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Citrate Transporters Play a Critical Role in Aluminium-stimulated Citrate Efflux in Rice Bean (*Vigna umbellata*) Roots

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• *Background and Aims* Aluminium (Al) stimulates the efflux of citrate from apices of rice bean (*Vigna umbellata*) roots. This response is delayed at least 3 h when roots are exposed to 50 μM Al, indicating that some inducible processes leading to citrate efflux are involved. The physiological bases responsible for the delayed response were examined here.
• *Methods* The effects of several antagonists of anion channels and citrate carriers, and of the protein synthesis inhibitor, cycloheximide (CHM) on Al-stimulated citrate efflux and/or citrate content were examined by high-pressure liquid chromatography (HPLC) or an enzymatic method.
• *Key Results* Both anion channel inhibitors and citrate carrier inhibitors can inhibit Al-stimulated citrate efflux, with anthracene-9-carboxylic acid (A-9-C, an anion channel inhibitor) and phenylisothiocyanate (PI, a citrate carrier inhibitor) the most effective inhibitors. A 6 h pulse of 50 μM Al induced a significant increase of citrate content in root apices and release of citrate. However, the increase in citrate content preceded the efflux. Furthermore, the release of citrate stimulated by the pulse treatment was inhibited by both A-9-C and PI, indicating the importance of the citrate carrier on the mitochondrial membrane and the anion channel on the plasma membrane for the Al-stimulated citrate efflux. CHM (20 μM) also significantly inhibited Al-stimulated citrate efflux, confirming that *de novo* protein synthesis is required for Al-stimulated citrate efflux.
• *Conclusions* These results indicate that the activation of genes possibly encoding citrate transporters plays a critical role in Al-stimulated citrate efflux.

Key words: Aluminium resistance, anion channel, citrate carrier, inhibitor, organic acid anions, protein synthesis, rice bean, toxicity, transporter, *Vigna umbellata*.

INTRODUCTION

Aluminium (Al) toxicity is one of the major constraints for reduced crop productivity in acid soils. Micromolar concentrations of Al can inhibit root elongation and consequently influence water and nutrient uptake, resulting in poor plant growth (Delhaize and Ryan, 1995; Kochian, 1995). On the other hand, some plant species have evolved sophisticated mechanisms to cope with Al stress. Much of the current evidence argues that Al-stimulated efflux of organic acids such as citrate, malate and oxalate from roots is an important Al resistance mechanism, although some reports demonstrated that organic acid effux plays a minor role such as in signalgrass (Wenzl *et al*., 2001) and spinach (Yang *et al*., 2005*a*) or is not the only mechanism for Al resistance such as in maize (Piñeros *et al*., 2005) and buckwheat (Zheng *et al*., 2005). Recently, an Al resistance gene from wheat, *ALMT1*, has been cloned, and identified as a gene encoding an Al-activated malate transporter, and expression of this gene in other genotypes increased malate efflux and enhanced Al resistance (Delhaize *et al*., 2004; Sasaki *et al*., 2004).

Two patterns of Al-stimulated efflux of organic acids have been proposed on the basis of the timing of efflux (Ma, 2000). In pattern I, no discernible delay is observed between the addition of Al and the onset of organic acid efflux such as in tobacco (Delhaize *et al*., 2001), wheat (Ryan *et al*., 1995) and buckwheat (Zheng *et al*., 1998). Thus, Al may activate an already expressed organic acid transporter in such species. However, in plant species such as rye (Li *et al*., 2000), triticale (Ma *et al*., 2000) and *Cassia tora* (Yang *et al*., 2005*b*), the efflux of organic acids was delayed by several hours, and Al might induce the expression of genes and synthesis of proteins involved in organic acid biosynthesis or transport which are necessary for efflux of organic acids across the root cells. However, there is not much direct experimental evidence to support this supposition. Recently, we demonstrated the different responses of buckwheat, a typical pattern I plant, and *C. tora*, a typical pattern II plant, to treatment with cycloheximide (CHM), a protein synthesis inhibitor, before, simultaneously and after Al exposure, and provided the first experimental evidence that both *de novo* synthesis and activation of an anion channel are needed for Al-induced efflux of citrate in *C. tora*, but that in buckwheat the plasma membrane protein responsible for oxalate efflux pre-existed (Yang *et al*., 2005*b*).

