ORIGINAL PAPER

Aluminum-inhibited root growth of rice seedlings is mediated through putrescine accumulation

Jen-Wu Wang · Ching Huei Kao

Abstract The effects of $AlCl_3$ on growth and polyamine levels of rice roots were investigated. When rice roots were treated with $AlCl_3$, root growth was markedly inhibited. $AlCl_3$ treatment resulted in a higher putrescine content and lower spermidine and spermine contents in rice roots. D-Argnine and α-methylornithine, inhibitors of putrescine biosynthesis, caused a reduced content of putrescine in rice roots under Al stress. $AlCl_3$ treatment also resulted in a decrease in diamine oxidase activity in rice roots. The growth of rice roots in the presence of $AlCl_3$ was recovered after the addition of D-arginine or α-methylornithine. The protective effect of D-arginine or α-methylornithine in counteracting $AlCl_3$-inhibited growth of rice roots is unlikely caused by reduction of Al uptake. Furthermore, the effect of the growth recovery in $AlCl_3$-treated rice roots by D-arginine or α-methylornithine was reversed by the addition of putrescine. Our results strongly suggest that putrescine accumulation is a factor causing growth inhibition of rice roots under Al tress. Evidence is also presented to show that lignification is responsible for putrescine- and $AlCl_3$-inhibited growth of rice roots.

Keywords Aluminum · Growth · Putrescine · Rice

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Introduction

The polyamines putrescine (PUT), spermidine (SPD), and spermine (SPM) are polycationic cellular molecules and are present in all living organisms. The involvement of polyamines in the growth of prokaryotic and eukaryotic cells was established through a series of mutants in *Escherichia coli* and *Saccharumyces cerevisiae* (Tabor and Tabor 1984). Bertosi et al. (1965) reported that polyamines stimulated the growth of explants from dormant tubers of *Helianthus tuberous*. Subsequently, polyamine contents and plant growth rates have been positively correlated in a wide variety of conditions in which high contents of polyamines are associated with rapidly growing tissues (Evans and Malmberg 1989).

In response to various types of environmental stress, plants accumulate polyamines, especially PUT (Boucherau et al. 1999; Galston et al. 1997; Kao 1997). K^+ deficiency may be the first stress condition that has been shown to increase PUT levels in plants (Richards and Coleman 1952;

Young and Galston 1984). Watson and Malmberg (1996) have shown that arginine decarboxylase (ADC), an enzyme critical for inducible PUT synthesis, is activated upon K^+ deficiency in *Arabidopsis*. Phosphate deprivation also increases PUT contents in tobacco, barley, and rice plants (Shih and Kao 1996; Sinclair 1967; Takahashi and Yoshida 1960). It has been shown that feeding PUT to cut leaves of barley produced the same symptoms found in K^+ deficient plant (Coleman and Richards 1956). It has also been demonstrated that PUT accumulation as a factor causing growth inhibition of suspension-cultured rice cells under K^+ deficiency and phosphate deprivation (Shih and Kao 1996; Sung et al. 1994). An inhibition of root growth has been described in genetically modified tobacco plants with high contents of PUT (Masgrau et al. 1997; Panicot et al. 2002). Moreover, Watson et al. (1998) reported that *Arabidopsis* mutants with low content of PUT had higher root growth rate than did wild-type plants. More recently, Camacho-Cristóbal et al. (2005) found that the inhibition of root growth caused by boron deprivation was associated high content of PUT in tobacco plants. All these results seem to suggest that high content of endogenous PUT is toxic for plant growth.

