

# Exogenous Ascorbic Acid and Glutathione Alleviate Oxidative Stress Induced by Salt Stress in the Chloroplasts of *Oryza sativa* L.

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The effects of exogenous ascorbic acid (AsA) and reduced glutathione (GSH) on antioxidant enzyme activities [superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR)] and the contents of malondialdehyde (MDA) and H<sub>2</sub>O<sub>2</sub>, as well as of endogenous AsA and GSH, in the chloroplasts of two rice cultivars, the salt-tolerant cultivar Pokkali and the salt-sensitive cultivar Peta, were investigated. Exogenous AsA and GSH enhanced SOD, APX, and GR activities, increased endogenous AsA and GSH contents, and reduced those of H<sub>2</sub>O<sub>2</sub> and MDA in the chloroplasts of both cultivars under salt stress (200 mM NaCl), but the effects were significantly more pronounced in cv. Pokkali. GSH acted more strongly than AsA on the plastidial reactive oxygen scavenging systems. These results indicated that exogenous AsA and GSH differentially enhanced salinity tolerance and alleviated salinity-induced damage in the two rice cultivars.

**Key words:** *Oryza sativa* L., Oxidative Stress, Salt Stress

## Introduction

Plants are constantly exposed to a wide range of abiotic and biotic stresses such as high salinity, drought, extremes in temperature, and heavy metals (Charu *et al.*, 2011). Among these stresses, salt stress is one of the major environmental factors that limit growth, development, and geographical distribution of plants and cause significant crop losses (Ashraf and Foolad, 2007). Anthropogenic activities lead to secondary salinization and global warming, which contributes to rising sea levels and increasing storm incidences, particularly in coastal areas, exacerbating the problem (Wassmann *et al.*, 2004). It is estimated that more than 6% of the world's land and 30% of irrigated areas already suffer from salinity problems (Chaves *et al.*, 2009). In China, salinization in areas of rice cultivation has recently increased to 1/5 of the total area (Ruan and Xu, 2002). Rice (*Oryza sativa* L.) is one of the world's major crops, which is widely cultivated and is very sensitive to salt stress (Fran-

cois and Maas, 1994). Several studies have shown that NaCl treatment inhibits the net photosynthetic rate and the electron transport activity, and damages the polypeptide composition of the thylakoid membrane and chloroplast ultrastructure (Yamane *et al.*, 2004; Moradi and Ismail, 2007; Wang *et al.*, 2009). Therefore, reducing the effects of salt stress on rice plants will help overcome barriers to increased crop production.

Environmental stresses, including salt stress, increase the formation of reactive oxygen species (ROS), *i. e.* <sup>1</sup>O<sub>2</sub>, OH<sup>•</sup>, H<sub>2</sub>O<sub>2</sub>, in cells (Parida and Das, 2005). Chloroplasts are the most important primary sites of ROS production in plants and extremely sensitive to salt stress (Sairam and Tyagi, 2004). To cope with oxidative stress, chloroplasts have developed defence systems including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR), and low-molecular weight antioxidants such as ascorbic acid (AsA) and glutathione (GSH) (Jaleel *et al.*, 2009). Enhanced antioxidant de-

fence is one of the mechanisms of plants to adapt to adverse environments including salt stress. Changes in the activities of antioxidant enzymes in response to salinity have been reported to differ between salt-tolerant and -sensitive cultivars of various crop plants (Sairam *et al.*, 2002; Meloni *et al.*, 2003; El-Shabrawi *et al.*, 2010). The low-molecular weight antioxidants AsA and GSH play an important role in scavenging and removing toxic products before membrane damage occurs in the chloroplasts (Szarka *et al.*, 2012). In this system, AsA is peroxidized to dehydroascorbate (DHA) by APX. DHA is then reduced by GSH, either enzymatically or non-enzymatically, leading to the formation of glutathione disulfide (GSSG). The re-reduction of GSSG to GSH is catalyzed by GR in an NADPH-dependent reaction (AsA-GSH recycling) (Foyer and Noctor, 2011). There are many reports on the induction of antioxidant enzyme activities by exogenous AsA and GSH leading to increased antioxidant levels in leaves, thus strengthening the tolerance of crop plants to salt stress (Chen and Liu, 2000; Shalata and Meumann, 2001). However, we know little about how the active oxygen scavenging system of chloroplasts responds to the addition of exogenous AsA and GSH.

In this study, we focused our attention on a pair of contrasting rice genotypes with similar general characteristics but marked differences in salt tolerance, which had been identified at the International Rice Research Institute (IRRI) and are described by Yan *et al.* (1992a, b). The two varieties were compared for the effects of exogenous AsA and GSH on the mentioned antioxidative enzymes, the endogenous levels of AsA and GSH, and on membrane lipid peroxidation in the chloroplasts.

