Pretreatment with Antioxidants Decreases the Effects of Salt Stress on Chloroplast Ultrastructure in Rice Leaf Segments (Oryza sativa L.)

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Abstract : We investigated the kinds of active oxygen species leading to the destruction of chloroplast ultrastructure in salt-stressed rice plants. After the seedlings were grown for 3 wks, leaf segments (5 mm square) were cut from the middle portion of the 5th leaves. Leaf segments were incubated in 200 mM NaCl under dark or light conditions for 24 hr. The chlorophyll content in the leaf segments drastically decreased in light between 12- and 24 hr in 200 mM NaCl, but, no reduction was observed in the dark. In electron microscopic studies, 200 mM NaCl caused swelling of thylakoids and destruction of thylakoid membranes in light. On the other hand, no ultrastructural changes were observed under dark condition. In one experiment, leaf segments were incubated in 200 mM NaCl for 24 hr in light after preincubation with antioxidants for 12 hr in light. Pretreatment with ascorbate and benzoate, which scavenge H2O2 and ·OH, respectively, effectively suppressed the reduction of chlorophyll content and the destruction of chloroplasts by NaCl in light. However, Tiron and DABCO, which scavenge O2· and 1O2, respectively, could not suppress the effects of salt stress in light. Fe-SOD activity was increased about eight time by salt stress (200 mM NaCl), but, catalase activity was reduced to 69% of the control and ascorbate peroxidase activity was not affected by NaCl. These results suggested that salt-induced injury in chloroplasts is dependent on light, and that H2O2 and ·OH are responsible for the deleterious effects of salt stress on chlorophyll content and chloroplast ultrastructure.

Key Words : Antioxidants, Chloroplasts, Oryza sativa L., Salt stress, Thylakoids, Ultrastructure.

Vast areas of land are unfit for agriculture because of low tolerance of crop plants to salinity. All over the world, about 20% of irrigated land has been suffering from secondary salinization and 50% of irrigation schemes are affected by salinity (Flowers et al., 1997). Rice (Oryza sativa L.) is a major crop and is sensitive to salt stress (Francois and Maas, 1994). Therefore, the barriers for increase of crop production could be overcome by reducing the effects of salt stress on rice plants.

The active oxygen species (AOS) such as singlet oxygen (1O2), superoxide anion (O2·), hydrogen peroxide (H2O2) and hydroxyl radical (·OH) are produced during normal aerobic metabolism by the interaction between O2 and electrons leaked from the electron transport chains in chloroplasts (Asada, 1999). These oxygen species are highly reactive and in the absence of any protective mechanism they lead to degradation of membrane lipids, proteins and nucleic acids (Fridovich, 1986). In order to prevent these oxidative stresses, chloroplasts have developed enzymatic and non-enzymatic systems for scavenging AOS (Asada, 1999). The non-enzymatic components scavenging AOS include carotenoids, ascorbic acid (AsA), glutathione and tocopherols. The antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.1). SOD is a major scavenger of O2· and converts O2· to H2O2 and O2. H2O2 is scavenged by CAT and a variety of peroxidases. Especially in chloroplasts, H2O2 is scavenged by APX using AsA as an electron donor as a part of the system for the protection of chloroplasts from oxidative damage (the ascorbate-glutathione cycle).

Stomatal closure is triggered by abscisic acid induced under salt stress (Fedina et al., 1994). Stomatal closure limits CO2 supply to the leaf, which leads to an overreduction of the photosynthetic electron transport chain (Osmond and Grace, 1995). The overreduction of electron transport chain induces generation of excess AOS (Asada, 1999). Although plants have protective mechanisms to reduce oxidative damage,
it is often observed that the balance between the production of AOS and the activity of AOS scavenging enzyme is upset under salt stress, leading to oxidative stress (Hernandez et al., 1995; 1999).