In an attempt to gain better understanding of the physiological and biochemical processes responsible for the efflux of organic acids, some reports have correlated this efflux

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with changes in enzyme activities involved in organic acid biosynthesis. However, the results remain ambiguous and still a matter of debate (Kochian *et al*., 2004). In the present study, we found that rice bean roots can specifically release citrate to alleviate Al toxicity, and the efflux was delayed by at least 3 h. In order to determine the key step involved in the Al-stimulated citrate efflux, several anion channel inhibitors and citrate carrier inhibitors as well as a protein synthesis inhibitor were used. Our results indicated that *de novo* protein synthesis (possibly of the citrate carrier and anion channel themselves) rather than citrate biosynthesis is the critical step leading to citrate efflux in roots of rice bean.

MATERIALS AND METHODS

Reagents

Niflumic acid (NIF), phenylisothiocyanate (PI), anthracene-9-carboxylic acid (A-9-C), phenylglyoxal (PG) and CHM were obtained from Wako Chemical (Osaka). Pyridoxal 5′-P (PP) and mersalyl acid (MA) were purchased from Sigma Chemical Company (St Louis. MO, USA). Stock solutions (10 mM) of NIF and PI were prepared in ethanol, CHM, PP and MA were prepared in de-ionized water and A-9-C was dissolved in 1 M NaOH.

Plant material and growth conditions

Seeds of rice bean [*Vigna umbellata* (Thunb.) Ohwi & Ohashi 'Jiangnan'] were collected from Quzhou (acid soil region, Zhejiang Province, China). Seeds were soaked in de-ionized water overnight, and germinated at 26 °C in the dark. After germination, the seeds were transferred to a floating tray with a net bottom suspended in a 5·0 L solution of 0·5 mM $CaCl_2$ (pH 4·5). The solution was renewed daily. On d 3, seedlings of a similar size were transplanted into a 1 L plastic pot (12 seedlings per pot) containing aerated nutrient solution. One-fifth strength of Hoagland solution was used, which contained the macronutrients in mM: KNO_3 (1·0), $Ca(NO_3)_2$ (1·0), $MgSO_4$ (0·4) and $(NH_4)H_2PO_4$ (0·2), and the micronutrients in μM: NaFeEDTA (20), H_3BO_3 (3), $MnCl_2$ (0·5), $CuSO_4$ (0·2), $ZnSO_4$ (0·4) and $(NH_4)_6Mo_7O_{24}$ (1). The solution was adjusted to pH 4·5 by HCl and renewed every other day. The plants were grown in a greenhouse for 2 weeks, and 2 days before the treatments the pots were moved to a controlled-environment room with a 14 h/26 °C day and a 10 h/22 °C night regime, a light intensity of 150 μmol photons m^{-2} s^{-1} and a relative humidity of 65 %. All the experiments were repeated at least once, and the results from a set of experiment are presented.

Collection of root exudates

Before various treatments, the roots were cleaned by placing them in 0·5 mM $CaCl_2$ solution at pH 4·5 overnight in the same pots. Seedlings (12 d old) were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$. Root exudates were collected every 3 h for 12 h. The collected exudates were passed through a cation-exchange column (16 mm × 14 cm) filled with 5 g of Amberlite IR-120B resin (H^+ form, Muromachi Chemical, Tokyo, Japan), followed by an anion-exchange column (16 mm × 14 cm) filled with 2 g of Dowex 1 × 8 resin (100–200 mesh, formate form). The organic acids retained on the anion-exchange resin were eluted by 15 mL of 1 M HCl, and the eluate was concentrated to dryness by a rotary evaporator (40 °C). The residue was redissolved in 1 mL of Milli-Q water and subjected to determination of organic acids.

