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BIOTIC AND ABIOTIC STRESS

Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum

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Abstract When seedlings of rice (*Oryza sativa* L.) cultivar Pant-12 were raised in sand cultures containing 80 and 160 μM Al^{3+} in the medium for 5–20 days, a regular increase in Al^{3+} uptake with a concomitant decrease in the length of roots as well as shoots was observed. Al^{3+} treatment of 160 μM resulted in increased generation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), elevated amount of malondialdehyde, soluble protein and oxidized glutathione and decline in the concentrations of thiols (-SH) and ascorbic acid. Among antioxidative enzymes, activities of superoxide dismutase (SOD EC 1.15.1.1), guaiacol peroxidase (Guaiacol POX EC 1.11.1.7), ascorbate peroxidase (APX EC 1.11.1.11), monodehydroascorbate reductase (MDHAR EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1) and glutathione reductase (EC 1.6.4.2) increased significantly, whereas the activities of catalase (EC EC 1.11.1.6) and chloroplastic APX declined in 160 μM Al^{3+} stressed seedlings as compared to control seedlings. The results suggest that Al^{3+} toxicity is associated with induction of oxidative stress in rice plants and among antioxidative enzymes SOD, Guaiacol POX and cytosolic APX appear to serve as important components of an antioxidative defense mechanism under Al^{3+} toxicity. PAGE analysis confirmed the increased activity as well as appearance of new isoenzymes of APX in Al^{3+} stressed seedlings. Immunoblot analysis revealed that changes in the activities of APX are due to changes in the amounts of enzyme protein. Similar findings were obtained when the experiments were repeated using another popular rice *cv*. Malviya-36.

Keywords Aluminum stress · Antioxidative enzymes · Lipid peroxidation · *Oryza sativa* · Oxidative stress

Abbreviations

chl-APX	Chloroplastic ascorbate peroxidase
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
DHAR	Dehydroascorbate reductase
Guaiacol POX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
SOD	Superoxide dismutase

Introduction

Aluminum is a major factor reducing crop production in acid soils throughout the world (Kochian 1995; Pereira et al. 2006). Al preferentially accumulates in the root tips; it affects cell wall and plasma membrane characteristics, enhances Fe mediated peroxidation of lipids, oxidation of proteins (De Gara et al. 1993), interferes with signal transduction (Arroyo-Serralta et al. 2005) and binds directly to DNA or RNA (Simonovicova et al. 2004). Inhibition of

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root growth is one of the earliest and most dramatic symptoms exhibited by plants suffering from Al^{3+} toxicity (Kochian 1995; Ma et al. 2004; Wang and Kao 2006). When roots of growing plants are exposed to Al^{3+}, an Al-specific signal transduction occurs between roots and shoots that leads to myriad of toxicity symptoms in shoots (Cakmak and Horst 1991). Although Al itself is not a transition metal and cannot catalyze redox reaction, the involvement of oxidative stress in Al-toxicity has been suggested in many plant species (Cakmak and Horst 1991; Jones et al. 2006). Even without an external supply of Fe, enhanced peroxidation of lipids is observed due to Al in pea (*Pisum sativum*) roots (Yamamoto et al. 2001). As Al induces the expression of diverse genes in plant species like wheat, maize, sugarcane, tobacco, *Arabidopsis* and many of these genes encode antioxidant enzymes such as glutathione S-transferase, peroxidase, superoxide dismutase (SOD; Ezaki et al. 2000; Simonovicova et al. 2004) a strong correlation appears between Al toxicity and oxidative stress in plants (Boscolo et al. 2003; Watt 2004).

To defend against oxidative damage, plants possess non-enzymic and enzymic antioxidants, which play an important role in regulating the concentrations of reactive oxygen species (ROS). Among ROS scavenging enzymes SOD removes O_2^- by disproportionating it to H_2O_2 and O_2 and exists in three isoforms. These isoforms include a copper-zinc containing protein, sensitive to CN^- and predominantly presented in chloroplast, a CN^- insensitive Mn-SOD predominantly associated with mitochondria and a CN^- insensitive Fe-SOD located in chloroplast (Ushimaru et al. 1999). H_2O_2 produced is directly eliminated by catalase in peroxisomes and by guaiacol peroxidase (Guaiacol POX) in cytosol, vacuole, cell wall as well as in extracellular space. H_2O_2 is eliminated by the action of ascorbate–glutathione cycle involving successive redox reactions of ascorbate, glutathione and NADPH, which are catalyzed by ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in concert (Noctor and Foyer 1998). In plant cells ascorbate–glutathione cycle has been shown to operate in cytosol and all organelles in which ROS detoxification is needed (Edjolo et al. 2001; Kuniak and Sklodowska 2005). Unlike O_2^- and H_2O_2 other ROS such as $^1O_2$ and OH are detoxified mainly through non-enzymic reactions involving antioxidant molecules including ascorbate and glutathione in hydrophilic environment and α-tocopherol and carotenoids in membranes (Noctor and Foyer 1998).

Many reports suggest that the proportion of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of antioxidant system (Noctor and Foyer 1998). Rice serves as a staple food for the majority of world population. Aluminum toxicity is one of the major factors limiting rice production in acid soils and in flooded soils with pH less than 5.0 (Dobermann and Fairhurst 2000). Although the implication of the oxidative stress in aluminum toxicity is well documented in roots of other plant species (Cakmak and Horst 1991; Boscolo et al. 2003), to date very little information is available on the involvement of oxidative stress in the photosynthetic organ, i.e., shoots. In recent years many research groups have studied the effect of aluminum toxicity in different plant systems, yet the effect of aluminum toxicity in terms of content of ROS and ascorbate–glutathione cycle has not been studied in detail. Therefore, the present study was undertaken with the objectives to examine the effect of increasing concentrations of Al^{3+} in sand cultures on uptake of Al^{3+}, content of ROS, possible induction of oxidative stress and the status of antioxidant defense system including ascorbate–glutathione cycle in roots as well as shoots of growing rice plants. The isoenzymic profile of major H_2O_2 decomposing enzyme APX was also examined in aluminum-stressed seedlings. To get further insight into the regulation of expression of cytosolic and chloroplastic APX genes, their protein products were studied by immunoblot analysis using a specific monoclonal antibody. To the best of our knowledge this is the first report where increase in the APX activity under aluminum toxicity has been related to the increase in the corresponding protein amount and appearance of new isoenzymes.

