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Changes in ROS-Scavenging Enzyme Activity in Rice (*Oryza sativa* L.) Exposed to High Salinity

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ABSTRACT: We studied changes in the biochemical and physiological status and ROS-scavenging enzyme (superoxide dismutase, catalase and peroxidase) activity in leaves and roots of rice (*Oryza sativa* L.) plants exposed to high salinity. Under salt stress, the reduction in RWC (relative water content) in leaves was relatively severe in comparison with that of roots. The proline content was also significantly higher in leaves of rice plants following salt treatment. The activities of CAT and POX in roots increased with increasing NaCl concentration, but the activity of SOD decreased. These results suggest that the increase of endogenous proline is closely associated with the increase of CAT and POX activities, which may play important roles in salt tolerance. Therefore, we conclude that the alleviation of oxidative damage and increased resistance to salinity may result from the presence of efficient antioxidative systems.

Key words: Catalase, Oxidative stress, Peroxidase, Rice, Salinity, Superoxide dismutase

INTRODUCTION

Increased salinity of croplands resulting from irrigation restricts the distribution of plants and induces adverse effects on plant metabolism in affected areas. In several regions, salinity has resulted in severe agricultural and environmental problems. Salinization has affected 19% of irrigated land and 21% of croplands in arid regions, acting as a major cause of land destruction (FAO 2000), as salinity limits plant growth and productivity.

Rice (*Oriyza sativa* L.) is the primary stable food for over two billion people in Asia, Africa, and Latin America (Salekdeh et al. 2002). Major environmental limitations on rice production include salinity and drought (Toenissen 1995). Salt stress, mainly caused by NaCl, is one of the most important abiotic stresses and seriously affects crop productivity and survival (Dhaliwhal and Arora 1999).

When plants are exposed to environmental stressors such as salinity, they experience functional disorders of metabolism including ionic imbalance, osmotic stress and oxidative stress (Hasegawa et al. 2000). As a consequence of ion imbalance and hyperosmotic stress, the primary effects of salt stress, secondary stressors such as oxidative damage may occur (Demiral and Türkan 2004). When exposed to drought or high salt content in the soil, many plants accumulate large quantities of proline (Mansour 2000). This osmoprotectant compound plays a major role in osmoregulation and osmotolerance (Gzik 1996, Rajasekaran et al. 1997, Gadallah 1999). However, its role in exerting resistance to salt stress is still debated.

A large body of evidence suggests that environmental stresses, especially drought and salt stress, increase the production of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), $O_2{\cdot}^-$ (superoxide), $^1O_2$ (singlet oxygen) and ·OH (hydroxyl) (Hernandez et al. 2001). ROS are toxic to cells and thus lead to oxidative damage to proteins, membrane lipids, DNA and other cell components (Foyer et al. 1994, McCord 2000, Mittler 2002). Higher plants have active oxygen-scavenging systems consisting of several antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and molecules of non-enzyme antioxidants (Bowler et al. 1992). The SOD are metalloenzymes which play a key role in protection against oxidative stress (Santos et al. 2000, Moran et al. 2003). SOD react with superoxide radicals ($O_2{\cdot}^-$) to produce H_2O_2 (Bowler et al. 1992, Gomez et al. 2004), which is still toxic and must be eliminated by conversion to H_2O in subsequent reactions. Three SOD isoforms are distinguished according to the metal cofactor and subcellular localization. CuZn-SOD is located in the cytosol and chloroplasts, Mn-SOD in mitochondria and Fe-SOD in chloroplasts. Since the H_2O_2 generated from SOD catalytic reactions is toxic, it is subsequently detoxified by POX and CAT into H_2O and O_2 (Gupta et al. 1993). POX and CAT are effective scavengers of H_2O_2, which causes damage to cell components. POX protects cells against the destructive influences of H_2O_2 by catalyzing its decomposition through oxidation of phenolic and enodiolic co-substrates (Lin and Kao 2002). CAT is present in the peroxisomes of

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nearly all aerobic cells, and, to a lesser extent, in mitochondria (Shigeoka et al. 2002), but is virtually absent in chloroplasts (Dionisio-Sese and Tobita 1998). It can protect the cell from H_2O_2 by catalyzing its decomposition into O_2 and H_2O.

