Plant Growth Regul (2007) 52:91–100
DOI 10.1007/s10725-007-9183-1

ORIGINAL PAPER

H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum

Baohui Ma · Jianmin Wan · Zhenguo Shen

Received: 4 October 2006 / Accepted: 22 March 2007 / Published online: 20 April 2007

Abstract The effect of Al stress on H_2O_2 production of rice (*Oryza sativa* L.) seedlings and difference in responses of antioxidant enzymes between Al-tolerant variety (Azucena) and Al-sensitive rice one (IR 64) were investigated. Aluminum-induced H_2O_2 production and malondialdehyde (MDA) content were more pronounced for IR 64 than for Azucena. In the presence of 2 mM Al, addition of 10 mM imidazole (inhibitor of NADPH oxidase) and 1 mM azide (inhibitor of peroxidase) significantly decreased H_2O_2 production by 16% and 43% for Azucena, and 21% and 68% for IR 64, respectively. Under Al treatment, the Al-tolerant variety Azucena had significantly higher activities of catalase, ascorbate peroxidase, dehydroascorbate reducase, glutathione peroxidase and glutathione reductase, and higher concentrations of reduced glutathione than the Al-sensitive one IR 64. Treatment with buthionine sulfoximine, a specific inhibitor of GSH synthesis, significantly increased H_2O_2 production in both varieties in the presence and absence of Al. In contrast, the treatment with GSH significantly decreased the production of H_2O_2 induced by Al stress. Results suggest that GSH may play an important role in scavenging H_2O_2 caused by Al stress.

Keywords Aluminum · Germination · Glutathione · Hydrogen peroxide · *Oryza sativa* L. · Root growth · Seedling

Abbreviations

Al	Aluminum
APX	Ascorbate peroxidase
BSO	Buthionine sulfoximine
CAT	Catalase
DHAR	Dehydroascorbate reducase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Aluminum is one of the most abundant elements in soils and becomes mobilized into the phytotoxic forms under acidic conditions (pH 5.0 and below). In acid soils, which comprise about 30–40% of the arable land in the world, Al toxicity is believed to be the major factor limiting crop growth and productivity (Kochian et al. 2004). Studies carried out in

B. Ma · Z. Shen (✉)
College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, P. R. China
e-mail: zgshen@njau.edu.cn

J. Wan
National Key Laboratory of Crop Genetics & Germplasm Enhancement, Nanjing 210095, P. R. China

different plant species have showed that Al is strongly phytotoxic and causes growth inhibition and even plant death, although the mechanisms involved in its toxicity are still not completely understood. The initial symptom of Al toxicity is an inhibition of root elongation, which can be observed within hours or minutes after exposure to Al at micromolar concentrations (Ma et al. 2004). Recently, numerous studies on Al toxicity have been carried out and several toxic effects of Al were described. For example, Al disrupted signal transduction pathways, in particular Ca^{2+} homeostasis and signaling, inhibited cell division and ion fluxes, disrupted cytoskeletal dynamics, and affected plasma membrane stability and function (Kochian et al. 2004). It seems that Al interacts with multiple sites of the root cells including cell wall, plasma membrane surface, cytoskeleton and nucleus.

In the previous works it was demonstrated that environmental stresses including changes in temperature, water deficiency and an excess of metallic ions cause molecular damage to plants, either directly or indirectly through the production of reactive oxygen species (ROS) (Richards et al. 1998; Mittler 2002). In order to protect from oxidative damage, plant cells have developed a wide range of endogenous defense mechanisms involving both enzymatic and non-enzymatic antioxidant systems (Howe and Schilmiller 2002; Mittler 2002). Enzymatic antioxidant systems include superoxide dismutases (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2), while non-enzymatic antioxidants include low molecular weight compounds such as ascorbic acid (AsA) and reduced glutathione (GSH). AsA and GSH can directly or indirectly interact with ROS and maintain the integrity of cell structures and the proper functions of various metabolic pathways (Noctor and Foyer 1998; Smirnoff 2000). Moreover, AsA and GSH also have roles in photosynthesis, redox signaling and growth regulation. During germination of some herbaceous plants, AsA content rapidly rise to cope with an increased level of ROS (De Gara et al. 1997). Changes in GSH content have also been reported in germinating seeds (Kranner and Grill 1993). However, little information is available about the relationship between the activities of antioxidant enzymes in germinating seeds as well as early seedlings and Al tolerance in plants.

