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Protective effect of ascorbic acid and glutathione on $AlCl_3$-inhibited growth of rice roots

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Abstract

The effect of $AlCl_3$ on the antioxidant system of rice roots and the role of applied antioxidants ascorbic acid (AsA) and glutathione (GSH) in $AlCl_3$-inhibited growth of rice roots were investigated. $AlCl_3$ treatment resulted in a rapid inhibition of root growth but had no effect on lipid peroxidation and antioxidative enzyme activities in rice roots. $AlCl_3$ treatment resulted in lower content of H_2O_2, AsA, and GSH than in controls. Exogenous AsA or GSH counteracted growth inhibition of rice roots induced by $AlCl_3$. $AlCl_3$ treatment increased syringaldazine peroxidase (SPOX) activities and lignin content in rice roots. Exogenous AsA or GSH prevented the decrease in H_2O_2 content and the increase in SPOX activities and lignin content in rice roots caused by $AlCl_3$. Results suggest that lignification induced by low AsA or GSH content may explain the mechanism of Al-inhibited growth of rice roots.

Additional key words: hydrogen peroxide, lignin, *Oryza sativa*, reactive oxygen species.

Introduction

The primary effect of aluminum (Al) toxicity is the inhibition of root growth (Tamás *et al.* 2006). Several mechanisms of Al toxicity have been proposed (Zheng and Yang 2005). However, the precise physiological and molecular bases are not completely understood (Matsumoto 2000, Kochian *et al.* 2005, Zheng and Yang 2005).

It has been shown that Al induces reactive oxygen species (ROS) and enhances lipid peroxidation in *Hordeum vulgare* (Sakihama and Yamasaki 2002, Šimonovicová *et al.* 2004a,b), *Oryza sativa* (Kuo and Kao 2003, Meriga *et al.* 2004), *Pisum sativum* (Yamamoto *et al.* 2001, 2002), *Triticum aestivum* (Darkó *et al.* 2004), and *Zea mays* (Boscolo *et al.* 2003). In *Arabidopsis* and cultured tobacco cells, Al induced the expression of several genes (*e.g.* for peroxidase and SOD) that are induced by oxidative stress (Ezaki *et al.* 1995, 1996, Richards *et al.* 1998). Thus a possible induction of oxidative stress by Al was suggested.

ROS can damage essential membrane lipids as well as proteins and nucleic acids (Inzé and Van Montagu 1995, Noctor and Foyer 1998). Levels of ROS in plant cells are normally controlled by protective antioxidant system. Various associations between Al and endogenous levels of antioxidant enzymes have been reported (Kuo and Kao 2003, Darkó *et al.* 2004, Meriga *et al.* 2004, Šimonovicová *et al.* 2004a). Darkó *et al.* (2004) demonstrated that the roots of Al-tolerant wheat exhibited more intensive growth, while accumulating less Al and ROS than Al-sensitive wheat under Al stress condition. They also found that among the antioxidant enzymes induced by Al stress, CAT and glutathione-S-transferase may play an important role in the detoxification of ROS in Al-tolerant wheat.

Ascorbic acid (AsA) and glutathione (GSH) have been implicated in the regulation of plant cell growth and division (*e.g.* Conklin *et al.* 1996, Córdoba-Pedregosa *et al.* 1996, 2005, May *et al.* 1998, Potters *et al.* 2000, 2002, Vernoux *et al.* 2000). Lukaszewski and Blevins (1996) demonstrated that increasing concentration of aluminum caused progressive inhibition of root growth and a parallel reduction in AsA concentration of *Cucurbita pepo*. Recently, Devi *et al.* (2003) reported that the higher content of AsA in Al tolerant cell line of tobacco than in Al sensitive cell line are responsible for its higher tolerance to Al. Yamaguchi *et al.* (1999) observed that total GSH concentration in tobacco cell suspension treated with a combination of Al and iron was lower than in the control cells. High content of GSH has been shown to be responsible for the tolerance

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; DHA - dehydroascorbate; d.m. - dry mass; GR - glutathione reductase; GSH - reduced glutathione; GSSG - glutathione disulfide; MDA - malondialdehyde; ROS - reactive oxygen species; SOD - superoxide dismutase; SPOX - syrinaldazine peroxidase.