The effects of salinity in plants have been well investigated on important metabolic processes such as ion uptake and transport, respiration, photosynthesis and the detoxification processes of AOS (Marschner, 1995; Dionisio-Sese and Tobita, 1998; Jayasundara et al., 2000; Mitsuya et al., 2003). In some studies, salt stress induced changes in chloroplast ultrastructure such as the swelling of thylakoids and the destruction of thylakoid membranes (Hernandez et al., 1995; Rahman et al., 2000; Mitsuya et al., 2003). Yamane et al. (2003) showed that ion toxicity or ionic imbalance but not osmotic effects of salt stress induced the swelling of thylakoids and the destruction of thylakoid membranes. These ultrastructural changes have been reported as a typical phenomenon of oxidative stress (Hernandez et al., 1995). However, it is still unclear what kinds of AOS induce the ultrastructural changes in rice plants.

Because the salt-tolerant plants have an efficient antioxidative systems (Gossett et al., 1994; Olmos et al., 1994; Hernandez et al., 1995; Shalata and Moshe, 1998), it is necessary to identify the kinds of AOS leading to the injuries under salt stress to clarify the mechanisms of salt-induced injury and to reduce the adverse effects of salinity.

**Materials and Methods**

1. **Plant materials**

   Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were surface sterilized with a 5% sodium hypochlorite solution for 5 min. After washing several times with distilled water, seeds were imbibed in a beaker containing distilled water in a culture room kept at 24±2°C until the appearance of the white tip of the coleoptiles.

   After imbibition, the seeds were sown on hydroponic culture medium containing nutrient solution according to Mae and Ohira (1981) and grown in a growth chamber under 12 hr photoperiod at 400-500 µmol m⁻² s⁻¹ and 28/25°C (day/night) for 3 wks.

2. **Treatment of leaf segments**

   After the seedlings were grown for 3 wks, leaf segments (5 mm square) were cut from the middle portion of the 5th leaves, which were fully expanded uppermost leaves of the plants. About 10 leaf segments were cut per leaf. The leaf segments were incubated in 200 mM NaCl dissolved in 5 mM MES buffer (pH 6.0) or in the buffer without NaCl as control in the dark or light conditions (230-250 µmol m⁻² s⁻¹) for 24 hr.

   In one experiment, antioxidants dissolved in 5 mM MES buffer (pH 6.0) were supplied to leaf segments by floating the segments for 12 hr under light condition. Then, the leaf segments were incubated in 200 mM NaCl without antioxidants under light intensity of 230-250 µmol m⁻² s⁻¹ for 24 hr. The pH of these solutions was adjusted to 6.0 with NaOH or HCl.

3. **Antioxidants**

   Tiron (1,2-dihydroxybenzene-3,5-disulfonate), a scavenger of O₂⁻ (Sakaki et al., 1983), sodium L-ascorbate (AsA), a scavenger of H₂O₂ and other active oxygen species (Noctor and Foyer, 1998), DABCO (1,4-diazabicyclo-[2,2,2]-octane), a scavenger of O₂¹⁻ (Rout and Shaw, 2001) and benzoate, a scavenger of ‘OH (Luna et al., 1994), were used as antioxidants.

4. **Measurement of chlorophyll content**

   The content of chlorophyll in the incubated rice leaf segments was determined by extracting the segments in 100% ethanol. Absorbances of the chlorophyll extract at 665 nm and 649 nm were measured spectrophotometrically and these readings were converted to chlorophyll content of the leaf using the equation of Knudsen et al. (1977).

5. **Enzyme extraction and assays**

   All operations were carried out at 0-4°C. For the determination of SOD activity, 60 leaf segments were frozen with liquid nitrogen and homogenized with 50 mM HEPES buffer (pH 7.6) and 0.1 mM Na₂EDTA. The homogenate was centrifuged at 15000 g for 15 min at 4°C to obtain crude extract. The crude extract was dialyzed against 5 mM HEPES buffer to remove low-molecular-weight compounds interfering with SOD activity determination.

   SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) (Beyer and Fridovich, 1987). For determination of total SOD activity a 3 mL reaction mixture containing 50 mM HEPES (pH 7.6), 0.1 mM Na₂EDTA, 50 mM Na₂CO₃ (pH 10.4), 13 mM methionine, 0.025% (w/v) Triton X-100, 75µM NBT, 2 µM riboflavin and an appropriate aliquot of enzyme extract was used. The reaction mixtures were irradiated for 10 min at light intensity of 40 µmol m⁻² s⁻¹. One unit SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. Activities of different forms of SOD were identified by using 3 mM KCN and 5 mM H₂O₂ as the final concentration in the reaction mixture (Yu and Rengel, 1999). Identical reaction mixtures with extracts that had not been illuminated were used to estimate background absorbance.

   For the determination of APX and CAT activities, 20 leaf segments were frozen in liquid nitrogen and homogenized with 50 mM potassium nitrate.
phosphate buffer (pH 7.8), containing 1 mM EDTA, 7 mM mercaptoethanol, 0.1% (w/v) Triton X-100, 5 mM sodium ascorbate and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15000 g for 10 min and the supernatant was used for assays. The activities of APX and CAT were measured following Nakano and Asada (1981) and Aebi (1974), respectively. APX activity was determined by measuring the oxidation of ascorbate at 300 nm. CAT activity was assayed by monitoring the consumption of H$_2$O$_2$ at 240 nm.

Protein in the supernatant was quantified by the Coomassie brilliant blue-dye binding method using bovine serum albumin as a standard according to Bradford (1976).

6. **Electron microscopy**

Electron microscopic studies were made using 10 leaf segments treated with NaCl as mentioned above. Small pieces of leaf segments were fixed in 5% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) and post fixed in 2% osmium tetroxide in the same buffer. Samples were dehydrated in a series of graded acetone and propylene oxide and embedded in Spurr’s resin.

To compare leaf segments with corresponding leaves of intact plants treated with NaCl, plants grown for 3 wks and treated with 200 mM NaCl in hydroponic culture for 24 hr were examined as above.

Ultrathin sections (70-90 nm in thickness) were cut with a diamond knife and placed on 150 mesh copper grid. The sections were stained with 2% uranyl acetate for 25 min followed by lead citrate for 5 min. Then the sections were examined on a Hitachi H600 transmission electron microscope at 100 kV. Photographs were taken at three or more random sites in at least 2 to 4 leaf segments and representative pictures were presented.

**Results**

1. **Changes in chlorophyll content and ultrastructure**

Fig. 1 shows the changes of chlorophyll content in leaf segments during the incubation in 200 mM NaCl under dark or light conditions. Chlorophyll content in the leaf segments incubated 200 mM NaCl was not significantly different from that in control until 12 hr. However, the chlorophyll content in the leaf segments decreased drastically between 12 and 24 hr incubation in 200 mM NaCl under the light condition.

On the other hand, chlorophyll content did not change for 24 hr incubation in 200 mM NaCl under dark condition.

Fig. 2A shows a typical chloroplast in a mesophyll cell of a control plant before preparing leaf segments. Fig. 2B shows a chloroplast in a mesophyll cell of a leaf segment after 24 hr incubation in 5 mM MES buffer. Both chloroplasts possessed well-developed grana and stroma thylakoids and no ultrastructural distortion was observed, suggesting that there were no ultrastructural damages due to incubation of leaf segments in 5 mM MES buffer for 24 hr.

NaCl induced a marked changes in chloroplast ultrastructure. Fig. 3A shows a chloroplast of a plant treated with 200 mM NaCl in hydroponic culture for 24 hr. Fig. 3B shows a chloroplast in a leaf segment treated with 200 mM NaCl for 24 hr. In both chloroplasts, the most notable ultrastructural changes were swelling of thylakoids (arrows) and destruction of thylakoid membranes. Similar ultrastructural changes were observed in the chloroplasts of both plants and leaf segments treated with NaCl, indicating that the treatment of leaf segments with NaCl serves as a model system to investigate NaCl effects on chloroplasts in intact plants.

2. **Changes in antioxidant enzyme activities**

Figs. 4A-D show the effects of salt stress on SOD activities. Salt stress induced a significant increase of Fe-SOD activity. After a 24 hr incubation in 200 mM NaCl, Fe-SOD activity was about 8-fold higher than the control (Fig. 4D). Mn-SOD activity increased slightly after a 12 hr incubation, but, CuZn-SOD was not influenced by NaCl treatment (Figs. 4B, C).