Location of secretion site

In order to study the spatial distribution of citrate exudation along the root, either apical 5 or 10 mm root segments of 3-d-old seedlings were excised. The segments were transferred into 8 mL centrifuge tubes containing 5 ml of 0·5 mM $CaCl_2$ solution (pH 4·5). Tubes with root segments were placed on a shaker for 1 h to remove organic acids leaked from cut cells. The washing solution was then removed from each tube and a further 5 mL of 0·5 mM $CaCl_2$ solution (pH 4·5) added to rinse the root segments. Al treatment was initiated by replacing the Ca solution with 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$ for 6 h.

Effect of anion channel and citrate carrier inhibitors

In anion channel inhibitor experiments, seedlings (12 d old) were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 0 or 50 μM $AlCl_3$ in the presence or absence of 20 μM NIF, PG or A-9-C. In citrate carrier inhibitor experiments, seedlings (12 d old) were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 0 or 50 μM $AlCl_3$ in the presence or absence of 20 μM PI, PP or MA. Root exudates were collected after 9 h treatments. In another experiment, seedlings (12 d old) were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$ for 6 h, and then transferred to the 0·5 mM $CaCl_2$ solution (pH 4·5) (+Ca), 0·5 mM $CaCl_2$ solution (pH 4·5) containing 20 μM A-9-C (+A-9-C), 20 μM PI (+PI) or 20 μM A-9-C plus 20 μM PI (+PI +A-9-C). Root exudates were collected every 3 h, and apical 5 mm root segments were excised for determination of endogenous citrate content.

Effect of protein synthesis inhibitor

Seedlings (12 d old) were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 0 or 50 μM $AlCl_3$ in the presence or absence of 20 μM CHM. After 9 h, root exudates were collected.

Determination of citrate

For endogenous citrate analysis, root apices were homogenized on ice, in 1 mL of 0·6 N perchloric acid. The extract was centrifuged at 15 000 *g* for 5 min, and 0·9 mL of supernatant was collected and neutralized with 80 μL of 5 M K_2CO_3. The neutralized solution was centrifuged at 15 000 *g* for 5 min, and the supernatant was used for the measurement of citrate.

Citrate in root tissues (Fig. 5B) and released from root apices (Fig. 2) was determined by an enzymatic method

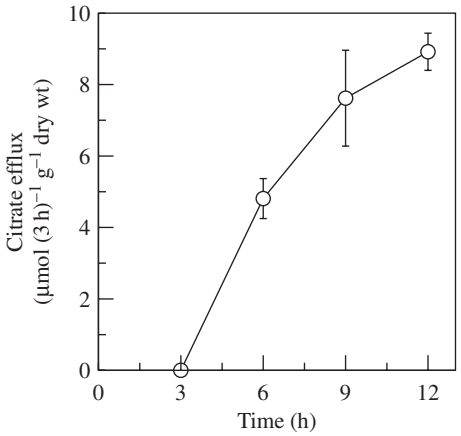

FIG. 1. Time course of Al-stimulated release of citrate in rice bean. Seedlings were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$. Root exudates were collected every 3 h after initiation of Al treatment. Organic acids were analysed by HPLC. Error bars represent ± s.d. (n = 3).

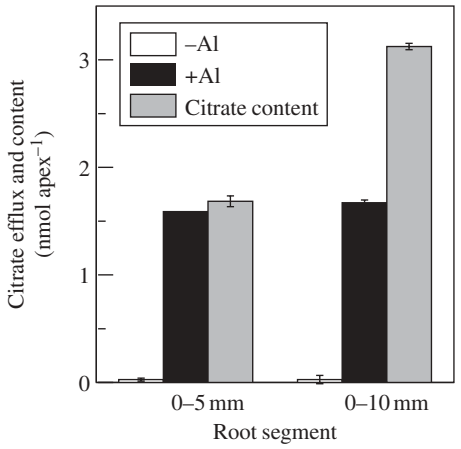

FIG. 2. Citrate exudation in excised root segments of rice bean. Either the terminal 5 or 10 mm of the root was used for citrate exudation experiments. Root segments were washed in 0·5 mM $CaCl_2$ solution (pH 4·5) for 1 h and then incubated in 0·5 mM $CaCl_2$ solution (pH 4·5) with 0 or 50 μM $AlCl_3$ for 6 h. Root segments in 0·5 mM $CaCl_2$ solution without Al (pH 4·5) after incubation were used for tissue extraction and citrate analysis. Citrate was determined enzymatically. Error bars represent ± s.d. (n = 3).