The increasing evidence showed that Al^{3+} ions enhance the peroxidation of phospholipids and proteins (Yamamoto et al. 1997, 2001; Boscolo et al. 2003). Increased formation of ROS (presumably superoxide anion) was also detected in Al-sensitive tobacco cells (Yamamoto et al. 2002). Darkó et al. (2004) reported that the roots of Al-sensitive wheat accumulated more superoxides and peroxides than Al-tolerant plants, leading to less oxidative damage of the latter. However, it is still unknown whether the Al ion directly induces ROS including H_2O_2 because the Al ion cannot by itself catalyze redox reactions. It has been suggested that the Al-enhanced Fe-mediated peroxidation of lipids leads to the loss of plasma membrane integrity and eventually cell death (Yamamoto et al. 1997). Studies on the gene expression induced by Al treatments also demonstrated that Al stress activates several genes encoding antioxidant enzymes such as peroxidase, SOD and glutathione S-transferase (Richards et al. 1998; Ezaki et al. 1996, 2000), indicating common mechanisms induced by Al treatment and oxidative stress. Recently, it was suggested that ROS formation is a secondary response of Al stress and the oxidative stress is not the primary cause of Al induced root growth inhibition (Yamamoto et al. 2001; Boscolo et al. 2003).

In the present study, the activities of some antioxidant enzymes, responsible for detoxifying ROS, in germinating rice seeds and early seedlings of two varieties with differential Al tolerance, Azucena (Al-tolerant) and IR 64 (Al-sensitive), were evaluated. The concentrations of H_2O_2 and GSH were also determined in germinating rice seeds and early seedlings. The aims were to examine the effect of Al stress on H_2O_2 production of germinating rice seeds as well as early seedlings and to investigate difference in responses of antioxidant enzymes between Al-tolerant variety and Al-sensitive rice one.

Materials and methods

Plant material and germination condition

Rice seeds (*Oryza sativa* L. an upland rice variety Azucena—Al-tolerant and a lowland rice variety IR 64—Al-sensitive) were surface sterilized with 5% sodium hypochlorite (NaClO) for 10–15 min and then

washed thoroughly with distilled water. The seeds were geminated in Petri dishes on two filter papers fully moistened with 0.5 mM $CaCl_2$ solution containing 0 (control), 1, 2, 4 mM $AlCl_3$ at pH 4.1 in the dark at 25°C. To test the effects of NADPH oxidase or peroxidase inhibitions, 10 mM imidazole or 1 mM azide was added to the solution at the beginning of Al treatment. For assessing the role of GSH in reducing Al toxicity, 1 mM buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, or 20 mg/l GSH was added at the beginning of Al treatment. After 72 h from the beginning of experiments, root length and H_2O_2 content were measured, and Al accumulation in root apices was observed. The seedling samples, including shoots, roots and germinated seeds, were immediately frozen in liquid nitrogen until analysis. Each experiment was repeated at least three times.

Observation of Al accumulation

Rice roots exposed to 0, 1, 2, 4 mM $AlCl_3$ for 72 h were briefly rinsed with distilled water and then the root apices (0–1 cm) excised with a razor. The roots were stained with 0.1% Eriochrome Cyanine R and then observed with stereo microscope (Zeiss stemi 2000-C).

Measurement of H_2O_2 and lipid peroxidation

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 0.5 g plant samples with 3 ml of phosphate buffer (50 mM, pH 6.8). The homogenate was centrifuged at 6,000*g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000*g* for 15 min. The intensity of the yellow color of supernatant at 410 nm was measured. The amount of H_2O_2 was calculated from the standardized H_2O_2 curve.

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968).

Determination of GSH and GSSG content

Total glutathione was extracted from 0.7 g plant samples with 3 ml 5% sulfosalicylic acid and centrifuged at 14,000*g* for 10 min. Total glutathione and GSSG were determined by the 5,5'-dithiobis-(2-nitrobenzoic acid)-GR recycling procedure (Griffith 1980). Changes in absorbance of the reaction mixtures were measured at 412 nm and the total glutathione content was calculated from a standard curve with GSH. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. A specific standard curve with GSSG was used. GSH was determined by subtraction of GSSG from the total glutathione content.

Measurement of GPX activity

About 0.5 g plant samples were ground in 50 mM Tris–HCl pH 7.4 with the addition of 1 mM PMSF (Phenylmethylsulfonyl fluoride). The extract was centrifuged at 15,000*g* for 30 min and the supernatant used as the protein source for GPX (EC 1.11.19) assays. The assay was carried out according to Lawrence and Burke (1976).