APX and CAT activities, both key enzymes for scavenging H$_2$O$_2$, were monitored during the incubation in 200 mM NaCl for 24 hr (Figs. 5A-B). CAT activity severely decreased during incubation in 200 mM NaCl for 24 hr. After 24 hr incubation, CAT activity was reduced to 69% of the control (Fig. 5B). APX activity gradually increased during the incubation with or without salt stress for 24 hr. The activity was not affected by the treatment with NaCl (Fig. 5A).
3. Effects of antioxidants

Figs. 6A-D show the changes in chlorophyll content during the incubation in 200 mM NaCl after preincubation in various antioxidants for 12 hr. The decrease of chlorophyll content of the leaf segments in 200 mM NaCl was affected by pretreatment with various antioxidants. Tiron, which scavenges $O_2^-$, slightly suppressed the decrease of chlorophyll content (Fig. 6A). Pretreatment with AsA and benzoate, which scavenge $H_2O_2$ and $\cdot OH$, respectively, suppressed the reduction of chlorophyll content (Figs 6B, D). Pretreatment with DABCO, which scavenges $^{1}O_2$ suppressed the decrease of chlorophyll content until 12 hr, but, had no effect after 24 hr (Fig. 6C). As a negative control for this experiment, the toxicity of antioxidants themselves was determined, but all the
Fig. 4. Changes in the activities of SOD isozymes during the incubation in 200 mM NaCl for 24 hr. Data are means±SE of 3 experiments. Differences from control values were significant at P<0.01 (b) (Duncan’s multiple range test).

Fig. 5. Changes in the activities of APX and CAT during the incubation in 200 mM NaCl for 24 hr. Data are means±SE of 3 experiments.

Antioxidants showed no adverse effects on chlorophyll content (data not shown).

Figs. 7A-D show chloroplasts in leaf segments incubated in 200 mM NaCl for 24 hr after preincubation in antioxidants for 12 hr. Pretreatment with AsA and benzoate suppressed the swelling of thylakoids and the destruction of thylakoid membranes caused by NaCl (Figs. 7B,D). On the other hand,
Tiron and DABCO could not suppress the chloroplast destruction (Figs. 7A,C). All antioxidants showed no adverse effects on chloroplast ultrastructure by themselves (data not shown).

Fig. 8 shows a chloroplast in a leaf segment treated with 200 mM NaCl under dark condition. Although leaf segments were incubated in 200 mM NaCl for 24 hr, no ultrastructural changes were observed under dark condition.

**Discussion**

Under the light condition NaCl induced the reduction of chlorophyll content (Fig. 1) and the destruction of chloroplasts in leaf segments (Fig. 3B), under the dark condition, however, chlorophyll content did not decrease and no ultrastructural changes were observed (Fig. 1 and Fig. 8). Mitsuya et al. (2003) also observed that thylakoid membranes of chloroplast in rice degraded in light but not in the dark although the leaf tissues contained comparable amount of Na⁺ and Cl⁻ in both light and dark.

These results suggest that salt-induced damages in chloroplasts, especially inner membranes, were caused by light-induced oxidative stress (Hernandez et al., 1995; 1999).

The chloroplasts of rice plants (*Oryza sativa* L.) have two SOD isozymes, Fe-SOD and CuZn-SOD (Kaminaka et al., 1997; 1999). Mn-SOD in chloroplasts has been identified as thylakoid-bound isozyme in spinach (Hayakawa et al., 1985). However, no genes for chloroplastic Mn-SOD have been characterized in rice plants. In the present study, Fe-SOD activity drastically increased after a 24 hr incubation in 200 mM NaCl (Fig. 4D). It is suggested that the incubation in 200 mM NaCl for 24 hr excessively induces O₂⁻ in chloroplasts.