## Materials and Methods

### *Plant material and experimental design*

*Oryza sativa* L. salt-tolerant cv. Pokkali and salt-sensitive cv. Peta were obtained from the International Rice Research Institute (IRRI, Manila, The Philippines). Seeds were surface-sterilized in 0.1% HgCl<sub>2</sub> for 10 min, rinsed well with running water, and immersed in distilled water for 24 h. After germination, seeds were sown in plastic pots with thin sand and planted outdoors. The seedlings were planted outdoors with a photoperiod of 12 h light/12 h dark from May to June 2011, at a rela-

tive humidity of 60/80%, and a photon flux density of 600–1400  $\mu\text{mol}/(\text{m}^2 \text{ s})$ . Seedlings at the three-leaf, one-spindle stage were cultivated with Kimura B solution [consisting of (mM): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (48.2), MgSO<sub>4</sub> (65.9), KNO<sub>3</sub> (18.5), KH<sub>2</sub>PO<sub>4</sub> (24.8), Ca(NO<sub>3</sub>)<sub>2</sub> (59.9), K<sub>2</sub>SO<sub>4</sub> (15.9)] in small turnover boxes and exposed to natural irradiation. The temperature was (30  $\pm$  2) °C/(24  $\pm$  2) °C during day/night. After the fourth leaf had fully expanded, the seedlings were cultured in four different nutrient solutions designated: (a) control: Kimura B solution without NaCl, GSH or AsA; (b) NaCl: Kimura B solution + 200 mM NaCl (selected according to the results of preliminary experiments); (c) GSH: Kimura B solution + 0.065 mM GSH (concentration determined in preliminary experiments); (d) NaCl + GSH: Kimura B solution + 200 mM NaCl + 0.065 mM GSH; (e) AsA: Kimura B solution + 5 mM AsA (as determined by preliminary experiments); and (f) NaCl + AsA: Kimura B solution + 200 mM NaCl + 5 mM AsA.

All solutions were renewed and ventilated thoroughly every 4 d during the experiment. The leaves of the seedlings were sampled after treatment for 2, 4, 6, and 8 d.

### *Isolation of chloroplasts*

Intact chloroplasts were isolated from leaves according to the method described by Ketcham *et al.* (1984) with slight modifications. Fresh leaves without the midribs were kept in the refrigerator at 4 °C for 30 min, then cut into pieces, and homogenized by grinding with four times the volume (w/v) of cold extraction medium [50 mM Tris-HCl, pH 7.6, 5 mM MgCl<sub>2</sub>, 10 mM NaCl, 0.4 M sucrose, 0.1% bovine serum albumin (BSA)]. The homogenate was filtered through eight-layered muslin cloth to remove large debris, then centrifuged in a TGLL-18G centrifuge (Taicang Instrumentarija, Taicang Jiangsu, China) at 1000  $\times g$  for 1 min. The pellets were resuspended in 6 ml of extraction medium, and loaded onto 20 ml of a Percoll step gradient from 40% to 80% (prepared in the extraction medium) and recentrifuged at 2000  $\times g$  for 2 min. After centrifugation, the lower band containing intact chloroplasts was removed with the help of a Pasteur pipette and washed twice by gentle resuspension in the extraction medium. Intactness of the Percoll-purified chloroplasts was found to be 90–95% as determined by the ferricyanide reduction method. The suspension was kept in the dark on ice for subsequent use.

### *Measurement of the chlorophyll content*

One ml of chloroplast suspension was mixed with 9.9 ml of 80% acetone and centrifuged at  $3000 \times g$  for 5 min. Absorption of the supernatant was determined at 645 and 663 nm, and chlorophyll content was calculated as described by Arnon (1949).

### *Measurement of the $H_2O_2$ content*

$H_2O_2$  levels were determined according to the modified method of Bernt and Bergmeyer (1974). The chloroplast suspension (1 ml) was mixed with 0.1 ml of 95% (v/v) hydrochloric acid, containing 20% (v/v) titanium tetrachloride, and 0.2 ml of 28% ammonia. After centrifugation at  $10,000 \times g$  for 10 min, the resulting pellet was repeatedly washed with acetone and then dissolved in 3 ml of 1 M  $H_2SO_4$ . The absorbance was measured at 410 nm and the  $H_2O_2$  content of the chloroplasts calculated from a standard curve of  $H_2O_2$ .

### *Measurement of the malondialdehyde content*

The malondialdehyde (MDA) content was analysed fluorometrically according to the method of Yagi (1976) based on the reaction with 2-thiobarbituric acid (TBA). The chloroplast suspension obtained was mixed with 29 mM TBA in 8.75 M acetic acid and heated at 95 °C for 1 h. After cooling, thiobarbituric acid reactive substances (TBARS) were extracted into *n*-butanol, and the fluorescence of the organic layer was measured at 531 nm (excitation) and 553 nm (emission). The concentration of lipid peroxidation products was expressed in nmol of TBARS per mg of chlorophyll, with 1,1,3,3-tetraethoxypropane used as a standard.

### *Antioxidant enzyme activity assays*

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Rao and Sresty, 2000). A blank reaction was performed using all components with the exception of the enzyme extract. One unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of the rate of nitroblue tetrazolium reduction at 560 nm.

The APX activity assay was performed as described by Nakano and Asada (1981). The 3-ml reaction solution contained 50 mM phosphate buffer (pH 7.0),

0.5 mM AsA, 0.1 mM  $H_2O_2$ , and 0.1 ml enzyme extract. APX activity was calculated by following the decrease in absorbance of AsA (extinction coefficient,  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ) within 1 min at 290 nm. The blank was determined by the addition of phosphate buffer instead of extract in the reaction mixture. One unit of APX activity was defined as the amount of enzyme required for catalyzing the consumption of 1  $\mu\text{mol}$  AsA/min.