Several studies have shown that salt tolerance may be improved if the free radicals formed by the accompanying activated oxygen damage are scavenged by an enhanced antioxidative defense system (Gossett et al. 1994, Hernandez et al. 1995, 2000, Schmer et al. 1995, Kennedy and De Fillippis 1999, Sreenivasulu et al. 2000, Benavides et al. 2000, Lee et al. 2001, Alscher et al. 2002, Mittova et al. 2002, 2003, Shigeoka et al. 2002). Dionisio-Sese and Tobita (1998) studied the activities of SOD and POX enzymes under NaCl stress in the leaves of four rice cultivars exhibiting different sensitivities to NaCl. There are conflicting reports about the responses of CAT activity to osmotic stress, as some works have shown enhanced CAT activity (Gosset et al. 1994, Vaidyanathan et al. 2003), whereas others have reported a salt-induced down-regulation (Shim et al. 2003).

Some physiological aspects of the POX-CAT-SOD system as a protective mechanism against salt stress remain unknown (Cavalcanti et al. 2004). Khedr et al. (2003) found that severe salt stress inhibited the activities of the antioxidant enzymes CAT and POX but the activities of these enzymes were significantly higher in the presence of proline than in its absence. However, most of these studies were performed with leaves, and little information is available about the activities of the POX-CAT-SOD system in the roots, which are usually the first organs directly exposed to salt stress (Azevedo-Neto et al. 2005).

In this work, we hypothesized that the SOD, POX and CAT enzymes will produce distinct responses in leaves and roots under salt stress, and we assessed a part of the enzymatic antioxidant system and investigated proline accumulation in rice leaves and roots subjected to oxidative damage induced by low and high NaCl levels.

METERIALS AND METHODS

Plant Materials and Induction of Salt Stress

Surface-sterilized seeds of rice (*Oryza sativa* L.) were germinated in water for 5 days at 25℃ under dark conditions. After germination, the seedlings were grown in a growth chamber for 15 days. The growth conditions were maintained at 25℃ with a relative humidity of 70% and a 14 h (200 μmol quanta m^{-2} s^{-1}) photoperiod. After 15 days, the seedlings were grown in vermiculite supplemented with varying concentrations of NaCl (100, 200 and 300 mM) for 24 h. After salt treatment, leaves and roots were used as experimental materials.

Measurement of Relative Water Content (RWC)

We determined the RWC using procedures modified from Weatherley (1950). After the fresh mass of leaves and roots was measured, they were allowed to float on water for 7 h. The turgid tissue was then quickly blotted dry prior to determining turgid mass. Dry mass was determined after oven drying at 70℃ for 72 h. The relative water content (RWC) was then calculated using the following formula (Smart and Bingham 1974)

$$RWC\ (\%) = [(\text{fresh weight} - \text{dry weight})/(\text{turgid weight} - \text{dry weight})] \times 100$$

Measurement of Proline Content

The free proline content of leaves and roots was determined using a procedure modified from Paleg et al (1984). Rice leaves (1 g) were homogenized in 5 mL of MCW (methanol:chloroform:water = 12:5:3) and centrifuged at 1,500 g for 10 min. After the addition of ninhydrin reagent to the supernatant, the resulting mixture was heated at 100℃ for 45 min in a water bath. The reaction was then stopped by dipping the mixture tube into an ice bath. The mixture was extracted with toluene, and the absorbance of the toluene fraction was read at 520 nm. Proline content was determined using a calibration curve and expressed as μmol proline g^{-1} fr wt.

Preparation of Enzyme Extracts

Leaves (1 g) frozen with liquid N_2 were ground to a fine powder in a mortar. Soluble proteins were extracted by suspending the powder in 4 mL of 100 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 1% PVP-40, and 0.5% Triton X-100. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000 g at 4℃ for 20 min. The supernatant was then used for determination of antioxidant enzyme activities following the method of Lee and Lee (2000). Protein content was measured according to Lowry et al. (1951).