Aluminum (Al) does not exert any known function in plant metabolism and belongs to the non-essential metal. Under neutral soil conditions, it exists in the non-phytotoxic insoluble form, whereas acidification of soil and soil water below pH 4.5 dramatically enhances release of the phytotoxic aluminum ion (MacDonald and Martin 1988). Since acid soils occupy up to 40% of world arable land (Kochian 1995), Al phytotoxicity may be considered as one of the major limiting factors of crop productivity in the world (Matsumoto 2000). The primary effect of Al toxicity is the inhibition of root growth; however, the mechanisms involved in this toxicity are far from clear (Matsumoto 2000). It is not known whether PUT accumulates in roots under Al stress. Neither do we know the relation between the PUT content and Al-inhibited root growth. In this paper, we described the possible role of PUT in Al-inhibited root growth of rice seedlings.

Materials and methods

Plant material

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium chlorite for 15 min and washed extensively with distilled water. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pre-treated at 37°C for 1-day under dark condition. Uniformly germinated seeds were then selected and transferred to a Petri dish (9 cm) containing filter paper moistened with 10 ml of distilled water and were allowed to grow at 27°C in darkness. Then, 2-day-old seedlings were treated with distilled water, $AlCl_3$ (0.5 mM) or test solutions at the desired concentrations as specified in the individual experiments. 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.

Al determination

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).

Polyamine determination

Roots were homogenized in 5 ml of 5% (w/v) perchloric acid. Polyamine contents were determined using high performance liquid chromatography (Waters 484, Milford, USA) after benzoylation as described previously (Chen and Kao 1991).

Extraction and assay of diamine oxidase (DAO)

For extraction of DAO, roots were homogenized with ice-cold phosphate buffer (50 mM, pH 7.8)

using a pestle and mortar. The homogenate was centrifuged at 10,000g for 20 min at 4°C. DAO activity was measured by the method of Naik et al. (1981). The incubation mixture contained 50 mM phosphate buffer (pH 7.8), 10 mM PUT, 0.1 mM pyridoxal phosphate and enzyme extract in a total volume of 4 ml. After incubation at 30°C for 1 h, the reaction was terminated using 1 ml 20% (w/v) trichloroacetic acid. After 30 min, the incubation mixture was centrifuged at 5,000g for 15 min. One ml of ninhydrin mixture (250 mg ninhydrin in 6 ml acetic acid and 4 ml phosphoric acid) was added to the supernatant. Color was developed at 100°C for 30 min. After adding 1 ml of acetic acid, absorbance was measured at 510 nm. In controls, trichloroacetic acid was added prior to the enzyme solutions. One unit of DAO was defined as increase of A_{510} per h.

Lignin determination

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 1,000g 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 ml acetyl bromide in acetic acid (1:3, v/v). Then 1 ml 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 ml of 2 N NaOH and 0.1 ml 7.5 M hydroxylamine hydrochloride were added, and the volume was made up to 10 ml with acetic acid. After centrifugation at 1,000g for 5 min, the absorbance of the supernatant was measured at 280 nm (A_{280}).

Statistical analysis

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 and discussion

AlCl$_3$ inhibits growth of rice roots

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. The reduction of root growth by AlCl$_3$ was evident 8 h after treatment (Fig. 1A). Al concentration in the control roots remained unchanged during 12 h of incubation (Fig. 1B). However, Al concentration in AlCl$_3$-treated roots increased with increasing duration of incubation
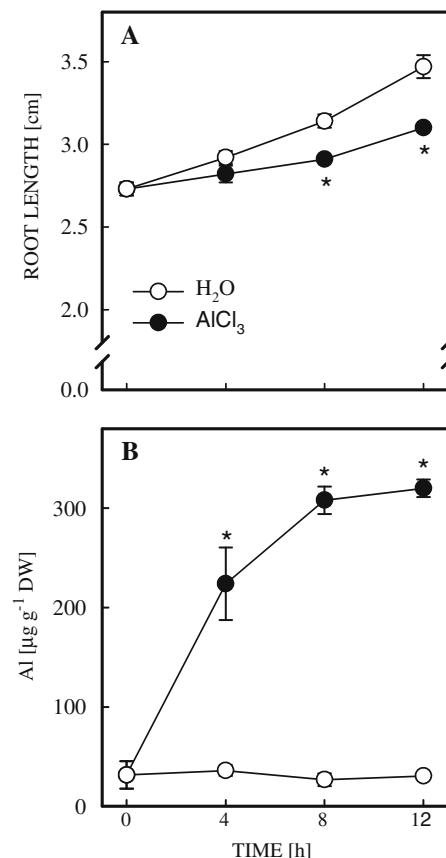