Materials and methods

Plant material and stress condition

Seedlings of a commonly grown rice cultivar of India Pant-12 were used for conducting all the experiments. Similar experiments were performed using another rice *cv*. Malviya-36. Seedlings were raised for 20 days in plastic pots containing purified quartz sand saturated with nutrient solution consisting of 500 μM $Ca(NO_3)_2$, 500 μM KNO_3, 250 μM NH_4NO_3, 125 μM $MgSO_4$, 11 μM H_3BO_3, 2 μM KH_2PO_4, 2 μM $FeCl_3$, 2 μM $MnCl_2$, 0.35 μM $ZnCl_2$ and 0.2 μM $CuCl_2$ as described earlier (Richharia et al. 1997). Sand cultures were maintained at pH 4.5. Nutrient solution served as control and nutrient solution supplemented with 1 and 5 mM $Al_2(SO_4)_3$ served as treatment solutions. As the amount of available toxic Al species is related to the ionic strength of the nutritive solution the actual concentrations of toxic Al^{3+} in the treatment solution were determined using aluminon reagent (Hsu 1963). Aluminon acetate buffer was prepared by diluting 120 ml glacial acetic acid to 900 ml with water and adding 24 g NaOH. In the resulting solution 0.35 g aluminon was added, and diluted

to 1 l. The pH of the solution was maintained at 4.5. In a quartz cuvette (1 cm path length) 1 ml sample was pipetted and to it 0.7 ml aluminon reagent was rapidly added and absorbance was monitored after 60 s at 540 nm in a Bausch and Lomb Spectronic 20 Spectrophotometer. Treatment solution containing 1 and 5 mM $Al_2(SO_4)_3$ thus corresponded to 80 and 160 μM of active Al^{3+} respectively. Pots were maintained in a green house at 28 ± 1°C with 80% relative humidity and 12 h light/dark cycle (irradiance 40–50 μmol m^{-2} s^{-1}). For the determination of Al content, 10 mm roots from the bottom and whole shoots were used, whereas for the rest of the studies whole roots and shoots were used.

Determination of root, shoot length and aluminum uptake

To examine the effect of Al^{3+} on growth of root and shoot, seedlings were uprooted at 5, 10, 15 and 20 days and length of roots and shoots were determined based on ten random samplings in triplicate. Aluminum was estimated in roots and shoots of 10- and 20-day-old rice seedlings according to Yamamoto et al. (1994) using atomic absorption spectrophotometer (ELICO SL 173, India).

Measurement of superoxide anion (O_2^-) and hydrogen peroxide

The rate of superoxide anion production was measured by observing the autooxidation of epinephrine in terms of rate of adrenochrome formation (Mishra and Fridovich 1972). Formation of O_2^- was expressed as ΔA (480 nm min^{-1} g^{-1} tissue fresh weight). Amount of H_2O_2 was measured colorimetrically using titanium sulfate. H_2O_2 content was calculated using extinction coefficient 0.28 mM^{-1} cm^{-1} and was expressed as nmol g^{-1} tissue fresh weight (Jana and Chaudhuri 1981).

Estimation of lipid peroxides, total soluble protein and thiol

The concentration of lipid peroxidation products was determined in roots and shoots in terms of thiobarbituric acid reactive substances (TBARS) according to Heath and Packer (1968). An extinction coefficient 155 mM^{-1} cm^{-1} was used to quantify the concentration of lipid peroxides together with the oxidatively modified proteins and expressed as nmol g^{-1} fresh weight. Total soluble protein was quantified according to the method of Bradford (1976) using bovine serum albumin (Sigma) as standard. Protein thiol content and non-protein thiol content were determined according to the method of deKok and Kuiper (1986).

Assay of antioxidant enzymes

For extraction of SOD about 200 mg of fresh root and shoot samples were homogenized using prechilled mortar and pestle in 5 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1.0 mM EDTA, 0.1 mM Triton X-100 and 2% polyvinyl pyrrolidone (PVP). The contents were centrifuged at 22,000*g* for 10 min at 4°C. The supernatant was dialyzed in cellophane membrane tubings against the cold extraction buffer for 4 h with three to four changes of the buffer, and after dialysis in the supernatant the SOD was assayed according to the method of Mishra and Fridovich (1972). One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. Activities of different isoforms of SOD were assayed in presence of 3 mmol l^{-1} KCN (Fe-SOD) and 5 mmol l^{-1} H_2O_2 (Mn-SOD) in reaction mixture (Giannopolitis and Ries 1972).

The extraction medium for catalase was similar to that of SOD except that 50 mM Tris–NaOH buffer (pH 8.0) was used. The activity of catalase was assayed according to the method of Aebi (1983). The rate of H_2O_2 decomposition was measured at 240 nm (extinction coefficient of 0.036 mM^{-1} cm^{-1}) and enzyme specific activity was expressed as μmol H_2O_2 oxidized min^{-1} mg^{-1} protein.

Guaiacol peroxidase was extracted in 5 ml of cold 50 mM Na-phosphate buffer (pH 7.0) and assayed according to the method of Egley et al. (1983). The enzyme specific activity was expressed as μmol H_2O_2 reduced min^{-1} mg^{-1} protein. The extraction media for MDHAR, DHAR and GR were similar to that of SOD. MDHAR activity was assayed according to Hossain et al. (1984). The reaction was followed by measuring the decrease in absorbance at 340 nm due to NADH oxidation. The activity of DHAR was determined according to Doulis et al. (1997). The enzyme specific activity was expressed as μmol dehydroascorbate reduced min^{-1} mg^{-1} protein. The activity of GR was assayed according to Foyer and Halliwell (1976). The enzyme specific activity was expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein.

Ascorbate peroxidase was extracted in 5 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ascorbic acid, 1 mM EDTA and 2% PVP added fresh just prior to use. The activity of APX was assayed according to Nakano and Asada (1987) using assay mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM ascorbic acid, 0.2 mM EDTA, 20 μM H_2O_2 and 50 μl enzyme extract in a total volume of 1 ml. The enzyme specific activity was expressed as μmol ascorbate oxidized min^{-1} mg^{-1} protein.

To study chloroplastic ascorbate peroxidase (chl-APX), chloroplasts were isolated from leaves of seedlings

according to the method of Atal et al. (1991). Freeze-thaw extracts of chloroplasts were prepared and chloroplastic APX was assayed as described earlier. In order to confirm that preparations are real chloroplasts and to examine the expression of chloroplastic APX protein, immunoblot analysis was done using mouse monoclonal antibody raised against spinach chloroplastic APX (Cosmo Bio Co. Ltd., Tokyo, Japan).

Isoenzymic profile and immunoblot analysis of APX

To study the influence of Al^{3+} toxicity on the isoenzymic pattern of APX, 20-day-old seedlings of rice *cv*. Pant-12 were used. Dialyzed enzyme extracts prepared from roots and shoots as well as from chloroplasts corresponding to 300 µg proteins were loaded on a 7.3% polyacrylamide resolving gel (De Gara et al. 1993). After electrophoresis at 4°C the gels were incubated for 15 min at room temperature in 0.1 M sodium phosphate buffer (pH 6.4) containing 4 mM ascorbate and 4 mM H_2O_2. The gels were washed with water and then stained for 10 min with 0.1% ferricyanide and 0.1% ferrichloride in 0.125 M HCl (De Gara et al. 1993). The isoenzymes appeared as colorless bands on Prussian-blue background.

