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Relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in roots of rice seedlings

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The present study examined the response of antioxidant systems to NaCl stress and the relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in roots of rice seedlings. NaCl treatment caused an increase in the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) in roots of rice seedlings, but had no effect on the activities of superoxide dismutase (SOD) and catalase (CAT). There were detectable differences in APX and GR isoenzymes between control and NaCl-treated roots. Levels of activity for SOD and CAT isoenzymes did not change in NaCl-stressed roots compared with the control roots. NaCl treatment produced an increase in H_2O_2, ascorbate (AsA), dehydro-ascorbate (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) levels. Treatment with 50 m*M* Na-gluconate (whose anion is not permeable to membrane) led to a similar Na^+ level in roots to that with 100 m*M* NaCl. It was found that treatment with 50 m*M* Na-gluconate affected H_2O_2, AsA, and DHA levels, APX and GR activities, OsAPX and OsGR mRNA induction in the same way as 100 m*M* NaCl. These observed changes seem to be mediated by Na^+ toxicity and not by Cl^- toxicity. On the other hand, it was found that NaCl, but not Na-gluconate and $NaNO_3$, caused an increase in GSH and GSSG levels, indicating that Cl^-, rather than Na^+, is responsible for the NaCl-increased GSH and GSSG levels in roots of rice seedlings.

Introduction

Soil salinity is one of the major abiotic stresses affecting plant growth and productivity globally. A consequence of salinity stress in plants is the excessive generation of active oxygen species (AOS) such as superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical (Dionisio-Sese and Tobita 1998, Hernández et al. 2001, Lee et al. 2001). Plants have evolved both enzymatic and non-enzymatic mechanisms for AOS scavenging (Bowler et al. 1992, Foyer et al. 1994 Asada 1999, Mittler 2002). Superoxide is a toxic by-product of oxidative metabolism. Thus, the dismutation of superoxide into H_2O_2 and O_2 by superoxide dismutase (SOD, EC 1.15.1.1), which can be divided into three classes based on the metal present in the active site: Cu/Zn-, Fe-, or Mn-SOD, is an important step in protecting the cell. In the ascorbate–glutathione cycle, ascorbate peroxidase (APX, EC 1.11.1.1) uses two molecules of ascorbate (AsA) to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA). MDHA is a radical with a short lifetime that, if not rapidly reduced, disproportionates to AsA and dehydroascorbate (DHA). MDHA can be reduced to AsA by MDHA reductases (EC 1.6.5.4) by using NADPH as electron donors. DHA is then reduced to AsA by the action of DHA reductase, using reduced glutathione (GSH), which is generated from oxidized glutathione (GSSG) by glutathione reductase (GR, EC 1.6.4.2) at the expense of NADPH. Catalase (CAT, EC 1.11.1.6) is implicated in the removal of H_2O_2. Low molecular weight antioxidants such as AsA and GSH are known to scavenge different types of AOS (Apse and Blumwald 2002).

Abbreviations – AOS, active oxygen species; APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; DPI, diphenyleneiodonium chloride; DW, dry weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

When growing in saline soil, roots have to cope with two types of stress. The first of these is an osmotic stress resulting from salt concentration in the soil that results in lowered water potential and a consequent loss of cell turgor in roots. The second is ionic stress induced by changes in the concentrations of Na^+, Cl^- or both in the root-growing medium and within root tissues. In addition to its known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress, all of which contribute to its deleterious effects (Gueta-Dahan et al. 1997, Hernández et al. 2001, Shalata et al. 2001). It has been shown that the physiological disturbances in citrus produced by salinity are associated with Cl^- build-up rather than with Na^+ accumulation (Romero-Aranda et al. 1998, Moya et al. 2003). Cramer et al. (1994) reported early variety differences in leaf growth rates of maize plants. They showed that a maize variety that was more sensitive to early growth inhibition by salinity accumulated less Na^+. Results from Montero et al. (1998) and Sibole et al. (1998) strongly suggest that bean is extremely sensitive to Na^+. Munns et al. (1988) reported that leaf growth of barley was reduced by higher NaCl treatments, but is not directly controlled by local concentration of Cl^-, suggesting that there is some influence that originates elsewhere in the plants. However, it is not known whether a positive correlation between Na^+ and Cl^- accumulation and induction of antioxidant systems may occur.

Data concerning the effect of NaCl stress on antioxidant systems in roots are scarce. In the present study, using roots of rice seedlings, the response of antioxidant systems to NaCl stress was investigated. In previous studies we have shown that NaCl stress caused an increase in H_2O_2 levels in the roots of rice seedlings (Lin and Kao 2001a) and this increase was attributed to Na^+ rather than Cl^- (Lin and Kao 2001b). Thus we also investigated the relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in the roots of rice seedlings.