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mechanism of tobacco cells (Devi *et al.* 2003) and wheat plant (Dong *et al.* 2002) to Al. All these results suggest that AsA and GSH play an important role in Al-inhibited growth.

In the present paper, we have studied the effect of $AlCl_3$ on the antioxidant system of rice roots and the role of antioxidant (AsA and GSH) in Al Cl_3-inhibited root growth of rice.

Materials and methods

Uniformly germinated rice (*Oryza sativa* L., cv. Taichung Native 1) caryopses were grown in a Petri dish (9 cm) containing filter paper moistened with 10 cm^3 of distilled water for 2-d at 27 °C in darkness. Then, 2-d-old seedlings were treated with distilled water or $AlCl_3$ solution. In previous work, we observed that increasing concentration of $AlCl_3$ from 0.25 to 0.5 mM at pH 4.0 progressively decreased root growth of rice seedlings and no further decrease was observed at 0.75 and 1 mM $AlCl_3$ (Wang and Kao 2004). Thus, 0.5 mM $AlCl_3$ was used in the present investigation. Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts and so seedlings grown in distilled water were used as the controls. Each Petri dish contained 10 seedlings and each treatment was replicated four times.

For the determination of Al, roots were dried at 65 °C for 48 h. Dried material was ashed at 550 °C for 20 h. Ash residue was incubated with 31 % HNO_3 and 17.5 % H_2O_2 at 70 °C for 12 h, and dissolved in 0.1 M HCl. Al was then quantified using an atomic absorption spectrophotometer (*Model AA-680, Shimadzu,* Kyoto, Japan).

The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 50 mg root tissue with 3 cm^3 of phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6 000 *g* for 25 min. To determine H_2O_2 content, 3 cm^3 of extracted solution was mixed with 1 cm^3 of 0.1 % titanium sulphate in 20 % (v/v) H_2SO_4. The mixture was then centrifuged at 6 000 *g* for 15 min. The absorbance was measured at 410 nm. Malondialdehyde (MDA) was extracted with 5 % (m/v) trichloroacetic acid and determined according to Heath and Packer (1968).

For extraction of enzymes, roots were homogenized with 0.1 M phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulting supernatant was used for the determination of enzyme activity. The whole extraction procedure was carried out at 4 °C. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in absorbance at 240 nm. One unit (U) of CAT was defined as the amount of enzyme which breaks down 1 nmol H_2O_2 per min. SOD was determined to Paoletti *et al.* (1986). One U of SOD was defined as the amount of enzyme which inhibits by 50 % the rate of NADH oxidation observed in blank. APX was determined according to Nakano and Asada (1981). The decrease in AsA concentration was followed at 290 nm. One U of APX was defined as the amount of enzyme which breaks down 1 μmol of AsA per min. GR was determined by the method of Foster and Hess (1980). One U of GR was defined as the amount of enzyme which decreases A_{340} (1 unit per min). Syringaldazine peroxidase (SPOX) was assayed according to Grison and Pilet (1985). The oxidation of syringaldazine was measured followed the absorbance decrease at 530 nm. One U of SPOX was defined as the amount of enzyme which decreases A_{530} (1 unit per min).

Contents of ascorbate (AsA) and dehydroascorbate (DHA) in 5 % (m/v) trichloroacetic acid extract and GSH and glutathione disulfide (GSSG) in 3 % sulfosalicylic acid extract were determined as described by Laws *et al.* (1983) and Smith (1985), respectively. The lignin content in roots was measured by the Sasaki *et al.* (1996) method, a method originally described by Morrison (1972). Roots were homogenized with a pestle and mortar in 95 % ethanol. The homogenate was centrifuged at 1 000 *g* for 5 min. The pellet was washed three times with 95 % ethanol and twice with a mixture of ethanol and hexane (1:2, v/v). The material was allowed to air dry and its lignin content measured. The dried sample was washed one time with 2 cm^3 acetyl bromide in acetic acid (1:3, v/v). Then 1 cm^3 acetyl bromide in acetic acid (1:3, v/v) was added to the pellet and incubated at 70 °C for 30 min. After cooling of the mixture to room temperature, 0.9 cm^3 of 2 M NaOH and 0.1 cm^3 7.5 M hydroxylamine hydrochloride were added, and the volume was made up to 10 cm^3 with acetic acid. After centrifugation at 1 000 *g* for 5 min, the absorbance of the supernatant was measured at 280 nm (A_{280}).