For immunoblot analysis, dialyzed enzyme extracts prepared from roots and shoots as well as from chloroplasts corresponding to 100 µg protein from control as well as Al stressed seedlings were separated on a 12.5% polyacrylamide gel according to the standard SDS-PAGE procedure of Laemmli (1970) and then transblotted on to a nitrocellulose membrane using an electroblot apparatus with the transfer buffer of Towbin et al. (1979). For cyt-APX, the membrane was incubated with a mouse monoclonal antibody [EAP 1, Ishikawa et al. (1996)] raised against the purified Euglena APX and for chl-APX, mouse monoclonal antibody raised against spinach chloroplastic APX (Cosmo Bio Co. Ltd., Tokyo, Japan) were used. The reacted proteins were detected with alkaline phosphatase conjugated goat antimouse IgG (Genei, Bangalore, India) as the secondary antibody and NBT/BCIP (Genei, Bangalore, India) as chromogenic substrate.

Image analysis

The photographs of activity gels and APX immunoblots were scanned with an image analyzer (model LAS-1000 Plus Fuji Photo film Co. Ltd., Japan).

Estimation of ascorbate and glutathione pool

The contents of reduced ascorbate (AsA), dehydroascorbate and total ascorbate (AsA + DHA) were determined spectrophotometrically according to the method of Law et al. (1983). The amounts of reduced glutathione, oxidized glutathione (GSSG) and total glutathione were determined according to the method of Griffith (1980).

Statistical analysis

All the experiments were performed in triplicate. The values in the tables and figures indicate the mean values ± s.d. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Effect of Al on root and shoot length

Figure 1a shows the effect of increasing concentrations of Al^{3+} on the length of root and shoot of 5-, 10-, 15- and 20-day-old rice seedlings. With 160 µM Al^{3+} in the growth medium about 39–46% reduction in the length of roots and

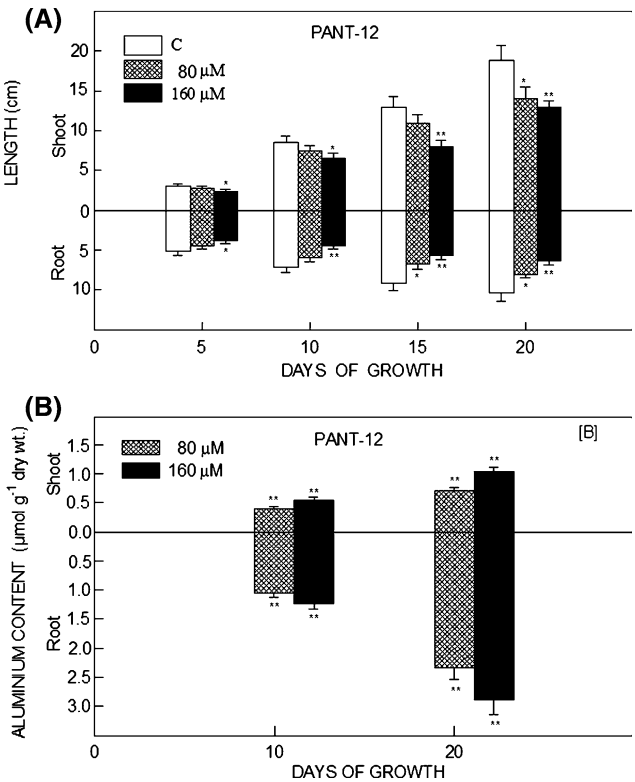

Fig. 1 Effect of increasing concentrations of aluminum (Al^{3+}) in sand cultures on **a** length of roots and shoots and **b** content of Al^{3+} in roots/shoots of growing seedlings of rice *cv*. Pant-12. Values are mean ± s.d. based on three independent determinations and *bars* indicate standard deviations. * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

about 32% reduction in the length of shoots were observed in 20-day-old seedlings. With increasing time of exposure to Al^{3+} (10–20 days) reduction in the length of the shoots and roots was observed.

Uptake and distribution of Al in growing rice seedlings

Figure 1b shows the concentration of absorbed Al in roots and shoots of 10- and 20-day-old Al^{3+} stressed rice seedlings. As it is evident, with increase in the age of seedlings, a higher content of Al^{3+} was noticed in the plant parts. Localization of absorbed Al^{3+} was always much greater in the roots than in the shoots. In 160 μM Al^{3+} treated rice seedlings 2.8–4.0 μmol Al^{3+} g^{-1} dry wt. was noticed in roots and 1.0–1.2 μmol Al^{3+} g^{-1} dry wt. in shoots.

Effect of Al on generation of reactive oxygen species

During the entire growth period of 5–20 days Al^{3+} treatment in situ led to a concomitant increase in the content of O_2^- in both the roots as well as the shoots of the rice seedlings. With 160 μM Al^{3+} treatment about 43–80% increase in O_2^- content was observed in the roots and 56–60% increase in the shoots of 15-day-old seedlings as compared to the contents in control seedlings (Fig. 2a). Al^{3+} treatment of 80 μM led to a decline in the amount of H_2O_2 as compared to control; however, with a higher Al^{3+} treatment of 160 μM an increase in H_2O_2 content was observed. About 8–18% decline in H_2O_2 content was observed in 80 μM Al^{3+} stressed rice seedlings whereas in 160 μM Al^{3+} stressed seedlings about 10–13% increase in

