhan 28 and 70-180 µM/min/mg in BAUdhan 3 after exposure to fungal pathogens at different time points (Figure 3). The POD activity is almost the same in both genotypes under physiological conditions. There was a significant increase in POD activity after pathogen inoculation in both BRRIdhan 28 and BAUdhan 3 as time progressed after inoculation. At 96 hai, the POD activity was almost two times higher in BAUdhan 3 than BRRIdhan 28 (Figure 3).

DISCUSSION

Genotyping of available rice germplasm using allele-specific markers was helpful to identify and validate major resistant genes and subsequently used in marker-assisted breeding to develop blast-resistant rice varieties. In this study, molecular genotyping of the selected resistant and susceptible rice genotypes with gene-specific markers (SSR markers) was done to validate ten (10) major blast-resistant genes: *Pi-9*, *Pi-1*, *Pi-5(t)*, *Piz-5*, *Pi-b*, *Pi-b*, *Pi-ta*, *Pi-33*, *Pi27(t)*, *Pitp(t) and Pi-k^h*. Here we found that resistant genotypes BAUdhan 3 possess all the blast-resistant genes but susceptible genotypes BRRIdhan 28 and BRRIdhan 29 scored only six *Pi-9*, *Pi-5(t)*, *Piz-5*, *Pi-b*, *Pi-ta and Pi27(t)*. Possibly four resistant genes namely *Pi-1*, *Pi-33*, *Pitp(t) and Pi-k^h* can distinguish the resistant BAUdhan 3 and susceptible BRRIdhan 28 cultivars. The variation in the number of resistance genes and their frequency were observed for a number of studies where it varies from 6-97% (Imam *et al.*, 2014) or 30-99% (Kim *et al.*, 2010). Rice germplasm possessed the presence of resistant genes was also reported by Singh *et al.* (2015). The induction of resistance due to the presence of *Pi9*, *Pita-2*, *Piz-t* genes was more effective in rice cultivars during blast disease (Imam *et al.*, 2014). Therefore, to introgress race-specific resistance R gene using DNA-based molecular markers, identification and diversity analysis of resistance genes in available germplasm is imperative (Jia *et al.*, 2003). This study identified SSR Markers RM 224, RM 72 and RM 206 using resistant and susceptible genotypes, which can be used to screen out resistant genotypes.

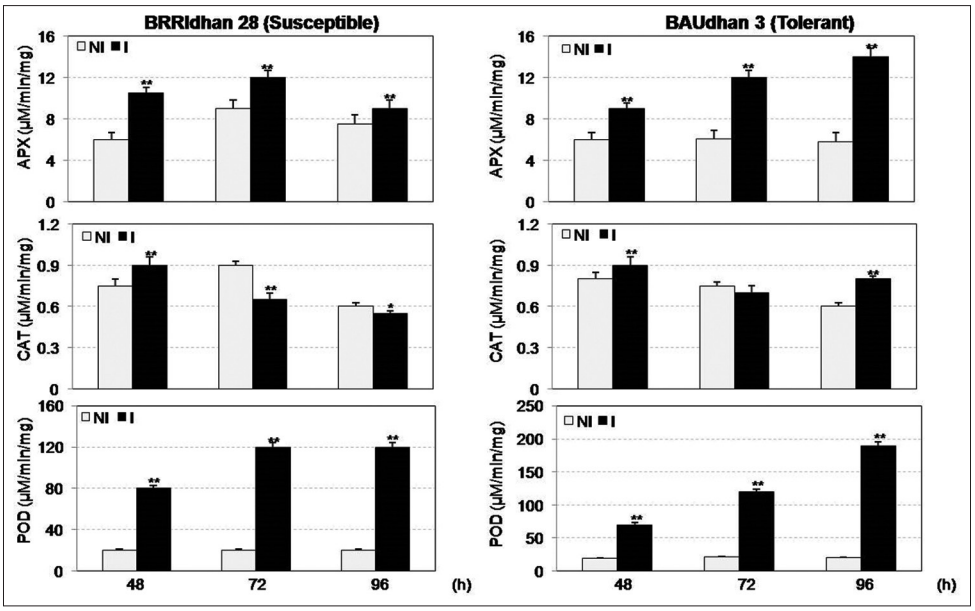

Figure 3: Ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) activity in leaves of rice plants of BRRIdhan 28 and BAUdhan 3 non-inoculated (NI) and inoculated plants (i) at 48, 72 and 96 hours after inoculation (hai). The vertical bar indicates activity profile of enzymes form non-inoculated and inoculated leaf with *M. oryzae*. The values indicate means ± SE (n=5). Asterisk indicates significant difference between inoculated and non-inoculated plants. *P<0.05, **P<0.01