Statistical differences between measurements ($n = 4$) on different treatment or on different times were analyzed by Duncan's multiple range test or Student's *t*-test.

Results

The reduction of root growth by $AlCl_3$ was evident 8 h after treatment (Fig. 1*A*). Al concentration in control roots remained unchanged during 12 h of incubation. However, Al concentration in $AlCl_3$-treated roots increased with increasing duration of incubation (Fig. 1*B*). The increase in Al concentration in $AlCl_3$-

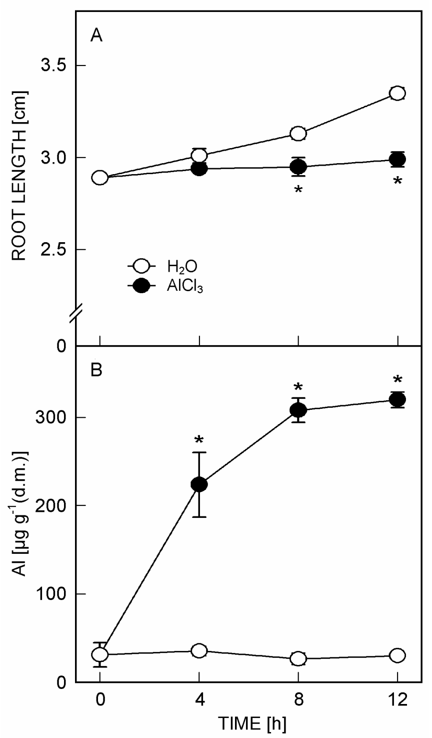

Fig. 1. Changes in root length (*A*) and Al concentration (*B*) in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) or H_2O (pH 4.0) for 4, 8 and 12 h. Means ± SD ($n = 4$). *Asterisks* indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$ according to Student's *t*-test.

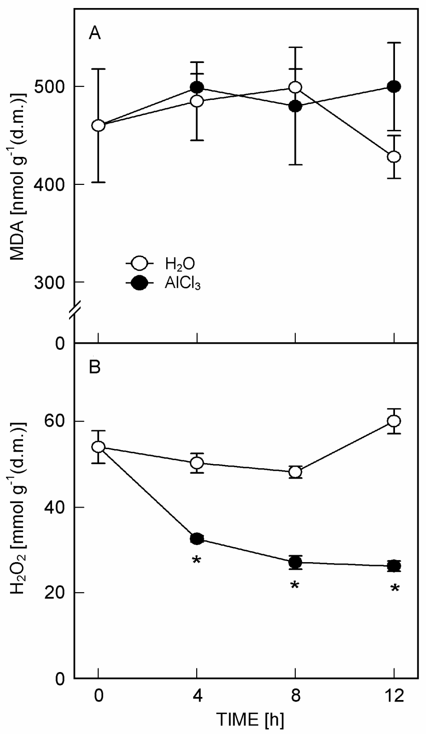

Fig. 2. Changes in MDA (*A*) and H_2O_2 (*B*) contents in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) or H_2O (pH 4.0) for 4, 8 and 12 h. Means ± SD ($n = 4$). *Asterisks* indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$ according to Student's *t*-test.