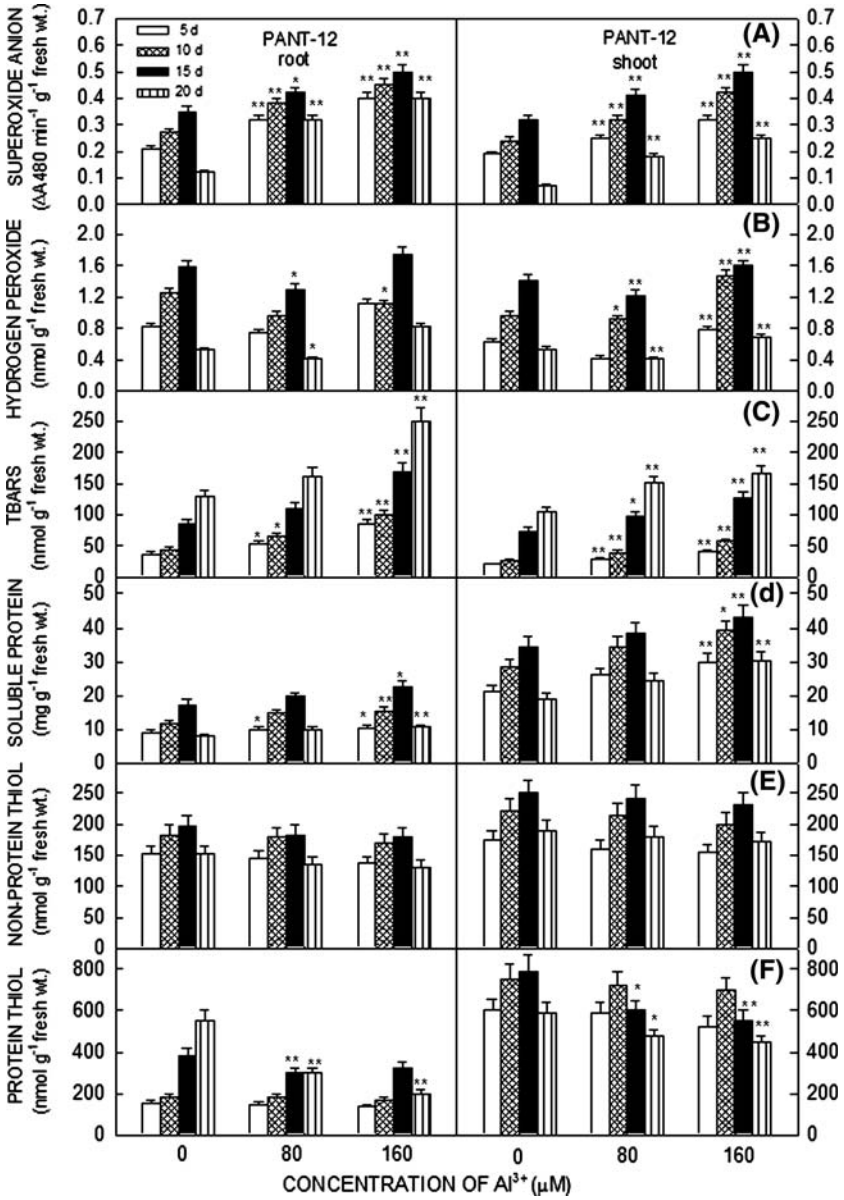

Fig. 2 Effect of increasing concentrations of Al^{3+} in the growth medium on the contents of **a** superoxide anion **b** hydrogen peroxide **c** thio barbituric acid reactive substances (TBARS) **d** soluble protein **e** non-protein thiol and **f** protein thiol in roots and shoots of seedling of rice *cv*. Pant-12 at different days of growth. Values are mean ± s.d. based on three independent determinations and *bars* indicate standard deviations. * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

H_2O_2 content was observed as compared to the content in control seedlings (Fig. 2b).

Effect of Al on lipid peroxidation, total soluble protein and thiol

Increasing concentrations of Al^{3+} in the growth medium led to a concomitant increase in lipid peroxidation in both the roots as well as the shoots of rice seedlings. With 160 μM Al^{3+} treatment about 99–137% increase in TBARS content was observed in the roots and 73–76% increase in the shoots of 15-day-old seedlings as compared to the contents in controls (Fig. 2c). A gradual increase in the amount of total soluble protein was observed in the seedlings with Al treatment. In the presence of 160 μM Al^{3+}, 15-day-old rice seedlings showed about 12–36% increase in the content of total soluble protein as compared to controls (Fig. 2d). The content of protein thiol as well as non-protein thiol decreased in the seedlings due to Al^{3+} treatment. With 160 μM Al^{3+} treatment in situ a decline of about 7–10% in non-protein thiol and about 11–20% in the content of protein thiol was observed in the 15-day-old rice seedlings as compared to the contents in controls (Fig. 2e).

Effect of Al on the activities of antioxidative enzymes

With increase in the concentrations of Al^{3+} in the growth medium, a concomitant increase in SOD activity was observed in both the roots as well as the shoots of rice seedlings. About 25–32% increase in total SOD activity was observed in the roots and 48% increase in the shoots of 160 μM Al^{3+} stressed 15-day-old seedlings. Similarly about 28–39% increase in the activity of Cu/Zn-SOD, 32–52% increase in the activity of Fe-SOD and 49–53% increase in Mn-SOD activity were observed in the shoots of 160 μM Al^{3+} stressed rice seedlings (Fig. 3a–e).

Seedlings treated with 80 μM Al^{3+} showed increased catalase activity compared to control seedlings, whereas with a higher Al^{3+} treatment of 160 μM, an inhibition in catalase activity was observed (Fig. 4a). About 9–17% increase in catalase activity was observed in roots and shoots of 15-day-old 80 μM Al^{3+} stressed rice seedlings whereas about 10–22% decline in enzyme activity was noticed in 160 μM Al^{3+} stressed rice seedlings. Increasing concentrations of Al^{3+} in the growth medium led to a concomitant increase in Guaiacol POX activity in roots as well as shoots (Fig. 4b). Seedlings treated with 160 μM Al^{3+} for 15 days showed about 26–76% increase in Guaiacol POX activity compared to control seedlings.

The enzymes of ascorbate–glutathione cycle MDHAR, DHAR, as well as GR showed significant increases in activity in Al^{3+} treated rice seedlings as compared to controls, and the increase was more pronounced in the

