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Effects of NaCl Stress on Germination, Antioxidant Responses, and Proline Content in Two Rice Cultivars

Young Geol Sohn[1], Byung Hyun Lee[2], Kyu Young Kang[2], and Jeung Joo Lee[3]*

[1]Namhae Substation, NHRI, RDA, 777 Dajeong, Idong, Namhae 668-812, Korea
[2]Division of Applied Life Sciences (BK21 program), Gyeongsang National University, Chinju 660-701, Korea
[3]Division of Plant Resources and Environment, Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, Korea

We investigated the physiological and biochemical bases for salt tolerance in two rice (*Oryza sativa* L.) cultivars -- relatively salt-tolerant 'Dongjin' and salt-sensitive 'Kumnam'. Salinized hydroponic cultures were studied at the germination and seedling stages. NaCl inhibited germination more severely in 'Kumnam' than in 'Dongjin'. Increasing the salt concentration also deterred growth to a larger extent in the former. Moreover, the leaves of 'Kumnam' exhibited greater increases in lipid peroxidation and Na^+ accumulation than those of 'Dongjin' under stress. The activities of constitutive and salt-induced superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (AP, EC 1.11.1.11) were also higher in 'Kumnam', while only catalase (CAT, EC 1.11.1.6) activity was slightly higher in stressed plants of 'Dongjin'. The positive correlation between leaf proline levels and NaCl concentration was more evident in 'Kumnam'. However, 'Dongjin' seeds, which had higher germinability in the presence of NaCl, also contained more proline. These results suggest that the higher salt tolerance in 'Dongjin' seedlings could be ascribed to their lower NaCl accumulations in the leaves. This presumably is due to reductions in the uptake or transport rates of saline ions to the shoots from the roots. Finally, we believe that the higher germination rate by 'Dongjin' is caused by its higher seed proline content.

Keywords: antioxidants, germination, proline, rice cultivars, salt stress

NaCl is the most important constituent of a saline environment. Salinity stress retards plant growth (Qadar, 1995; Lutts et al., 1996; Alan, 1999) by influencing several vital facets of plant metabolism, such as transpiration, CO_2 assimilation in light, respiration, cell growth and division, hormonal balance, nitrogen metabolism, enzyme level, water availability, ion uptake (Hsiao, 1973), and osmotic adjustment (Crammer et al., 1990; Delauney and Verma, 1993; McNeil et al., 1999). Salinity also induces numerous disorders in seeds and propagules during germination (de Villiers et al., 1994; Khan and Ungar, 1997; Cuartero and Fernández-Muñoz, 1999). The negative effects of these various environmental stresses is assumed to be at least partially due to the generation of active oxygen species (AOS) and/or the inhibition of the system that defends against them (Elstner, 1982; Smirnoff, 1993; Bor et al., 2003). AOS include superoxide anion ($\dot{O}_2^-$), the hydroxyl radical (• OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). The increase in AOS, which are also produced during normal aerobic metabolism, seems to occur in response to all abiotic stresses, including chilling (Lee et al., 2004), heavy metal toxicity (Ali et al., 2002), drought (Smirnoff, 1993), and salt (Hernandez et al., 1994; Gossett et al., 1996). Failure to quench or inactivate the AOS may lead to the degradation of macromolecules in cells as membrane lipids, proteins, and DNA (Elstner, 1982; Smirnoff, 1993).

Plants possess a battery of antioxidative mechanisms to detoxify and eliminate these AOS. The defense systems include hydrophilic (ascorbic acid, glutathione), hydrophobic (α-tocopherol, carotenoids) antioxidants, and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (AP, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), and glutathione reductase (EC 1.6.4.2) (Shalata and Tal, 1998). Resistance to environmental stresses is usually correlated with a more efficient antioxidative system (Cakmak and Marschner, 1992; Smirnoff, 1993; Olmes et al., 1994).