Materials and methods

Plant material

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pre-treated at 37°C for 1-day under dark condition. Uniformly germinated seeds were then selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No. 1 filter paper (Whatman, UK) moistened with 10 ml of distilled water or NaCl at the desired concentration as specified in the individual experiments. Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts and so seedlings grown in distilled water were used as the controls. For the experiments to examine the relative importance of Cl^- in regulating NaCl-induced antioxidative system in roots, Na-gluconate and $NaNO_3$ were used. Each Petri dish contained 20 germinated seeds and each treatment was replicated four times. The germinated seeds were allowed to grow at 27°C in darkness.

H_2O_2 determination

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing roots with 3 ml of phosphate buffer (50 m*M*, pH 6.8) including 1 m*M* hydroxylamine. The homogenate was centrifuged at 6000 *g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride (Aldrich, Milwaukee, WI) in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6000 *g* for 15 min. The intensity of yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.25 $\mu mol^{-1} cm^{-1}$. The H_2O_2 level was expressed on the basis of dry weight (DW).

Enzyme extraction and assays

For extraction of enzymes, root tissues were homogenized with 0.1 *M* sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of APX activity, 2 m*M* AsA was added to the extraction buffer. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulting supernatant was used for the determination of enzyme activity. The whole extraction procedure was carried out at 4°C. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 $mM^{-1} cm^{-1}$ at 240 nm) for H_2O_2. One unit of CAT was defined as the amount of enzyme, which breaks down 1 nmol H_2O_2 per min. SOD was determined according to Paoletti et al. (1986). One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NADH oxidation observed in blank. APX was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as a decline in the optical density at 290 nm and activity was calculated using the extinction coefficient (2.8 $mM^{-1} cm^{-1}$ at 290 nm) for ascorbate. One unit of APX was defined as the amount of enzyme that breaks down 1 µmol of ascorbate per min. GR was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreases 1 A_{340} per min. The activities of all enzymes were expressed on the basis of DW.

Gel activity analysis

Roots (0.1–0.2 g) were homogenized in 50 m*M* potassium phosphate (pH 7). The homogenate was centrifuged for 15 min at 13 000 *g* at 4°C. The supernatant was

collected and protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard. Samples containing 50 μg protein, with the addition of bromophenol blue and glycerol, were subjected to non-denaturing PAGE, essentially as described by Laemmli (1970), except that SDS was omitted. For the analysis of APX activity, 2 m*M* AsA was added to the extraction buffer, the carrier buffer and the gel was pre-run for 10 min before the samples were loaded (Mittler and Zilinskas 1993). The gel, equilibrated with 50 m*M* sodium phosphate (pH 7) containing 2 m*M* AsA in the presence of 5 m*M* KCN for 30 min, was incubated in a solution composed of 50 m*M* sodium phosphate (pH 7), 2 m*M* AsA and 2.3 m*M* H_2O_2 for 20 min. The gel was washed in the buffer with gentle agitation for 1 min and submerged in a solution of 50 m*M* sodium phosphate buffer (pH 7) containing 28 m*M* *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 2.45 m*M* nitroblue tetrazolium (NBT). CAT activity was detected by incubating the gels in 3.27 m*M* H_2O_2 for 5 min, rinsing them in water, and staining them in a solution of 1% potassium ferricyanide and 1% ferric chloride for 5 min (Scandalios 1968). GR activity was detected by incubating the gels in 50 ml of 0.25 *M* Tris-HCl buffer (pH 7.5) containing 10 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10 mg 2,6-dichlorphenolindophenol, 3.4 m*M* GSSG and 0.5 m*M* NADPH (Madamanchi et al. 1992).

SOD isoenzymes were separated by isoelectric focusing on a 10% non-denaturing polyacrylamide gel containing 2% ampholine (pH 3–10, Amersham, NJ), based on the method described by Beauchamp and Fridovich (1971). Aliquots of enzyme extract (50 μg protein) were loaded in the gel. Loading buffers were 0.01 *M* phosphoric acid for the anode and 0.02 *M* NaOH for the cathode, respectively. Gel was pre-focused for 20 min at 60 V without samples, then focused overnight at 200 V. After completion of electrophoresis, the gel was soaked in a solution containing 2.45 m*M* NBT for 15 min, followed by incubation in 50 m*M* potassium phosphate buffer (pH 7.8) containing 28 μ*M* riboflavin and 28 m*M* TEMED under dark conditions for 15 min. The gel was then exposed to light for 15 min. For identification of individual SOD isoenzymes, the gel was treated with either 8 m*M* KCN or 8 m*M* H_2O_2 in the 50 m*M* potassium phosphate buffer (pH 7.0) for 30 min before staining for SOD activity.

