Antioxidant Capacity and Damages Caused by Salinity Stress in Apical and Basal Regions of Rice Leaf

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Abstract: We investigated the mechanisms of increased sensitivity to Na⁺ in the apical and basal regions of the rice leaf under salinity. Three-week-old plants were treated with 200 mM NaCl in hydroponic culture for 3 d. Segments 6 cm in length were obtained from the apical and basal regions of the fully expanded uppermost leaves (6th leaf blades) as old and young tissues, respectively. In the plants exposed to 200 mM NaCl, Nitro blue tetrazolium (NBT) reducing activity, and H₂O₂ and Malondialdehyde (MDA) contents significantly increased, accompanied by the swelling of thylakoids and destruction of thylakoid membranes in the apical regions. However, no indication of oxidative damages was observed in the basal region, even though the Na⁺ content in the basal region was comparable to that in the apical region. In the apical region, the capacity to scavenge H₂O₂ was lower than that in the basal region due to decrease in the constitutive levels of ascorbate peroxidase and guaiacol peroxidase. In addition, the activities of antioxidant enzymes except superoxide dismutase and guaiacol peroxidase decreased drastically after 48 hr of exposure to NaCl. By contrast, the activities of catalase and glutathione reductase in the basal region increased compared with those in the control, and other antioxidant enzymes did not decrease under salinity during the experimental period. These results suggest that the capacity to scavenge reactive oxygen species decreased with age, and thus the apical region of the leaf blade suffered severer damage by Na⁺ than the basal region.

Key words: Age, Antioxidant enzyme, NaCl, Reactive oxygen species, Rice.

Rice (Oryza sativa), one of the most important cereal crops in tropical and temperate regions of the world, is sensitive to salinity and NaCl at a concentration as low as 50 mM is lethal at the seedling stage (Yeo et al., 1990). Na⁺ and Cl⁻ are carried in the transpiration stream to the leaves and accumulated therein, which leads eventually to their sequential death (Flowers and Yeo, 1981). Comparison among rice subspecies and varieties differing in salinity tolerance has shown that salinity tolerance correlates with the ability to exclude Na⁺ from the shoot and maintain a low Na⁺/K⁺ ratio (Lee et al., 2003; Ren et al., 2005). Thus, exclusion or intracellular compartmentation of Na⁺ into the vacuole by transgenic methods could increase salinity tolerance in the rice plant (Fukuda et al., 2004; Martínes-Atienza et al., 2007). However, other mechanisms may be involved in salinity tolerance in many plant species including rice, especially at high salinity (Munns and Tester, 2008).

The relationship between the concentration of salt in leaves and the damage caused by salt is poorly understood. A negative correlation between salinity tolerance and Na⁺ accumulation in leaves is often observed (Yeo and Flowers, 1983; Munns and Tester, 2008). However, Mitsuya et al. (2002) reported that the damage in the 4th leaf of rice appeared from the apical region of the leaf blade, whereas the Na⁺ content in the apical region was less than that in the basal region. In gramineous leaves, the basal region of a leaf blade is younger than the apical region (Esau, 1943). Mitsuya et al. (2003) examined the sensitivity of leaf tissues at different nodal positions of rice plants and reported that the old lower leaves were more sensitive than the young upper leaves even though both tissues contained comparable amounts of Na⁺. Therefore, the tolerance to salinity in the tissue may decrease with advancing age, because the old tissue has a lower threshold value for Na⁺ than the young tissue (Mitsuya et al., 2002; 2003).