Osmoprotectants raise osmotic pressure in the cytoplasm, and play important adaptive roles in stabilizing proteins and membranes when salt levels or tempera-

*Corresponding author; fax +82-55-758-5110
e-mail jjl@gshp.gsnu.ac.kr

tures are unfavorable (McNeil et al., 1999). The three types of osmoprotectant are: 1) betaines and allied compounds, 2) polyols and sugars, and 3) amino acids such as proline. A widespread phenomenon, proline accumulation in plants exposed to salt or water stress has been correlated in many species with their adaptation to osmotic stress (Stewart and Lee, 1974; Blum and Ebercon, 1976; Delauney and Verma, 1993; Rhodes et al., 1999). However, its role in imparting resistance to salt stress is controversial, with some researchers suggesting that it is a symptom of stress injury rather than an indicator of stress tolerance (Hanson et al., 1977; Moftah and Michel, 1987; Liu and Jhu, 1997). Therefore, the role of proline accumulation in salt stress must be more precisely investigated. Rice is a moderately salt-tolerant crop, with varieties that exhibit a range of sensitivities to salinity (Dubey and Rani, 1989). This current study was designed to determine, aside from their growth, the differences between two cultivars in coping with the effects of salt stress on their germination rates, lipid membrane peroxidation, Na^+ contents, antioxidant enzyme activities, and proline accumulation.

MATERIALS AND METHODS

Plant Materials and NaCl Stress Treatments

Seeds of rice (*Oryza sativa* L.) cultivars 'Dongjin' and 'Kumnam' were sterilized with 0.05% prochloraz emulsifiable concentrate for 24 h and rinsed thoroughly with tap water to remove the pesticide. To evaluate the inhibition of germination by NaCl stress, 100 seeds each were spread on Whatman No. 2 filter paper in 9-cm-diam Petri dishes. To these, either 5 ml of distilled water or NaCl solution was added. The seeds were germinated for 6 d in the dark at 30°C. After 2, 3, and 5 d, 5 ml of distilled water (control) or an NaCl solution (45, 90, 180, or 270 mM) was added to the dishes. The number of germinated seeds was counted each day for 6 d.

To evaluate how NaCl stress inhibits seedling growth, seeds were germinated on metal mesh in a plastic container moistened with distilled water. They were then reared hydroponically in a controlled growth chamber (16-h photoperiod at 30/22°C day/night and 70% RH) with a light intensity of 250 mE $m^{-2}s^{-1}$ from fluorescent and metal halide lamps. The nutrient solution, slightly modified from that of Cakmak and Marschner (1992), contained 0.88 mM K_2SO_4, 1 mM $Ca(NO_3)_2$, 1 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.1 mM KCl, 40 μM FeEDTA, 10 μM H_3BO_4, 1 μM $MnSO_4$, 1 μM ZnSO4, 0.1 μM $CuSO_4$, and 0.01 μM $(NH_4)_6MoO_{24}$. This solution was adjusted to pH 5.8 with 1 N HCl, and was exchanged every 2 d. After 15 d, the plants were salinized with a nutrient solution containing 0, 45, or 90 mM NaCl. Leaves and roots were sampled at 0, 3, and 6 d after treatment with the salt. To analyze for lipid peroxidation, antioxidant enzyme activities, and proline accumulation, leaf samples were harvested, weighed, and stored at -70°C. All experiments were independently repeated at least three times.

Growth Measurements

Growth ratios were calculated for randomly selected salt-treated rice seedlings. After blotting, fresh weights and shoot lengths were determined by measuring their increments from beginning to end of the NaCl treatment.

Na^+ Determination

Leaf samples were oven-dried at 90°C for 48 h, then ground with a glass stick in test tubes. Na^+ was extracted by shaking in 1 N HCl for 24 h and filtering with Whatman No. 2 paper. Sodium concentrations in properly diluted extracts were determined via inductively coupled plasma spectrophotometry.

Lipid Peroxidation

Lipid peroxidation was defined by the content of malondialdehyde (MDA), according to the method of Du and Bramlage (1992). A 0.5-g sample of frozen leaves was ground to a fine power with liquid nitrogen and extracted with 5 ml of cold ethanol. The crude extract preparation was centrifuged at 12,000g for 20 min. A mixture of trichloroacetic acid, thiobarbituric acid, butylated hydroxytoluene, and an aliquot of supernatant was heated and the reaction was stopped by quickly placing the mixture in an ice-bath. The cooled mixture was centrifuged, and the absorbance of the supernatant was measured at 400, 500, and 600 nm. Thiobarbituric acid-reactive substances (TBARS) measured as MDA, a degraded product of the lipid. The concentration of was MDA determined obtained from the absorbance by using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Antioxidative Enzyme Assays