GR activity was measured according to Gamble and Burke (1984). The 1-ml reaction mixture contained 0.1 mM NADPH, 40 mM Tricine-NaOH (pH 7.8), and 0.2 ml of supernatant. The reaction was initiated by the addition of 0.5 mM oxidized GSH, and the rate of NADPH oxidation was monitored at 340 nm. For the blank, Tricine-NaOH buffer instead of enzyme extract was added to the reaction mixture. GR activity was expressed as  $\mu\text{mol}$  of substrate oxidized per min per mg chlorophyll.

### *Measurement of the antioxidant content*

AsA and GSH were extracted according to Lu *et al.* (1999). The chloroplast suspension (1 ml) was homogenized in 3 ml of 5% trichloroacetic acid (TCA) solution. The extract was centrifuged at  $15,000 \times g$  for 10 min, and the volume of the supernatant was adjusted to 4 ml with 5% TCA before assaying the contents of the antioxidants.

GSH levels were measured according to the method of Griffith (1980). A chloroplast suspension (50  $\mu\text{l}$ ) was mixed with 5% sulfosalicylic acid (150  $\mu\text{l}$ ) and centrifuged. Fifty  $\mu\text{l}$  of the supernatant were mixed with 700  $\mu\text{l}$  of 3 mM NADPH, 100  $\mu\text{l}$  of 10 mM DTNB [5,5-dithiobis-(2-nitrobenzoic acid)], and 150  $\mu\text{l}$  of 125 mM phosphate buffer (pH 6.5) containing 6.3 mM EDTA. The reaction was initiated by adding 10  $\mu\text{l}$  of GR (5 U/ml), and the change in absorbance at 412 nm was monitored. A standard curve was prepared with GSH for calculating the amounts of GSH in the samples.

AsA content was determined following the modified method of Hodges *et al.* (1996). A 200- $\mu\text{l}$  sample of the suspension was added to 200  $\mu\text{l}$  of 150 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 7.4) and 200  $\mu\text{l}$  of water. To a second 200- $\mu\text{l}$  sample of suspension, 200  $\mu\text{l}$  of buffer and 100  $\mu\text{l}$  of 10 mM dithiothreitol were added, and after thorough mixing and standing at room temperature for 15 min, 100  $\mu\text{l}$  of 0.5% (w/v) *N*-ethylmaleimide were added. Both samples were mixed by vortexing and incubated at room temperature for 30 s. To each

sample 400  $\mu\text{l}$  of 10% (w/v) TCA, 400  $\mu\text{l}$  of 44% (v/v)  $\text{H}_3\text{PO}_4$ , 400  $\mu\text{l}$  of 4% (w/v) bipyridyl in 70% (v/v) ethanol, and 200  $\mu\text{l}$  of 3% (w/v)  $\text{FeCl}_3$  were added. After vortex-mixing, samples were incubated at 37 °C for 60 min, and the absorbance at 525 nm was measured. A standard curve in the range of 0–40 nmol of AsA was used for calibration.

### Protein content

Protein content was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

### Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Trading Co. Ltd., Shanghai, China).

### Statistical analysis

Data presented are the mean values of three replicates, and were analysed using a one-way ANOVA model from the SPSS 13.0 package for Windows (SPSS Inc., Chicago, IL, USA). The significance of differences between control and treatment mean values was determined by Duncan's multiple range tests.  $P$  values < 0.05 were considered significant.

## Results

### *Effects of exogenous GSH and AsA on $\text{H}_2\text{O}_2$ and MDA contents in the chloroplasts of plants under salt stress*

As shown in Fig. 1, the  $\text{H}_2\text{O}_2$  content in the chloroplasts of both rice cultivars increased under NaCl stress compared to the respective controls, but addition of GSH or AsA inhibited this increase. On day eight under salt stress with 0.065 mM GSH, the  $\text{H}_2\text{O}_2$  contents of both rice cultivars Pokkali and Peta were lowered by 11% and 17.5%, respectively, compared to salt alone. Similarly, eight days after the addition of AsA to both culture solutions, the  $\text{H}_2\text{O}_2$  content decreased to 81% and 75.2% compared with that of the NaCl treatment.

MDA is a product of membrane lipid peroxidation caused by ROS, and is considered an important indicator of damage to cell membranes. In our study, the pattern of the MDA content was similar to that of  $\text{H}_2\text{O}_2$  under salt stress (Fig. 2). Under non-salt stress conditions, the MDA content of both cultivars decreased after treatment with either GSH or AsA, compared with the control. In response to the NaCl treatment, the MDA content in the chloroplasts of both cultivars increased quickly, but the increase was reduced in the presence of exogenous GSH or AsA in the culture solution. Thus, on day eight of GSH and AsA treatments, the MDA content of cultivar Pokkali was 71.8% and 69.5%, respectively, of that in the presence of NaCl