Fig. 1 Changes in root length (**A**) and Al concentration (**B**) in rice roots treated with AlCl$_3$ (0.5 mM, pH 4.0) or H$_2$O (pH 4.0). Means ± SE ($n = 4$). Asterisks indicate values that are significantly different between H$_2$O and AlCl$_3$ treatments at $P < 0.05$

(Fig. 1B). The increase in Al concentration in AlCl$_3$-treated roots was evident 4 h after treatment (Fig. 1B).

Changes of polyamines in AlCl$_3$-treated rice roots

To characterize the role of polyamines in the growth of rice roots under Al stress, the contents of polyamines in rice roots in the presence or absence of AlCl$_3$ were determined. The chromatography analysis indicated the presence of PUT, SPD, and SPM in rice roots. The content of PUT in the control roots remained unchanged for the first 8 h of treatment but increased slightly at 12 h (Fig. 2A). AlCl$_3$-treated rice roots had a higher content of PUT than the control roots at 4, 8 and 12 h after treatment (Fig. 2A). In contrast, AlCl$_3$ treatment had a lower content of SPD than the control roots at 12 h (Fig. 2B), which occurred after the onset of growth inhibition of rice roots caused by AlCl$_3$ (Fig. 1A). Thus, it is unlikely that SPD plays any role in Al-inhibited root growth. The content of SPM in AlCl$_3$-treated roots was also lower than that in the control roots at 4 and 8 h (Fig. 2C). If the lower content of SPM is responsible for the growth inhibition in rice roots under Al stress, then AlCl$_3$-treated rice roots might be expected to recover their growth by the addition of SPM. However, no such recovery was observed (data not shown).

Since PUT accumulation in rice roots under Al stress is preceded root growth inhibition, PUT may be a factor causing growth inhibition in rice roots. If this suggestion is correct, PUT is expected to inhibit the growth in the control rice roots. Addition of PUT to the control rice roots indeed resulted in a reduction of root growth (Fig. 3). This agrees with the already reported toxic effect of exogenous PUT treatments to various organisms including plants (Davis and Ristow 1991; DiTomaso et al. 1989; Guarino and Cohen 1979; Shevyakova 1966; Strogonov et al. 1972).

AlCl$_3$ affects the biosynthesis and catabolism of PUT

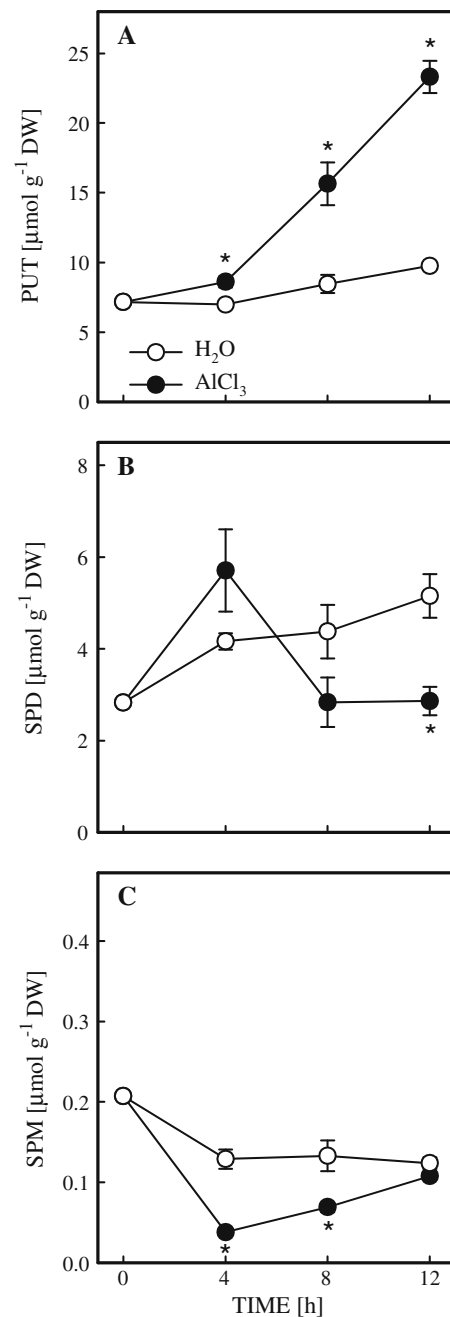The PUT may be formed in plants directly from ornithine via ornithine decarboxylase (ODC) or