A 0.5-gram sample of frozen leaves was ground to a fine powder with liquid nitrogen and extracted with ice-cold 50 mM potassium phosphate buffer (pH 7.0) that contained 5 mM ethylenediaminetetraacetic acid, 0.2 mM ascorbic acid, and 10% insoluble polyvinylpolypyrrolidone (w/v). The homegenate was centrifuged at 12,000g for 20 min at 4°C and the supernatant was used for determinations of enzyme activities (Kenyon and Duke, 1985). All activities were measured at 25°C in a final volume of 1 ml, using aliquots of the supernatants. Activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured by the method described by McCord and Fridovich (1969). The assay mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM xanthine monosodium salt, 10 mM cytochrome C, an aliquot of xanthine oxidase (EC 1.2.3.2) that causes a 0.025 increment in absorbance at 550 nm within 60 s, and an aliquot of crude enzyme. One unit of SOD activity was defined as the amount of enzyme required to reduce 50% of the absorbance that was increased by xanthine oxidase. Activity of ascorbate peroxidase (AP, EC 1.11.1.11) was measured by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate, and an aliquot of crude enzyme (Nakano and Asada, 1981). Activity of catalase (CAT, EC 1.11.1.6) was assayed, according to the method of Blume and McClure (1980), by monitoring the decrease in absorbance at 240 nm due to H_2O_2 oxidation ($E=0.04\ mM^{-1}cm^{-1}$). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 5 mM H_2O_2, and an aliquot of crude enzyme.

Proline Content

Proline content was determined according to the method of Bates et al. (1973). First, 100 seeds each of 'Dongjin' and 'Kumnam' were left uncoated after being dried for 4 weeks at ambient temperature. They were imbibed with 15 ml of 3% aqueous sulfosalicylic acid at 4°C for 24 h, then ground with sea sand. Second, to assess the proline content in the leaves, 0.5 g of frozen tissue was ground with liquid nitrogen and homogenized in 10 ml of 3% sulfosalicylic acid. Homogenates of the seeds and leaves were centrifuged at 12,000g for 20 min at 4°C. After the supernatant was added to acetic acid and ninhydrin, then boiled for 1 h, its absorbance was read at 520 nm to determine the level of proline.

RESULTS

Inhibition of Germination by NaCl

Without exposure to NaCl stress, germination for 'Dongjin' rice seed was faster than for 'Kumnam' (Fig. 1), but rates for both decreased with rising levels of salinity. This inhibitory effect was more pronounced in 'Kumnam' than in 'Dongjin'. For example, after 6 d of salt stress, germination was still >83% for 'Dongjin' in the presence of 270 mM NaCl, but, for 'Kumnam', was only about 52% and 11% in 180 and 270 mM NaCl, respectively.

Growth Inhibition by NaCl

As salt concentrations increased, gains in fresh

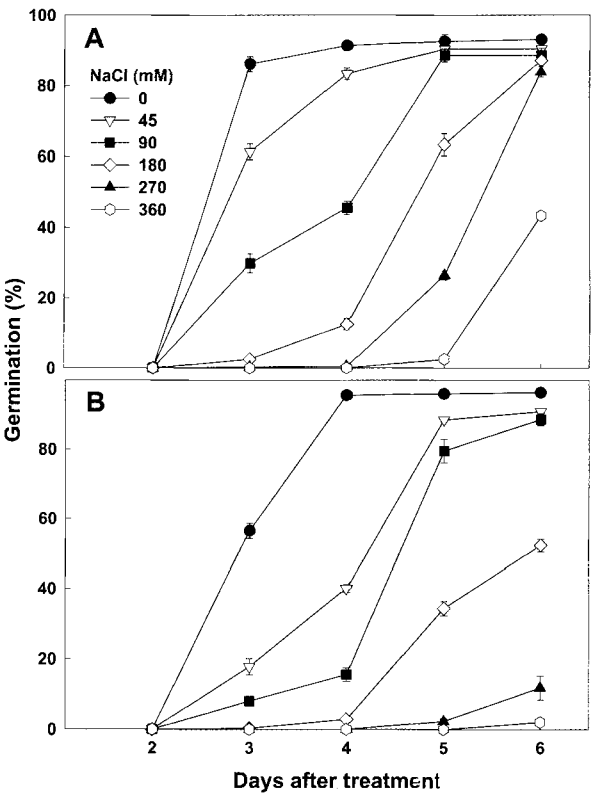

Figure 1. Effects of salinity on cumulative germination of 'Dongjin' **(A)** and 'Kumnam' **(B)** rice cultivars. One hundred seeds each were germinated at 30°C for 6 d in dark incubator. Data represent means ± S.E. of four replicates.