Assay of Enzyme Activities

Determination of SOD activity was performed according to Beyer and Fridovich (1987). 25 mL of the reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57mM nitroblue tetrazolium (NBT) and an appropriate volume of plant extract. The reaction was initiated by light illumination. One unit of SOD is defined as the amount of enzyme which causes a 50% decrease in the SOD-inhibitory NBT reduction. SOD activity was determined at 560 nm. CAT activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm following the method of Lee and Lee (2000). The reaction mixture contained 50 mM potassium

phosphate buffer (pH 7.0) and plant extract in a 3 mL volume. The reaction was initiated by adding 10 mM H_2O_2. One unit of catalase is defined as the amount of enzyme which liberates half the oxygen peroxide from 10 mM H_2O_2 solution in 100 s at 25℃. POX activity was determined by monitoring the formation of guaiacol dehydrogenation product (extinction coefficient 6.39 mM cm^{-1}) at 436 nm following the method of Lee and Lee (2000). 3.18 mL of reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol and plant extract. The reaction was initiated by adding 0.1 mM H_2O_2. One unit of peroxidase specific to guaiacol is defined as the amount required to oxidize the guaiacol from 0.3 mM guaiacol and 0.1 mM H_2O_2 per min at 25℃ at pH 7.0.

PAGE Analysis of Enzyme Activities

SOD, CAT and POX activity was determined following the procedure of Lee and Lee (2000). Bromophenol blue and glycerol were added to plant extracts containing equal amounts of protein to a final concentration of 12.5% and subjected to discontinuous PAGE under non-denaturing, nonreducing conditions, except that SDS was omitted and the gels were supported by 10% glycerol. Electrophoresis was performed at 4℃ for 4 h with a constant current of 30 mA. SOD activity was detected following the modified procedure of Beauchamp and Fridovich (1971). Identification of SOD isoforms was achieved by incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM KCN or 5 mM H_2O_2 for 30 min before staining for SOD. CAT activity was detected by incubating the gels in 0.01% H_2O_2 for 10 min, rinsing them in water, and staining them in a solution of 1% $FeCl_3$ and $K_3[Fe(CN)_6]$ for 10 min (Woodbury et al. 1971). Staining of POD isozymes was achieved by incubating the gels in sodium citrate buffer (pH 5.0) containing 9.25 mM ρ-phenylenediamine and 3.92 mM H_2O_2 for 15 min (Olson and Varner 1993)

RESULTS

Salt-induced Metabolic Changes

The relative water content (RWC) in leaves and roots of rice was measured following NaCl treatment (Fig. 1). RWC for rice leaves decreased with increasing concentrations of NaCl. Decrements of 24% and 14% in comparison with control plants were observed at concentrations of 200 and 300 mM NaCl, respectively, whereas no change in RWC was observed at a concentration of 100 mM NaCl. In rice roots, significant differences in RWC were not observed between the treatment conditions. Declines in RWC with salt treatment might have resulted from changes in osmotic balance in rice leaves.

Proline is generally assumed to serve as a cellular compatible solute that increases to maintain the osmotic balance between the cell and its surroundings. The content of proline in rice leaves and roots under NaCl treatment is shown in Fig. 2. Salt stress caused an increase of about 30～80% in the free proline content of rice leaves, and the proline content of rice roots increased about 46% in plants treated with 200 mM NaCl, whereas the proline contents of rice roots subject to other treatments were not different from that of control roots.