(Delhaize *et al*., 1993), and citrate in root exudates was analysed by high-performance liquid chromatography (HPLC) according to Zheng *et al*. (2005).

RESULTS

Effect of Al on citrate efflux

Despite many kinds of organic acids present in root tissues, only a large amount of citrate was released from the roots of rice bean when exposed to 50 μM Al (other organic acids were not detected), but the efflux was delayed by at least 3 h after the initiation of Al treatment (Fig. 1). This delay of citrate efflux by Al stress is a typical pattern II response. Al-stimulated citrate efflux was largely confined to the apical 5 mm of roots, and the citrate content in the apical 10 mm root was approx. 2-fold greater than in the apical 5 mm root (Fig. 2). On a root length basis, approx. 95 % of the citrate was released from the apical 5 mm of roots compared with the apical 10 mm of roots.

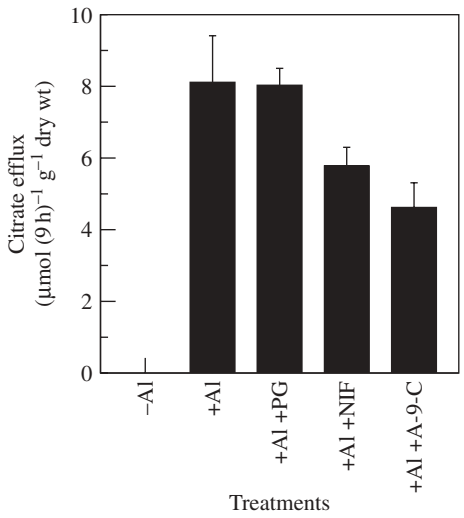

FIG. 3. Effect of anion channel inhibitors on Al-stimulated release of citrate in rice bean. Seedlings were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$ in the presence or absence of each inhibitor (20 μM). Root exudates were collected after 9 h of treatment. Organic acids were analysed by HPLC. Error bars represent ± s.d. (n = 3).

Effect of anion channel and citrate carrier inhibitors on citrate efflux

Anion channel inhibitors (NIF, A-9-C and PG at 20 μM) produced different effects on Al-stimulated citrate efflux. PG had no effect, while NIF and A-9-C inhibited the release by 29 and 43 %, respectively (Fig. 3). Three kinds of citrate carrier inhibitors (PP, PI and MA at 20 μM), which have been reported as inhibitors of citrate carrier on the mitochondrial membrane (Genchi *et al*., 1999), also showed different effects on citrate efflux. MA and PI inhibited the release by 34 and 45 %, respectively, but PP had no effect (Fig. 4).

A 6 h pulse with 50 μM Al also induced significant citrate efflux after 3 h, but it was decreased after that (Fig. 5A). However, the efflux of citrate was significantly inhibited by 20 μM PI and A-9-C, and even more when exposed to both (Fig. 5A). The citrate content in root apices increased with exposure time, and was maintained at least for 6 h when transferred to Ca solution (Fig. 5B), but the citrate content in root apices treated with Ca solution did not change over time (data not shown). Furthermore, PI and A-9-C had no effect on the citrate content of root apices (Fig. 5B), indicating that the decreased citrate efflux was not due to the citrate content but to the inhibitory effects of PI and A-9-C on citrate transporters.

Effect of protein synthesis inhibitor on citrate efflux

When compared with Al treatment, the efflux of citrate was inhibited by 87 % after 9 h of treatment with 20 μM CHM (Fig. 6).