Isolation of OsAPX and OsGR cDNA

Rice cDNA clones encoding putative *OsAPX* and *OsGR* were isolated through the RT-PCR approach using root RNA as template. Total RNA was isolated from hydroponically cultivated rice roots using the TRIZOL reagent and following the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Using 1 μg of template RNA, *OsAPX* (0.8 kb) and *OsGR* (1.6 kb) cDNA, were amplified by Super Script®· One-Step RT-PCR with Platinum® Taq (Invitrogen), respectively. The gene-specific primers were designed according to the cDNA sequences published in NCBI genebank (accession number: *OsAPX*, AY254495; *OsGR*, D78136). The primer pairs used for the RT-PCR were: for *OsAPX*, 5′-TTCGAATTCTCCACCC-GCAGCCATGGCGA-3′ (*Eco*RI site underlined), and 5′-ACTGTCTAGAACGGGCAATGTACTAGGCAGT-3′ (*Xba*I site underlined); for *OsGR*, 5′-AGCGAATTCTT-GAGGATCCATGGC-3′ (*Eco*RI site underlined), and 5′-TCATCTAGACCAGAGTACACTTTGCC-3′ (*Xba*I site underlined). The PCR products were subcloned into the *Eco*RI and *Xba*I sites of pBluescript (Strategene, CA) to generate pBS/APX and pBS/GR, respectively.

RNA gel blot analysis

RNA gel blot analysis was employed to study *OsAPX* and *OsGR* transcription levels in roots following different treatments. Samples of 7 μg of total RNA was size fractionated on 1% agarose gel containing 10 m*M*sodium phosphate buffer (pH 6.5), transferred to a nylon filter, and hybridized with ^{32}P random primer labelled *OsAPX* DNA (0.8 kb) or *OsGR* DNA (1.6 kb) probe (Thomas 1983). All hybridization reagents were prepared as described in Sambrook et al. (1989). The hybridization signals were scanned (Microtek ScanMaker 8700 scanner, software QUANTITY ONE; Bio-Rad Hercules, CA), and the ratio of *OsAPX* and *OsGR* mRNA to 18S rRNA was determined. The results are expressed as the degree of increase over mRNA levels for the untreated roots.

Determinations of AsA, DHA, GSH and GSSG

AsA and DHA in 5% (w/v) trichloroacetic acid extract and GSH and GSSG in 3% sulfosalicylic acid extract were determined as described by Laws et al. (1983) and Smith (1985), respectively.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or on different times were analysed following the Duncan's multiple range test or Student's *t*-test.

Results

The effect of NaCl concentration on antioxidant enzymes (SOD, CAT, APX, and GR) is presented in Fig. 1. Increasing concentrations of NaCl from 50 to 150 m*M* progressively increased the activities of APX and GR (Fig. 1B and C), but had no effect on the activities of SOD and CAT (Fig. 1A and D).

SOD isoenzymes were identified using 8 m*M* KCN to inhibit Cu/Zn-SOD or 8 m*M* H_2O_2 to inactivate both Cu/Zn-SOD and Fe-SOD (Fridovich 1986). As shown in Fig. 2A, one isoenzyme of SOD in rice roots was identified as Mn-SOD, and the other three were identified as Cu/Zn-SOD. No Fe-SOD isoenzyme was

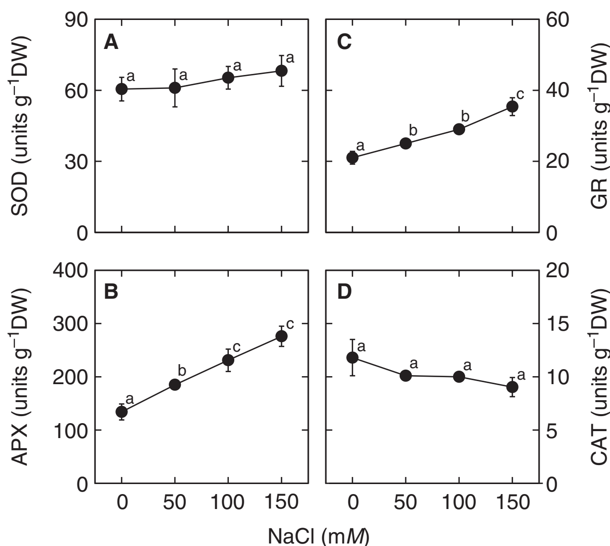

Fig. 1. Effect of NaCl concentration on the activities of antioxidant enzymes in the roots of rice seedlings. Enzyme activities were assayed after 5 days of treatment. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

observed in rice roots. There was no detectable difference in the activity of SOD isoenzymes between control and NaCl-stressed roots (Fig. 2A). Two isoenzymes of CAT in rice roots were identified (Fig. 2B). However, no detectable difference in the activity of CAT isoenzymes was observed between control and NaCl-stressed roots (Fig. 2B).