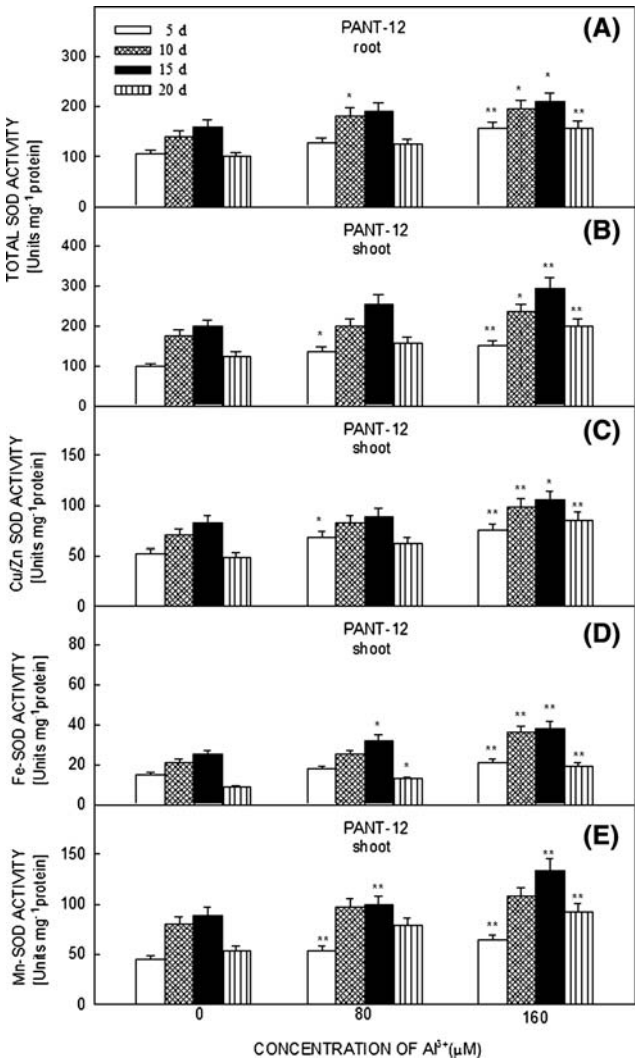

Fig. 3 Effect of increasing concentrations of Al^{3+} in the growth medium on the specific activities of total superoxide dismutase (total-SOD) in **a** roots and **b** shoots and **c** Cu/Zn-SOD **d** Fe-SOD and **e** Mn-SOD in shoots of seedlings of rice *cv*. Pant-12 at different days of growth. Values are mean ± s.d. based on three independent determinations and *bars* indicate standard deviations. * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

80 μM Al^{3+} treated seedlings as compared to the 160 μM Al^{3+} treated seedlings (Fig. 4c–e). The activity of APX increased in 160 μM Al^{3+} treated seedlings. Seedlings treated with 160 μM Al^{3+} for 20 days showed about 82–150% increase in APX activity as compared to control seedlings (Table 1). The activity of chl-APX decreased in seedlings treated with 160 μM Al^{3+} (Table 1).

Effect of Al on isoenzymic profiles and amount of APX proteins

Two major activity bands of APX (APX 1 and APX 2) were revealed in enzyme preparations from shoots (Fig. 5

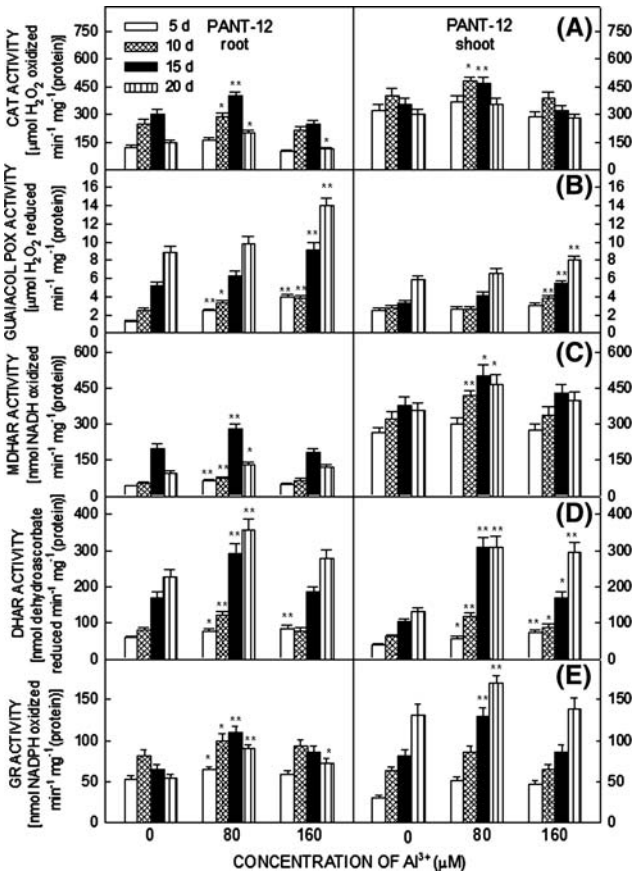

Fig. 4 Effect of increasing concentrations of Al^{3+} in the growth medium on the specific activities of **a** catalase (CAT) **b** guaiacol peroxidase (Guaiacol POX) **c** monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and **d** glutathione reductase (GR) in roots and shoots of seedlings of rice *cv*. Pant-12 at different days of growth. Values are mean ± s.d. based on three independent determinations and *bars* indicate standard deviations. * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

a) and only one enzymic band was detected in chl-APX preparation (Fig. 5b) from control as well as Al stressed seedlings. With increase in the concentrations of Al^{3+} treatment the intensity of APX 1 increased in shoots, whereas the intensity of activity band APX 2 as well as of chl-APX increased in 80 μM Al^{3+} stressed seedlings but the intensity declined in 160 μM Al^{3+} treated seedlings (Fig. 5d, e). In the roots of control seedlings four major activity bands (APX 1′, APX 2′, APX 3′ and APX 4′) were seen and the intensities of bands 2′, 3′ and 4′ increased with increase in the concentration of Al^{3+} treatment. Two new APX isoenzymic bands APX 5′ and APX 6′ appeared in the enzyme preparations from the roots of 160 μM Al^{3+}-treated seedlings (Fig. 5c, f).

Immunoblot analysis of APX revealed only one band of cytosolic APX (cyt-APX 1) in shoots and two (cyt-APX 1′ and cyt-APX2′) in roots of control as well as Al^{3+} stressed seedlings and the amount of these cyt-APXs was greater in Al^{3+} stressed seedlings as compared to controls. Al^{3+} treatment of 160 μM led to 55% increase in the amount of cyt-APX 1, 39% increase in the amount of cyt-APX 1′ and 60% increase in the amount of cyt-APX 2′ (Fig. 6a–d). Immunoblot analysis of chl-APX in extracts prepared from chloroplasts revealed only one band in control as well as Al^{3+} stressed rice seedlings. Al^{3+} treatment of 80 μM led to about 20% increase in the amount of chl-APX whereas 160 μM Al^{3+} led to about 55% decline in chl-APX protein (Fig. 6e, f).

Effect of Al on ascorbate and glutathione pool

Seedlings treated with Al^{3+} had decreased amounts of reduced ascorbate (AsA), total ascorbate (DHA + AsA), a decreased AsA/DHA ratio and an elevated amount of dehydroascorbate during the entire 5–20 days' growth period (Fig. 7a–d). In 160 μM Al^{3+} treated 15-day-old seedlings about 60–74% decline in AsA/DHA was observed as compared to the control seedlings. In 80 μM Al^{3+} treated 20-day-old rice seedlings significant increases in the amount of reduced glutathione as well as total glutathione were observed, whereas no significant change in the amount of GSSG as well GSH/GSSG ratio could be observed as compared to controls. With a high Al^{3+} concentration of 160 μM, significant increase in the amounts of GSSG and decline in GSH/GSH ratio was observed, whereas no significant change was observed in the amount of GSH and total glutathione (Fig. 7e–h). Al^{3+} concentration of 80 μM resulted in about 30–40% increase in the amount of GSH and 28–35% increase in the amount of total glutathione in 20-day-old seedlings, whereas under similar conditions 160 μM Al^{3+} stressed seedlings showed 25–36% increase in GSSG and about 36–38% decline in GSH/GSSG as compared to the levels in the control seedlings.