Salt-induced Enzyme Activities

Total SOD activity in leaves increased with salt treatment, but

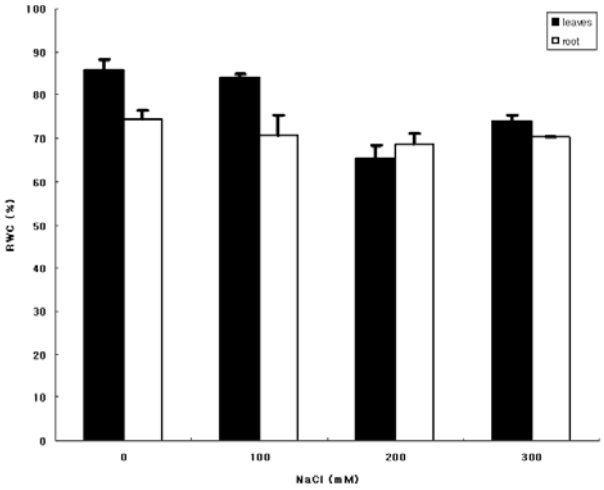

Fig. 1. Relative water contents (RWC) of leaves and roots of rice plants exposed to various concentrations of NaCl for 24 h. Values represent mean ± S.D. (n=6).

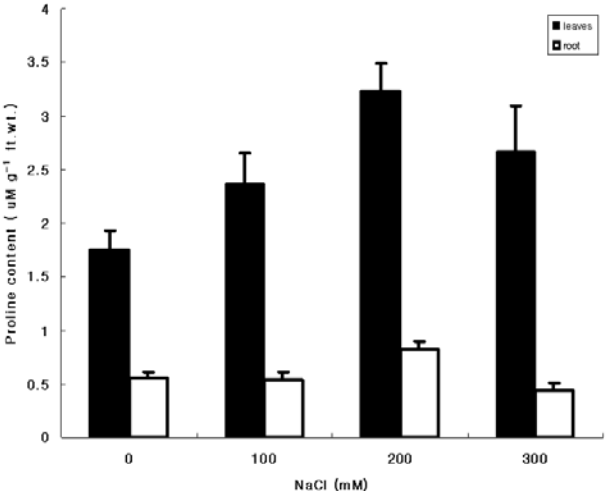

Fig. 2. Proline contents of rice leaves exposed to various concentrations of NaCl for 24 h. Values represent mean ± S.D. (n=6).

SOD activity in roots showed a different pattern (Fig. 3A). SOD activity in leaves increased about 61% at 100 mM NaCl relative to control leaves, and increased of 27% and 45% at concentrations of 200 and 300 mM NaCl, respectively. However, in roots, SOD activity decreased with increasing NaCl. Compared to control roots, an approximately 50% reduction in SOD activity was observed ay 100 mM NaCl treatment. Therefore, under salt stress, the activity of SOD in leaves increased, whereas in roots SOD activity decreased. The gel analyses revealed six isoforms in rice leaves (Fig. 3B). When preincubated with specific inhibitors, the leaves produced 4 bands of CuZn-SOD and 2 bands of Mn-SOD isoforms, but no Fe-SOD forms. Salt stress induced the expression of the CuZn-SOD isoforms, but not Mn-SOD isoforms. Thus, the increase of SOD activity in leaves of rice corresponds to increased CuZn-SOD activities under salt stress.

The POX activity in leaves of rice plants also increased in response to increased NaCl concentration, showing a 43% increase at the concentration of 300 mM NaCl (Fig. 4A). At 100 and 200 mM NaCl, rice leaves showed 30% and 34% increases in POX activity, respectively. The roots also showed a remarkable increase in the activity of POX under salt stress. POX activity was higher in roots than in leaves of rice, showing a 116% increase in the presence of NaCl relative to the control treatment. The gel analyses of POX isoforms revealed three isoforms in rice leaves (Fig. 4B). POX-1 was strongly stimulated by salt, and the POX-2 and POX-3 isoforms also showed increased expression under salt stress.

Rice leaves showed a 71% increase in CAT activity at 100 mM of NaCl, but showed a 28% reduction at 300 mM NaCl. (Fig. 5A). This result shows that lower concentrations of NaCl induced CAT activity whereas higher concentrations of NaCl inhibited CAT activity in leaves. CAT activity in rice roots showed a similar pattern. CAT activity in roots increased in the 100 and 200 mM NaCl treatments (31 and 24%, respectively), but showed a 14% reduction in the 300 mM NaCl treatment. The analysis of CAT isoforms by 7% PAGE demonstrated that the CAT-2 and CAT-3 isoforms increased in the 100 mM NaCl treatment in leaves (Fig. 5B). Increases in CAT activity in the 100 mM NaCl treatment was therefore likely to result from the activation of CAT-2 and CAT-3. At 300 mM NaCl, however, inhibition of CAT-2 and CAT-3 was observed in leaves. These results suggest that CAT-2 and CAT-3 in leaves are sensitive to salt stress, and that the activation of CAT