The effect of NaCl on the changes in the isoenzymes of APX is presented in Fig. 3A. There was detectable difference in the activity APX-1, a major isoenzyme, between control and NaCl-treated roots. NaCl stress did not affect APX-2 activity in comparison with controls. As shown in Fig. 3B, the roots of rice seedlings contained two isoenzymes of GR, and the major isoenzyme of GR was named GR-1, with the minor isoenzyme being named GR-2. NaCl stress had significant effect on the activation of both GR-1 and GR-2.

When 2-day-old-rice seedling roots were treated with 150 m*M* NaCl, the levels of H_2O_2 and the activities of APX and GR increased with the time of incubation (Fig. 4A–C). After 1 day of NaCl treatment significant increases in AsA, DHA, and GSH levels occurred in comparison with control roots (Fig. 5). AsA and DHA levels gradually increased during the treatment, reaching a maximum after 3 days of salt stress (Fig. 5A and B). The increase in GSSG levels was also higher in the NaCl-treated roots than in their respective control, and it occurred after 2 days of salt-treatment (Fig. 5D).

To test whether Cl^- toxicity is involved in the induction of antioxidant systems, experiments were performed to compare the effect of NaCl with that of Na-gluconate, whose anion is not able to permeate through the membrane (Blumwald and Poole 1985). Our previous work indicated that treatment with 50 m*M* Na-gluconate had a similar level of Na^+ but a much lower level of Cl^- in roots in comparison with levels for 100 m*M* NaCl (Lin and Kao 2001b). The increase in the level of H_2O_2 and the activities of APX and GR generated by 50 m*M* Na-gluconate was similar to that by 100 m*M* NaCl (Fig. 4D–F). We also demonstrated that roots of rice seedlings treated with 50 m*M* Na-gluconate had similar levels of AsA and DHA to those treated with 100 m*M* NaCl (Fig. 6A and B). These results strongly suggest that Cl^- is not required for the NaCl-induced levels of H_2O_2, AsA, and DHA, as well as for the increase in APX and GR activities in roots of rice seedlings. Figure 6C and D show that 100 m*M* NaCl treatment increased GSH and GSSG levels in comparison with controls. However, 50 m*M* Na-gluconate and 100 m*M* $NaNO_3$ treatments had no effect on GSH and GSSG levels in roots of NaCl-treated rice seedlings. These results implied that Cl^- rather than Na^+ is responsible for the increase in GSH and GSSG levels in roots of rice seedlings. The effect of 50 m*M* Na-gluconate and 100 m*M* NaCl on the expression of genes of APX and GR are shown in Fig. 7A and B. The steady-state transcript level of *OsAPX* and *OsGR* increased after 3 days of NaCl and Na-gluconate treatments. There was no detectable difference in transcript levels of *OsAPX* and *OsGR* between NaCl- and Na-gluconate-treated roots, indicating that Cl^- is not required for NaCl-induced expression of genes of APX and GR in rice roots.

Discussion

The role of APX and GR in the H_2O_2 scavenging in plant cells has been well established in the ascorbate–glutathione cycle (Bowler et al. 1992). Lee et al. (2001) showed that NaCl stress resulted in a higher activity of APX in rice leaves but not in rice roots. In shoot cultures of rice, activity of APX was similar whether the shoots were grown in the presence or absence of NaCl (Fadzilla et al. 1997). In contrast, there was an early increase in GR activity in NaCl-exposed shoot cultures of rice (Fadzilla et al. 1997). Hernández and Almansa (2002) demonstrated that APX activity did not change and GR activity increased in pea leaves during short-term NaCl stress. Here, we show that both activities and isoenzymes of APX and GR are enhanced by NaCl in the roots of rice seedlings (Figs 1B and C, 3A and B).

Superoxide is a toxic by-product of oxidative metabolism. Thus, the dismutation of superoxide into H_2O_2 and O_2 by SOD is an important step in protecting the cell. In this study, three isoenzymes of Cu/Zn-SOD and one isoenzyme of Mn-SOD were observed in rice roots (Fig. 2A). However, no Fe-SOD isoenzyme was detected in gel activity assay. Lee et al. (2001) observed that NaCl treatment induced a significant increase of SOD activity in rice leaves, however, the activity of SOD in rice roots was not affected by NaCl. A significant increase of SOD activity occurred in pea leaves after short-term NaCl stress (Hernández and Almansa 2002). We observed