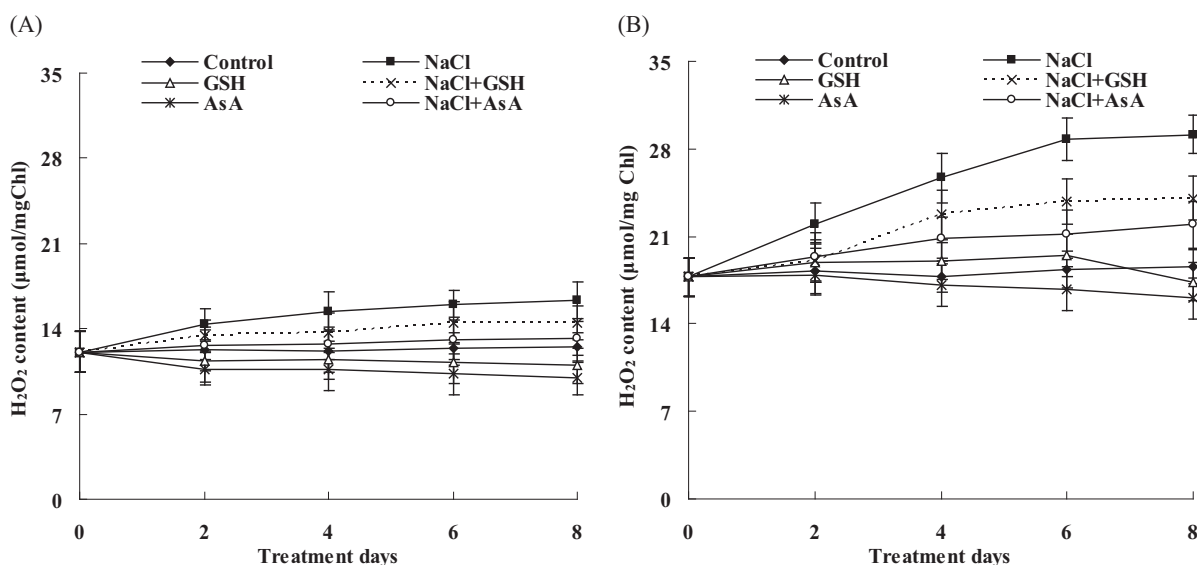


Fig. 1. Effects of exogenous GSH and AsA on the  $\text{H}_2\text{O}_2$  content in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

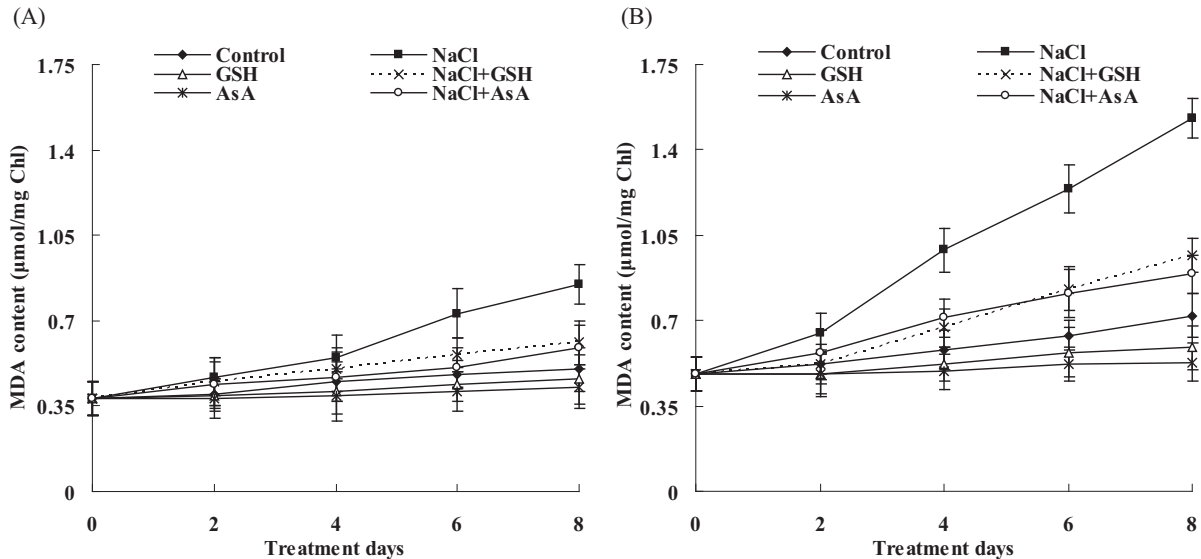


Fig. 2. Effects of exogenous GSH and AsA on the MDA content in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

alone. Comparable results were obtained with cultivar Peta, *i.e.* the MDA content in the NaCl + GSH and NaCl + AsA treatments decreased by 36.6% and 41.8%, respectively, compared with that of the NaCl treatment.

#### *Effects of exogenous GSH and AsA on SOD, APX, and GR activities in the chloroplasts under salt stress*

SOD activities in chloroplasts of the cultivars Pokkali and Peta exposed to GSH were much higher than those of the respective controls throughout the experiment, while there were no significant differences between AsA-treated and untreated plants (Fig. 3). When the two cultivars were treated with NaCl, SOD activities decreased significantly ( $P < 0.05$ ), and the effect was counteracted by exogenous GSH or AsA. By day eight of the NaCl + GSH treatment, SOD activities of the cultivars Pokkali and Peta increased to 160% and 337%, respectively, compared to the respective activities after NaCl treatment. SOD activities of the NaCl + AsA treatment after eight days were 128% and 245%, respectively, of those of the NaCl-treated plants.

When rice cultivars Pokkali and Peta were treated with exogenous GSH, APX activities increased by 26% and 13.4%, respectively, compared with the control, while the corresponding values for AsA treatment

were 31.4% and 28%, respectively (Fig. 4). APX activities of both cultivars increased at first and subsequently decreased with NaCl + GSH or AsA treatment, but still remained higher than in the NaCl-treated plants. At the end of treatment (day eight), APX activities in the cultivars Pokkali and Peta treated with NaCl + GSH were 132.6% and 148.1%, respectively, of those in the treatment with NaCl alone. The corresponding values for NaCl + AsA treatment were 149% and 175%.