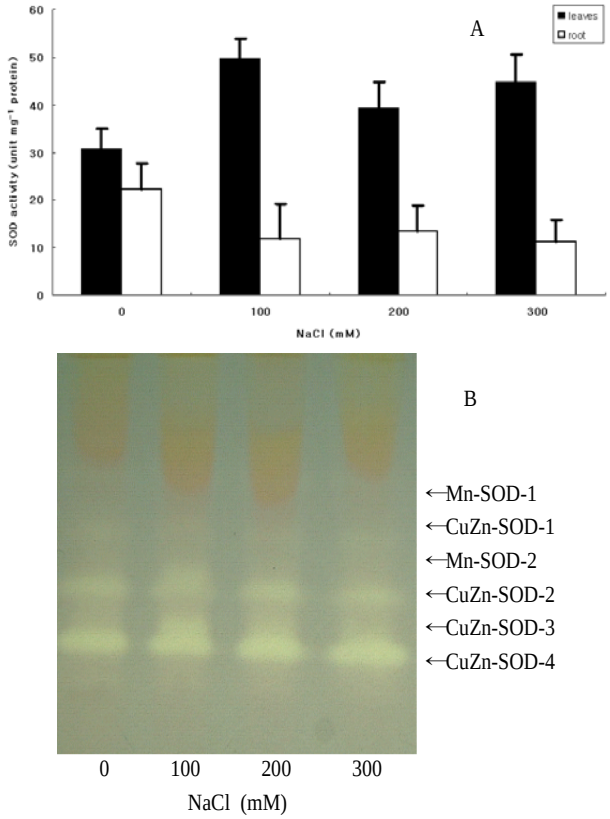

Fig. 3. (A) Activities of superoxide dismutase (SOD) in leaves and roots of rice plants following exposure to various concentrations of NaCl for 24 h. Values represent mean ± S.D. (*n*=6). (B) Native gel stained to show the activities of SOD in rice leaves exposed to various concentrations of NaCl for 24 h.

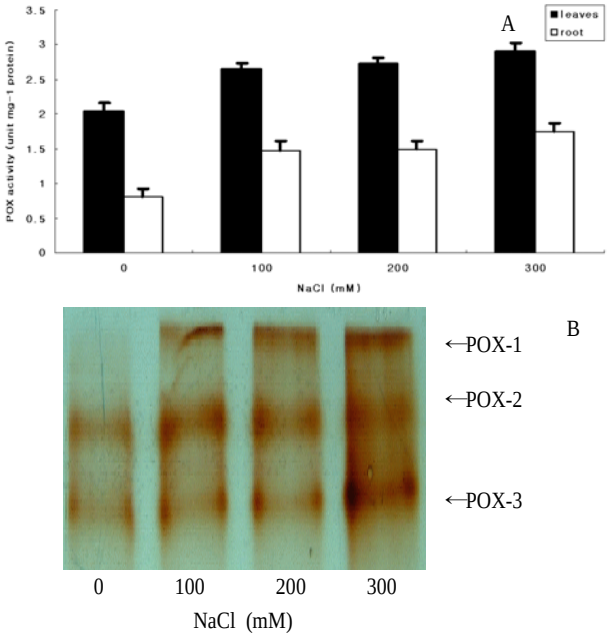

Fig. 4. (A) Activities of peroxidase (POX) in leaves and roots of rice plants following exposure to various concentrations of NaCl for 24 h. Values represent mean ± S.D. (*n*=6). (B) Native gel stained to show the activities of POX in rice leaves exposed to various concentrations of NaCl for 24 h.

