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ORIGINAL ARTICLE

Redox homeostasis, antioxidant defense, and methylglyoxal detoxification as markers for salt tolerance in Pokkali rice

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Abstract To identify biochemical markers for salt tolerance, two contrasting cultivars of rice (*Oryza sativa* L.) differing in salt tolerance were analyzed for various parameters. Pokkali, a salt-tolerant cultivar, showed considerably lower level of H_2O_2 as compared to IR64, a sensitive cultivar, and such a physiology may be ascribed to the higher activity of enzymes in Pokkali, which either directly or indirectly are involved in the detoxification of H_2O_2. Enzyme activities and the isoenzyme pattern of antioxidant enzymes also showed higher activity of different types and forms in Pokkali as compared to IR64, suggesting that Pokkali possesses a more efficient antioxidant defense system to cope up with salt-induced oxidative stress. Further, Pokkali exhibited a higher GSH/GSSG ratio along with a higher ratio of reduced ascorbate/oxidized ascorbate as compared to IR64 under NaCl stress. In addition, the activity of methylglyoxal detoxification system (glyoxalase I and II) was significantly higher in Pokkali as compared to IR64. As reduced glutathione is involved in the ascorbate–glutathione pathway as well as in the methylglyoxal detoxification pathway, it may be a point of interaction between these two. Our results suggest that both ascorbate and glutathione homeostasis, modulated also via glyoxalase enzymes, can be considered as biomarkers for salt tolerance in Pokkali rice. In addition, status of reactive oxygen species and oxidative DNA damage can serve as a quick and sensitive biomarker for screening against salt and other abiotic stresses in crop plants.

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Introduction

Environmental factors greatly influence plant growth and development. A major deviation from the optimal conditions can lead to a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological processes. Stress-induced cellular and metabolic changes result in enhanced accumulation of toxic compounds in cells that include reactive oxygen species (ROS) (Suzuki and Mittler 2006). Exposure of plants to stress activates plasma membrane-bound NADPH-dependent superoxide synthase (Sagi and Fluhr 2006) producing superoxide ($^{-}O_2.$), which, in turn, is converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 can further be converted by the metal-mediated reaction into one of the most damaging and reactive molecule known in the nature 'hydroxyl radical' ($^{\bullet}OH$). Once the $^{\bullet}OH$ is produced, disruption of metabolic systems is almost certain leading to the programmed cell death (Halliwell and Gutteridge 1989). ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, proteins, and DNA affects key components of cell metabolism (Casano et al. 1994). Plants have evolved a complex antioxidant system to counter the harmful effects of ROS. The titer of ROS in any cellular component is governed by this antioxidant system composed of the ascorbate–glutathione cycle that includes SOD and a number of other antioxidant enzymes (Noctor and Foyer 1998).

In addition to the generation of ROS, accumulation of a toxic compound, 'methylglyoxal', has been reported under salt stress condition (Yadav et al. 2005) and detoxification

of this toxic compound might be a strategy for tolerance against various abiotic stresses (Singla-Pareek et al. 2003, 2006). Up-regulation of antioxidant and glyoxalase systems provides protection against NaCl-induced oxidative damage in plants (Hoque et al. 2008). Rice plants can tolerate low-salinity conditions whereas Pokkali—a salt-tolerant species of rice—can grow under high-salt conditions. In this study, we have examined the differences in the redox status, antioxidant defense machinery, and detoxification of methylglyoxal in two contrasting cultivars of rice with a focus on finding the relationship between detoxification of ROS, MG, and salinity tolerance in Pokkali rice.

Materials and methods

Plant material and growth conditions

Plants of two rice genotypes, namely, Pokkali and IR64, were grown under controlled conditions in greenhouse, maintained at 28±2°C and 16 h photoperiod. Seeds were first surface sterilized by sodium hypochlorite (1%) for 2 min and allowed to germinate in vermiculite-containing plastic trays. After seed germination, MS basal liquid nutrient medium (half-strength) was supplied after seeds were germinated. Fifteen-day-old plants were administered salt treatment by saturating the medium with NaCl solution (0 and 200 mM). Samples were harvested after 0, 6, 24, and 48 h.

Estimation of aqueous peroxide (H_2O_2)

Aqueous peroxide content of the rice plants was determined using modified FOX assay as suggested by Evans et al. (1999). Tissue (0.5 g) was homogenized with activated charcoal (0.1 g) in trichloroacetic acid (5 ml of 5% w/v). The homogenate was filtered through filter paper (Whatman No. 1) and centrifuged at 8,000 × *g* for 10 min. Quantitative measurement of aqueous peroxide in the supernatant was performed using FOX assay. FOX reagent was prepared by mixing 1 ml of reagent 'a' (25 mM ammonium ferrous sulfate prepared in 2.5 M sulfuric acid), 50 μl of reagent 'b' (0.25 M xylenol orange prepared in HPLC-grade methanol), and 90 ml of reagent 'c' (9.69 mg of butylated hydroxytoluene prepared in 90 ml of HPLC-grade methanol). The total volume made up to 100 ml with distilled water. All solutions were prepared fresh and used within 2 h. Plant extract (0.2 ml) was taken in a tube and FOX reagent (1 ml) was added. Contents were stirred for 1 min followed by incubation at room temperature for 15 min. Absorbance was taken at 560 nm. A standard curve was prepared, taking varying concentrations of H_2O_2 to calculate the amount of aqueous peroxide. This experiment was performed in triplicate.

Oxidative DNA damage

Oxidative DNA damage was quantified using Oxiselect Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc., USA), which is based on the competitive enzyme immunoassay and detects the quantity of 8-hydroxydeoxyguanosine (8-OHdG). DNA was extracted from the rice leaves using standard C-TAB method. DNA samples (100 μg) were converted to single-stranded DNA by incubating them at 95°C for 5 min followed by rapid cooling on ice. Denatured DNA was further digested to nucleosides by incubating with nuclease P1 (10 units; Fermentas Inc., USA) in 20 mM sodium acetate (pH 5.2) for 2 h at 37°C followed by alkaline phosphatase (CIAP, Fermentas Inc.) treatment in 100 mM Tris buffer (pH 7.5) for 1 h at 37°C. Reaction mixture was centrifuged for 5 min at 6,000 × *g*, and supernatant was used for the 8-OHdG ELISA assay, following the manufacturer's instructions.