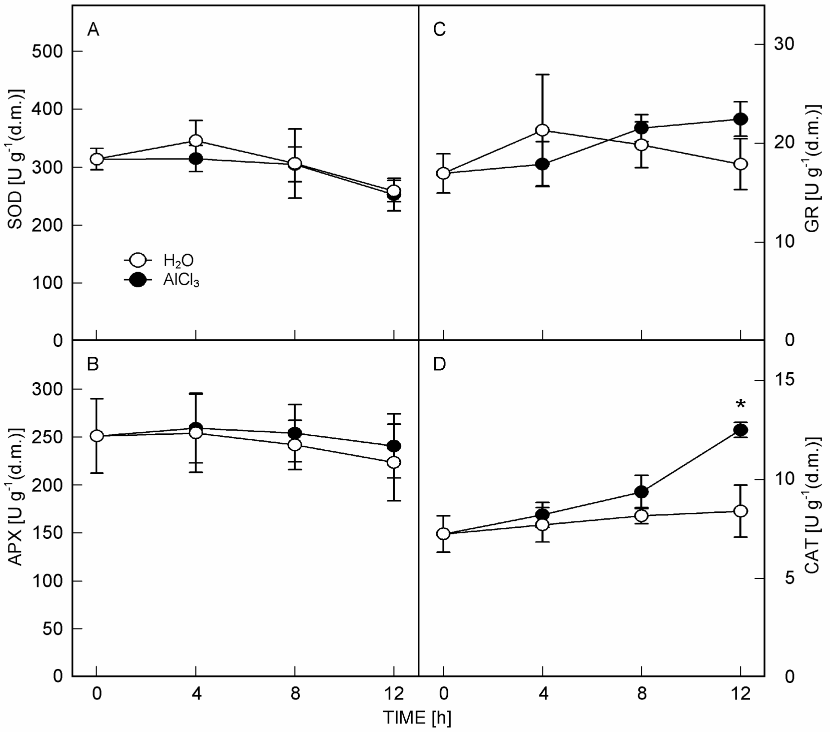

Fig. 3. Changes in antioxidative enzyme activities in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) or H_2O (pH 4.0) for 4, 8 and 12 h. Means ± SD ($n = 4$). *Asterisks* indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$ according to Student's *t*-test.