The reactive oxygen species (ROS) such as singlet oxygen (¹O₂), superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (·OH) are produced even in normal aerobic metabolism. Since these oxygen species are highly reactive, plants have developed antioxidant defense systems in order to prevent oxidative damage (Asada, 1999). However, the time-dependent shift in the antioxidant/pro-oxidant balance in favor of oxidative stress occurs with advancing age (Munné-Bosch and Alegre, 2002; Ohe et al., 2005). The decrease in antioxidant

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Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GPX, guaiacol peroxidase; GR, glutathione reductase; H₂O₂, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro blue tetrazolium; O₂⁻, singlet oxygen; O₂⁺, superoxide anion; ·OH, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase.
capacity with advancing age leads to an increase in
the sensitivity to environmental stresses (Thompson,
1988). However, little is known as to whether the
decline in the antioxidant capacity with age accelerates
the Na⁺ sensitivity in rice plants. Here, we investigated
the possible mechanisms responsible for the different
sensitivity to Na⁺ of the two tissues at different
developmental stages in salt-sensitive rice plants, to
obtain information for better understanding of the
mechanisms involved in salt stress injury.

Materials and Methods

1. Plant materials and stress treatment
   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 at 24±2°C
   until the appearance of the white tip of the coleoptile.
   After imbibition, the seeds were sown on hydroponic
   culture containing nutrient solution according to Mae
   and Ohira (1981) and grown for 3 wk. The plants were
   cultured in a growth chamber (14-hr photoperiod
   (day/night). The 3-wk-old plants were transferred to 200 mM
   NaCl in hydroponic culture at 0900. After exposure to
   NaCl for 0, 12, 24, 48 and 72 hr 6 cm long segments
   were sampled from the apical and basal regions of the
   fully expanded uppermost leaves (6th leaf blades) as
   a representative of old and young tissues, respectively.
   For the measurements of antioxidant enzymes, the leaf
   segments were immediately frozen with liquid N₂ and
   preserved at –80°C until use.

2. Measurement of Na⁺ and K⁺ contents
   The leaf segments were bottled dry, and the dry
   weight was determined after the leaf segments were
   dried at 70°C for 48 hr and cooled in a desiccation
   chamber. Na⁺ and K⁺ in the leaf segments were
   extracted with 1 mL of 1 M HCl at room temperature
   for 48 hr. The extraction media were further diluted
   100-1000 fold with distilled water before measurement.
   The Na⁺ and K⁺ contents were measured by atomic
   absorption spectrometry (Shimadzu Co. Ltd., AA-
   6400F) in the emission mode.

3. Measurement of nitro blue tetrazolium (NBT)
   reducing activity, and H₂O₂ and malondialdehyde
   (MDA) contents
   After the fresh weight of the leaf segment was
determined, the NBT-reducing activity was measured to
determine the O₂⁻ generation according to the method
published by Kuzniak et al. (1999). H₂O₂ was measured
according to the method described by Orendi et al.
(2001). The extent of lipid peroxidation in the leaf
segments was estimated by measuring the amount of
MDA using the method of Hodges et al. (1999).

4. Electron microscopy
   Electron microscopic studies were made using the
leaf tissue at the distance of 6 cm from leaf tip or base.
Small pieces of leaves were fixed according to Yamane
et al. (2008).
   Ultrathin sections (70–90 nm in thickness) were
   cut with a diamond knife and placed on 150 mesh
copper grid. The grids were stained with 2% uranyl
acetate for 25 min followed by lead citrate for 5
min. Then the sections were examined on a Hitachi
H7500 transmission electron microscope at 100 kV
and photographed with CCD camera (Advanced
Microscopy Technique, USA) connected to the
electron microscope. Photographs were taken at three
or more random sites in at least 3 leaf segments and
representative pictures were presented.

5. Extraction and assay of antioxidant enzymes
   All operations were carried out at 0–4°C. For the
determination of superoxide dismutase (SOD),
ascorbate peroxidase (APX) and catalase (CAT)
activities, the extracts were prepared according to
Yamane et al. (2004). SOD, APX and CAT activities
were assayed according to Beyer and Fridovich
(1987), Nakano and Asada (1981) and Aebi (1984),
respectively. For the determination of guaiacol
peroxidase (GPX) activity, extracts were prepared and
the activity was assayed by the method described by
Lee and Lin (1995). The GPX activity was represented
by the sum of soluble and cell wall bound GPX
activities. For the assay of glutathione reductase
(GR) activities, the leaf segments were homogenized
with 1.0 mL ice-cold 50 mM potassium phosphate
buffer (pH 7.8) containing 1.0 mM EDTA, 7.0 mM
mercaptoethanol, 0.1% (w/v) Triton X-100 and 1.0%
(w/v) polyvinylpyrrolidone. The homogenate was
centrifuged at 18,000 g for 15 min and the supernatant
was used for assays. GR activity was assayed according
to Lee et al. (2001).
   Protein in the supernatant was quantified by the
Coomassie brilliant blue-dye binding method
according to Bradford (1976).