Ascorbic acid content

Ascorbic acid content was measured according to Dutilleul et al. (2003) with minor modifications. Fresh leaf tissues were homogenized with metaphosphoric acid (3%) containing EDTA (1 mM) and centrifuged at 8,000×*g*. An aliquot of supernatant (0.1 ml) was taken and citrate phosphate buffer (1 ml of 0.1 M, pH 2.1) was added to this. Absorbance was taken at 265 nm. To this, ascorbate oxidase (1 unit) was added, and after 5 min, absorbance was again noted at 265 nm. The difference in two readings of absorbance indicated the reduced ascorbate content utilized specifically by ascorbate oxidase. In another set, DTT (0.1 ml of 0.1 M) was added and absorbance was read at 265 nm after 5 min. Again, 1 unit of ascorbate oxidase was added, kept at room temperature for 5 min. Absorbance was taken in the same way, which indicated the total ascorbate content. Oxidized ascorbate content was determined by subtracting reduced ascorbate from total ascorbate. Calibration curve was prepared by using graded concentration of L-ascorbic acid in 3% HPO_3. We also calculated the ratio of reduced ascorbate and oxidized ascorbate from the curve.

Glutathione content estimation was according to Griffith (1981) with minor modifications. Fresh leaves were homogenized in metaphosphoric acid [3% (w/v) containing EDTA (2 mM)] and centrifuged at 8,000 × *g*. For estimation, supernatant (0.25 ml) was taken and to it potassium phosphate buffer (1 ml of 0.1 M, pH 8.0) containing EDTA (1 mM) and 10 mM 5,5′-dithio-bis (0.1 ml of 2-nitrobenzoic acid) were added. After incubation at room temperature for 15 min, the absorbance was read at 412 nm. This absorbance accounted for the reduced glutathione (GSH). In a separate set, the same reaction was further allowed to take place with the addition of $MgCl_2$ (100 μl of 3 mM), NADPH (100 μl of

0.15 mM) and glutathione (0.1 units) reductase. Absorbance was again noted at 412 nm, which indicated the content of total glutathione. Oxidized glutathione (GSSG) was calculated by subtracting GSH content from total glutathione content. Calibration curve was prepared by using graded concentration of GSH in metaphosphoric acid (3%) separately using identical conditions for both the sets.

Enzyme assays

Enzyme extractions were carried according to Larkindale and Huang (2004a, b). Harvested leaves (0.25 g) were crushed into fine powder in liquid nitrogen. Further, this powder was homogenized in cold phosphate buffer (1.5 ml of 100 mM, pH 7.0) containing polyvinylpyrrolidone (1% PVP) and EDTA (1 mM) and centrifuged at 10,000 × *g* for 15 min at 4°C. Supernatant was stored on ice for all enzymatic assays; spectrophotometric and in-gel activity analysis were performed using native PAGE. In case of APX assay, the extraction buffer was supplemented with L-ascorbate (2 mM).

SOD [EC 1.15.1.1] activity was estimated using xanthine–xanthine oxidase system as suggested by Beyer and Fridovich (1987) with modifications. Reagents used for assay included potassium phosphate buffer (50 mM, pH 7.8), catalase (Sigma), nitroblue tetrazolium (2.24 mM, NBT), xanthine (2.36 mM), and xanthine oxidase (Sigma). SOD activity was assayed based on its ability to compete with NBT for superoxide anions generated by the xanthine–xanthine oxidase system, which, in turn, results in the inhibition of reduction of NBT. Catalase (0.1 units) was added to the assay mixture to avoid the H_2O_2-mediated possible inactivation of CuZn-SOD. Diluted enzyme extract (100 μl) was taken, and to it, potassium phosphate buffer (800 μl), NBT (50 μl), catalase (0.1 units), and xanthine oxidase (0.1 units) were added. Reaction was initiated by adding 50 μl of xanthine. Change in absorbance was read at 560 nm up to 2 min. A blank reaction was performed using all the components but sample extracts to get the maximum color. Enzyme activity was calculated in units (i.e., amount of enzyme required to inhibit NBT reduction by 50% under specified conditions of the assay) and expressed as units min^{-1} mg^{-1} protein.

Catalase (CAT) [EC 1.11.1.6] estimation was according to Aebi (1983). Diluted enzyme extract (100 μl) and potassium phosphate buffer (1.0 ml of 50 mM, pH 7.0) were mixed. Reaction was initiated by adding H_2O_2 (100 μl of 100 mM). Change in absorbance was recorded at 240 nm at an interval of 15 s for 2 min. Enzyme activity was expressed as units min^{-1} mg^{-1} protein and a change in absorbance by 0.01 corresponded to 1 unit of enzyme activity.

Ascorbate peroxidase (APX) [EC 1.11.1.11] activity was measured by the method of Nakano and Asada (1981). Diluted enzyme extract (0.1 ml) and potassium phosphate buffer (1 ml of 50 mM, pH 7.0) containing ascorbic acid (0.5 mM) were taken. The reaction was started by the addition of H_2O_2 (0.1 ml of 1 mM), and the decrease in absorbance was recorded at 290 nm after and at 15-s intervals for 2 min. A change in absorbance by 0.01 corresponded to 1 unit of enzyme activity. Enzyme activity was expressed as units min^{-1} mg^{-1} protein.

Glutathione reductase (GR) [EC 1.11.1.9] activity was estimated by the method of Smith et al. (1988) with some modifications. The reagents used were potassium phosphate buffer (100 mM, pH 7.6) containing EDTA (1 mM), NADPH (5 mM), 5,5′-dithio-bis (2-nitrobenzoic acid) [6 mM DTNB], and oxidized glutathione (0.2 mM GSSG). In potassium phosphate buffer (1.0 ml), diluted enzyme extract (100 μl), DTNB (50 μl), and GSSG (100 μl) were added. Reaction was initiated by addition of 50 μl of NADPH. Change in absorbance at 412 nm was followed at 15-s intervals up to 2 min. A change in absorbance by 0.01 corresponds to 1 unit and enzyme activity was expressed as units min^{-1} mg^{-1} protein.

Peroxidase (POX) [EC 1.11.1.7] activity was estimated by the oxidation of guaiacol at 470 nm as suggested by Rao et al. (1996). For assay, diluted enzyme extract (100 μl) was taken and to it potassium phosphate buffer (0.9 ml of 100 mM, pH 7.0) containing EDTA (1 mM) and guaiacol (0.1 ml of 10 mM) was added. Reaction was initiated by adding H_2O_2 (100 μl of 10 mM). The change in absorbance was recorded at 470 nm, after 15-s intervals, up to 2 min. Change of 0.1 absorbance has been taken as 1 unit and enzyme activity was expressed as units min^{-1} mg^{-1} protein.