Discussion

There is an increasing evidence suggesting that oxidative stress is a key damaging factor in plants exposed to a variety of stressful conditions including metal toxicity and that the plants resist oxidative stress by inducing the activities of antioxidative enzymes (Cuypers et al. 2002; Verma and Dubey 2003). Therefore the present study was undertaken to contribute to the understanding of the relationship between Al toxicity, oxidative stress and antioxidative defense system. Though the experiments were initially conducted using seedlings of rice *cv*. Pant-12, similar findings were obtained when the experiments were repeated using another rice *cv*. Malviya-36.

Table 1 Effect of 160 μM Al^{3+} in situ on the activities of ascorbate peroxidase (μM ascorbate oxidized min^{-1} mg^{-1} protein) in roots and shoots and chloroplastic ascorbate peroxidase (μM ascorbate oxidized min^{-1} mg^{-1} protein) extracted from shoots of rice *cv*. Pant-12

Cultivar	Age of seedlings	Al^{3+} treatment	Ascorbate peroxidase (μM ascorbate oxidized min^{-1} mg^{-1} protein)		Chloroplastic–ascorbate peroxidase (μM ascorbate oxidized min^{-1} mg^{-1} protein)
			Root	Shoot	Shoot
Pant-12	10 days	0	0.78 ± 0.05	0.56 ± 0.03	4.60 ± 0.27
		160 μM	1.58 ± 0.09**	1.38 ± 0.08**	4.20 ± 0.21
	15 days	0	1.68 ± 0.16	0.96 ± 0.08	4.96 ± 0.49
		160 μM	2.36 ± 0.26**	1.68 ± 0.17	4.74 ± 0.47
	20 days	0	1.42 ± 0.08	0.48 ± 0.03	3.90 ± 0.19
		160 μM	3.55 ± 0.20*	0.91 ± 0.05**	3.72 ± 0.19

Data represent mean values ± s.d. based on three independent determinations

*, **Represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

It was interesting to note that with increasing exposure time to Al^{3+}, the growing seedlings readily absorbed Al, and Al was then transported to above-ground parts, though its localization was greater in the roots indicating that the roots are the primary sites of Al accumulation. The concentration of Al in cultivated plants is usually higher in the roots than in the shoots. Small quantities of Al are transported or accumulated in the shoots (Kinraide et al. 1992; Meriga et al. 2003). Meriga et al. (2003) proposed that a greater accumulation of Al in the roots than in the shoots might be because of inactivation of Al in the roots, which seems to prevent Al transport to the shoots. A significant decrease in the length of roots and shoots was observed in Al stressed seedlings as compared to controls. The reduction in root length is a common feature associated with Al toxicity in many crops (Cakmak and Horst 1991; Kochian 1995; Ma et al. 2004). Until now, several hypotheses have been suggested for Al toxicity mechanisms, such as interaction with cell wall (Pereira et al. 2006), alterations in root cytoskeleton (Sivaguru et al. 1999), changes in cytosolic free Ca^{2+}, rapid callose formation (Sivaguru et al. 2005) and production of ROS (Jones et al. 2006). The decrease in shoot length of Al-stressed seedlings could be due to Al-specific signal transduction between roots and shoots (Larsen et al. 1997).

In our studies, with the increase in the concentration of Al^{3+} in the growth medium there appeared to be an increase in the content of O_2^-, TBARS, total soluble proteins and decline in thiol groups in growing rice seedlings. These results suggest that Al^{3+} toxicity is associated with the

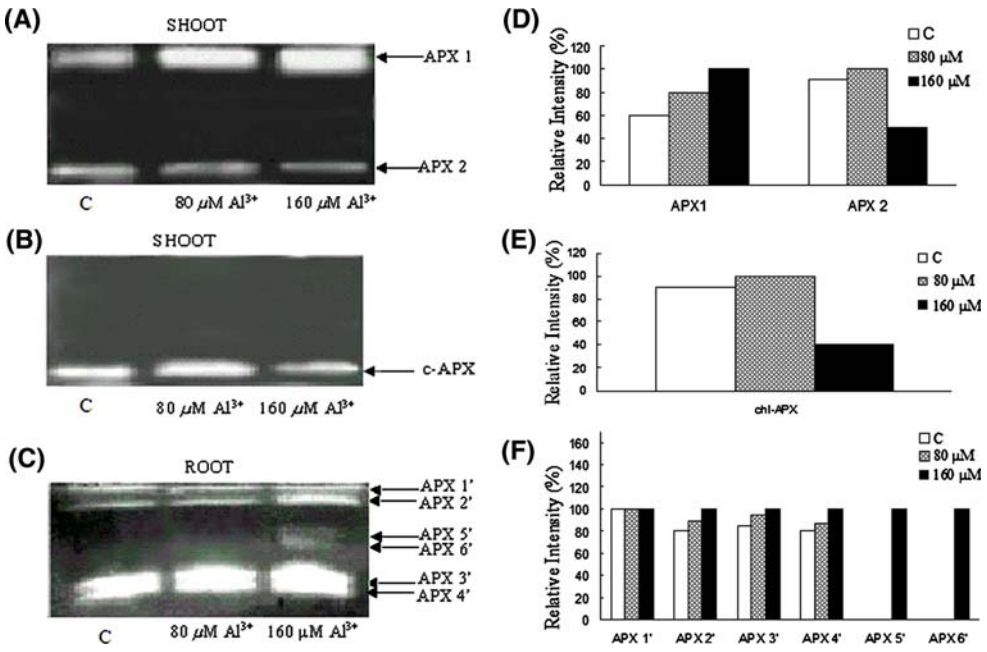

Fig. 5 Isoenzyme profile of ascorbate peroxidase extracted from **a** shoots **b** chloroplasts and **c** roots of 20-day-old control as well as 80 and 160 μM Al^{3+} stressed seedlings of rice *cv*. Pant-12. Intensity of activity bands **d** APX 1, APX 2 **e** chl-APX **f** APX 1′, APX 2′, APX 3′, APX 4′, APX 5′ and APX 6′ were quantified using an image analyzer model LAS 1000 Plus, Fuji Photo Film Co. Ltd., Japan