The activity of CuZn-SOD isozyme, a part of which is located in chloroplast (Kaminaka et al., 1997), however, was not increased by NaCl (Fig. 4B). In transgenic tobacco plants, in which an Fe-SOD from *Arabidopsis thaliana* is overexpressed, the activities of cytosolic and chloroplastic CuZn-SODs...
remained unchanged under salt stress (Van Camp et al., 1996). This report suggested that the transgenic Fe-SOD interferes with a signal pathway leading to the induction of CuZn-SOD isozymes, because the concentration of $O_2^-$ in the chloroplasts was lowered by transgenic Fe-SOD. A similar result was obtained in the rice plants overexpressing yeast Mn-SOD in chloroplasts (Tanaka et al., 1999). These results suggest that salt stress in the present study lowered the concentration of $O_2^-$ in the chloroplasts by activating Fe-SOD, and that CuZn-SOD was not activated by salt stress.

SOD scavenges $O_2^-$ in chloroplasts and its enzymatic action results in the formation of $H_2O_2$. In the present study, $H_2O_2$ was probably generated at higher rate in the salt-treated chloroplasts than in the control, because Fe-SOD was activated by treatment with NaCl for 24 hr. $H_2O_2$ generated in chloroplasts is generally scavenged by APX (Asada, 1999). Whereas APX activity gradually increased during the 24 hr
incubation in NaCl solution its level did not increase over the control (Fig. 5A). It is thought that this leads to an accumulation of excess H$_2$O$_2$ in chloroplasts and increases the risk of the formation of ·OH by a metal-catalyzed site specific Haber-Weiss reaction (Halliwell and Gutteridge, 2000). AsA and benzoate suppressed the reduction of chlorophyll content and the destruction of chloroplasts (Figs. 6B, D and 7B, D). AsA scavenges various AOS including H$_2$O$_2$. Benzoate is a scavenger of ·OH. Therefore it is suggested that, in rice plants, the imbalance of the antioxidant enzymes between Fe-SOD and APX induces excess H$_2$O$_2$ and H$_2$O$_2$-derived ·OH, and that the reduction of chlorophyll content and ultrastructural changes were induced by these AOS.

In the present study, NaCl caused deactivation of CAT (Fig. 5B). Similar results are observed in pea and rice plants (Corpas et al., 1993; Lee et al., 2001). Copas et al. (1993) suggested that CAT deactivation induces an increase in peroxisomal H$_2$O$_2$ concentration and H$_2$O$_2$ might diffuse from the peroxisomes into the cytosol. The diffusion of H$_2$O$_2$ from peroxisomes to the cytosol is dangerous to the plant cell and other organelles, because H$_2$O$_2$ can give rise to the production of ·OH (Halliwell, 1982). Therefore, the decrease in CAT activity observed in the present study may also relate to the chloroplast damage.

Tiron, which scavenges O$_2^-$, could not effectively suppress the reduction of chlorophyll and the destruction of chloroplasts (Figs. 6A and 7A), although O$_2^-$ was perhaps generated excessively in chloroplasts under NaCl stress. A similar result was obtained in Hydrilla verticillata (Rout and Shaw, 2001). The rate constant of Tiron with O$_2^-$ has been studied by pulse radiolysis and found to be 1.0×10$^7$ M$^{-1}$s$^{-1}$ (Bors et al., 1979). The rate constant for reaction of O$_2^-$ with SOD is about 2.0×10$^5$ M$^{-1}$s$^{-1}$ (Halliwell and Gutteridge, 2000). It is suggested that as Fe-SOD was drastically increased after a 24 hr incubation in 200 mM NaCl, the most of O$_2^-$ was catalyzed by Fe-SOD to H$_2$O$_2$ before Tiron could scavenge O$_2^-$ Therefore, Tiron had little effect on the reduction of chlorophyll content and the destruction of chloroplasts.

The present study suggests that the imbalance in the antioxidant activities between Fe-SOD and H$_2$O$_2$ scavenging enzymes induces the accumulation of excess H$_2$O$_2$ and H$_2$O$_2$-derived ·OH, and that H$_2$O$_2$ and ·OH are responsible for the deleterious effects of salt stress on chlorophyll content and chloroplast ultrastructure.

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Fig. 8. Ultrastructure of chloroplasts in leaf segments after the incubation in 200 mM NaCl for 24 hr under dark condition. Bar=1 µm.
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