Measurement of SOD, CAT, APX, GR, DHAR activities

Frozen plant samples (0.5 g) were homogenized with 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) with the addition of 1 mM AsA in the case of APX and DHAR assay in a chilled pestle and mortar. The homogenate was centrifuged at 15,000*g* for 20 min and the supernatant was used for determination of enzymatic activity. The whole extraction procedure was carried out at 4°C. APX activity was determined by monitoring the decrease at 290 nm as ascorbate was oxidized, as described by Nakano and Asada (1981). CAT activity was determined by following the consumption of H_2O_2 at 240 nm as described by Aebi (1984). The assay of DHAR activity was carried out by measuring the increase in absorbance at 265 nm due to ASA formation (Doulis et al. 1997). GR activity was determined by following the oxidation of NADPH at 340 nm according to the method of Schaedle and Bassham (1977). Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

Protein analysis

Protein was estimated according to Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Data were analyzed using the programs of SPSS 11.5 (the Statistical Package for the Social Science for Windows 11.5). All the values reported in this paper are the means of three replicates. Statistical assays were carried out by analysis of variance (ANOVA) test. Significant differences among means were determined by LSD at $P < 0.05$.

Results

Effect of Al on root length

Three days after the start of germination, root lengths were measured (see Fig. 1). Treatment with 1 mM Al for 3 d had no significant effect on the root elongation of both varieties. Increasing Al concentration from 1 mM to 4 mM resulted in a significant decrease in root elongation of both varieties. A more pronounced decrease of root growth was observed for IR 64 than for Azucena. The overall root growth after 3 d of germination at 2 and 4 mM concentration of Al decreased to 70% and 60% of the control plants for the variety Azucena, and 35% and 26% for the variety IR 64, respectively. It was evident that more Al was bind to the surface of the root apex of IR 64 than to that of Azuena after the roots were stained with 0.1% Erichrome Cyanine R solution (Fig. 2).

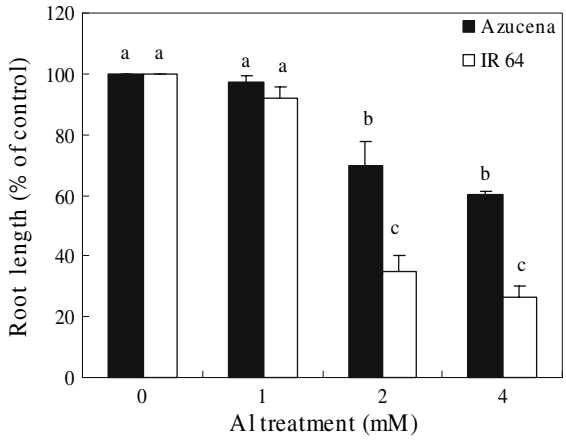

Fig. 1 Root growth of rice after seed germination with different Al treatment for 72 h. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

Effect of Al on the production of H_2O_2 and lipid peroxidation

Figure 3 shows Al stimulated H_2O_2 production by both rice varieties the 3rd day after germination. Aluminum-induced H_2O_2 production was more pronounced for IR 64 than for Azucena. The 1 mM Al treatment had no significant effect on H_2O_2 production by the seedlings of Azucena, but significantly increased production by the seedlings of IR 64. In the presence of 4 mM Al, H_2O_2 production in the Azucena increased by about 50%, and in IR 64 by 70%, respectively, in comparison with that in the control group.

In the presence of 2 mM Al, addition of 10 mM imidazole (inhibitor of NADPH oxidase) or 1 mM azide (inhibitor of peroxidase) significantly decreased H_2O_2 production (Fig. 4). Compared with the Al treatment alone, H_2O_2 production showed decreases of 16% and 43% with imidazole and azide for Azucena, and 21% and 68% for IR 64, respectively.

In the absence of Al, treatment of 1 mM BSO had no significant effect on root elongation of both varieties (Fig. 5). In the presence of 2 mM Al, addition of BSO to germination medium significantly decreased the root elongation of both varieties. Treatment with BSO significantly increased H_2O_2 production in the seedlings of both varieties in the presence and absence of Al (Fig. 6). In contrast, the treatment with 20 mg/l GSH significantly decreased the production of H_2O_2 induced by Al stress (Fig. 7).