6. Statistical analysis
   A completely randomized design was used with
two different regions of a leaf under two principal
treatments (control and NaCl treatment) and five
harvesting times for each treatment. Three to four
replicates (plants) were used per treatment. Data
were statistically analyzed using ANOVA followed by
Tukey’s HSD test (SPSS 14.0; SPSS Chicago, IL, USA).
Significant differences were analyzed based on P<0.05
between four means at each harvesting time.
Results

1. Element content

Fig. 1 shows the changes in Na⁺ and K⁺ contents in the tissues in the apical and basal regions of the leaves. The Na⁺ and K⁺ contents in both regions in the control were at similar levels (Fig. 1A, B). NaCl treatment induced an increase in the accumulation of Na⁺ in both regions (Fig. 1A). The accumulation of Na⁺ occurred first in the apical region, followed by the basal region (Fig. 1A). After 48 hr of NaCl treatment, the accumulation level of Na⁺ became similar in both regions (Fig. 1A). The effect of salinity on K⁺ content appears to differ with the leaf region. The content of K⁺ in the apical region was not changed by salinity during the experimental period (Fig. 1B). However, the content of K⁺ in the basal region gradually decreased under salinity (Fig. 1B). After 72 hr of NaCl treatment, K⁺ content in the basal region decreased to about 50% of the control, leading to a higher Na⁺/K⁺ ratio in the

![Graph showing Na⁺ and K⁺ content and Na⁺/K⁺ ratio](image-url)
basal region than in the apical region (Fig. 1C).

2. NBT-reducing activity, and H$_2$O$_2$ and MDA contents

NBT-reducing activity, measured as an indicator of O$_2^-$ generation, H$_2$O$_2$ content and MDA content in the apical region of leaf tended to be higher than those in the basal region in the control plants (Fig. 2). Induction of an increase in H$_2$O$_2$ content in the apical region was rapid (after 12 hr) and more pronounced after 24–72 hr of NaCl treatment compared with that in the control (Fig. 2B). NBT reducing activity and MDA contents in the apical region significantly increased after 24–72 hr of NaCl treatment compared with that in the control (Fig. 2A, C). However, NBT reducing activity, and H$_2$O$_2$ and MDA contents in the basal region was not changed by salinity compared

![Graph showing NBT-reducing activity, H$_2$O$_2$ content, and MDA content in the apical and basal regions of the leaf in control and NaCl-treated rice plants.](image)

Fig. 2. NBT-reducing activity (A), H$_2$O$_2$ content (B) and MDA content (C) in the apical and basal regions of the leaf in control and NaCl-treated rice plants during the experimental period. Values are means±SE of 4 replicates. Different letters above the bars indicate a significant difference at P<0.05 between four means at each harvesting time (Tukey’s HSD).
with those in the control during the experimental period (Fig. 2).

3. Chloroplast ultrastructure

Fig. 3A and 3B show electron micrographs of typical chloroplasts in mesophyll cells in the apical and basal regions of the leaves in the control plant, respectively. Both chloroplasts possessed well-developed granal and stromal thylakoids and no ultrastructural damage was observed (Fig. 3A, B). Fig. 3C, D show chloroplasts in a mesophyll cell in the apical and basal regions of a leaf treated with 200 mM NaCl in hydroponic culture for 24 and 72 hr, respectively. Swelling of thylakoids and destruction of thylakoid membranes were observed in the apical region after 24 hr of NaCl treatment (Fig. 3C). The chloroplasts were damaged more severely after 48 hr of NaCl treatment (data not shown). However, such damage was not observed in the basal region even in the plants treated with 200 mM NaCl for 72 hr (Fig. 3D).