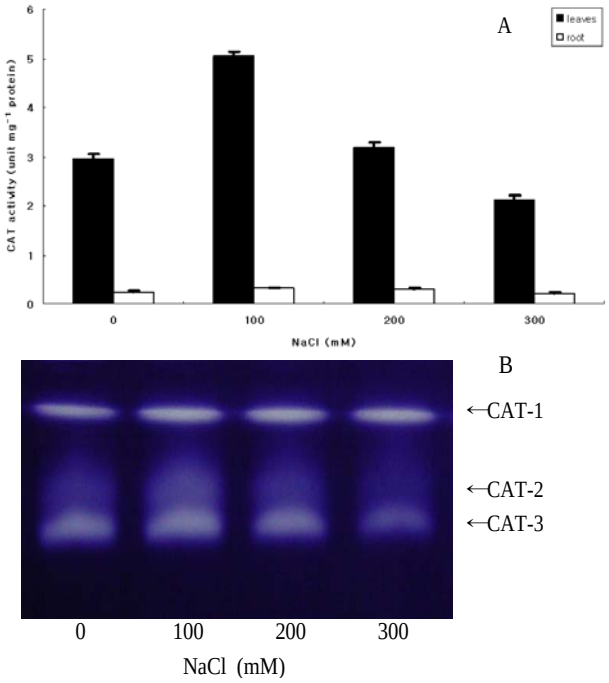

Fig. 5. (A) Activities of catalase (CAT) in leaves and roots of rice plants following exposure to various concentrations of NaCl for 24 h. Values represent mean ± S.D. (n=6). (B) Native gel stained to show the activities of CAT in rice leaves exposed to various concentrations of NaCl for 24 h.

is induced at moderate salt concentrations, but is inhibited at higher salt concentrations.

DISCUSSION

Salinity in irrigation water and agricultural land negatively affects agriculture because it limits the distribution of plants and induces a wide range of adverse metabolic responses in higher plants (Demple and Amabile-Cuevas 1991). Salt stress disrupts homeostasis of water potential and ionic balance in plants cells. Changes in the water state of plants cause initial growth retardation (Dionisio-Sese and Tobita 1998). The relative water content (RWC) in leaves was decreased by NaCl treatment (Fig. 1), which indicates that salt stress caused depression of plant growth.

To maintain their osmotic potential under salt stress, most plants accumulate micromolecular solutes such as sugars, amino acids, and ammonium. In particular, accumulation of free proline is a typical response under stress (Mansour 2000, Su and Wu 2004). The free proline content changed significantly in rice leaves under salt stress (Fig. 2). The increase in proline levels induced by stress is most likely the effect of an increase in metabolism and reduced loss due to oxidation (Dash and Panda 2001, Kavi et al. 2005). Proline is generally assumed to serve as a cellular compatible solute to maintain the osmotic potential between the cell and its surroundings (Pollard and Wyn Jones 1979). The reduced relative water content and increased proline content in plant tisuses were particularly remarkable in the 200 mM treatment, and reflect the damage that salt stress can induce, and the related increase in synthesis of defensive materials. Proline may serve as an osmoprotectant, and may play an important role in protecting enzymes from denaturation, increasing water-binding capacity and acting as a hydroxyl radical scavenger under stress conditions (Su and Wu 2004). The production of ROS such as superoxide, singlet oxygen and hydroxyl radicals increases under stress, and salt can be fatal to crops by inducing the generation and accumulation of ROS to a level that results in disturbance of cellular homeostasis and induces oxidative stress (Peltzer 2002). ROS is effectively scavenged by detoxification mechanisms including the antioxidant enzymes SOD, CAT and POX. The activities of antioxidant enzymes such as SOD, CAT and POX increase in the presence of proline (Khedr et al. 2003, Chen and Dickman 2005), and an increase in the activity of antioxidant enzymes alleviates salt stress. Our results also showed that SOD and POX activities increased with salt stress in rice leaves. When plants are subjected to stress, the first ROS scavenging enzyme active in the enzymatic mechanism is SOD. SOD dismutes active oxygen radicals into H_2O_2 and plays a key role in cellular defenses against ROS (Scandalios 1993). The H_2O_2 produced is then scavenged by CAT and POX. In the present work, total SOD activity increased with salt treatment (Fig. 3). The increase of SOD in leaves is closely related to a higher ability to scavenge active oxygen radicals under salt stress. The increase in SOD activity in rice leaves at 100 mM occurred faster than the increase in proline. This shows that SOD activity increases to promote salt tolerance very quickly, whereas the salt tolerance conferred by the increase in proline levels requires more time. In roots, however, there was not an increase in SOD activity with salt treatment. The osmotic or ionic effects of Na^+ and Cl^- might directly affect the SOD protein integrity and subsequently cause reduction in SOD activity in roots as demonstrated by Hernandez et al. (1994). Azevedo-Neto et al. (2006) have reported that salt stress increased SOD activity in leaves and reduced it in roots of both salt-tolerant and salt-sensitive maize. Several other researchers have also reported that activities of anti-oxidative enzymes increased when plants were exposed to salt stress (Singha and Choudhuri 1990, Arntzen 1995, Fadzillah et al. 1996, Gossett et al. 1996, Lee and Lee 2000). POX activity increased with increasing NaCl concentration in rice leaves and roots relative to the control treatment (Fig. 4), and the activity of isoform-1 (one of three POX isoforms in rice) in particular increased substantially (Fig. 4B). The increase in POX activity might be responsible for the elimination of H_2O_2