Fig. 2 Changes in the contents of polyamines in rice roots treated with AlCl$_3$ (0.5 mM, pH 4.0) or H$_2$O (pH 4.0). Means ± SE ($n = 4$). Asterisks indicate values that are significantly different between H$_2$O and AlCl$_3$ treatments at $P < 0.05$

indirectly from the decarboxylation of arginine via arginine decarboxylase (ADC). To understand whether PUT accumulation in rice roots
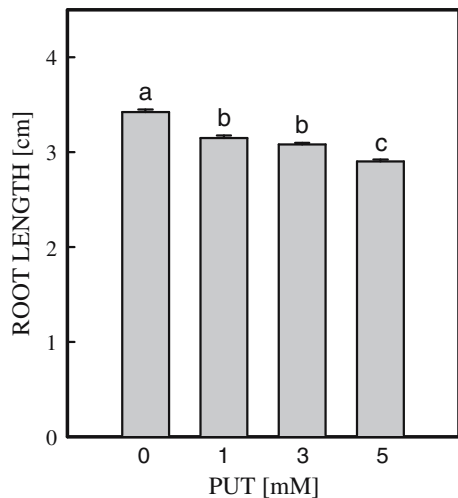
under Al stress is a result of an enhancement in PUT biosynthesis, we tested the effect of inhibitors of PUT biosynthesis (D-Arg and MO) on the content of PUT in rice roots under Al stress. Both D-Arg and MO decreased the content of PUT induced by $AlCl_3$ (Fig. 4A). Although the activities of ADC and ODC, enzymes responsible for the biosynthesis of PUT (Evans and Malmberg 1989), were not measured, the results suggest, although indirectly, that $AlCl_3$ may affect the biosynthesis of PUT.

PUT content can also be regulated by the catabolism of PUT. Diamine oxidase (DAO) catalyzes the catabolism of PUT to pyrroline, hydrogen peroxide, and ammonia (Boucherau et al. 1999). However, it is not known whether the increase in PUT content under Al stress is attributable to the decrease in DAO activity. The presence of DAO in rice roots has been demonstrated previously (Lin and Kao 2002). In the present study, we observed that $AlCl_3$ treatment indeed resulted in a reduction of DAO activity in rice roots (Fig. 4B), indicating that PUT content in rice roots under Al stress is also regulated by DAO activity. We believe that this is the first report showing that both biosynthesis and catabolism of PUT are responsible for PUT accumulation in rice roots under Al stress.
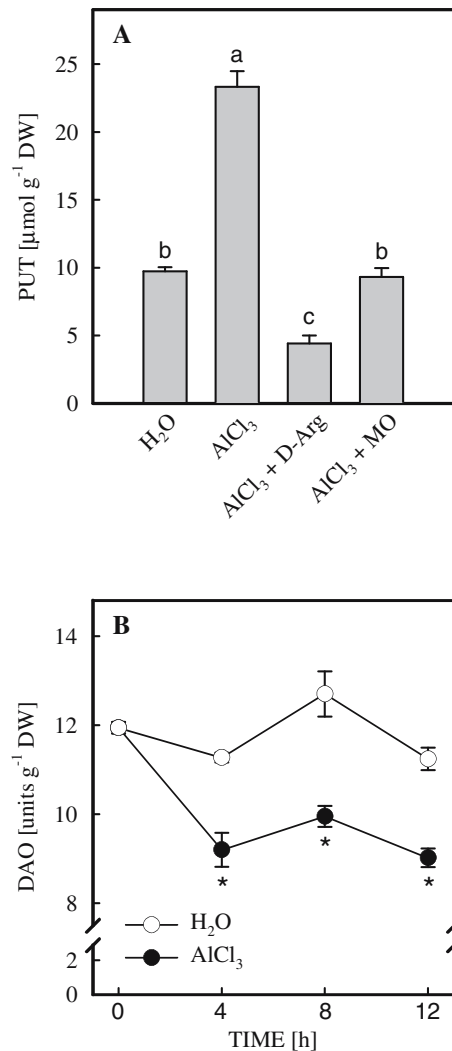