4. Effects of salinity on antioxidant enzymes

Fig. 4A shows the activity of SOD, which is a major scavenger of $\text{O}_2^-$ and converts $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and $\text{O}_2$. The SOD activity in control plants at each harvesting time was slightly higher in the apical region than in the basal region of the leaf (Fig. 4A). The activity in the apical region after 48 hr of NaCl treatment was enhanced about 2-fold than in the control (Fig. 4A). On the other hand, SOD activity in the basal region was not changed by salinity compared with that in the control during the experimental period (Fig. 4A).

Fig. 4B-D show the activities of major $\text{H}_2\text{O}_2$ scavenging enzymes such as APX, GPX and CAT. The activities of APX and GPX in control plants at each harvesting time were lower in the apical region than in the basal region (Fig. 4B, C). CAT activity in control plants at each harvesting time was higher in the apical region than in the basal region (Fig. 4D). NaCl treatment induced the changes in the levels of these $\text{H}_2\text{O}_2$ scavenging enzymes, even though GPX activity in both regions was hardly influenced by salinity (Fig. 4C). APX and CAT activities in the apical region did not differ from the control after 12 and 24 hr of the treatment with NaCl (Fig. 4B, D). However, both activities decreased drastically after 48 hr of the treatment with NaCl (Fig. 4B, D). APX activity in the basal region under salinity did not change compared with that in the control during the experimental period (Fig. 4B). CAT activity in the basal region
GR activity in the apical region, while the activity decreased drastically after 48 hr of NaCl treatment (Fig. 4E). On the other hand, GR activity in the basal region increased by the NaCl treatment during the experimental period (Fig. 4E).

Discussion

Lipid peroxidation is an effective indicator of cellular oxidative damage (Moran et al., 1994). Treatment with NaCl induced the increase in MDA content of the tissues in the apical region of the leaf (Fig. 2B). Chloroplasts are considered to be the most sensitive of the organelle to salt stress, because this organelle is the most powerful source of ROS production (Foyer et al., 1994). In fact, prominent swelling of thylakoids, which is a typical symptom of oxidative damage, is induced at the early stage of the damage by salinity (Hernández et al., 1995; Mitsuya et al., 2003; Yamane et al., 2004; Omoto et al., 2009). The loss of chloroplast integrity was observed in the apical region at 24 hr of NaCl treatment (Fig. 3C). By contrast, any indications of oxidative stress were not observed in the basal region during the experimental period (Fig. 2). The content of Na⁺ in the basal region was similar to that in the apical region after 48 hr of NaCl treatment (Fig. 1A). These results indicate that the damage is not correlated merely with Na⁺ content (Mitsuya et al., 2003).

One of the mechanisms of salt tolerance in rice is Na⁺ exclusion and an increased absorption of K⁺ to maintain a low Na⁺/K⁺ ratio in the shoot (Gregorio and Senadhira, 1993). K⁺ content in the basal region under salinity decreased with increasing Na⁺ content, leading to higher Na⁺/K⁺ ratio than in the apical region (Fig. 1C). However, the excess generation of H₂O₂ and salt-induced damage was observed in the apical region but not in the basal region (Figs. 2, 3). These results suggest that salt-induced damage is not correlated with Na⁺/K⁺ ratio within a leaf.