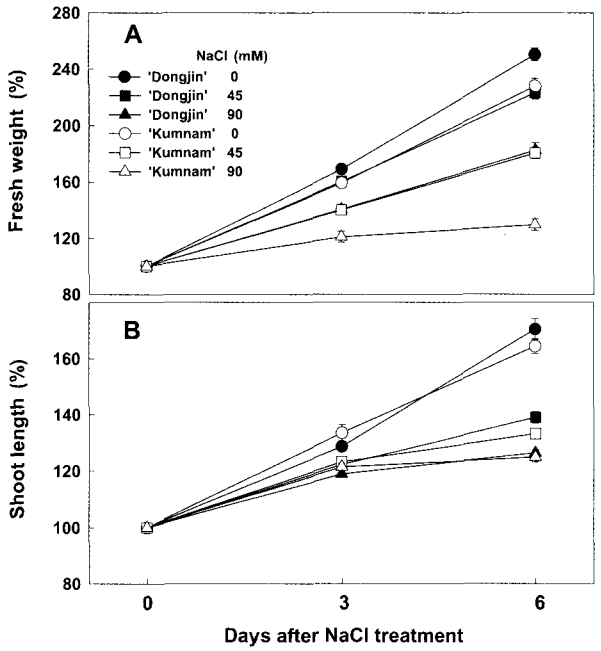

Figure 2. Effects of salinity on fresh weights **(A)** and shoot lengths **(B)** of 'Dongjin' and 'Kumnam' rice. Salt stress was induced by adding NaCl to culture medium of 15-d-old seedlings. Weights and lengths were measured before and after 3 or 6 d of treatment. Data represent means ± S.E. of eight replicates from two independent experiments.

weight declined for both rice cultivars, but, compared with the controls, growth of 'Dongjin' plants was less inhibited than 'Kumnam' under such stress (Fig. 2), indicating that salt tolerance was greater with 'Dongjin'. Shoot lengths were adversely affected by NaCl for both cultivars, although not to the same extent as the reduction seen in their fresh weights.

Lipid Peroxidation

Membrane lipid peroxidation was estimated in leaves after 6 d of NaCl treatment (Fig. 3), with MDA content being used as its index. Whereas at 45 mM NaCl, MDA levels were slightly but equally elevated in both cultivars, at a concentration of 90 mM NaCl, peroxidation increased by 156% in 'Dongjin' and 240% in 'Kumnam' compared with the control plants.

Na^+ Concentration

Over time, rates of Na^+ accumulation were higher in 'Kumnam' than in 'Dongjin' during salinity stress (Fig. 4). After 3 d of treatment, the sodium content in 'Kumnam' was 279% and 137% higher than in 'Dongjin' at 45 and 90 mM NaCl, respectively. After 6 d, its accumulation in 'Kumnam' was 223% higher than

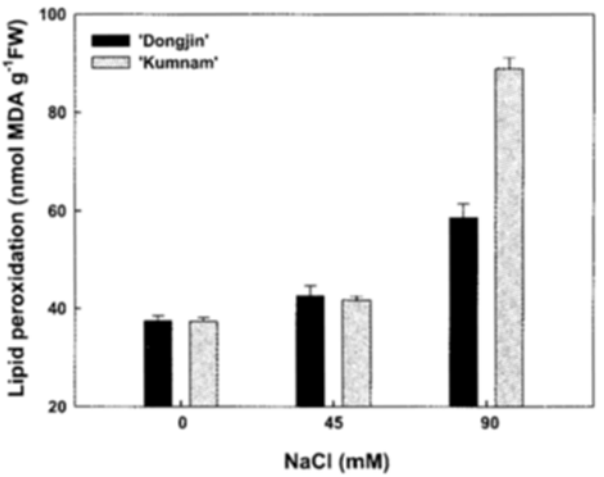

Figure 3. Effects of salinity on lipid peroxidation in leaves of 'Dongjin' and 'Kumnam' rice seedlings. Malondialdehyde (MDA) content was measured after 6 d of NaCl treatment. Data represent means ± S.E. of four replicates.