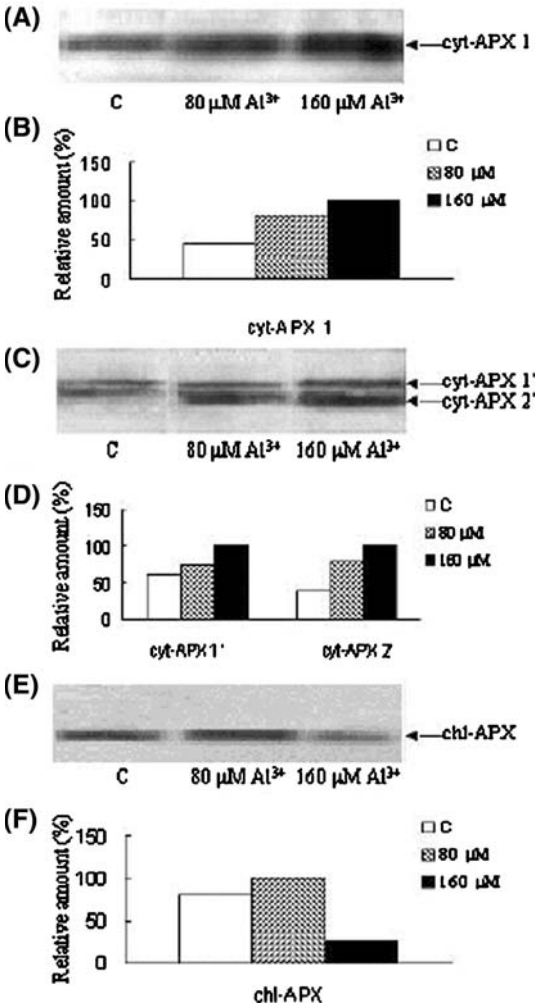

Fig. 6 Immunodetection of APX proteins extracted from shoots, roots and chloroplasts of 20-day-old control as well as 80 and 160 μM Al^{3+} stressed seedlings of rice *cv*. Pant-12. **a** Shoot extracts were electrophoresed on SDS polyacrylamide gels and subjected to immunoblot analysis. Blots were treated with monoclonal antibody (EAP 1) raised against the cytosolic APX isoform from Euglena. **b** Amount of cyt-APX 1 was quantified with an image analyzer in control as well as Al^{3+} stressed seedlings. **c** Root extracts were electrophoresed on SDS polyacrylamide gels and subjected to immunoblot analysis. **d** Amount of cyt-APX 1′ and cyt-APX 2′ was quantified with an image analyzer in control as well as Al^{3+} stressed seedlings. **e** Chloroplastic extracts were electrophoresed on SDS polyacrylamide gels and subjected to immunoblot analysis. Blots were treated with mouse monoclonal antibody raised against spinach chloroplastic APX. **f** Amount of chl-APX was quantified with an image analyzer in control as well as Al^{3+} stressed seedlings

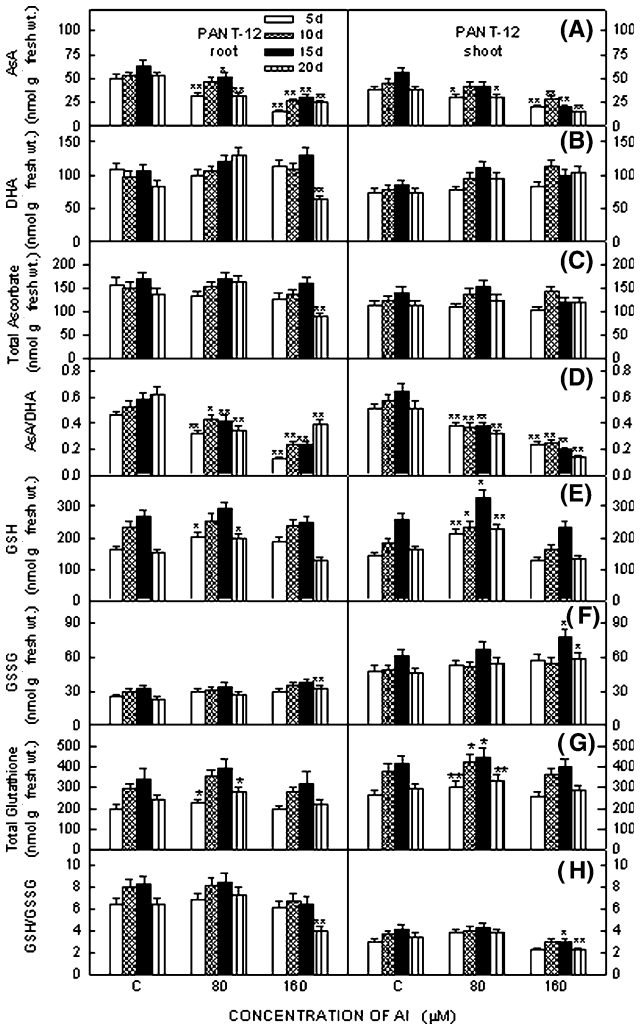

Fig. 7 Effect of increasing concentrations of Al^{3+} in the growth medium on the content of **a** ascorbate **b** dehydroascorbate **c** total ascorbate **d** ratio of ascorbate to dehydroascorbate (AsA/DHA) **e** reduced glutathione **f** oxidized glutathione **g** total glutathione and **h** ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) in roots and shoots of seedling of rice *cv*. Pant-12 at different days of growth. Values are mean ± s.d. based on three independent determinations and *bars* indicate standard deviations. * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test

induction of oxidative stress in rice plants, as peroxidation of membrane lipids as well as loss of thiol groups are indicators of oxidative stress. Similar to our results, induction in lipid peroxidation was observed by Yamamoto et al. (2001) in pea roots and Cakmak and Horst (1991) in soybean roots; however, Boscolo et al. (2003) observed protein oxidation rather than lipid peroxidation due to Al toxicity in maize roots and suggested that the target of oxidative stress varies depending on the plant species and that lipids are not the primary cellular target of oxidative stress. A decline in the content of H_2O_2 in rice seedlings treated with 80 μM Al^{3+} as observed in our studies, might be due to the consumption of H_2O_2 in oxidation processes such as lipid peroxidation or detoxification via the increased activities of enzymes of the antioxidant defense system. However, an elevated amount of H_2O_2 in the seedlings treated with 160 μM Al^{3+} coupled with greater lipid peroxidation and decline in thiol content under such conditions suggests that Al^{3+} treatment of 160 μM induces

a severe oxidative stress in rice plants, where the antioxidative defense system seemingly fails to combat the oxidative damage.