Glutathione peroxidase (GPX) [EC 1.11.1.9] was assayed according to Inoue et al. (1999) with minor modifications. GPX activity was measured in a reaction mixture (1.0 ml) composed of potassium phosphate buffer (0.5 ml of 100 mM, pH 7.6) with EDTA (1 mM), GSH (0.1 ml of 10 mM), NADPH (0.1 ml of 2 mM), GR (0.2 units), and enzyme extract (0.1 ml). The reaction mixture was kept at 25°C for 5 min, and the reaction was started by addition of *t*-butyl hydroperoxide (0.1 ml). Change in absorbance at 340 nm was followed at 15-s intervals up to 2 min. A change of 0.1 absorbance has been taken as 1 unit. Enzyme activity was expressed as units min^{-1} mg^{-1} protein.

Glyoxalase I activity was estimated as described by Deswal and Sopory (1999) with modifications. For assay, phosphate buffer (0.6 ml of 100 mM, pH 7.6) containing EDTA (1 mM) was taken and extract (0.1 ml), methylglyoxal (0.1 ml of 35 mM), and $MgCl_2$ (0.1 ml of 20 mM) were added in the given sequence. Reaction was performed at 25°C by addition of GSH (0.1 ml, 17 mM). Increase in absorbance due to the formation of *S*-lactoylglutathione was noted at 240 nm after 30-s intervals for 2 min. A change in absorbance by 0.1 corresponded to 1 unit of enzyme activity expressed as min^{-1} mg^{-1} protein.

Glyoxalase II activity was assayed according to Maiti et al. (1997). For assay, MOPS buffer (0.8 ml of 100 mM, pH 7.1) and extract (0.1 ml) were mixed. Reaction was started by adding *S*-lactoylglutathione (0.1 ml of 6 mM). Decrease in absorbance due to the consumption of *S*-lactoylglutathione was noted at 240 nm after every 30-s intervals for 2 min. A change in absorbance by 0.1 corresponded to 1 unit of enzyme activity expressed as units min^{-1} mg^{-1} protein.

In-gel analysis of ROS scavenging enzymes

Changes in proteins having isoenzymic activity of the ROS scavenging enzymes were studied using PAGE under non-reduced, non-denatured conditions at 4°C according to Laemmli (1970). Native PAGE analysis was performed for various enzymes involved in the ascorbate–glutathione cycle on a gel (10%) with protein load of 50 μg in each well. Specific procedures for running and staining of gels for different enzymes are given below.

Staining of gels for SOD activity was according to Rucinska et al. (1999). Gels were soaked in NBT (2.45 mM) for 20 min followed by immersion in a solution containing TEMED (28 mM), riboflavin (3 μM), and potassium phosphate (50 mM, pH 7.8) for 15 min. The gel was then placed on dry white illumination tray for 5 to 15 min. During illumination, gel became uniformly blue except at positions containing SOD. Illumination was discontinued after maximum contrast between the achromatic zones and general blue color was achieved.

Gel was pre-run for 30 min using electrode buffer containing 2 mM ascorbate before the samples were loaded as suggested by De Gara et al. (1997). After completion of electrophoresis run, gels were incubated at room temperature for 15 min in potassium phosphate (100 mM, pH 6.4) containing ascorbate (4 mM) and H_2O_2 (4 mM). The gel was washed with distilled water and stained with a solution of HCl (0.125 M) containing ferricyanide (3 mM) and ferrichloride (3.5 mM) at room temperature for 10 min. After development of achromatic bands against the dark blue background, gel was photographed using white illumination tray.

After electrophoresis, gel was incubated in a solution composed of guaiacol (10 mM), potassium phosphate buffer (50 mM, pH 7.0), and H_2O_2 (10 mM) for 10 min. Gel showed dark brown bands and was photographed immediately.

Staining for GR isoforms was performed using the principle employed by Smith et al. (1988). The gel was incubated in Tris–HCl buffer (100 mM, pH 7.6) containing oxidized glutathione (4.0 mM GSSG), NADPH (1.5 mM), and 5,5'-dithiobis (2 mM, 2-nitrobenzoic acid) (DTNB) for 20 min. Yellow colored bands showing the activity of GR were photographed immediately. To obtain maximum contrast, we inverted the colors.

Staining for GPX was according to Kho et al. (2004). The gel was submerged in a Tris–HCl buffer (50 mM, pH 7.9) containing GSH (13 mM) and H_2O_2 (2 mM) for 15 min, followed by incubation in a solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1.2 mM, MTT) and phenazine methosulfate (1.6 mM, PMS). Photographs of achromatic bands on a purple background showing the activity of GPX should be taken immediately. To obtain maximum contrast, we inverted the colors.

Proline content in leaves was estimated strictly following the method of Trotel et al. (1996) while Na and K content was measured using standard procedure with flame photometer (Elico, India) and Na/K ratio was calculated.

Total protein content in the leaves extract was determined (Bradford 1976).

Results

Hydrogen peroxide and oxidative DNA damage

Quantitative estimation of H_2O_2 levels in leaf tissues from 15-day-old plants of sensitive and tolerant cultivars of rice was performed using FOX assay (Fig. 1a). An increase in H_2O_2 content was evident in both the cultivars under NaCl stress as compared to control plants that reached its maximum at the 6-h time point. However, after 6 h, the H_2O_2 content decreased yet remained higher in comparison to control plants. H_2O_2 accumulation in leaf was higher in IR64 than in Pokkali at all time points observed.

Oxidative DNA damage was estimated in terms of 8-OHdG content, in two cultivars of rice. Figure 1b showed that oxidative DNA damage increased in direct proportion to NaCl stress. However, this damage was significantly less in Pokkali as compared to IR64 at all stress points studied.

Redox homeostasis

Ratio of GSH/GSSG provides a clear picture of the comparative profiles of two cultivars under NaCl stress (Fig. 2a). Under control condition, the GSH/GSSG ratio was 1.439 in IR64 and 1.756 in Pokkali. NaCl stress led to a significant decrease in GSH/GSSG ratio, which figured as 1.245 (IR) and 1.462 (Pokkali) after 48 h of exposure. A ratio of the reduced form of ascorbate to its oxidized form was calculated (Fig. 2b). Results clearly suggest a notable decrease in the ratio of reduced/oxidized ascorbate as early as 6 h of exposure to NaCl stress, which remained constant until 48 h in both the cultivars. However, this ratio was almost similar in control plants from both Pokkali and IR64 cultivars. In the case of NaCl-stressed plants after the 48-h time point, Pokkali showed a higher GSH/GSSG ratio (0.389) in comparison to IR64 (0.253).