As shown in Fig. 5, when treated with either exogenous GSH or AsA, there was a continuous rise in GR activities in both cultivars, which were significantly higher than those of the respective controls ( $P < 0.05$ ). By day eight, GR activities in the two cultivars treated with GSH increased by 33.9% and 28.8%, respectively, compared with the respective controls, while the corresponding values for AsA treatment were 21.3% and 116.7%. Under salt stress, GR activity tended to first increase and subsequently decrease in both cultivars when treated with either GSH or AsA; however, values remained higher than in NaCl-treated plants. At day eight of the NaCl + GSH treatment, GR activities in cultivars Pokkali and Peta had increased by 22.2% and 74.2%, respectively, compared with the NaCl treatment. Likewise, GR activities in the two cultivars treated with NaCl + AsA increased to 106% and 147.1%, respectively.

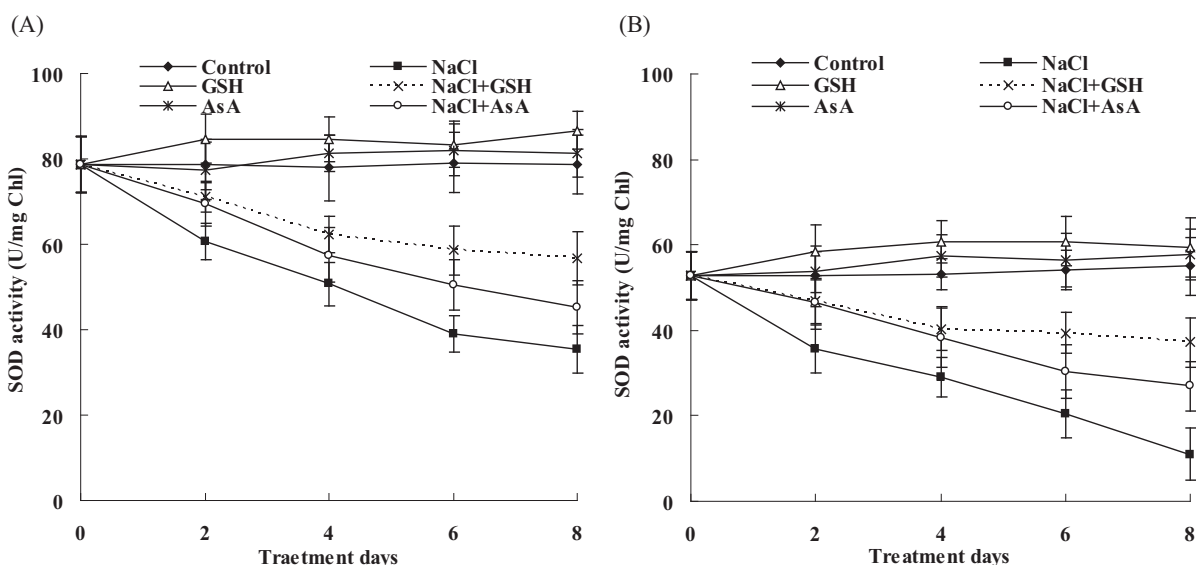


Fig. 3. Effects of exogenous GSH and AsA on the SOD activities in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

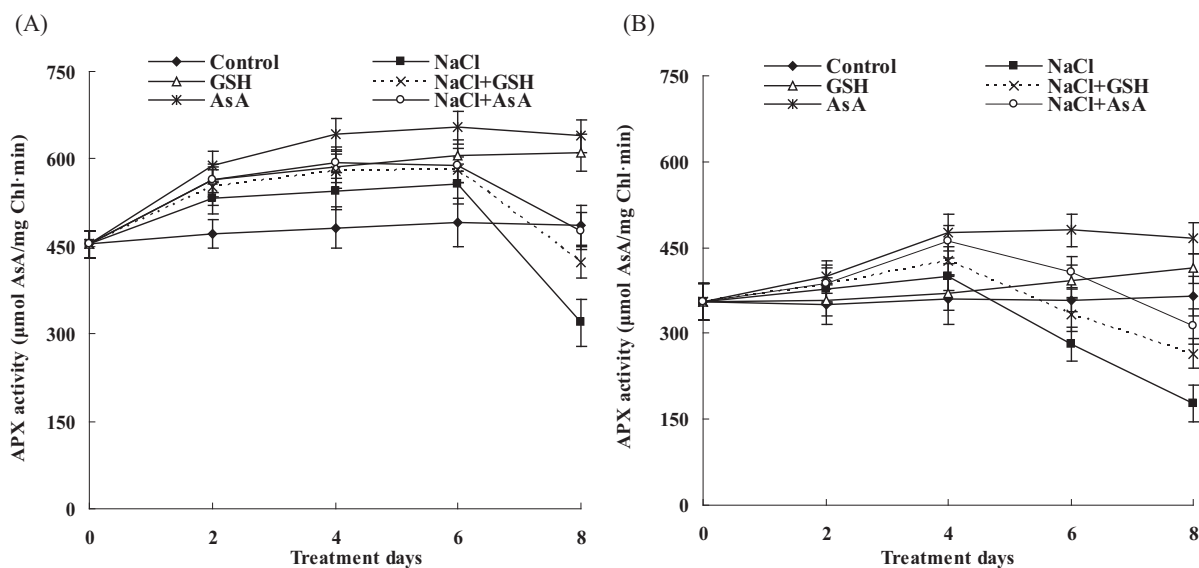


Fig. 4. Effects of exogenous GSH and AsA on the APX activity in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