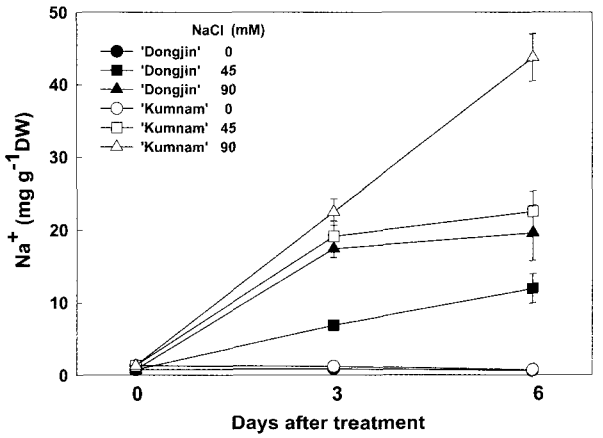

Figure 4. Effects of salinity on Na^+ accumulation in leaves of 'Dongjin' and 'Kumnam' rice seedlings. Na^+ content was measured before and after 3 or 6 d of NaCl treatment. Data represent means ± S.E. of four replicates.

in 'Dongjin' at 90 mM NaCl, indicating that 'Dongjin' absorbed less Na^+.

Antioxidant Enzyme Activities

The constitutive activity of SOD was higher in 'Kumnam' than in 'Dongjin' (Fig. 5A). When the two cultivars were treated at 45 and 90 mM NaCl, SOD activity of 'Dongjin' increased 17% above the level of the controls, but was almost unchanged in 'Kumnam'. However, at 90 mM, activity was 20% higher in 'Kumnam' than in 'Dongjin'. Likewise, constitutive activity of AP was higher in 'Kumnam' when plants were grown without NaCl stress (Fig. 5B). In

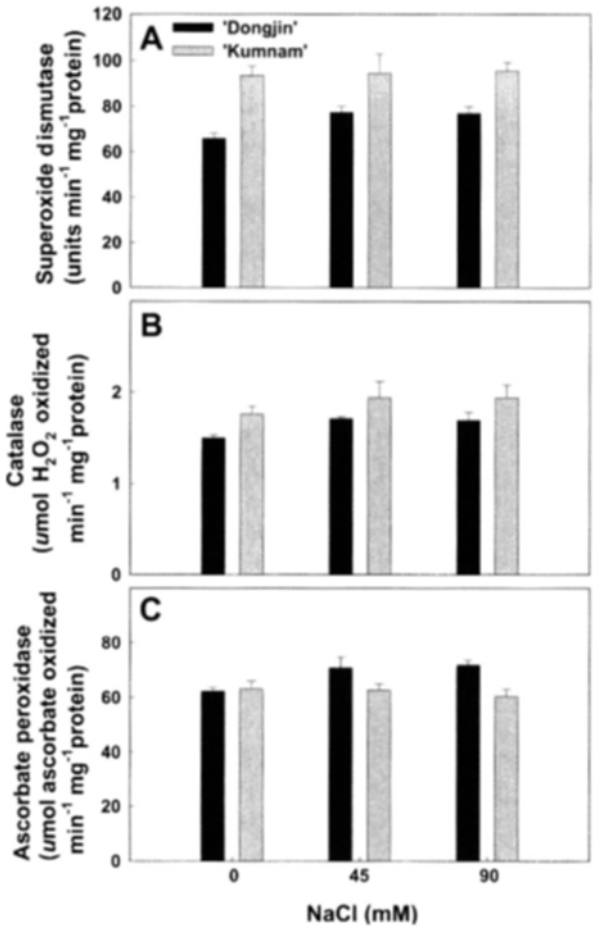

Figure 5. Effects of salinity on specific activities of superoxide dismutase **(A)**, ascorbate peroxidase **(B)**, and catalase **(C)** in leaves of 'Dongjin' and 'Kumnam' rice seedlings. Enzyme activities were measured after 6 d of NaCl treatment. Data represent means ± S.E. of four replicates.