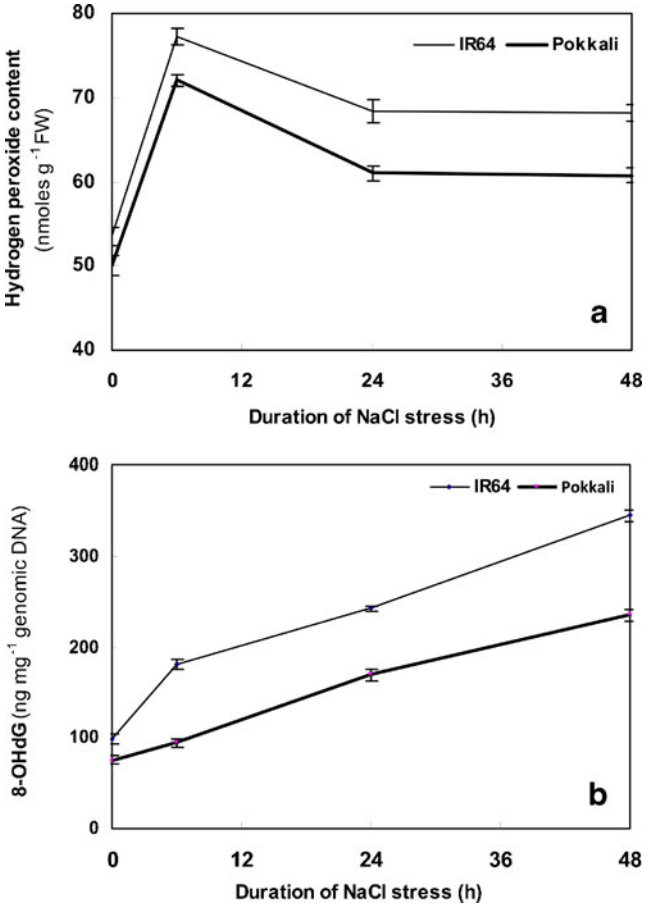

Fig. 1 Hydrogen peroxide content (**a**) and oxidative DNA damage (**b**) in two cultivars of rice under 200 mM NaCl stress. *Vertical bars* indicate standard error of means

ROS scavenging system

Activities of enzymes involved in the ascorbate–glutathione cycle were studied in IR64 and Pokkali seedlings (Fig. 3). In similar conditions, we also studied the isoenzyme patterns by specific activity staining of these enzymes and results presented here include only those that had significant differences in profile (Fig. 4).

SOD activity was higher in Pokkali in comparison to IR64 at all time points of NaCl stress and in control plants. After 48 h exposure to NaCl, decrease in activity of SOD was 44.01% in IR64 as compared to control, whereas in Pokkali, it was only 22.17% (Fig. 3a). The activity staining by native PAGE gel for SOD enzymes confirmed these results. There were three clear bands in both the cultivars (Fig. 4). It is also confirmed that almost all the isoforms of SOD down-regulated in IR64 and up-regulated in Pokkali, thus altogether making a huge difference in superoxide dismutation. However, this situation is a little bit different from the activity measurement wherein decreased activity was found in both the cultivars under salt stress (Fig. 3a).

Catalase activity decreased under NaCl stress up to 6 h in both cultivars (Fig. 3b), but it increased thereafter. A clear difference in CAT activity was evident in the two cultivars throughout the course of experiment. Pokkali showed higher catalase activity in unstressed as well as stressed plants as compared to that of IR64. At 24 h of NaCl exposure, CAT activity tended to move towards the control level and IR64 showed a 6.40% decrease in CAT activity while Pokkali registered a 6.02% increase in the CAT activity at the same time point. At the 48-h time point, the CAT activity more or less showed control levels in both cultivars; however, the absolute values were significantly higher in Pokkali than that in IR64.

Ascorbate peroxidase activity decreased in NaCl-exposed plants of both the cultivars; however, there was a greater decrease in IR64 as compared to its tolerant counterpart Pokkali (Fig. 3c). At 48 h of exposure, IR64 showed a 42.19% decrease while it was only 22.48% in Pokkali. In confirmation to the above findings, the isoenzyme profile of APX in native PAGE gel (Fig. 4) showed a decrease in APX activity in IR64 at all the time

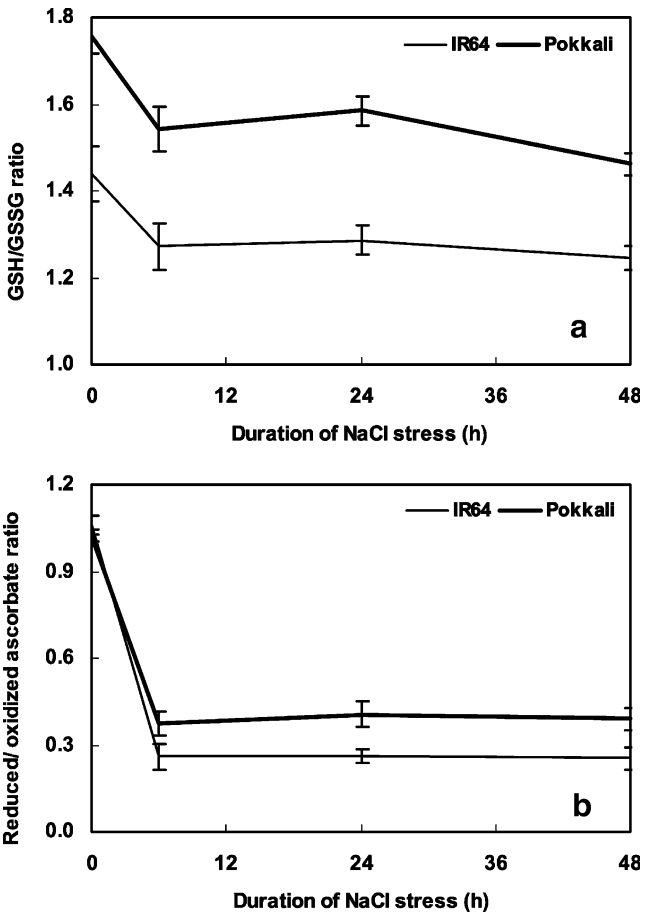

Fig. 2 Redox homeostasis in two cultivars of rice under 200 mM NaCl stress. **a** GSH/GSSG ratio. **b** Ratio of reduced/oxidized ascorbate. *Vertical bars* indicate standard error of means