#### *Effects of exogenous GSH and AsA on GSH and AsA contents in the chloroplasts of plants under salt stress*

Under salt stress, GSH contents in the chloroplasts of the cultivars Pokkali and Peta decreased greatly, but increased by 27 % and 50.5 %, respectively, in the pres-

ence of exogenous GSH, compared with NaCl treatment (Fig. 6). Although exogenous AsA treatment increased the GSH content in the chloroplasts of the cultivars Pokkali and Peta under non-salt stress conditions, such an effect was not seen in salt-treated plants.

AsA contents in the chloroplasts of the cultivars Pokkali and Peta treated with exogenous GSH were much higher than those in the respective controls (Fig. 7). On day eight of the treatment, the AsA content of the two cultivars had increased to 112.3% and

137.5%, respectively, compared to the controls. When the plants were treated with NaCl + GSH, AsA contents first increased and later declined, as was observed with the NaCl treatment. However, the AsA contents of cultivars Pokkali and Peta treated with

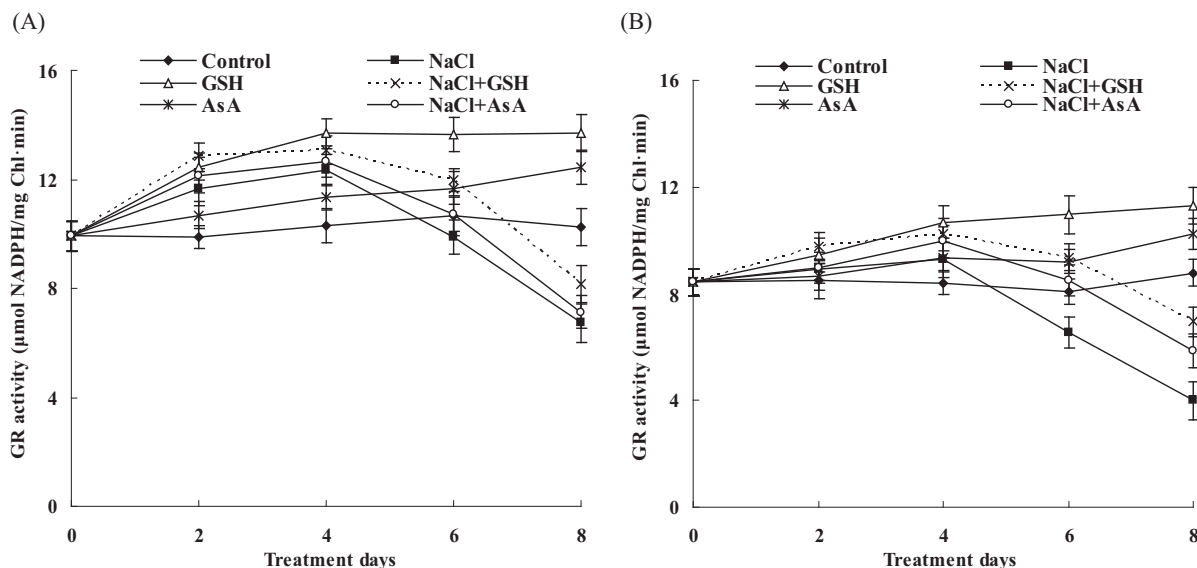


Fig. 5. Effects of exogenous GSH and AsA on the GR activity in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

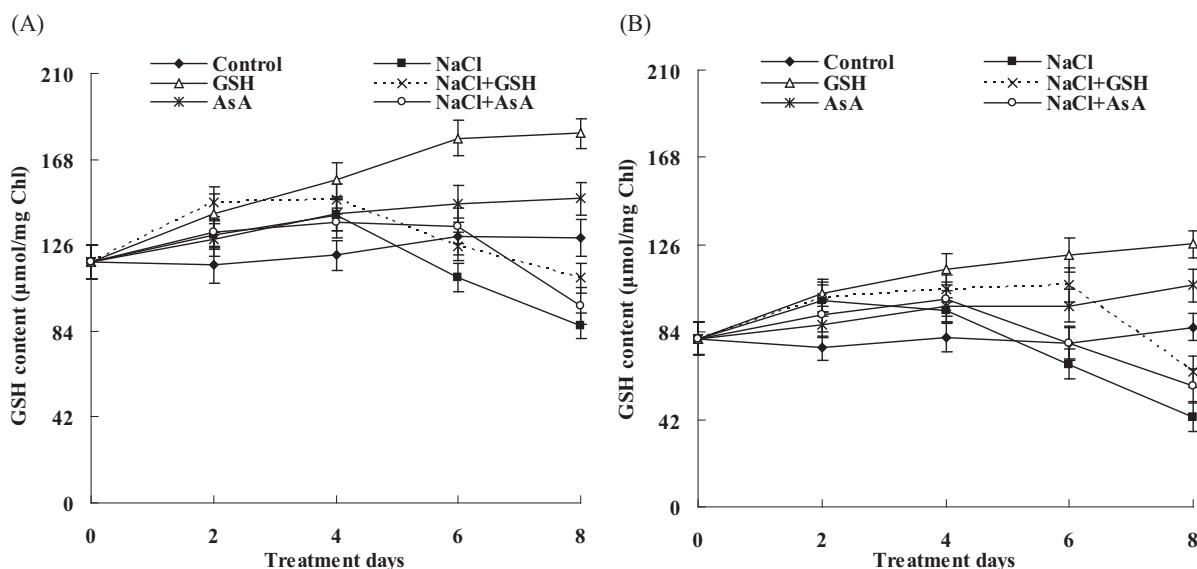


Fig. 6. Effects of exogenous GSH and AsA on the GSH content in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