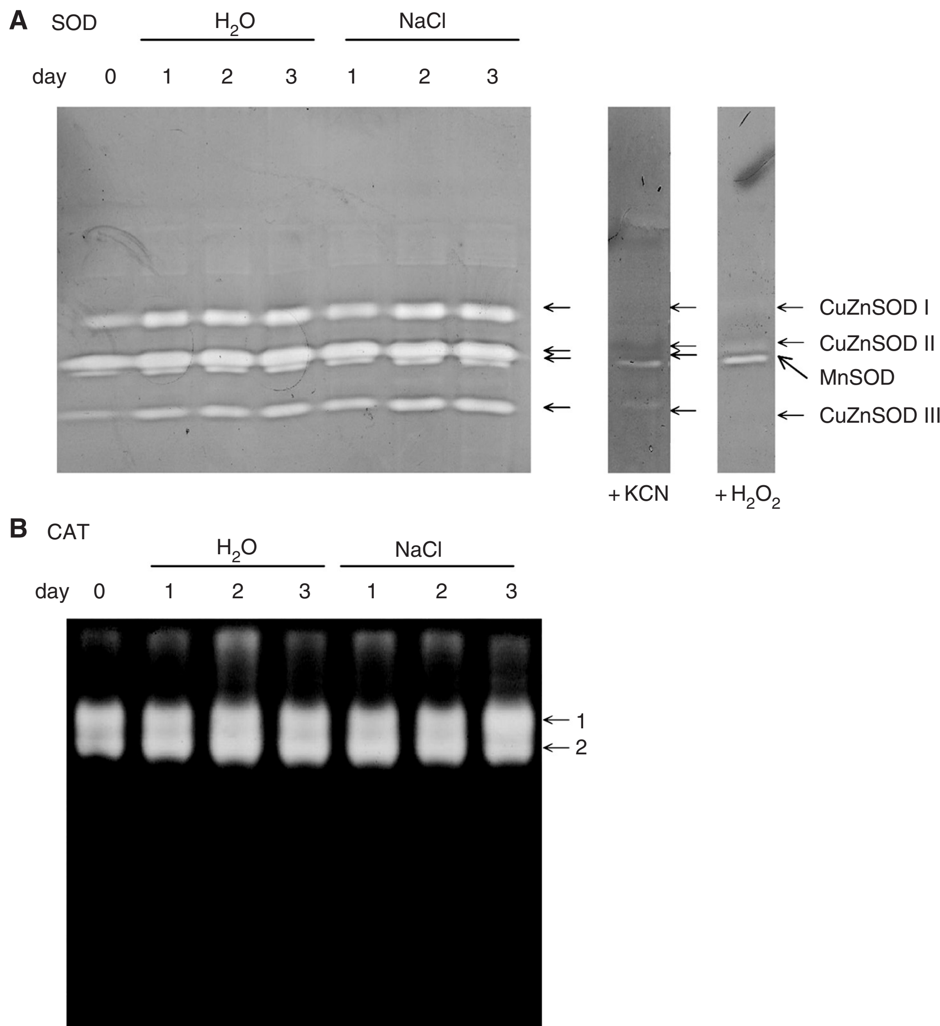

Fig. 2. Changes in the isoenzymes of SOD (A) and CAT (B) in the roots of rice seedlings in the presence and absence of NaCl. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and NaCl (150 m*M*) for 1, 2 and 3 days, respectively. The concentrations of KCN and H_2O_2 were 8 m*M*. Arrows indicate different isoenzymes in rice roots.

that NaCl had no effect on the activity of SOD and isoenzymes of SOD in rice roots (Figs 1A and 2A).

CAT is known to dismutate H_2O_2 into H_2O and O_2. In *Nicotiana plumbaginifolia*, Savouré et al. (1999) found that NaCl-stimulated catalase activity through activation of the *Cat2* and *Cat3* genes. Fadzilla et al. (1997) reported that NaCl had no effect on CAT activity in rice shoots. On the other hand, decrease in CAT by NaCl has been shown in rice leaves (Dionisio-Sese and Tobita 1998, Lee et al. 2001). In this study, we observe that NaCl does not influence the activity and isoenzymes of CAT in rice roots (Figs 1D and 2B). Data obtained in the present study seem to suggest that, if anything, CAT and SOD play a less important role in scavenging AOS in roots of rice seedlings under NaCl stress conditions.

Gene expression in response to environmental stress is usually studied at the level of steady-state mRNA abundance because this gives a more precise estimate of antioxidant gene activation than enzyme activity. Expression of *APX* and *GR* has been reported to be enhanced in plants by NaCl treatment (Kaminaka et al. 1998, Savouré et al. 1999, Kawasaki et al. 2001). However, López et al. (1996) demonstrated that APX activity, rather than mRNA level, was enhanced in NaCl-stressed *Raphanus sativus* plants. Hernández et al. (2000) found that transcript levels for cytosolic GR and APX were strongly induced in the NaCl-tolerant pea variety but not in the NaCl-sensitive pea variety. In the present investigation, we observe that both NaCl and Na-gluconate treatments result in an enhancement of the expression of *OsAPX* and *OsGR* in the roots of rice seedlings (Fig. 7A and B). Our results reveal that *OsAPX* and *OsGR* gene expression is up-regulated by NaCl in rice roots.