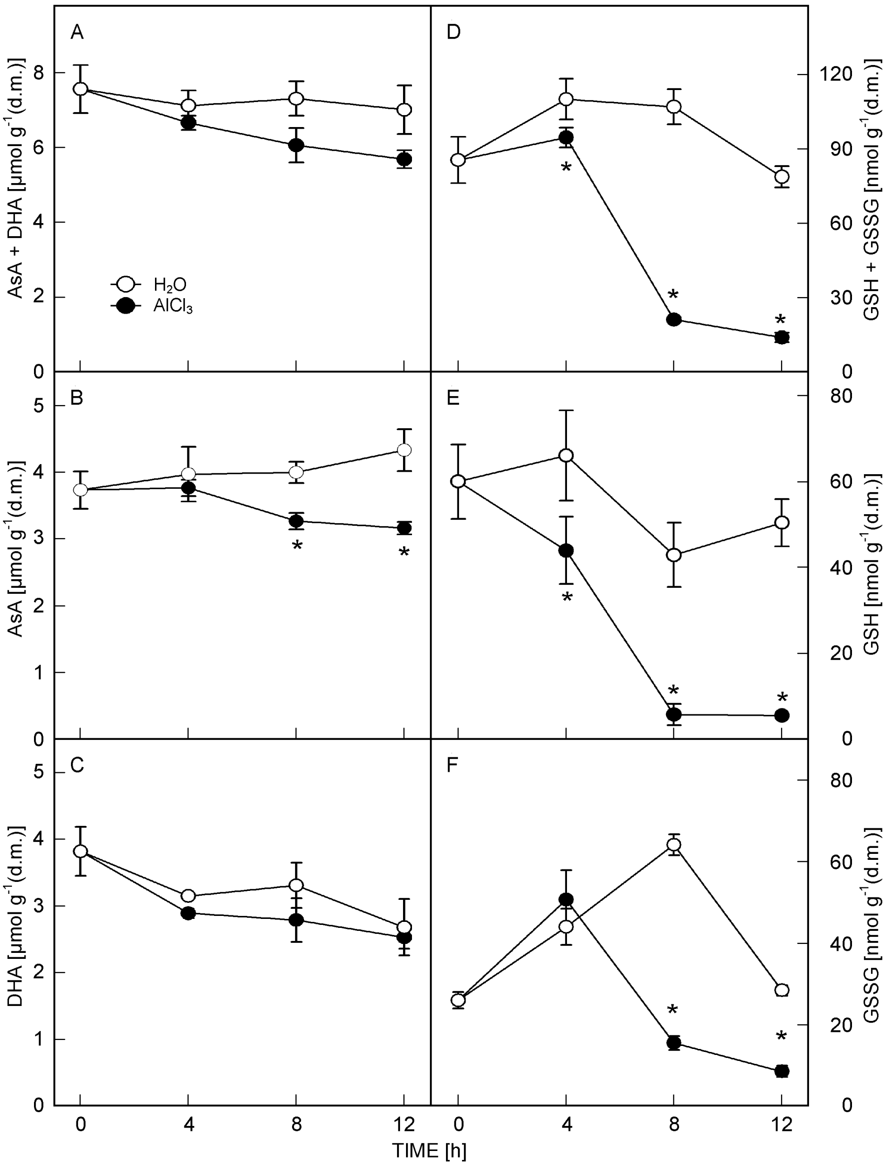

Fig. 4. Changes in antioxidants in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) or H_2O (pH 4.0) for 4, 8, and 12 h. Means ± SD ($n = 4$). *Asterisks* indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$ according to Student's *t*-test.

treated roots was evident at 4 h after treatment (Fig. 1*B*).

MDA is routinely used as an indicator of lipid peroxidation. No difference in MDA content was observed between H_2O- and $AlCl_3$-treated roots (Fig. 2*A*). However, H_2O_2 content in $AlCl_3$-treated roots was lower than that in control roots (Fig. 2*B*). For the activities of SOD, APX, and GR, no difference was observed between $AlCl_3$- and H_2O-treated roots (Fig. 3*A*,*B*,*C*). The increase in CAT activity in $AlCl_3$-treated roots was only observed 12 h after treatment (Fig. 3*D*).

When 2-d-old rice seedling roots were treated with 0.5 mM $AlCl_3$, AsA content was significantly lower than in control roots (Fig. 4*B*). However, $AlCl_3$ had no effect on AsA + DHA and DHA contents in roots (Fig. 4*A*,*C*). It was also observed that GSH, GSSG, and GSH + GSSG contents in $AlCl_3$-treated roots were lower than those of control roots (Fig. 4*D*,*E*,*F*). If AsA or GSH plays an important role in regulating $AlCl_3$-induced growth inhibition of rice roots, then growth of roots in $AlCl_3$ is expected to be enhanced by adding AsA or GSH. Adding AsA, which increased AsA but not GSH content (Fig. 5*A*,*B*), or GSH, which increased GSH but not AsA content (Fig. 5*A*,*B*), significantly enhanced growth of roots treated with $AlCl_3$ for 12 h (Fig. 5*C*). This protective effect on $AlCl_3$-inhibited root growth was also observed in a long (48 h) AsA or GSH treatment (Fig. 6*A*).

Since AsA or GSH was added simultaneously with $AlCl_3$, thus AsA- or GSH-reduced growth inhibition of rice roots caused by $AlCl_3$ may be mediated through

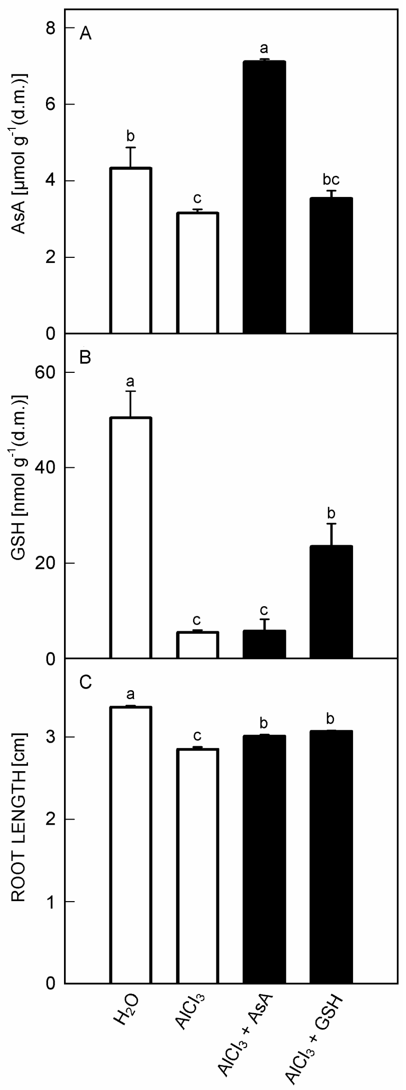

Fig. 5. Effect of AsA (0.5 mM, pH 4.0) and GSH (0.5 mM, pH 4.0) on the contents of AsA (*A*) and GSH (*B*) in roots and root growth (*C*) of rice seedlings in the presence of $AlCl_3$ (0.5 mM, pH 4.0). All measurements were made 12 h after treatment. Values with the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