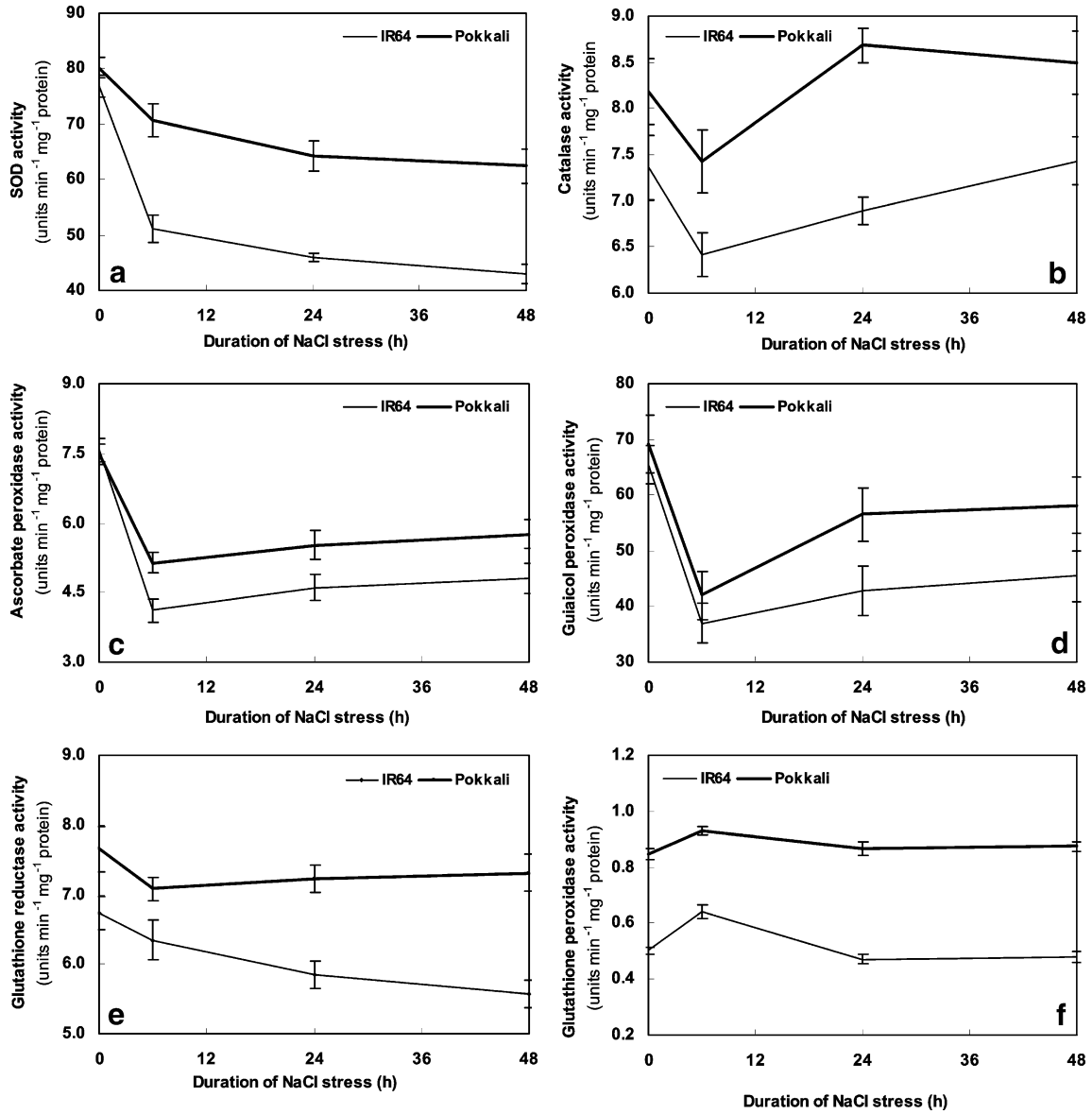

Fig. 3 Activity of enzymes involved in antioxidant defense system in two cultivars of rice under 200 mM NaCl stress. *Vertical bars* indicate standard error of means

points of NaCl stress. However, in variation to the above results, there was an increase in intensity in the two clear visible bands in Pokkali throughout the course of salt stress treatment. Comparisons between the two cultivars clearly revealed higher activity of APX in Pokkali as compared to IR64 at all stress points studied.

POX activity decreased sharply at the 6-h time point of NaCl exposure in IR64 (43.49%) and in Pokkali (39.28%). However, after that, POX activity showed recovery, and at 48 h, the decrease in POX activity (30.58%) was observed in IR64, while in Pokkali, the decrease was only 15.81% as compared to unstressed control plants (Fig. 3d). In-gel activity staining of POX revealed many isoforms in both the cultivars (Fig. 4). However, the uppermost and the lowest bands appeared almost similar in both the cultivars, yet clear differences were observed in middle bands that were of higher intensity in Pokkali throughout NaCl exposure.

Glutathione reductase activity decreased in both cultivars when exposed to NaCl stress in comparison to control plants (Fig. 3e). In IR64, GR activity decreased further as the exposure time of NaCl stress progressed. On the other hand, in Pokkali, after an initial drop at 6 h, GR showed a significant recovery. At 48 h, decrease was 17.31% in IR64 while it was 4.51% in Pokkali. We also studied the in-gel activity staining for this enzyme (Fig. 4). Three isoforms of GR that were evident in both the cultivars showed down-regulation in