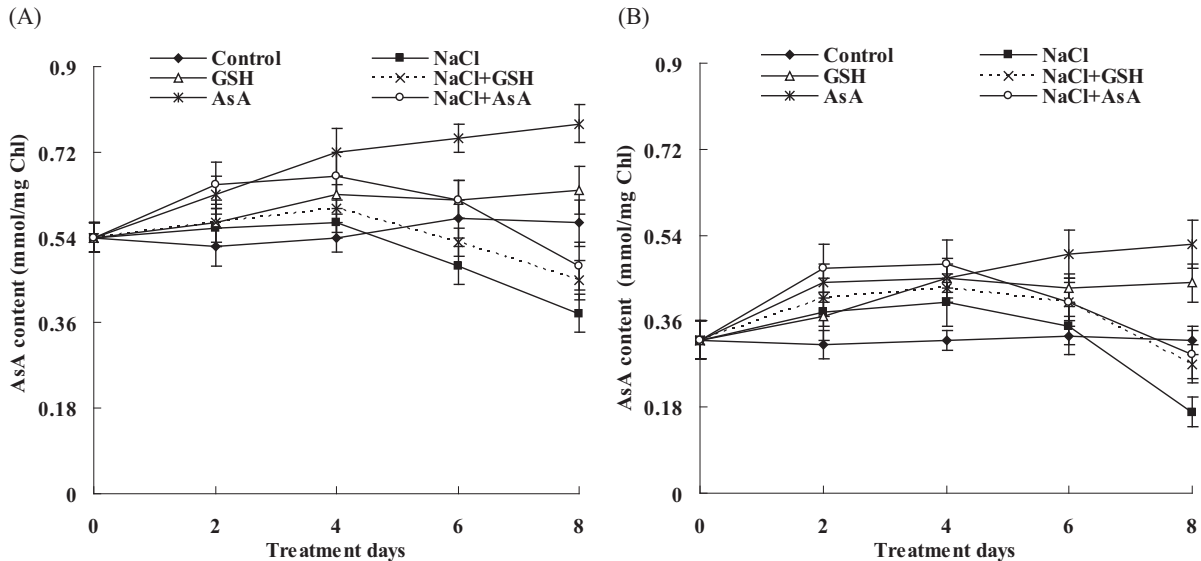


Fig. 7. Effects of exogenous GSH and AsA on the AsA content in chloroplasts of the rice cultivars (A) Pokkali and (B) Peta under salt stress. Values represent the mean  $\pm$  SE ( $n = 3$ ).

GSH were higher than those seen in the NaCl treatment. On day eight of the treatment, AsA contents of the chloroplasts of the cultivars Pokkali and Peta treated with NaCl + GSH had increased by 18.4 % and 58.8 %, respectively, compared with NaCl alone. Similar results were obtained for the exogenous AsA treatment.

## Discussion

Numerous stress factors, among them salinity, disturb the pro- and antioxidant balance in cells (Parida and Das, 2005). Overproduction of ROS is potentially harmful to all cellular components and negatively affects the cell metabolism. The most important primary site of ROS production in plants, both under normal and stress conditions, are chloroplasts (Sairam and Tyagi, 2004). In the absence of any protective mechanism, ROS can damage the chloroplast structure and function (Yamane *et al.*, 2004; Moradi and Ismail, 2007; Chaves *et al.*, 2009; Wang *et al.*, 2009). The antioxidative enzymes SOD and APX reduce oxidative damage to chloroplasts.  $O_2^-$  generated in photosystem I (PSI) can be immediately dismutated to  $H_2O_2$  by thylakoid-bound SOD.  $H_2O_2$  is scavenged by several enzymes of the AsA-GSH cycle. In this cycle, APX reduces  $H_2O_2$  to  $H_2O$  by oxidizing AsA to DHA. The latter is again reduced to AsA by GSH

which thereby is oxidized to GSSG. GR catalyzes the reduction of GSSG to GSH by NADPH. This leads to the scavenging of ROS, which are eventually detoxified to  $H_2O$  and  $O_2$  without producing another ROS (Asada, 1999). Therefore, AsA and GSH are essential antioxidants and ROS scavengers in chloroplasts. In the experiments presented here, the GSH content in the chloroplasts of the cultivars Pokkali (salt-tolerant) and Peta (salt-sensitive) increased significantly by the addition of GSH to the plants under non-salt stress conditions, meaning that exogenous GSH can be absorbed and transported to the leaves, and is then directed to the chloroplasts (Fig. 7). Moreover, exogenous GSH enhanced the GR activity in the chloroplasts of both rice varieties (Fig. 5). This suggests that GSH itself can stabilize the structure of biological macromolecules and protect the sulfhydryl (-SH) groups of enzymes and structural proteins from oxidation (Navari-Izzo *et al.*, 1997). Furthermore, the high GSH content in the chloroplasts promoted the AsA-GSH cycle thereby increasing the AsA content and raising the activities of APX, MDAR (monodehydroascorbate reductase), and GR in the chloroplasts. The combined effects of these enzymes result in the removal of ROS (Foyer and Noctor, 2011). As a result, lipid peroxidation of the chloroplast membranes is maintained at a low level.