The level of lipid peroxidation products, determined as malondialdehyde (MDA) with the TBA test, increased significantly with increasing Al in solution. The increase was more pronounced in IR 64 than in Azucena (Fig. 8).

Effect of Al on activities of antioxidative enzymes

In comparison to the control plants, a decreasing trend in CAT activity was found in both varieties as the Al concentration rose (Fig. 9). In contrast to CAT, APX and GR activities were stimulated by Al

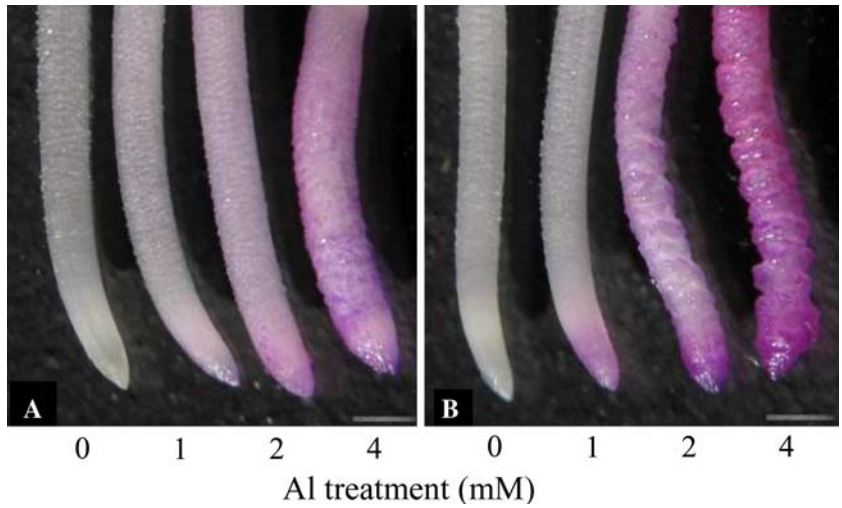

Fig. 2 Al accumulation in the Azucena (**A**) and IR 64 (**B**) root apices after seed germination with different Al treatment for 72 h. The roots were stained with 0.1% Eriochrome Cyanine R. Bar = 1 mm

treatment in Azucena, reaching the maximum values at 2 mM Al. While in IR 64, these activities did not change. The responses of GPX, using glutathione as a substrate, to Al treatment were different in the two varieties. In seedlings of Azucena, the activities of GPX increased as a result of treatment with 1 and 2 mM of Al but not with 4 mM of Al. However, in IR 64 seedlings, the GPX activities decreased following treatment with 2 and 4 mM of Al, and no decrease of GPX activity was observed at 1 mM Al. The activity of DHAR decreased with increasing Al in solution in IR 64, but it remained at the control value for every Al concentration tested in Azucena. Aluminum treatment had no significant effect on SOD activity in Azucena and IR 64. Interestingly, the activities of these enzymes were significantly higher in Azucena than in IR 64 except of SOD.

Effect of Al on glutathione level

Compared to the control, Al at 1 mM did not have any significant effect on the concentrations of GSH in the seedlings of both the rice varieties (Fig. 10). There was a significant increment in GSH concentrations when the seeds were exposed to 2 mM Al treatment. The increase was most pronounced in

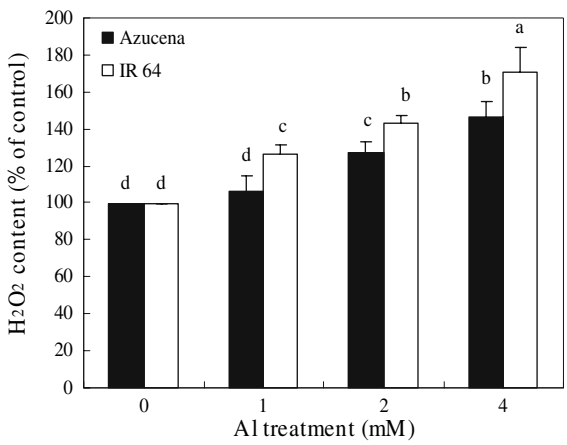

Fig. 3 Hydrogen peroxide content of rice after seed germination with different Al treatment for 72 h. Mean ± SE values (n = 3) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