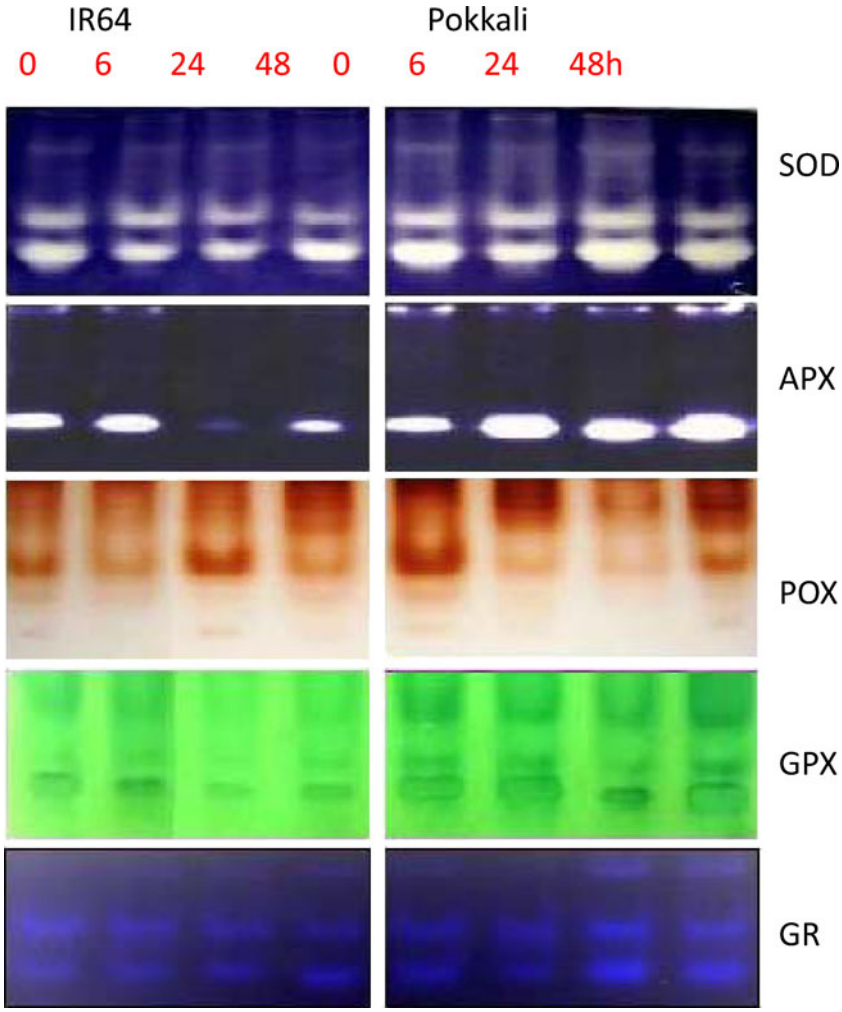

Fig. 4 Isoenzyme patterns of antioxidative enzymes in two cultivars of rice under 200 mM NaCl (GPX and GR are shown in inverted colors)

IR64 when subjected to NaCl exposure; however, in Pokkali, the activity of all the three isoforms got up-regulated especially on the longer exposure of the plants to NaCl.

Unlike the other enzymes, a rise noticed in GPX activity at 6 h in both the cultivars (Fig. 3f), which decreased afterwards, is of significance. At 48 h of NaCl stress, the GPX activity decreased in IR64 by 26.61%, while in Pokkali, the activity was actually higher by 3.8% than that in the control plants. In-gel activity staining for GPX also provided similar results (Fig. 4). NaCl stress clearly alleviated the intensity of all the bands in IR64 but that was not the case in Pokkali, which showed an upper edge over its counterpart.

Activity of glyoxalase I and II

Activity of glyoxalase I decreased under NaCl stress in comparison to control plants in both the cultivars, and such a decrease was observed throughout the exposure to NaCl (Fig. 5a). Nevertheless, the magnitude of decrease in glyoxalase I activity was less in Pokkali as compared to IR64. At 48 h of NaCl stress, the decrease was maximum in comparison to control plants, figuring 35.31% and 22.99% in IR64 and Pokkali, respectively. Activity of glyoxalase II also decreased in both the cultivars under salt stress. However, IR64 registered significantly more decrease as compared to Pokkali (Fig. 5b). After 48 h of NaCl stress, activity of glyoxalase II in Pokkali returned to its control level while IR64 registered a decrease of 24%, making a significant difference in the two cultivars.

Proline content and Na/K ratio

Proline content was estimated in leaves of two cultivars of rice under salt stress. Results presented in Fig. 6a showed that proline content increased with the duration of stress treatment. Up to 6 h, accumulation of proline was observed to be similar in both the cultivars, but after that, significantly higher proline accumulation was observed in Pokkali as compared to IR64.

Na/K ratio increased with the duration of NaCl exposure in both sensitive and tolerant cultivar (Fig. 6b); however, the degree of increase was higher in IR64. Pokkali

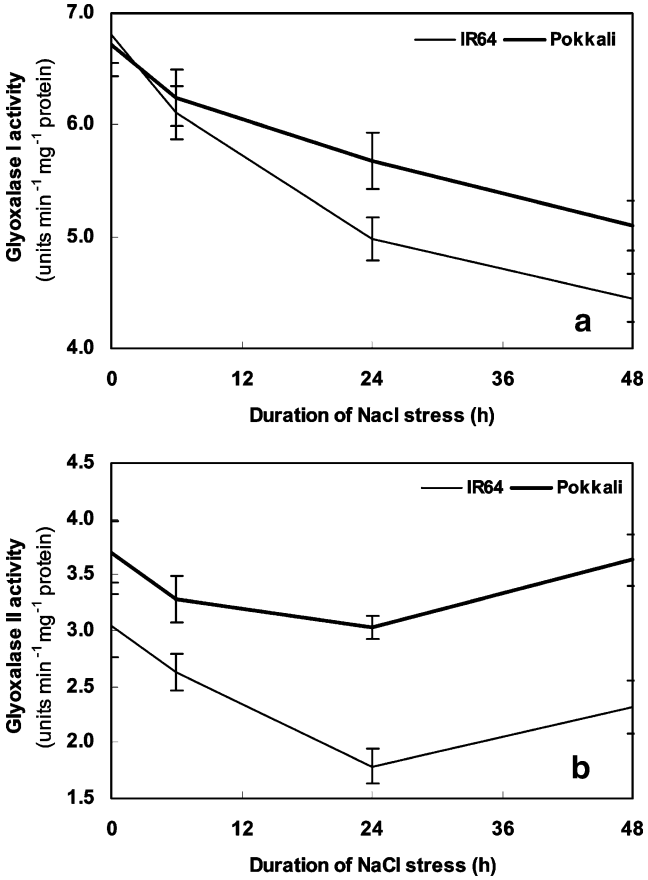

Fig. 5 Activity of enzymes involved methylglyoxal detoxification pathway in two cultivars of rice under 200 mM NaCl stress. **a** Glyoxalase I. **b** Glyoxalase II. *Vertical bars* indicate standard error of means

maintained a lower Na/K ratio as compared to IR64 at every comparison point.

Discussion

One of the prime objectives of this work was to find out the factors that contribute to salt tolerance in Pokkali rice. With this perspective, various biochemical parameters were studied. Ray and Islam (2008) observed a significant difference in Na/K ratio in seven different accessions and found that Pokkali maintained the lowest Na/K ratio under salt stress. Ability of plants to retain K ions in order to maintain Na/K homeostasis has always been a key feature of salt-tolerant crop plants (Munns 2002; Khan et al. 2009). However, despite a lower Na/K ratio in Pokkali, we observed that the difference in ionic status (in terms of Na/K) was marginal in the two cultivars, which indicates that avoidance mechanism cannot account for the observed NaCl tolerance and thus other endurance mechanisms might be responsible for the tolerance in Pokkali rice.

Proline, a compatible solute involved in osmotic adjustment, plays a role in stress tolerance (Jiménez-Bremont et al. 2006). In this study, though proline accumulation was found to be higher in Pokkali, the differences in accumulation in the two cultivars were not visible up to 6 h of NaCl stress and may be a means of osmotic adjustment at a later stage of NaCl exposure. Earlier, Sabu et al. (1995), in a study involving Pokkali, indicated that accumulation of proline is not indicative of salt tolerance characteristics in rice. Thus, in this study, the difference between the two cultivars with respect to proline accumulation and Na/K ratio seems to be insufficient to account solely for the observed salt tolerance of the Pokkali, yet a supportive role of these two cannot be ruled out.