Accumulation of reactive oxygen species (ROS) during pathogen infection was caused by an imbalance between ROS production and its degradation, leading to oxidative stress (Lima *et al.*, 2002; Magbanua *et al.*, 2007). To minimize the adverse effect of oxidative injury, plants induce the activity of ROS scavenging enzymes to maintain the levels constant (Filha *et al.*, 2011). In this study, the higher activities of APX and POD in the leaves of the inoculated plants of BAUdhan 3 compared with inoculated plants of BRRIdhan 28 at 96 hai were observed. In contrast, the MDA concentration was higher in the leaves of the inoculated plants of BAU dhan-3 than in non-inoculated plants only at 48 hai. The POD activity is not only involved in the removal of excess H_2O_2 but also plays an important role against pathogen infection due to participation in lignin biosynthesis (Rauyaree *et al.*, 2001). APX is involved in the removal of H_2O_2 from mitochondria, and chloroplast (Quan *et al.*, 2008) and the change in transcript level upon pathogen infection is well documented (Agrawal *et al.*, 2002a). In our study, the APX activity was increased in both genotypes after inoculation but the increase rate was higher in resistant genotypes BAUdhan 3. In the case of barley upon infection of *Blumeria graminis*, the transcript and activity of APX and POD were also altered in inoculated leaves (Hückelhoven *et al.*, 2001; Harrach *et al.*, 2008). Peroxidase activity (POD) was more during the infection of *Rhizoctonia cerealis* (Hong-xia *et al.*, 2011) and blast infection in wheat (Filha *et al.*, 2011). The results of the present study about POD activity are in agreement of above results. The CAT activity was decreased in BRRIdhan 28 with the progression of the disease, but not in the case of BAUdhan 3. In BAUdhan 3, CAT was higher in inoculated leaves at 96 hai, suggesting a major role of CAT in rice resistance to blast. Higher CAT activity was also observed in maize plants resistant of A. *flavus* (Magbanua *et al.*, 2007). Sometimes, plants tend to accumulate more H_2O_2 by reducing catalase activity as H_2O_2 also acts as a primary signaling component (Magbanua *et al.*, 2007), indicating their role in disease resistance is complex (Kuzniak & Skłodowska, 2005). The lack of effective antioxidative system caused severe damage in tomatoes during Fusarium wilt which is mainly governed by reduced CAT activity (Mandal *et al.*, 2008). MDA content in BRRIdhan 28 at 96 hai is high due to higher accumulation of H_2O_2 which causes large cellular damage inside the cell and makes it susceptible one. *Fusarium oxysporum* (Mandal *et al.*, 2008) infection also causes the over-accumulation of H_2O_2 in tomatoes. Due to the absence of an active and efficient antioxidative system, overaccumulation of ROS cause higher production of MDA during stress condition, which is very much toxic and cause lipid peroxidation in rice (Ou *et al.*, 1985). Higher activities of APX and POD in inoculated plants of BAUdhan 3 compare to inoculated plants of BRRIdhan28 resulted in the lower concentration of H_2O_2, thereby limiting cellular damage. The results show the presence of a more active antioxidative system in resistant cultivars, which can remove excess ROS produced due to *M. oryzae* infection, reduce cellular damage and contribute to more resistance.

CONCLUSION

Rice blast disease caused by *Magnaporthe oryzae* is the most dangerous fungal disease. In Bangladesh chemical fungicide is the only way to manage rice blast disease. The plants ability to face blast disease challenges involves a complex set of responses. This is the first study to investigate the biochemical responses and molecular marker validation of Bangladeshi rice genotypes to blast disease. The activity of different antioxidant systems was induced differentially in resistant and susceptible genotypes upon pathogen infection. The content of reactive

oxygen species (H_2O_2) and cellular damage indicator (MDA) was less in resistant genotypes than in susceptible ones. The resistant genotypes BAUdhan 3 also displayed higher levels of antioxidant enzyme activity. The results show a more effective antioxidative system to overcome oxidative injury during the infectious process of *M. oryzae* limits the cellular damage, thereby contributing to greater resistance to blast. Further research that aims to investigate the antioxidative system in the most productive rice cultivars will play a pivotal role in the development of biochemical markers that can be used in breeding programs to select resistant or tolerant cultivars that can be grown in areas in which severe epidemics of blast occur. In addition, ten SSR markers on different linkage groups were also profiled and revealed that validation of genomic DNA with RM224, RM72 and RM206 could be effectively exploited to screen the rice genotypes for blast resistance.

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