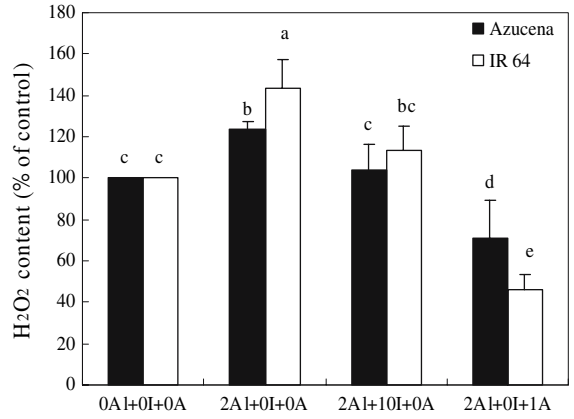

Fig. 4 Effect of 10 mM imidazole (I) as inhibitor of NADPH oxidase and 1 mM azide (**A**) as inhibitor of peroxidase on H_2O_2 content of rice in the presence or absence of 2 mM Al. Mean ± SE values (n = 3) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

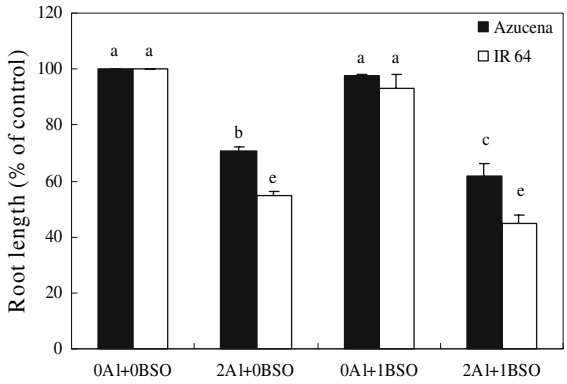

Fig. 5 Effect of 1 mM BSO as inhibitor of GSH synthesis on root growth of rice in the presence or absence of 2 mM Al. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

Azucena. However, by 4 mM Al GSH levels declined below those obtained at 2 mM Al. The responses of oxidized glutathione (GSSG) to Al in varieties Azucena and IR 64 were different. Considerable increase of GSSG concentration was observed in the seedlings of IR 64 exposed to Al treatments. In the seedlings of Azucena, GSSG concentration remained approximately constant. The presence of Al in the medium increased the GSH/GSSG ratio in seedlings of Azucena. In contrast, the GSH/GSSG ratio declined significantly in seedlings of IR 64. The GSH concentration and GSH/GSSG ratio were higher in Azucena than in IR 64.

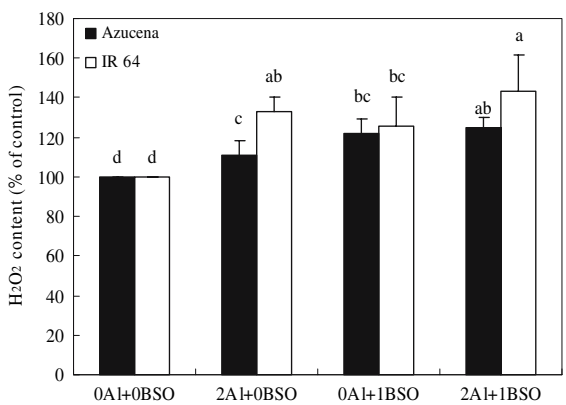

Fig. 6 Effect of 1 mM BSO as inhibitor of GSH synthesis on H_2O_2 content of rice in the presence or absence of 2 mM Al. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

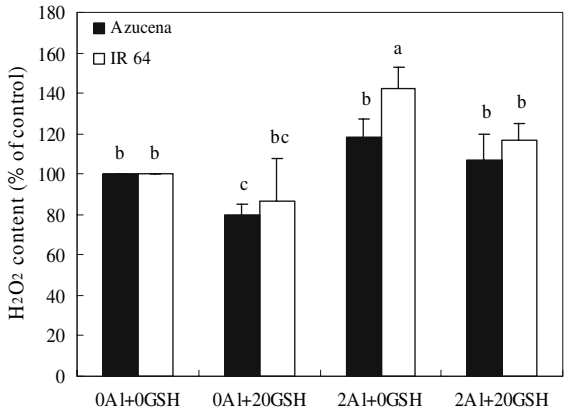

Fig. 7 Effect of 20 mg/l GSH supplied externally on H_2O_2 content of rice in the presence or absence of 2 mM Al. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

Discussion

During germination of orthodox seeds, cells switch from quiescent to very active metabolism. The recovery of respiratory activity inevitably produces ROS. In the present study, the increase in level of H_2O_2 was Al concentration dependent in rice seedlings treated with Al and was more significant for Al-sensitive rice variety IR 64 than for Al-resistant variety Azucena. Although the mechanism of Al-induced H_2O_2 generation is not clearly known, the result indicated that an oxidative stress was involved