Accumulation of H_2O_2 leads to oxidative DNA damage

Hydrogen peroxide being a stable compound is an indication of the status of ROS that regulates basic acclimatory, defense, and developmental processes in plants. H_2O_2 is one of the

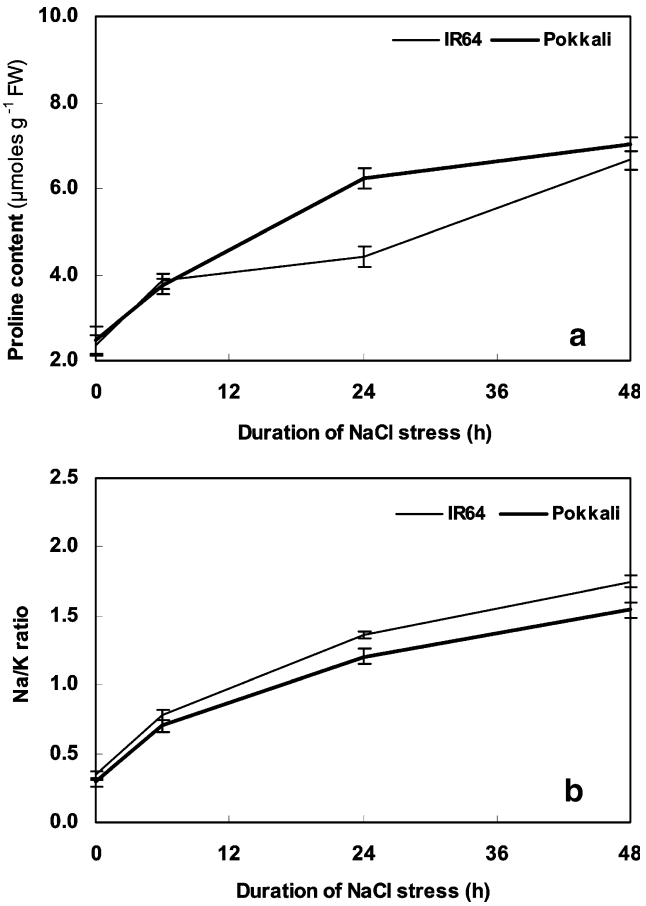

Fig. 6 Proline accumulation (**a**) and Na/K ratio (**b**) in two cultivars of rice under 200 mM NaCl stress. *Vertical bars* indicate standard error of means

crucial ROS produced in response to different environmental factors including salt and ionic stress (Levine et al. 1994). In this study, H_2O_2 accumulation was higher in IR64 than in Pokkali under salt stress (Fig. 1a). The difference may be ascribed to an efficient scavenging of the H_2O_2 in tolerant Pokkali cultivar as compared to its sensitive counterpart, IR64, which is also evident from literature in various crop species (Hernandez et al. 2000). Results suggested that H_2O_2 level came down after 6 h in both the cultivars, which possibly may be due to the activation of the antioxidant defense mechanism, discussed in the succeeding sections. A transient increase in H_2O_2 may be a signal for activation of a ROS-protective mechanism for acclimation to environmental stress (Prasad et al. 1994). Foyer and Noctor (2005) suggested that ROS is involved in oxidative signal transduction, which, in turn, triggers the antioxidant's defense system. Hung et al. (2005) reported that H_2O_2 functions as a stress signal in plants. We feel that an increase of H_2O_2 during early hours (6 h) of stress exposure indicates its role in signaling.

An increase in the degree of oxidative DNA damage was observed in both rice cultivars (Fig. 1b). There is a constant generation of free radicals and other ROS in vivo especially under stressful environment, which cause oxidative damage to biomolecules including nucleic acids, proteins, and lipids. DNA is the most biologically significant target of oxidative attack, and among numerous types of oxidative DNA damage, the formation of 8-OHdG is a ubiquitous marker for oxidative stress as it cannot be further metabolized. From these results, it is obvious that accumulation of H_2O_2 in IR64 is higher in comparison to that of Pokkali at all the time points. However, if this directly accounts for higher oxidative DNA damage, it needs to be further studied.

Maintenance of redox homeostasis contributes to salt tolerance

Glutathione and associated redox reactions play a central role in acclimation of plants to their environment (Noctor et al. 2002). Reduced form of glutathione protects the cell from oxidative damage based on its redox buffering action and abundance in the cell (Ogawa 2005). The GSH/GSSG redox buffer thus provides a basis for redox homeostasis by acting as an electron donor for both scavenging of reactive oxygen and metabolic reactions such as reduction of hydroperoxides and lipidperoxides. In the present work, a higher ratio of GSH/GSSG was observed in salt-tolerant cultivar 'Pokkali' as compared to its sensitive counterpart 'IR64' (Fig. 2a). Higher glutathione concentration in a salt-resistant rice cultivar in comparison to its susceptible counterpart further supports the role of glutathione in salt tolerance (Vaidyanathan et al. 2003). A comparative study performed in cultivated tomato (*Lycopersicon esculentum*) and its salt-tolerant wild relative *Lycopersicon pennellii* (Mittova et al. 2003) revealed that salt-tolerant species maintained a higher GSH/GSSG ratio. Tausz et al. (2004) have suggested that higher concentrations of glutathione and high GSH/GSSG ratio would confer better antioxidative protection and considered as an acclimation criterion.

Ascorbate, a reductant for the enzyme ascorbate peroxidase, is another antioxidant that plays a central role in the ascorbate–glutathione cycle. Pokkali maintained a higher ratio of reduced ascorbate to its oxidized form during salt stress condition. Decrease in ascorbate content and lower ratio of its reduced to oxidized form under salt stress may be due to the rapid oxidation and slow synthesis of ascorbic acid or decreased conversion rates of monodehydroascorbate, dehydroascorbate, and finally ascorbate. Decrease in ascorbate in response to salt stress has been reported in salt-sensitive cultivars of pea (Hernandez et al. 2000) and foxtail millet (Sreenivasulu et al. 2000). A similar decrease in ascorbate content along with an enhanced ratio of dehydroascorbate to ascorbate during salt stress was reported in cultivars of cotton, indicating a shift in redox status towards oxidation (Gossett et al. 1996) and such a shift was ascribed to the univalent oxidation of ascorbate by superoxide. Ascorbate can be oxidized by a direct reaction with superoxide or by serving as a reductant of chromoxyl radical of oxidized tocopherol, which, in turn, disrupts lipid peroxidation reactions by reacting with superoxide and by scavenging hydroxyl, peroxyl, and alkoxyl radicals (Halliwell and Gutteridge 1989). Together, results suggest that redox homeostasis within plant tissues shifts towards oxidation during salt stress and interferes with many defense reactions, especially the operation of the ascorbate–glutathione cycle. Thus, ascorbate may play a potential role in coping up with stressful environmental insults like salinity even in Pokkali rice.