from the cytosol. The activity of POX has been reported to increase under salt stress and higher activity has been found in salt-sensitive rice cultivars than in salt-tolerant ones (Mittal and Dubey 1991). POX has also been reported to be involved in the processes of lignification and suberization (Dionisio-Sese and Tobita 1998, Jbir et al. 2001). Lin and Kao (2001) demonstrated that reductions in root growth with increasing NaCl concentration were correlated with increases in cell wall POX activity. CAT activity increased in leaves exposed to salt stress, except in the 300 mM NaCl treatment (Fig. 5). Increases in CAT activity under salt stress have also been reported by other researchers (Baccouch et al. 1998, Ali et al. 2002). CAT is an iron porphyrin enzyme which hydrolyzed H_2O_2 into H_2O and O_2 (Gupta et al. 1993). Along with hydroperoxidases and SOD, CAT is an effective scavenger of ROS which cause damage to the cytoplasm. Of the CAT isoforms, isoforms 2 and 3 showed the greatest increase in activity in the 100 mM NaCl treatment (Fig. 5B). Bor et al. (2003) showed that protection against salinity was achieved as a result of higher constitutive activities of SOD and constitutive and induced activities of POX, APX and CAT in the leaves of wild sugar beet species. Demiral and Turkan (2004) also reported that increased CAT activity in rice seedlings is associated with effective H_2O_2 dismutation into water and molecular oxygen outside the chloroplasts under conditions of high salinity. Similarly, CAT and POX activities increased in rice leaves under salt stress. These results are consistent with the results of Khedr et al. (2003) and Hoque et al. (2006). Hoque et al. (2006) reported that CAT and POX activities increased in the presence of proline under salt stress conditions. In this study, we observed an increase in proline contents under salt stress. It seems that the increase in proline influences the activity of CAT in rice leaves under salt stress. Meanwhile, POX activities increased more in roots than in leaves, which suggests that POX plays a more important role in the salt tolerance mechanism of rice roots than other enzymes.

Resistance to oxidative stress may be closely related to salt tolerance (Gosset et al. 1994, Gueta-Dahan et al. 1997, Hernandez et al. 2000, Mittova et al. 2002, Badawi et al. 2004, Cavalcanti et al. 2004). According to several reports, exogenous proline can effectively detoxify H_2O_2 by enhancing the activities of CAT and POX under salt stress (Hoque et al. 2006). Accordingly, our results suggest that the increase in endogenous proline is closely associated with the increase in CAT and POX activities, and may play an important role in salt tolerance. We conclude that the alleviation of oxidative damage and increased resistance to salinity may involve the deployment of an efficient antioxidative system.

As shown in our results, osmotically active metabolites, specific proteins and free-radical-scavenging enzymes are all involved in resistance to salt stress in plants. This increased resistance to salt stress is essential for plant growth and productivity in saline habitats, and also may permit plant survival following ecological disturbance.

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