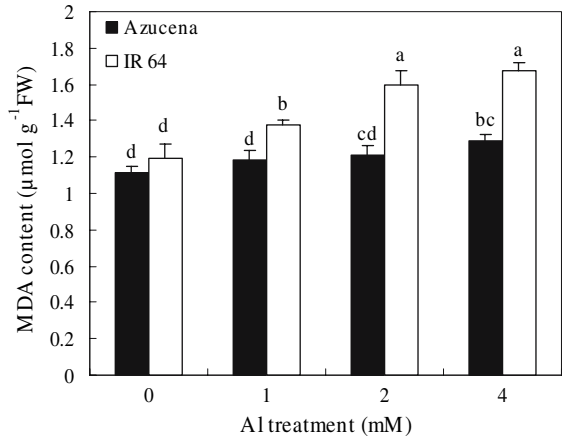

Fig. 8 Malondialdehyde content in rice after seed germination with different Al treatment for 72 h. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

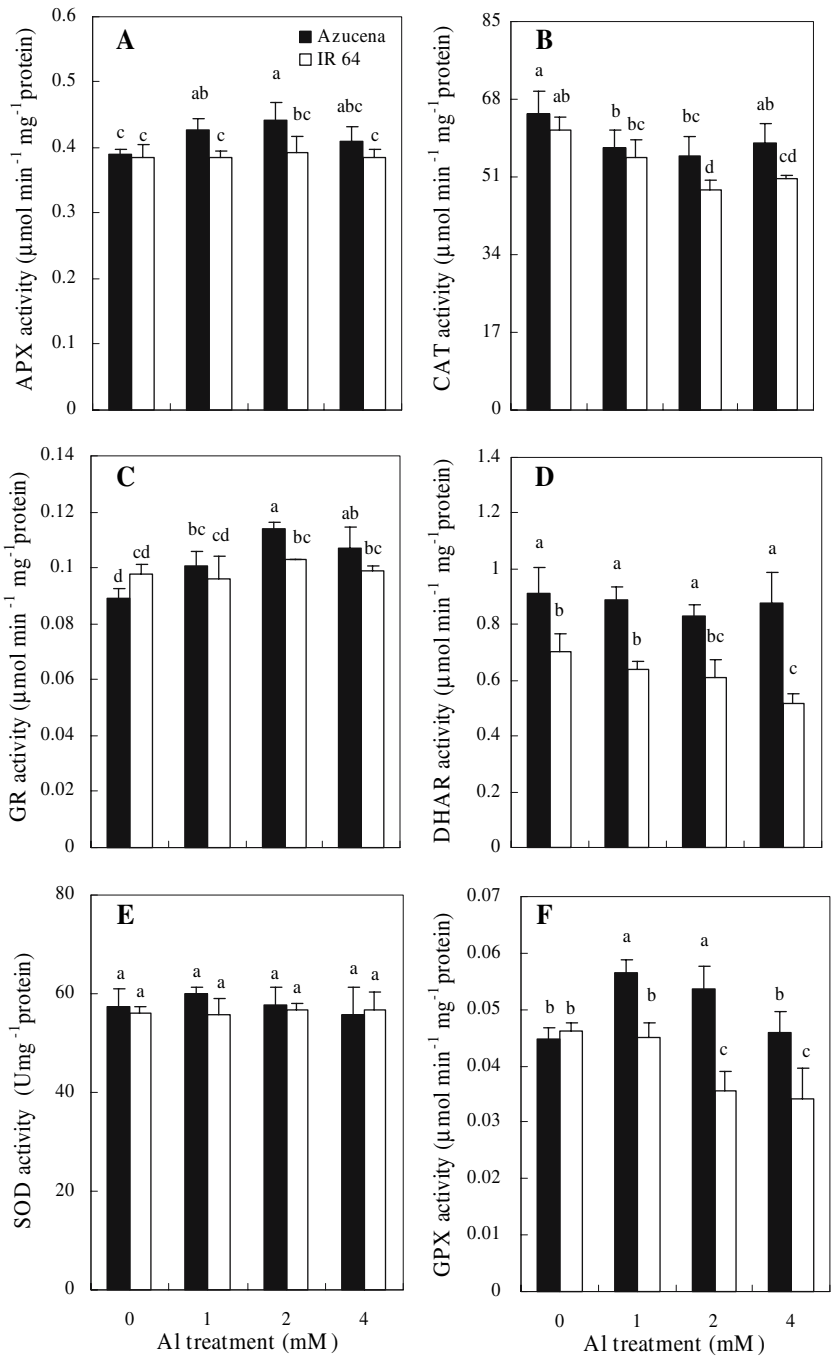

Fig. 9 APX (**A**), CAT (**B**), GR (**C**), DHAR (**D**), SOD (**E**) and GPX (**F**) activities of rice after seed germination with different Al treatment for 72 h. Mean ± SE values (n = 3) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