blockage of Al uptake. Al concentration in roots treated with $AlCl_3$ was similar to that treated with $AlCl_3$ + AsA (Fig. 6*B*). Although Al concentration in roots treated with $AlCl_3$ + GSH was lower than that treated with $AlCl_3$ alone (Fig. 6*B*), the amount of Al [about 550 μg g^{-1}(d.m.)] found in roots treated with $AlCl_3$ + GSH is still high enough to inhibit growth of rice roots (Fig. 1*B*). Thus, the protective role in counteracting $AlCl_3$-inhibited growth of roots is unlikely caused by blockage of Al uptake. This conclusion is supported further by the observations that the protective effect of AsA or GSH was also observed when rice roots were exposed to AsA or GSH and $AlCl_3$ separately (Fig. 7).

It was observed that both lignin content and

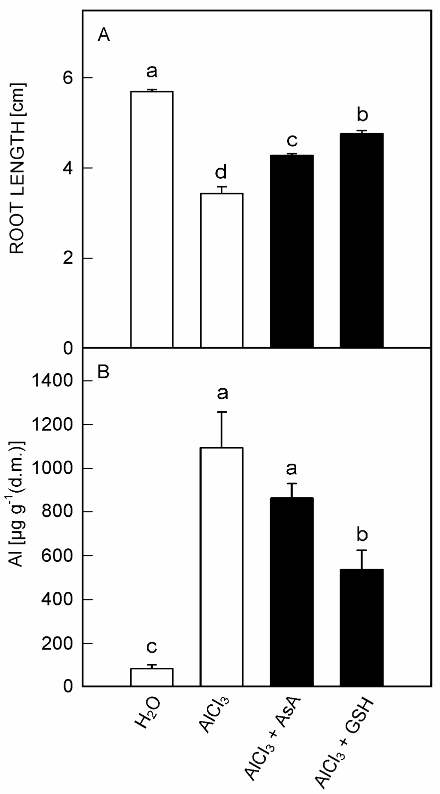

Fig. 6. Effect of AsA and GSH on root growth (*A*) and Al concentration (*B*) in roots of rice seedlings treated with $AlCl_3$. Two-d-old rice seedlings were treated with distilled H_2O (pH 4.0) and 0.5 mM $AlCl_3$ (pH 4.0), 0.5 mM $AlCl_3$ + 0.5 mM AsA (pH 4.0) and 0.5 mM $AlCl_3$ + 0.5 mM GSH (pH 4.0) for 48 h. Values with the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

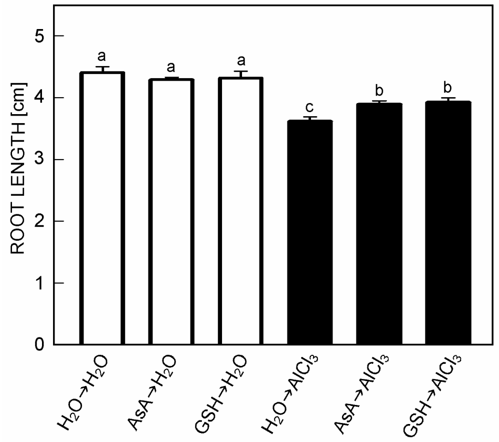

Fig. 7. Effect of pre-treatments of AsA and GSH on root growth of rice seedlings exposed to $AlCl_3$. Two-d-old rice seedlings were pre-treated with distilled water (pH 4.0), 0.5 mM AsA (pH 4.0) and 0.5 mM GSH (pH 4.0), respectively, for 12 h and then treated with distilled water or 0.5 mM $AlCl_3$ for 12 h. Values with the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

syringaldazine peroxidase (SPOX) activity in rice roots increased during $AlCl_3$ treatment (Fig. 8*A*,*B*). The decrease in H_2O_2 content, the increase in lignin content, and the increase in SPOX activity caused by $AlCl_3$ were significantly prevented by adding AsA or GSH (Fig. 9).