Under salt stress, exogenous GSH significantly inhibited the decline of the SOD and APX activities



in Pokkali and Peta chloroplasts (Figs. 3 and 4). It also increased the GR activity and the AsA/GSH content in the chloroplasts (Fig. 5), which significantly reduced salt stress-induced peroxidation of membrane lipids, as evidenced by the marked decrease in the MDA content of the chloroplasts as compared to those from salt-stressed plants (Fig. 2). In addition, SOD, APX, and GR activities were higher in Peta as compared to Pokkali chloroplasts. Likewise, increases in the contents of AsA and GSH, and decreases in  $H_2O_2$  and MDA contents were also greater in Peta than in Pokkali cultivars when supplemented with exogenous GSH. This result indicates that stimulation of the ROS metabolism by exogenous GSH was more effective in the chloroplasts of the salt-sensitive as compared to the salt-tolerant cultivar. Our results also show that salt stress plus GSH and salt stress alone induce similar changes in the GR activity and GSH content in Pokkali and Peta chloroplasts, *i. e.* values increased at first and then decreased, indicating that the changes in the GR activity were related to the GSH content (Figs. 5 and 6), in accordance with the AsA-GSH cycle of the chloroplasts. Previous studies have reported an increase in the GSH content of plants resistant to drought and heat, which is due to the increase in GSH synthesis and/or the reduction of GSH degradation (Burke and Hatfield, 1987; Kocsy *et al.*, 2001). After four days under salt stress, the  $H_2O_2$  content in the chloroplasts, particularly in cultivar Peta, increased significantly, likely resulting in an increase in the DHA content within the chloroplasts. Hence more GSH will be needed to regenerate AsA in the AsA-GSH cycle, resulting in the observed decrease of the GSH content.

In the chloroplasts, AsA can directly or indirectly scavenge  $H_2O_2$ ,  $^1O_2$ ,  $OH^\cdot$ , and other ROS (Munne-Bosch and Alegre, 2002), whereby APX is a key enzyme in the removal of  $H_2O_2$ . Hence, the higher the concentration of AsA, the more substrate is available to APX to destroy  $H_2O_2$  in the chloroplasts (Asada, 1999). Our results show that, under salt stress, exogenous AsA reduced the  $H_2O_2$  content in the chloroplasts of the two rice cultivars and thereby reduced membrane lipid peroxidation (Figs. 1 and 2). These results are consistent with other reports (Shalata and Meumann, 2001; Zhang and Kirkham, 1996). Our experiments illustrate that exogenous AsA adjusts the antioxidant enzyme activity and content of the AsA-GSH cycle in the chloroplasts under salt stress. In addition,

exogenous AsA raised the SOD, APX, and GR activities and the AsA content, while it reduced the  $H_2O_2$  and MDA levels (Figs. 1–5), thus alleviating damage inflicted on the chloroplasts of both cultivars by overproduction of ROS induced by salt stress. A similar result was observed with exogenous GSH, *i. e.* protection of the ROS scavenging system in the chloroplasts under salt stress. In both rice cultivars, exogenous GSH was more effective than AsA in promoting SOD, APX, and GR activities, higher AsA and GSH contents, and lower  $H_2O_2$  and MDA levels, suggesting that exogenous GSH stimulates the AsA-GSH cycle more efficiently.

Over the experimental period, in the chloroplasts of the salt-tolerant cultivar Pokkali, the AsA content increased by about 40 % and the  $H_2O_2$  content decreased by approximately 40 % compared to the salt-sensitive cultivar Peta (Figs. 1 and 6), resulting in a 20–40 % higher MDA content in the Peta chloroplasts (Fig. 2). Hydrophilic AsA is found in the thylakoid lumen and stroma of chloroplasts (Asada, 1999), whereas lipophilic  $\alpha$ -tocopherol ( $\alpha$ -Toc) and  $\beta$ -carotene reside in the thylakoid membrane (Munne-Bosch and Alegre, 2002). Thus, AsA could protect  $\alpha$ -Toc and  $\beta$ -carotene from oxidation by removing or preventing the formation of  $OH^\cdot$  on the thylakoid membrane (Melis, 1999). Moreover, in the thylakoid membrane interface, AsA is assumed to regenerate  $\alpha$ -Toc from its  $\alpha$ -tocopheryl radical (Munne-Bosch and Alegre, 2002). In addition,  $\alpha$ -Toc and AsA act synergistically in eliminating  $^1O_2$ ,  $O_2$ , and  $OH^\cdot$  in chloroplasts. Because of the lower AsA content in the chloroplasts of the salt-sensitive cultivar Peta,  $\alpha$ -Toc is presumably less protected, resulting in higher lipid peroxidation of thylakoid membranes than in cultivar Pokkali.

Salt stress-induced photooxidative stress has been found here to be alleviated by exogenous AsA and GSH, resulting in improved salt-tolerance. The endogenous contents of AsA and GSH may be considered biomarkers for salt tolerance in rice (EI-Shabrawi *et al.*, 2010).

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