in the toxicity of Al during germination of rice seeds and early growth of seedlings exposed to Al treatments. It has been demonstrated that Al generates oxidative stress in *Arabidopsis thaliana* (Richards et al. 1998), *Triticum aestivum* L. (Darkó et al. 2004), *Nicotiana tabacum* and *Pisum sativum* (Yamamoto et al. 2002), by inducing production of ROS. In pumpkin (*Cucurbita pepo* L.) roots, maximum accumulation of Al in the root tip was correlated with increased production of H_2O_2 (Dipierro et al. 2005). Tamás et al. (2004) observed a considerable increase of H_2O_2 production in

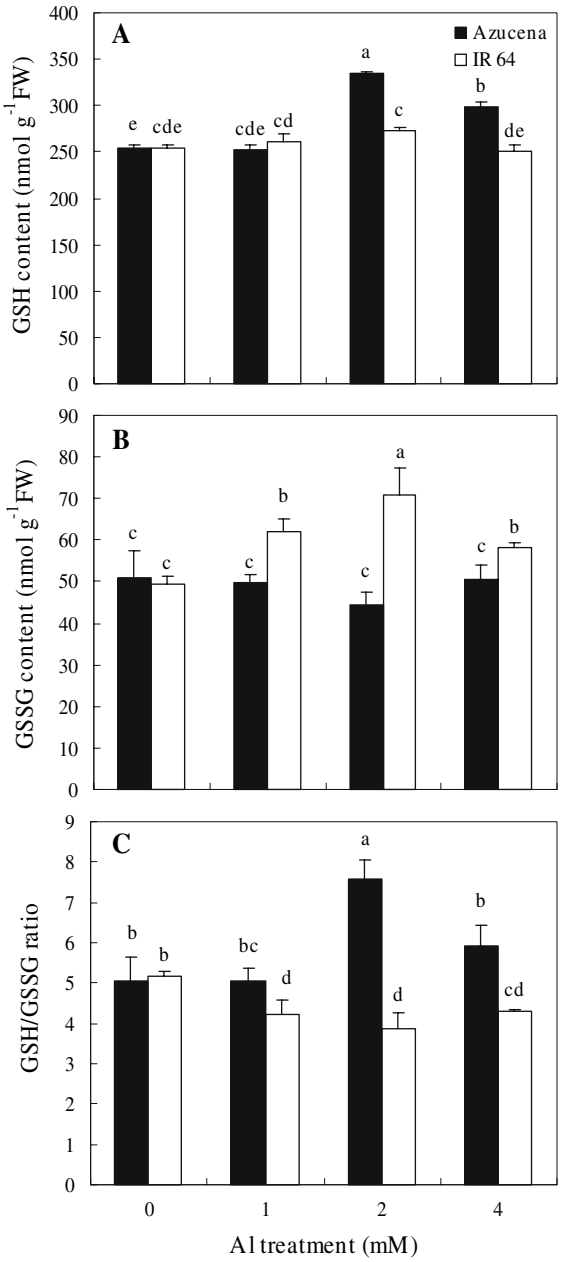

Fig. 10 GSH (**A**), GSSG (**B**) content and GSH/GSSG ratio (**C**) of rice after seed germination with different Al treatment for 72 h. Mean ± SE values ($n = 3$) followed by the same letter are not significantly different according to LSD test ($P < 0.05$)

germinating barley seeds treated with Al in comparison to control. In plants, the polymerization of hydroxy cinnamyl alcohols to lignin is catalyzed by peroxidases (POD) in the presence of H_2O_2. Lin and Kao (2001) suggested that elevated production of H_2O_2 of rice root during osmotic stress is probably involved in ferulic POD-catalysted cell wall stiffening which subsequently reduces root growth. High levels of H_2O_2 can also accelerate processes like Haber–Weiss reaction, resulting in formation of hydroxyl radicals that can cause lipid peroxidation (Neill et al. 2002). Seedlings of Al-tolerant variety Azucena exhibited a lower level of MDA, reflecting a lower extent of lipid peroxidation. Results suggested that Al-inhibited root growth of the sensitive variety IR 64 at least partly attributed to the toxic effects of H_2O_2. Some studies have shown that H_2O_2 can act as a mobile signal, alerting the plant to various environmental stresses. The role of enhanced production during germination of rice seed and early growth of seedlings in response to Al toxicity requires further experiments.