Fig. 4 Effect of D-Arg and MO on the content of PUT (**A**) in rice roots treated with $AlCl_3$ (0.5 mM, pH 4.0) and changes in DAO activity (**B**) in the roots of rice seedlings in the presence or absence $AlCl_3$ (0.5 mM, pH 4.0). The concentrations of D-Arg and MO were 5 mM. PUT content was determined 12 h after treatment. Means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. Asterisks indicate values that are significantly different between H_2O and $AlCl_3$ treatments at $P < 0.05$

Effect of D-Arg and MO on the growth
of $AlCl_3$-treated rice roots

To investigate the role of PUT in root growth, the effect of D-Arg and MO on the growth of rice roots treated with or without Al was examined. Under normal condition without Al, D-Arg and

MO caused an inhibition of root growth (Fig. 5A). Both D-Arg and MO, which decreased PUT content induced by AlCl$_3$ (Fig. 4A), significantly enhanced growth of roots treated with AlCl$_3$ for 12 h (Fig. 5B). This protective effect of AlCl$_3$-inhibited root growth was also observed in a long (48 h) D-Arg or MO treatment (Fig. 5A). These results suggest that PUT accumulation is a factor related to growth inhibition of rice roots under Al stress. Furthermore, the growth recovery
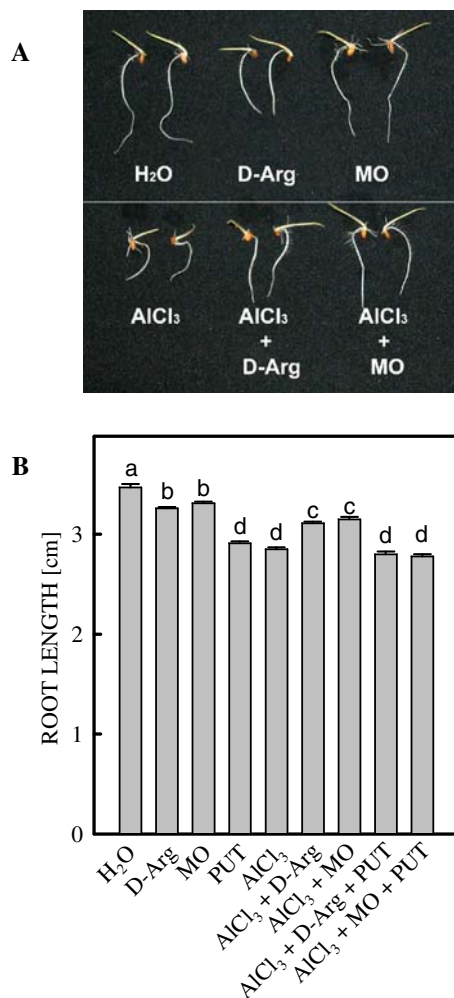
in AlCl$_3$-treated rice roots by D-Arg or MO was reversed by the addition of PUT (Fig. 5B).