With increasing amounts of Al^{3+} in the growth medium, a concomitant increase in the activities of all the isoforms of SODs (Cu/Zn-SOD, Fe-SOD, Mn-SOD) as well as of total SOD was observed in the seedlings. The increased activities of SOD could be either due to increase in the existing quantity of the enzyme, or due to synthesis of a new isoenzyme of SOD. The activities of Cu/Zn-SOD, Fe-SOD and Mn-SOD have been shown to increase in plant cells under stressful conditions, which appear to be part of a defense mechanism under oxidative stress generated in cytosol, mitochondria and chloroplasts (Ushimaru et al. 1999). The increase in catalase activity in rice seedlings treated with 80 μM Al^{3+} suggests that H_2O_2 may diffuse from chloroplasts to peroxisomes under such conditions leading to induced catalase activity. In our studies catalase did not appear to be an efficient scavenger of H_2O_2 under higher concentration of Al^{3+} treatment, as the decline in the activity of CAT was observed in 160 μM Al^{3+} stressed rice seedlings. The decline in CAT activity in highly Al stressed seedlings might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under such conditions (Ushimaru et al. 1999). The enhancement in the activity of peroxidase in Al^{3+} stressed rice plants as observed in our experiments suggests the role of peroxidases in removing excess H_2O_2 produced under Al toxicity. Several studies have suggested the involvement of Guaiacol POX in metal-imposed oxidative stress (Cuypers et al. 2002; Verma and Dubey 2003). Similar to our results Cakmak and Horst (1991) and Boscolo et al. (2003) also reported increase in SOD and peroxidase activity under aluminum toxicity in soybean roots and maize roots, respectively, however, unlike our result Boscolo et al. (2003) reported no change in catalase activity under aluminum toxicity in maize roots.

The enhanced activities of enzymes of ascorbate-glutathione cycle MDHAR, DHAR and GR observed in Al^{3+} stressed rice seedlings appear to be due to the need of maintaining a favorable redox status, by maintaining a sufficient amount of reduced ascorbate and reduced glutathione and to overcome the possible problems of oxidation. However, this enhancement was more pronounced in 80 μM Al^{3+} treated rice seedlings as compared to those treated with 160 μM Al^{3+}. Previously, we have reported the increased activity of APX in rice seedlings growing under 80 μM Al^{3+} (Sharma and Dubey 2004); here we report that APX activity further increases in rice seedlings even under 160 μM Al^{3+} treatment, suggesting the important role of APX in scavenging H_2O_2 under stressful conditions. Among H_2O_2 decomposing enzymes APX has higher affinity for H_2O_2 than CAT and Guaiacol POX (Wang et al. 1999). Activity of chl-APX however declined in 160 μM Al^{3+} stressed rice seedlings.

In order to verify whether Al-induced enhancement of APX activity was accompanied by any change in the isoform pattern, native gel electrophoresis was done followed by specific gel staining to detect APX isoenzymes. Results indicated the presence of two major isoenzymic bands in shoots (APX 1 and APX 2) and four in roots (APX 1′, APX 2′, APX 3′ and APX 4′) of control seedlings. APX occurs in several isoforms localized in different cellular compartments like cytosol, microbodies (including glyoxysomes and peroxisomes), chloroplasts and mitochondria (Asada 1994). Our results showed increased intensity of band APX 2 in shoots of 80 μM Al^{3+} treated seedlings but its intensity declined under 160 μM Al^{3+} treatment. These results are in conformity with the data obtained spectrophotometrically for chl-APX activity. Further, this activity band (APX 2) corresponds to the single-activity band of chl-APX obtained when chloroplastic protein was subjected to polyacrylamide gel electrophoresis followed by APX activity staining. Immunoblot analysis done for the chl-APX using enzyme preparations from chloroplast and mouse monoclonal antibody raised against spinach chloroplastic APX, revealed that Al^{3+} treatment of 80 μM led to an increase in the amount of chl-APX protein but this amount declined in 160 μM Al^{3+} treated seedlings. Activity band APX 1 however, showed consistent increase in intensity with increasing concentrations of Al^{3+} treatment. This APX may represent the cytosolic form as the chl-APX has a very short lifetime in ascorbate-depleted medium (Asada 1994) (Table 1). Further, immunoblot analysis revealed that the amount of cytosolic APX protein having molecular weight 28.5 kDa (Sharma and Dubey 2004) was higher in Al^{3+} stressed seedlings as compared to control seedlings (Fig. 6a, b). Based on our earlier studies related to purification and molecular weight determination of APX 1 and APX 2 isoforms from seedlings of rice *cv*. Pant-12 (Sharma and Dubey 2004), it is apparent that the cytosolic APX protein band obtained in immunoblot analysis from shoots corresponds to activity band APX 1 of shoot extracts. Al^{3+} treatment of 160 μM also led to increase in the intensity of activity bands APX 2′, APX 3′ and APX 4′ in roots as well as appearance of two new isoenzymes APX 5′ and APX 6′. The differential appearance of the APX isoforms may be due to differential response of various forms of APX to increasing intensity of stress conditions. Similar to shoots, the protein content of roots corresponding to cytosolic APX was also higher in Al^{3+} stressed seedlings as compared to controls (Fig. 6c, d). These results lead to two possible interpretations–firstly, the genes encoding different APX isoforms may be induced differentially by Al that might act directly on trans-acting factors for specific genes or through signal transduction.

Secondly, the expression of the APX genes may be regulated post transcriptionally as has been reported for cytosolic APX expression in pea plants subjected to drought stress (Mittler and Zilinskas 1994).

In our experiments Al^{3+} treatment led to a decline in ascorbate (reduced form) and an increase in dehydroascorbate content in the rice seedlings. The decrease in ascorbate may result from the inhibition of biosynthetic pathways or through an increase in catabolism through an accumulation of the oxidized form of the ascorbate DHA and through its degradation into oxalate and tartarate (Noctor and Foyer 1998; Dipierro et al. 2005). Guo et al. (2005) observed enhanced Al resistance in rice roots fed with ascorbate and suggested that this possibly might be a result of increased production of oxalate, which acts as a metal chelator. Although a significant part of the ascorbate pool is regenerated enzymatically via MDHAR, DHAR or non-enzymatically, DHA is always produced because of rapid disproportionation of MDHA. Possible accumulation of DHA can increase its irreversible conversion to the potentially toxic L-diketogulonic acid (Noctor and Foyer 1998). The increased amount of reduced glutathione in rice seedlings growing under 80 μM Al^{3+} appears to be due to increased GR activity, which represents the process of regulation directed toward reduction of glutathione, which is oxidized during the reduction of DHA to AsA via DHAR. However, 160 μM Al^{3+} treated rice seedlings showed an increased amount of oxidized glutathione and a decline in GSH/GSSG. Following stress, the inevitable rise of ROS acts as signaling molecule for the change in redox status of the glutathione, toward its oxidized form.

In conclusion results of the present study suggest that Al^{3+} toxicity is associated with induction of oxidative stress in the roots as well shoots of growing rice seedlings marked by elevated contents of O_2^-, H_2O_2, lipid peroxides and decline in thiol content and redox ratios of glutathione and ascorbate. Among the antioxidative enzymes SOD, Guaiacol POX and APX appear to play a key role in the antioxidative defense mechanism under Al^{3+} toxicity conditions. The isoenzyme profile and the immunoblot analysis of APX revealed that Al-induced enhancement of APX activity was accompanied by a change in the isoenzyme pattern, and of these isoenzymes, the cytosolic form of APX plays a major role in combating oxidative damage.

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