Discussion

Several of our observations suggest that Al treatment does not lead to oxidative stress in rice roots: *1*) no accumulation of H_2O_2 was observed in $AlCl_3$-treated rice roots (Fig. 2*B*); *2*) $AlCl_3$ treatment had no effect on lipid peroxidation in rice roots (Fig. 2*A*); *3*) no general up-regulation of antioxidative enzymes by $AlCl_3$ was observed (Fig. 3). It is clear that our results are in contrast with those researchers, who demonstrated that Al induced oxidative stress in plants (Yamamoto *et al.* 2001, 2002, Sakihama and Yamasaki 2002, Boscolo *et al.* 2003, Kuo and Kao 2003, Darkó *et al.* 2004, Meriga *et al.* 2004, Šimonovicová *et al.* 2004a,b). Using leaves of the same rice cultivar as used in the present study (Kuo and Kao 2003), we observed that $AlCl_3$ was able to induce oxidative stress. It appears that Al-induced oxidative stress in rice plants is organ-specific.

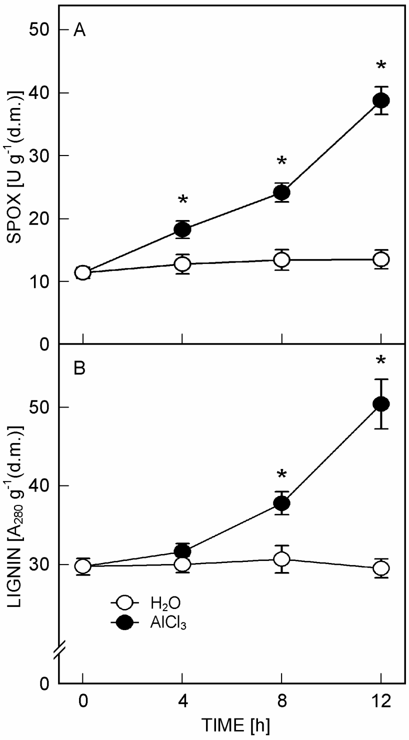

Fig. 8. Changes in SPOX activity (*A*) and lignin content (*B*) in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) or H_2O (pH 4.0) for 4, 8 and 12 h. Means ± SD ($n = 4$). *Asterisks* indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$ according to Student's *t*-test.

It has been documented that AsA or GSH plays a crucial role in plant growth (*e.g.* Conklin *et al.* 1996, Córdoba-Pedregosa *et al.* 1996, 2005, Sánchez-Fernández *et al.* 1997, May *et al.* 1998, Potters *et al.* 2000, 2002, Vernoux *et al.* 2000). In the present study, two lines of evidence indicated that AsA or GSH seems to be involved in root growth inhibition of rice seedlings caused by $AlCl_3$. Firstly, treatment of $AlCl_3$ decreased AsA or GSH content in rice roots (Fig. 4*B*,*E*). Secondly, the growth of roots in $AlCl_3$ can be enhanced by adding AsA or GSH (Fig. 5*C*, 6*A*). Low content of AsA has