Since D-Arg or MO was added simultaneously with AlCl$_3$, thus D-Arg or MO-reduced growth inhibition of roots caused by AlCl$_3$ may be mediated through binding Al to carboxyl group of D-Arg or MO, which in turn reduces the uptake of Al. Al concentration in roots treated with AlCl$_3$ was not significantly different from that treated with AlCl$_3$ + D-Arg or AlCl$_3$ + MO (Fig. 6). Thus, the protective role of D-Arg or MO in counteracting AlCl$_3$-inhibited growth of roots is unlikely caused by reduction of Al uptake. This conclusion is supported further by the observations that the protective effect of D-Arg or MO was also observed when rice roots were exposed to D-Arg or MO and AlCl$_3$ separately (Fig. 7).

The presence of D-Arg under Al stress decreased PUT content less than the control roots (Fig. 4A). However, root growth inhibition by Al was not recovered to the control level in the presence of D-Arg (Fig. 5). These results indicate that Al-inhibited root growth of rice cannot be explained solely by PUT accumulation, other possibilities cannot be completely excluded.
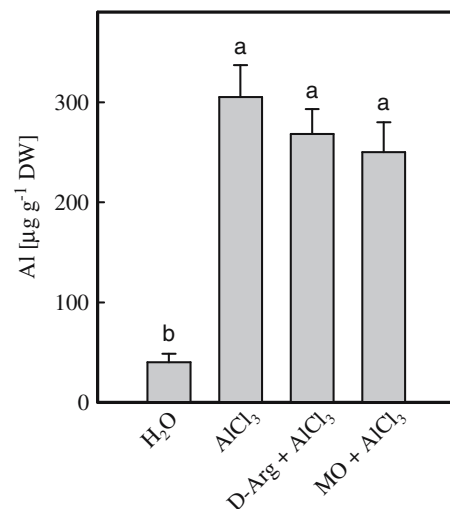

Fig. 5 Effect of D-Arg, MO, and PUT on root growth of rice seedlings treated with or without AlCl$_3$ (0.5 mM, pH 4.0). The concentrations of D-Arg, MO, and PUT were 5 mM. Picture was taken 48 h after treatment (**A**) and root growth was measured 12 h after treatment (**B**). Means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$

Fig. 6 Effect of D-Arg, MO, and PUT on Al concentration in rice roots treated with or without AlCl$_3$ (0.5 mM, pH 4.0). The concentrations of D-Arg, MO, and PUT were 0.5 mM. Al concentration was measured 12 h after treatment. Means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$
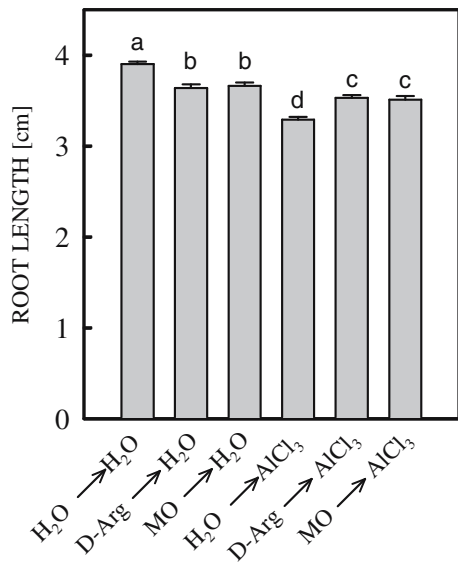
Fig. 7 Effect of pre-treatment of D-Arg and MO on root growth of rice seedlings treated with or without AlCl₃ (0.5 mM, pH 4.0). Two-day-old rice seedlings were pretreated with distilled water (pH 4.0), 0.5 mM D-Arg (pH 4.0) and 0.5 mM MO (pH 4.0), respectively, for 12 h and then treated with distilled water or 0.5 mM AlCl₃ (pH 4.0) for 12 h. Means ± SE (n = 4). Values with the same letter are not significantly different at P < 0.05