In plant cells, H_2O_2 can be generated by specific enzymes such as SOD, NADPH oxidase, xanthine oxidase, amine oxidase and cell wall peroxidase (Neill et al. 2002). Tamás et al. (2004) suggested that Al-induced enhanced H_2O_2 production during germination of barley seeds is catalyzed by NADH-dependent cell wall peroxidase. This was supported by the present result that the H_2O_2 production of germinating rice seeds and early seedlings was more sensitive to azide as peroxidase inhibitor than to imidazole as inhibitor of NADPH oxidase, suggesting that a peroxidase might be implicated in the production of H_2O_2 induced by Al.

Under most conditions, H_2O_2 in plants can be efficiently scavenged either by CAT or by the ascorbate-glutathione cycle where APX reduces it to H_2O. Our results showed that, among the enzymes involved in H_2O_2 removal under Al stress, CAT activity was the highest. GPX also catalyzes the reduction of H_2O_2 by GSH. As compared to those of the Al-sensitive variety IR 64, H_2O_2 content was lower in Al-tolerant variety Azucena under Al stress. It seems that H_2O_2 is quenched more efficiently in the Azucena than in IR 64, which is further confirmed by the fact that the activities of the H_2O_2 detoxifying enzymes were higher. Azucena showed an increase in activities of APX and GPX without decreasing GSH concentrations. For CAT, although similar activity decrease was observed in both varieties exposed to Al, the activity also was higher in Azucena compared to that of IR 64. Moreover, the Azucena maintained a higher activity of GR than IR 64 in all Al treatments,

indicating that GR might also contribute to the observed Al tolerance.

APX-mediated detoxification of H_2O_2 is coupled with ascorbate oxidation. AsA is then regenerated from its oxidized form via the oxidation of GSH (Noctor and Foyer 1998). Efficient recycling of GSH is ensured by GR activity. Although AsA was not monitored in the present study, the increase in APX, GR and higher DHAR activities of Al-treated Azucena can maintain AsA and GSH turnover and activation of the H_2O_2 scavenging AsA-GSH pathway. Compared to the Al-sensitive variety IR 64, the Al-tolerant one Azucena had significantly higher GSH concentration under Al treatment. Overexpression of GR in plants increases the antioxidant capacity and the resistance to oxidative stress (Kocsy et al. 2001). Increased GSH levels have been measured in extracts of several plants subjected to different stresses (Kocsy et al. 1996; Ruiz and Blumwald 2002). GSH is also involved in scavenging H_2O_2 by GPX, besides by contributing AsA regeneration (Noctor and Foyer 1998). Therefore, the increased levels of GSH may contribute to the Al tolerance in plants by enhanced oxidation of GSH to GSSG by dehydroascorbate reductase yielding AsA, sequentially protect cells from metal-related oxidative stress damage (Schützendübel and Polle 2002). Moreover, it is well established that not only GSH concentration, but also GSH/GSSG ratio is important to maintain cell redox status (May et al. 1998). Glutathione half-cell reduction potential correlated much better with seed viability than concentrations of GSH and GSSG alone (Kranner et al. 2006). In the present study, GSH/GSSG ratio increased in Al-tolerant rice variety and decreased in Al-sensitive one exposed to Al stress. The GR capacity was insufficient to maintain the redox status of the glutathione pool in IR 64. When GSH was supplied to plants externally, there was a diminished H_2O_2 production in IR 64. The treatment of BSO, a specific inhibitor of GSH biosynthesis, shows that decreased GSH levels correlated with increased production of H_2O_2, demonstrating the putative protective function of GSH. Kocsy et al. (2000) reported that inhibition of GSH synthesis reduced chilling tolerance in maize. These results showed that GSH played an important role in controlling the cell redox status and scavenging H_2O_2 caused by Al stress.

Acknowledgements This research project was supported by the National Natural Science Foundation of China (No. 30228023) and the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institutions of MOE.

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