Table 1. Proline content of 100 seeds each from two rice cultivars, 'Dongjin' and 'Kumnam'. Data represent means ± S.E. of eight determinations obtained from two independent extractions.

Cultivars	Proline (μmol g^{-1} seed)
Dongjin	1.62 ± 0.05
Kumnam	0.63 ± 0.05

'Dongjin' and 'Kumnam', AP activity increased to 14% and 10%, respectively, above the control after treatment with 45 mM NaCl, but not with 90 mM NaCl. Although the constitutive activity of CAT was nearly the same for both cultivars (Fig. 5C), when grown at 45 and 90 mM NaCl, its level was 13% and 15% higher, respectively, in 'Dongjin' seedlings than in the controls. However, no NaCl-induced rise in CAT activity was detected in 'Kumnam' plants.

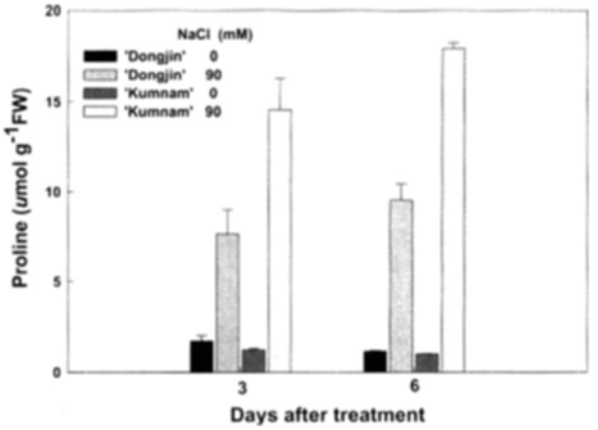

Figure 6. Effects of salinity on proline accumulation in leaves of 'Dongjin' and 'Kumnam' rice seedlings. Proline was measured after 3 or 6 d of NaCl treatment. Data represent means ± S.E. of four replicates.

Proline Accumulation

For 'Dongjin', proline content was 257% higher than that calculated for seeds of 'Kumnam' (Table 1). Proline accumulations in leaf tissues were slightly higher in 'Dongjin' than in 'Kumnam', and were greatly increased by NaCl stress, although with markedly different patterns (Fig. 6). For example, after 3 and 6 d of salt treatment, proline content in 'Dongjin' leaves was 443% and 821% higher, respectively, than in the control, but was 1174% and 1774% more in 'Kumnam', respectively, than in the controls.

DISCUSSION

Mohammed and Sen (1990) have suggested that germination is inhibited in saline media because of osmotic stress or specific ion toxicity. This response is reversed when seeds are transferred from salt solution to distilled water. In this study, germination was better for 'Dongjin' than for 'Kumnam' during the period of salinity treatment (Fig. 1), which suggests that the former is more tolerant of NaCl-induced stress. It is generally assumed that a plant's resistance to salinity rests with its ability to restrict or prevent the entry of Na^+ from roots to the leaves. Commonly, higher sodium content disrupts the nutrient balance and osmotic regulation, thereby causing specific ion toxicity (Alan, 1999). In this study, the salt-sensitive 'Kumnam' (Fig. 2) showed a more pronounced accu-

mulation of Na^+ in its leaves than did the salt-tolerant 'Dongjin' at high NaCl concentrations (Fig. 4). These results are in accord with those of other studies that have shown an inverse relationship between Na^+ concentration and salt tolerance (Yeo and Flowers, 1983).