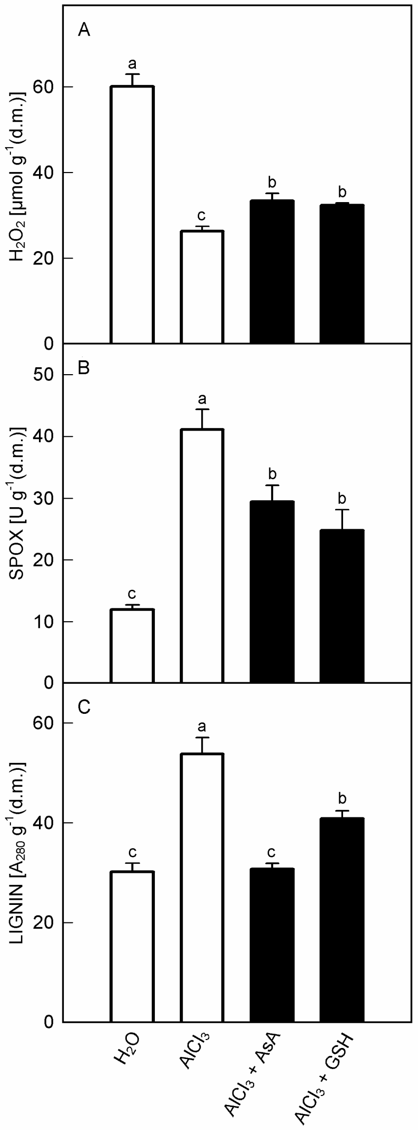

Fig. 9. Effect of AsA (0.5 mM, pH 4.0) and GSH (0.5 mM, pH 4.0) on the contents of H_2O_2 (*A*), the activity of SPOX (*B*), and the content of lignin (*C*) in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0). All measurements were made 12 h after treatment. Values with the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

also been described to be responsible for Al-inhibited growth of *Cucurbita pepo* (Lukaszewski and Belvins 1996) and *Nicotana tabacum* (Devi *et al*. 2003). The tolerance mechanism to Al in tobacco cells (Devi *et al*. 2003) and wheat plant (Dong *et al*. 2003) has been shown to be due to high GSH content. It appears that low AsA or GSH content is responsible for $AlCl_3$-inhibited root growth of rice seedlings.

The lower content of AsA or GSH in $AlCl_3$-treated rice roots is possible due to the reduction of the rate of AsA or GSH synthesis. However, the possibility that utilization, regeneration, catabolism and transport of AsA or GSH are altered by $AlCl_3$ in rice roots cannot be excluded.

Both AsA and GSH can function as antioxidants in plant cells. It has been shown that high content of AsA is responsible for tolerance mechanism of tobacco cells to Al by protecting cells from lipid peroxidation (Devi *et al*. 2003) and GSH is able to protect cells from either peroxidation or H_2O_2 commonly enhanced by Al (Devi *et al*. 2003, Dong *et al*. 2002, Yamaguchi *et al*. 1999). However, $AlCl_3$ treatment did not increase lipid peroxidation and decreased H_2O_2 content in rice roots (Fig. 2). Thus, $AlCl_3$-inhibited growth of rice roots is unlikely mediated through decreased antioxidant capacity of AsA or GSH.

In the present study we observed that $AlCl_3$ treatment resulted in a lower content of H_2O_2 in rice roots than controls (Fig. 2*B*). No accumulation of H_2O_2 was also observed in drought, excess Fe- and NaCl-treated leaves (Moran *et al*. 1994, Lin and Kao 2000, Fang *et al*. 2001). Lignification is part of cell differentiation and irreversibly inhibits cell elongation. It has been shown that lignification in the elongation region coincided with the extent of inhibition of root growth by Al in two wheat cultivars that differed in their sensitivity to Al (Sasaki *et al*. 1996). Here, we show that $AlCl_3$ treatment resulted in an increase in lignin content (Fig. 8*B*). H_2O_2 is required for lignin synthesis. It is possible that H_2O_2 is being utilized in the formation of lignin in $AlCl_3$-treated rice roots. This would explain why H_2O_2 content decreased in rice roots exposed to $AlCl_3$. It has been shown that syringaldazine, a hydrogen donor, has a particularly high affinity for peroxidation associated with lignification (Goldberg *et al*. 1983). In the present study, SPOX activity in $AlCl_3$-treated root was also observed to be higher than that in control roots (Fig. 8*A*). The increase in SPOX activity caused by $AlCl_3$ was also observed to be prior to that in lignin content (Fig. 8). All these results strongly suggest that lignification is responsible for Al-inhibited growth of rice roots.

Our data indicated that AsA and GSH prevented the decrease in H_2O_2 content, the increase in lignin content, and the increase in SPOX activity in $AlCl_3$-treated rice roots (Fig. 9). It appears that lignification induced by low AsA or GSH content may explain the mechanism of Al-inhibited growth of rice roots. Veljovic-Jovanovic *et al*. (2001) suggested that low AsA in the *vtc-1* mutant of *Arabidopsis*, which is deficient in AsA biosynthesis, will create an environment that markedly favors cell wall cross linking. Thus, the possibility that Al-inhibited growth of rice roots mediated through cell wall cross linking cannot be excluded.

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