ROS scavenging system play a key role in salt tolerance

SOD constitutes the first line of defense against ROS and catalyzes the dismutation of O_2^- to H_2O_2. In the present study, higher activity of SOD (Fig. 3a) in Pokkali along with up-regulation of all isoforms of SOD (Fig. 4) clearly suggests that Pokkali possesses more efficient superoxide dismutation. Earlier studies have shown that salt-tolerant rice plants exhibited an elevated level of SOD activity, whereas relatively low activity was found in sensitive plants (Dionisio-Sese and Tobita 1998, Bhattacharjee and Mukherjee 1997). Kayupova and Klyshev (1984) reported a decrease in SOD activity by destruction/inactivation of SOD in NaCl-stressed plants by removing/replacing the metal moiety from the enzyme. In the present work, higher activity of SOD in Pokkali in comparison to IR64 as shown by total activity as well as by activity staining pattern

clearly suggests that SOD accounts for better salt tolerance of Pokkali in comparison to IR64.

Catalase is one of the H_2O_2 detoxifying enzymes and mostly associated with peroxisomes where it removes H_2O_2 formed during photorespiration. In the present study, Pokkali showed higher activity in unstressed as well as in stressed conditions. The differential behavior of these two cultivars on time scale indicates the early adaptive potential of Pokkali in terms of CAT activity. In accordance, a significant difference in the CAT activity in leaves of salt-tolerant and salt-sensitive cultivars of pea is reported during salt stress condition (Corpas et al. 1993).

Ascorbate peroxidase is one of the most important antioxidant enzymes in plants that detoxify hydrogen peroxide using ascorbate as reductant. APX exists in several isoforms found in various compartments of plant cells and shows differential expression and regulation under environmental stress factors. During this study, APX activity decreased in NaCl-exposed plants of both rice cultivars (Fig. 3c). However, a clear up-regulation was observed in two isoforms of APX in Pokkali, while in IR64, a down-regulation was evident during salt stress conditions (Fig. 4). In soybean, APX activity decreased (30–100%) during 200 mM NaCl stress depending on the exposure time (Comba et al. 1998). It was suggested that a decrease in enzyme activity may be due to an imbalance between production of ROS and the quenching ability of antioxidants.

Peroxidases are another group of non-chloroplastic enzymes that detoxify H_2O_2 in the cell cytosol. Pokkali maintained significantly higher POX activity throughout NaCl stress exposure (Fig. 3d) as well as in the case of in-gel activity staining of its different isoforms (Fig. 4). Peroxidases are nonspecific in using electron donor such as guaiacol and pyrogallol depending on species and environmental conditions; thus, it is difficult to draw any conclusion on the basis of POX activity. However, our results indicate higher status of POX activity in Pokkali in comparison to IR64, which may contribute to an efficient removal of H_2O_2 in cell compartments other than chloroplast.

Glutathione reductase exhibited a contrasting trend in two cultivars during salt stress. A decrease in its activity and down-regulation of all isoforms of GR was found in IR64 while the reverse was evident in Pokkali (Figs. 3e and 4). Glutathione reductase plays a crucial role in photo protection against oxidative stress by maintaining the glutathione in its reduced state, which is necessary for regeneration of ascorbate into its reduced form, thus ensuring smooth operation of the ascorbate–glutathione cycle. Our results are in agreement with that of other workers who found diminished activity of the GR in sensitive cultivar whereas the reverse was evident in the case of tolerant pea cultivar (Hernandez et al. 2000). Uddin et al. (2007) identified mutant lines of rice that showed high tolerance to salinity and ascribed this trait to higher induction of APX, GR, and SOD. These rice mutant lines indicated that they have the ability to up-regulate their antioxidative system in order to cope up with salt-induced stress.

Glutathione peroxidase uses reduced glutathione as reductant, thus providing an alternate line of defense in addition to reaction catalyzed by APX. Higher activity of GPX in Pokkali as compared to IR64 (Figs. 3f and 4) is suggestive of more efficient antioxidant defense system in Pokkali and may be one of the contributing factors conferring salt tolerance. High level of GPX protein in tobacco plants helps in the maintenance of membrane integrity and increased tolerance to oxidative stress (Kazuya et al. 2004). The specific expression pattern of the phospholipid hydroperoxide glutathione peroxidase gene in salt-induced tolerant foxtail millet seedlings suggests that its product plays a crucial role in the defense reaction against salt-induced oxidative damage (Sreenivasulu et al. 2004).

Detoxification of methylglyoxal

Glutathione is involved in detoxification of methylglyoxal (a reactive ketoaldehyde) via a reaction catalyzed by glyoxalase I producing *S*-D-lactoylglutathione, which, in turn, is utilized by glyoxalase II, which completes the conversion of methylglyoxal to D-lactate and liberates free GSH. In the present study, we checked if there is a relationship between antioxidant defense and the methylglyoxal detoxification pathway. Pokkali exhibited higher activity of both the enzymes involved in detoxification of methylglyoxal in comparison to IR64, throughout NaCl exposure (Fig. 5). The toxic compound methylglyoxal builds up under stress conditions and its detoxification might be a strategy for conferring tolerance against various abiotic stresses (Hoque et al. 2008). The engineering of glyoxalase pathway in transgenic tobacco (*Nicotiana tabacum*) for improved salinity and heavy-metal tolerance is reported (Singla-Pareek et al. 2003, 2006, 2008). Recently, Roy et al. (2008) reported that *Arabidopsis thaliana* plants transformed with glyoxalase I gene from *Brassica* conferred salt tolerance. Maintenance of a higher GSH/GSSG ratio in glyoxalase overexpressing transgenic tobacco, which shows improved salt tolerance, further strengthens the involvement of glutathione in salt tolerance mechanism (Yadav et al. 2005). In the light of results obtained in the present study and supporting evidences from literature, we infer that Pokkali possesses better components for detoxification of the toxic compound methylglyoxal and, hence, may contribute to its better tolerance against NaCl stress. As glutathione is involved in both the pathways, viz., the ascorbate–glutathione cycle as

well as detoxification of methylglyoxal, it may lead to a cross talk between these two important pathways.

In conclusion, our results comprehensively suggest that Pokkali has constitutively higher levels of antioxidants, like GSH and ascorbate, and antioxidant enzymes and also efficient methlyglyoxal detoxification glyoxalase system, in comparison to sensitive IR64, thus providing a threshold of "anti-stress" defense mechanisms that can be activated 'up to the requisite level' under stress conditions in shorter time than in its sensitive counterpart.

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