AsA is a major antioxidant in photosynthetic and non-photosynthetic tissues which reacts directly with AOS and is utilized as a substrate for APX-catalysed H_2O_2 detoxification (Noctor and Foyer 1998). GSH is involved in AsA regeneration and functions also as a direct scavenger of AOS (Noctor and Foyer 1998). Elevated AsA and GSH levels have been measured in plants exposed to NaCl (Meneguzzo et al. 1999, Khan et al. 2002). However, there are other reports indicating that AsA and GSH levels decreased in plants in response to NaCl stress

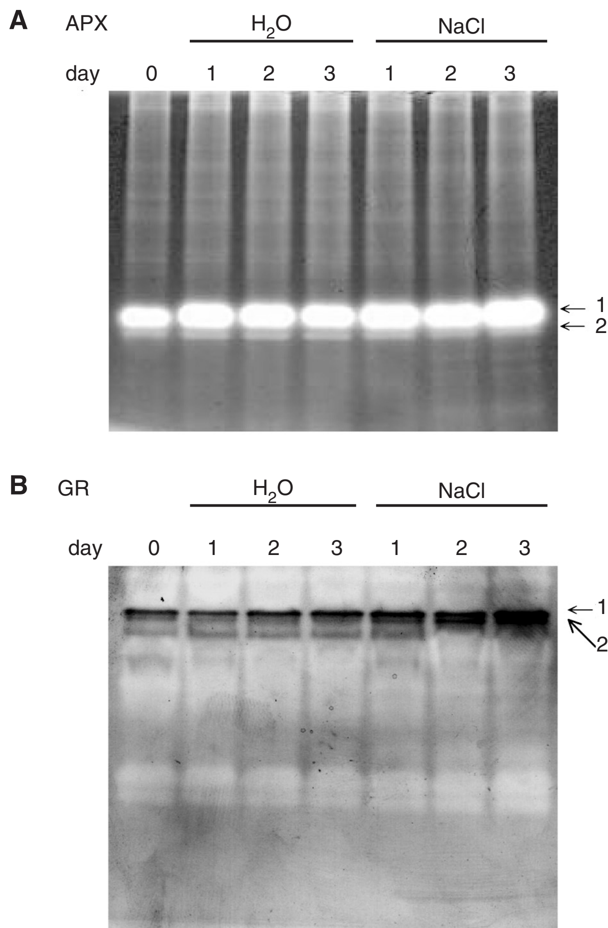

Fig. 3. Changes in the isoenzymes of APX (A) and GR (B) in roots of rice seedlings in the presence and absence of NaCl. Rice seeds were germinated in germinated in distilled water for 2 days and then were transferred to distilled water and NaCl (150 m*M*) for 1, 2 and 3 days, respectively. Arrows indicated different isoenzymes in rice roots.

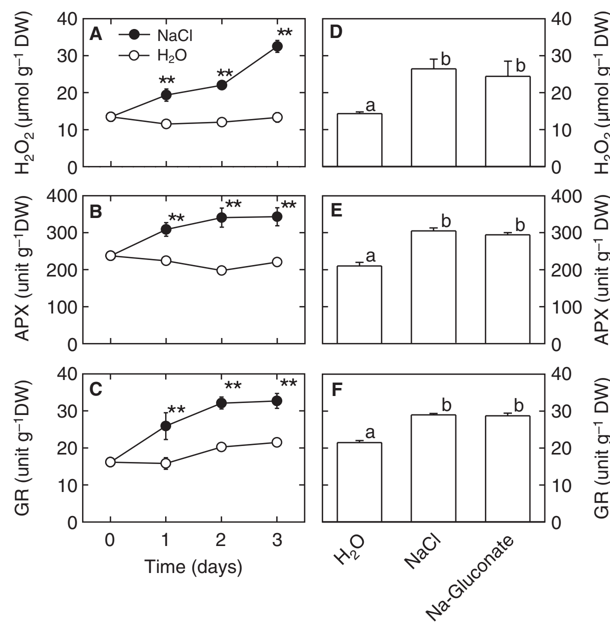

Fig. 4. Changes in the levels of H_2O_2 (A) and the activities of APX (B) and GR (C) in the roots of rice seedlings in the presence and absence of NaCl (150 m*M*) and the effect of NaCl (100 m*M*) and Na-gluconate (50 m*M*) on the levels of H_2O_2 (D) and the activities of APX (E) and GR (F) in the roots of rice seedlings. Rice seeds were germinated in distilled water for 2 days and then transferred to distilled water and NaCl (150 m*M*) for 1, 2 and 3 days, respectively (A–C) or transferred to NaCl (100 m*M*) and Na-gluconate (50 m*M*) for 3 days (D–F). Bars represent standard errors ($n = 4$). ** represents values that are significant between H_2O and NaCl treatments at $P < 0.01$. Values with the same letter are not significantly different at $P < 0.05$.