Treatment with NaCl also increases lipid peroxidation or induces oxidative stress in plant tissues (Hernandez et al., 1994; Olmes et al., 1994; Gossett et al., 1996). Here, we monitored the extent of peroxidative damage by measuring the amount of MDA produced when polyunsaturated fatty acids in the membrane underwent peroxidation. Although the same level of MDA was measured in 'Dongjin' and 'Kumnam' without salt treatment, its content rose with NaCl stress (Fig. 3). Unlike for 'Dongjin', MDA increased noticeably in 'Kumnam' when exposed to 90 mM NaCl, perhaps because of reduced activities by antioxidant enzymes. Bor et al. (2003) have suggested that the wild salt-tolerant beet, *Beta maritima*, exhibits a better protective mechanism against oxidative damage by maintaining higher constitutive and salt-induced activities of antioxidant enzymes, such as SOD, AP, and CAT, than does the relatively sensitive sugar beet, *Beta vulgaris*. In addition, Scandalios (1993) has reported that SOD and CAT are the most effective antioxidant enzymes in preventing cellular damage. The reduction of lipid peroxidation caused by elevated activities of antioxidant enzymes during salinity stress has also been reported from a wild tomato species (Shalata and Tal, 1998). Our results showed that the constitutive activity of SOD and AP were lower in 'Dongjin' than in 'Kumnam' (Fig. 5A and B), although both increased proportionately with higher NaCl concentrations. However, CAT constitutive activity was similar in both cultivars (Fig. 5C), and was slightly increased only in 'Dongjin' after salt treatment. Therefore, based on our analyses of their activities, we believe that these antioxidant enzymes (SOD, AP, and CAT) had little effect on reducing the damage caused by salinity stress in our two rice cultivars.

In response to drought and salinity stresses, many plant species accumulate high levels of proline, which is thought to function in stress adaptation (Stewart and Lee, 1974; Smirnoff and Cumbes, 1989; McCue and Hanson, 1990; Delauney and Verma, 1993; Shalata and Tal, 1998). However, the lack of correlation between proline level and salt tolerance in certain species has also led to the conclusion that this accumulation is merely a symptom of injury and does not enhance tolerance (Moftah and Michel, 1987; Liu and Jhu, 1997). This increase in free proline content is more pronounced in salt-susceptible than salt-resistant rice cultivars (Lutts et al., 1996; Oh et al., 2003). Here, we demonstrated that proline accumulations in both cultivars were closely associated with the NaCl concentration (Fig. 6), being significantly higher in 'Kumnam' than in 'Dongjin' after 3 and 6 d of salinity stress. This experimental evidence showed that their capacity to accumulate proline under NaCl stress was not an indicator of salt tolerance nor a protective value, but merely a consequence or a symptom of the stress.

In contrast, the content of constitutive proline in seeds was higher in 'Dongjin' than in 'Kumnam', the former also having a higher germination rate under salinity stress (Fig. 1). Poljakoff-Mayber et al. (1994) have also reported that NaCl stress causes free proline to accumulate in germinating seeds. Proline may, therefore, be beneficial to the germination process, first by reducing osmotic inhibition and second by providing substrates for the growth of embryonic tissues (Lin and Kao, 1996). Under increasing levels of salinity, germinating seeds of salt-tolerant rice cultivars contain higher levels of proline and other free amino acids than do salt-sensitive cultivars (Dubey and Rani, 1989). Furthermore, because NaCl stimulates proline accumulation in the germinating seeds of *Koteletzkya virginica*, that amino acid may be considered a compatible solute (Poljakoff-Mayber et al., 1994). Thus, if seeds already possess a high level of proline, their germinability may be improved by it under NaCl stress. This response was proven true in the current study as well, where the constitutive proline content in seeds was positively correlated with germination rates for salt-stressed rice.

In this study, we found substantial differences between the growth and germination responses of two rice cultivars. In the presence of NaCl, the salt-sensitive 'Kumnam', which had high Na^+ accumulations, showed a rapid increase in lipid peroxidation but a large decline in its growth rate. The salt-tolerant 'Dongjin', however, had lower Na^+ accumulations and lipid peroxidation, and its growth was not reduced to the extent found with 'Kumnam'. Our data for constitutive and salt-induced activities of SOD, AP, and CAT also suggest that those antioxidant enzymes did not play an important role in conferring salt tolerance in 'Dongjin'. Likewise, the significantly higher level of stress-induced proline accumulation in 'Kumnam' clearly demonstrated that the level of that amino acid in NaCl-treated plants was inversely correlated with their ability to withstand salinity stress.

Therefore, we conclude that leaf proline contents are not useful indicators of salt tolerance in rice. The higher degree of tolerance by 'Dongjin' at the seedling stage might be ascribed to lower accumulations of salt in the leaf, presumably a result of reducing the uptake or transport rates of saline ions to the shoot from the root. Finally, the higher germination rate of 'Dongjin' seeds could be attributed to their higher proline contents. Further research is necessary to investigate the osmoregulatory role of proline in seeds and in the vegetative portions of plants.

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