PUT and AlCl₃ increase the content of lignin

Lignification, the process of collective formation of phenylpropanoid macromolecules and polymerization of cinnamyl alcohols, 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 AlCl₃ treatment resulted in an increase in lignin content (Fig. 8). Adding PUT, which inhibited growth of rice roots (Fig. 3), significantly increased lignin content in rice roots (Fig. 8). Similarly, adding D-Arg and MO, which counteracted growth inhibition of rice roots caused by AlCl₃ (Fig. 5), prevented increase in lignin content in AlCl₃-treated rice roots (Fig. 8). Furthermore, the decrease in lignin content in AlCl₃-treated rice roots by D-Arg or MO was reversed by the addition of PUT (Fig. 8). It appears that lignification is responsible for PUT- and AlCl₃-inhibited growth of rice roots. The facts that D-Arg almost
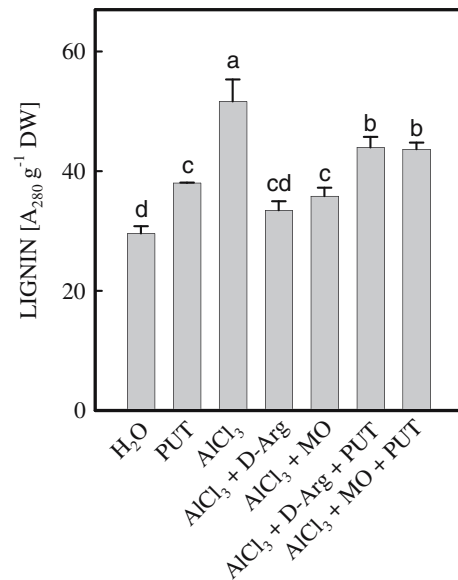

Fig. 8 Effect of D-Arg, MO, and PUT on the content of lignin in the roots of rice seedlings treated with AlCl₃ (0.5 mM, pH 4.0). The concentrations of D-Arg, MO, and PUT were 5 mM. Lignin level was measured 12 h after treatment. Means ± SE (n = 4). Values with the same letter are not significantly different at P < 0.05

completely reduced the increase in lignin content caused by AlCl₃ but did not completely recover the root growth (Figs. 5A, 8) suggest that lignification is not the only mechanism for Al-inhibited growth of rice roots. Our results do not explain the mechanism of Al- or PUT-induced lignin formation. Further study in this direction may allow better understanding of the toxic effect of Al on rice roots.

Conclusion

It has been shown that PUT accumulation is a factor causing growth inhibition of suspension-cultured rice cells under K⁺ deficiency and phosphate deprivation (Sung et al. 1994; Shih and Kao 1996). Here, we show that AlCl₃-inhibited growth of rice roots is mediated though PUT accumulation. Clearly, PUT in excess of the content normally found in rice cells could be a factor, among others, inhibiting growth of rice cells. Our results are also consistent with the findings that the abnormal phenotypes induced by boron

deficiency (Camacho-Cristóbal et al. 2005) or by genetic manipulation, such as over-expression of ADC (Masgrau et al. 1997; Watson et al. 1998; Panicot et al. 2002), are linked to high contents of endogenous PUT. Although other possibilities cannot be completely ruled out, the results of this investigation indicate that the observed growth reduction of rice roots under Al stress is due to, at least in part, PUT accumulation via ADC, ODC, and DAO. Under normal condition without Al, D-Arg and MO treatments lead to the inhibition of rice root growth (Fig. 5A), suggesting that at least some amount of PUT seems to be necessary for normal growth. Judging from the data presented here, PUT could be both a necessary factor and an inhibitory factor for the growth of rice roots, which may be depending on the content of PUT in rice roots.

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