The increase in ROS and lipid peroxidation (Munné-Bosch and Alegre, 2002; Ohe et al., 2005) and loss of chloroplast integrity such as a disorientation of grana stacks and swelling of thylakoids are first signs of the decrease in antioxidant defenses (Smart, 1994). The generation of ROS and the increase in MDA content in control plants had the tendency to be higher in the apical region than in the basal region (Fig. 2), though chloroplast integrity in the apical region was preserved during the experimental period (Fig. 3A). The levels of antioxidant enzymes such as APX and GPX in control plants were significantly lower in the apical region than in the basal region (Fig. 4B, C). When plants were affected by 200 mM NaCl, H₂O₂ content in the apical region immediately increased (Fig. 2B). These results suggest that the capacity to scavenge H₂O₂ is low in the apical region compared with that of the basal region, and the decrease in the

![Fig. 4. Changes in the activities of antioxidant enzymes in the apical and basal regions of the leaf in control and NaCl-treated rice plants during the experimental period. A: SOD. B: APX. C: GPX. D: CAT. E: GR. Values are means±SE of 3 (APX and CAT) or 4 (SOD, GPX and GR) replicates. Different letters above the bars indicate a significant difference at P<0.05 between four means at each harvesting time (Tukey’s HSD).](image-url)
capacity may be, at least partly, induced by the low constitutive levels of APX and GPX. In our previous study, damage caused by salinity was induced by H$_2$O$_2$ and 'OH derived from H$_2$O$_2$ in rice plants (Yamane et al., 2004). Thus, the decrease in H$_2$O$_2$ scavenging capacity could be one of the reasons that the apical region suffers severer damage than the basal region.

The basal region has an enhanced antioxidant capacity under salinity due to the increase in the activities of CAT and GR (Fig. 4D, E). CAT is predominately located in the peroxisomes and is the most important enzyme scavenging H$_2$O$_2$ produced by photorespiration (Van Breusegem et al., 2002). However, CAT is not limited to the removal of peroxisomal H$_2$O$_2$ (Willekens et al., 1997; Foyer and Noctor, 2000). This enzyme appears to be critical for maintaining the redox balance during oxidative stress and is indispensable for stress tolerance in some C$_3$ plants (Willekens et al., 1997). In our previous study, NaCl induced severe chloroplast damage with a decrease in CAT activity, while APX activity was maintained during the stress treatment (Yamane et al., 2004). These results suggest that the activation and the maintenance of CAT activity under salinity are most important factors against salt-induced oxidative stress.

GR activity might limit the flux of the water-water cycle under stress conditions (Foyer et al., 1995). The increase in GR activity leads to an increase in the reduction state of the glutathione pool and therefore to an increase in ascorbate regeneration (Foyer et al., 1995). Ascorbate is an important non-enzymatic antioxidant, which was shown to scavenge O$_2^-$, H$_2$O$_2$ and 'OH (Yu, 1994). Thus, it is suggested that GR is important for reducing O$_2^-$ in the basal region under salinity. Another possibility is that the elevated levels of GR activity could increase the ratio of NADP$^+$/NADPH, thereby ensuring the availability of NADP$^+$ to accept electrons from the photosynthetic electron transport chain (Jung, 2004). Under such a situation, the flow of electrons to O$_2$ and therefore, the formation of O$_2^-$ can be minimized. In the present study, the generation of NBT-reducing substance was suppressed (Fig. 2A) and SOD activity did not increase in the basal regions (Fig. 4A). On the other hand, the reduction of GR activity in the apical region under salinity corresponded to the increase in SOD activity (Figs. 4A, E). These results suggest that an increase in GR activity under salinity may play an important role in suppressing the generation of O$_2^-$. In conclusion, the basal region of leaf in rice plants has the ability to scavenge H$_2$O$_2$ by enhancing the activity of CAT and maintaining the high constitutive levels of APX and GPX than those in the apical region under salinity. In addition, GR in the basal region may suppress the generation of O$_2^-$ under salinity. On the other hand, the apical region has the ability to scavenge O$_2^-$ by increasing the activity of SOD, but H$_2$O$_2$ scavenging enzymes such as APX and CAT decreased under salinity. In gramineous leaves, the basal region of a leaf blade is younger than the apical region (Esau, 1943). Therefore, these results suggest that the decline in the activities of antioxidant enzymes with advancing age leads to lower tolerance to salt-induced oxidative stress, which might increase the Na$^+$ sensitivity.

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