(Hernández et al. 1999, 2000, Shalata et al. 2001). This study shows that both AsA and DHA levels increased in NaCl stress in rice roots (Fig. 5A and B). It appears that the increase in AsA levels in rice roots treated with NaCl depends on the rates of its synthesis as well as on the rates of its regeneration. In addition to APX activity, ascorbate oxidase catalyses the oxidation of AsA to DHA. The definitive role of ascorbate oxidase is not clear, although it has been suggested that the enzyme may participate in a redox system involving AsA (Weis 1975). It is mostly likely that NaCl caused an increase in ascorbate oxidase activity in rice roots which in turn resulted in an increase in DHA level (Fig. 5B). GSH is a major water-soluble antioxidant species. It has been shown that salinity induced glutathione synthesis in *Brassica napus* (Ruiz and Blumwald 2002). The increase in GR activity (Fig. 4C) and in GSH contents (Fig. 5C) observed in NaCl-treated rice roots suggests that GSH contents may be regulated by its synthesis and regeneration. GSH can, directly or by means of glutathione peroxidase, react with AOS and lipid peroxides to GSSG (Noctor et al. 1998). Glutathione peroxidase is induced in plants in response to stress (Eshdat et al. 1997). It is probable that the increase

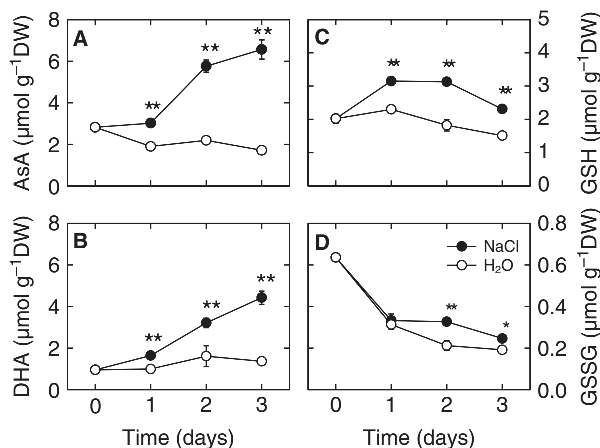

Fig. 5. Changes in the levels of AsA (A), DHA (B), GSH (C) and GSSG (D) in the roots of rice seedlings in the presence and absence of NaCl (150 m*M*). Rice seeds were germinated in distilled water for 2 days and then transferred in distilled water and NaCl (150 m*M*) for 1, 2 and 3 days, respectively. Bars represent standard errors ($n = 4$). * and ** represent values that are significant between H_2O and NaCl treatments at $P < 0.05$ and $P < 0.01$, respectively.

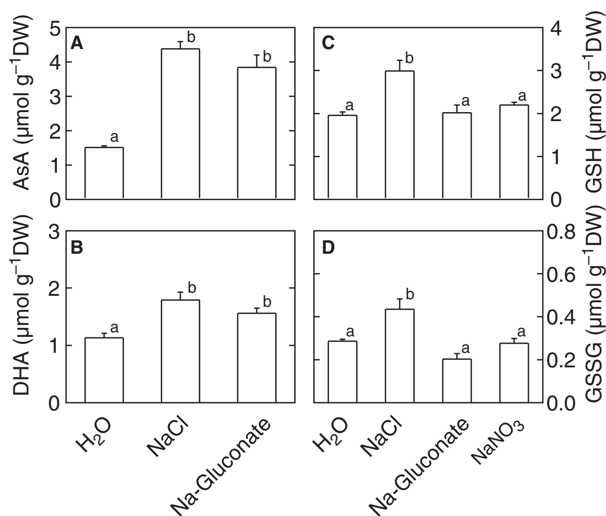

Fig. 6. The effect of NaCl (100 m*M*) and Na-gluconate (50 m*M*) or $NaNO_3$ (100 m*M*) on the levels of AsA (A), DHA (B), GSH (C) and GSSG (D) in the roots of rice seedlings. Rice seeds were germinated in distilled water for 2 days and then transferred to NaCl (100 m*M*), Na-gluconate (50 m*M*), and $NaNO_3$ (100 m*M*) for 3 days. Values with the same letter are not significantly different at $P < 0.05$.

in GSSG level in NaCl-treated rice roots (Fig. 5D) is mediated through GSH oxidation. Further research on the effect of NaCl on ascorbate oxidase and glutathione peroxidase is likely to be highly rewarding.

Environmental stresses are known to cause oxidative stress within plant cells. The accumulation of H_2O_2 has been observed in response to chilling (Okuda et al. 1991, Prasad et al. 1994, Fadzilla et al. 1996, O'Kane et al. 1996), heat (Dat et al. 1998, Gong et al. 2001), UV radiation (Murphy and Huerta 1990), excess light (Karpinski et al. 1997), and anoxic stress (Blokhina et al. 2001). The accumulation of H_2O_2 has been observed in response to NaCl in *Lycopersicon esculentum* (Mittova et al. 2004), *Pisum sativum* (Hernández et al. 1993, 2001), *Morus alba* (Sudhaker et al. 2001), and *Lens culinaris* (Bandeoğlu et al. 2004). Lee et al. (2001) showed that NaCl treatment resulted in an accumulation of H_2O_2 in the leaves but not in the roots of rice plants. Here, we show that H_2O_2 levels increase in NaCl-treated roots of rice seedlings (Fig. 4A and D). NaCl-induced accumulation of H_2O_2 in rice leaves has been suggested to be due to NaCl-enhanced SOD and NaCl-deactivated CAT activities (Lee et al. 2001). This does not seem to be the case in the roots of rice seedlings, because NaCl had no effect on SOD and CAT activities (Figs 1A and D, 2A and B). In several model systems investigated in plants, the accumulation of H_2O_2 appears to be mediated by the activation of a plasma-membrane-bound NADPH oxidase complex (Orozco-Cardenas and Ryan 1999, Pei et al. 2000, Zhang et al. 2001, Jiang and Zhang 2002). Diphenyleneiodonium chloride (DPI) and imidzaole are known to inhibit plasma-membrane NADPH oxidase (Cross 1990, Orozco-Cardenas and Ryan 1999, Pei et al. 2000, Jiang and Zhang 2002). We have preliminary data indicating that DPI (0.1 μM) and imidazole (5 m*M*) can prevent the increased production of H_2O_2 induced by NaCl (Tsai and Kao, unpublished observation). It appears that NaCl-induced accumulation of H_2O_2 is mediated through the activation of NADPH oxidase in rice roots. NADPH oxidase does not appear to be the only source of H_2O_2 generation in rice roots, because NaCl-induced cell wall-bound NADH peroxidase and diamine oxidase activities, which are devoted to H_2O_2 generation, have been detected in the roots of rice seedlings (Lin and Kao 2001a).

In previous work, we have shown that increasing concentrations of NaCl from 50 to 150 m*M* progressively increased both Na^+ and Cl^- levels in roots of rice seedlings (Lin and Kao 2001b). Of particular interest in the present investigation are the findings that Cl^- is not involved in the NaCl-induced H_2O_2 (Fig. 4D), AsA and DHA contents (Fig. 6A and B), as well as the NaCl-induced in APX and GR activities (Fig. 4E and F), and the NaCl-enhanced expression of *OsAPX* and *OsGR* (Fig. 7A and B), and Cl^- rather than Na^+ is required for the NaCl-induced GSH and GSSG contents (Fig. 6C and D). How the Cl^- contributes to the GSH and GSSG production remains to be investigated. When growing in saline soils, roots also encounter osmotic stress resulting from salt concentration in the soil that results in lowered water potential and consequent loss of cell turgor in roots. Furthermore, it has been shown that ABA accumulates in plants under salt stress (Montero et al. 1997, 1998). Thus, it is of great interest to know the relative importance of osmotic stress and endogenous ABA in NaCl-induced antioxidant systems in the roots of rice seedlings. Work in this direction is currently in progress.

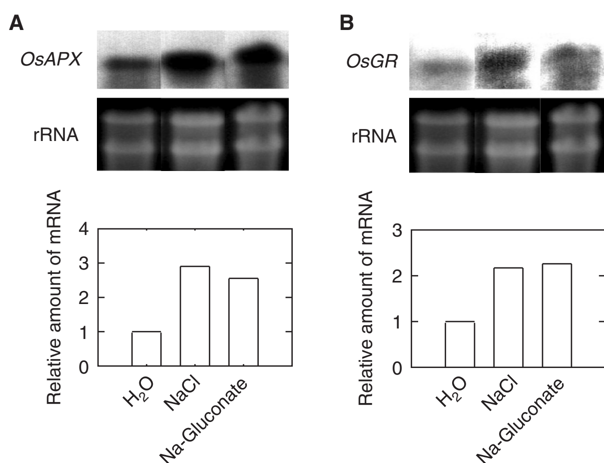

Fig. 7. Effect of NaCl and Na-gluconate on the mRNA abundance of *OsAPX* (A) and *OsGR* (B) in the roots of rice seedlings. Rice seeds were germinated in distilled water for 2 days and then transferred to NaCl (100 m*M*) and Na-Glucaonte (50 m*M*) for 3 days, respectively. Northern blots of total RNA samples isolated from root samples were hybridized with *OsAPX* (A) and *OsGR* (B). The values of mRNA of *OsAPX* and *OsGR* were adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the reaction with the roots in water was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsAPX* and *OsGR*.

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