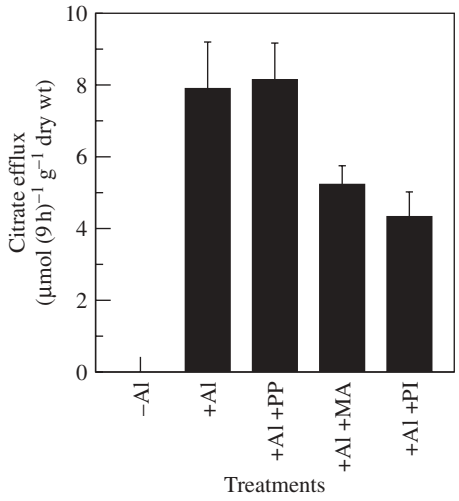

FIG. 4. Effect of citrate carrier inhibitors on Al-stimulated release of citrate in rice bean. Seedlings were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing 50 μM $AlCl_3$ in the presence or absence of each inhibitor (20 μM). Root exudates were collected after 9 h of treatment. Organic acids were analysed by HPLC. Error bars represent ± s.d. (n = 3).

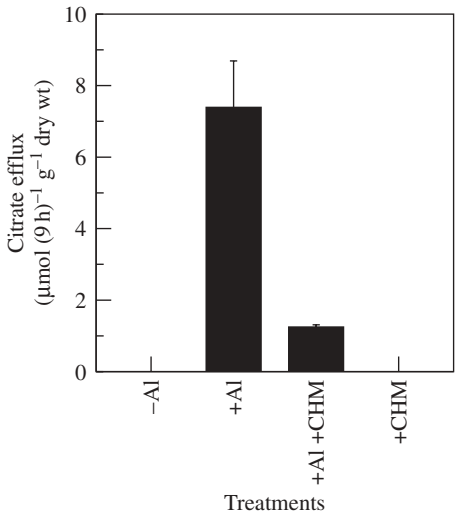

FIG. 6. Effect of the protein synthesis inhibitor CHM on Al-stimulated release of citrate in rice bean. Seedlings were exposed to 0·5 mM $CaCl_2$ solution (pH 4·5) containing no Al (–Al), 50 μM $AlCl_3$ (+Al), 50 μM $AlCl_3$ plus 20 μM CHM (+Al +CHM) or 20 μM CHM (+CHM). Root exudates were collected after 9 h of treatment. Organic acids were analysed by HPLC. Error bars represent ± s.d. (n = 3).

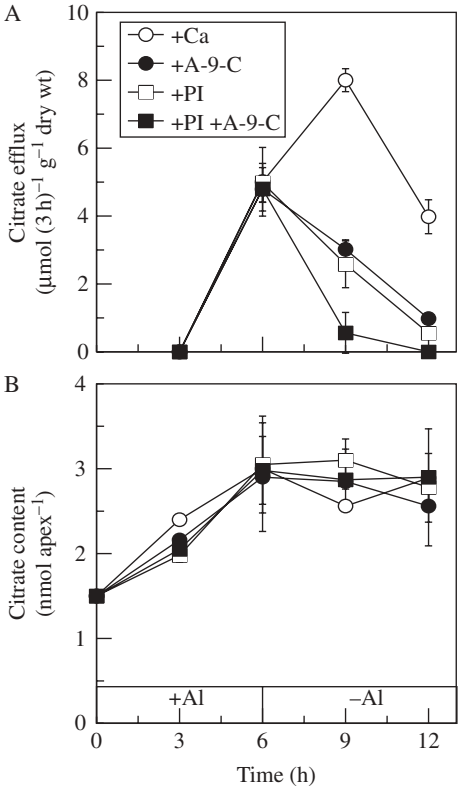

FIG. 5. Effect of A-9-C and PI on the citrate efflux (A) and content (B) after 6 h pulse treatment with 50 μM $AlCl_3$. Seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM $AlCl_3$ for 6 h and then transferred to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 20 μM A-9-C, 20 μM PI or both. Root exudates were collected every 3 h for determination of citrate efflux, and apical 5 mm root apices were excised for endogenous citrate content analysis. Error bars represent ± s.d. (n = 3).

DISCUSSION

Al-stimulated efflux of organic acids has been considered an important mechanism leading to Al resistance. We found that citrate was released from the roots of rice bean, but could not be detected during the first 3 h exposure to Al stress (Fig. 1). Furthermore, neither P deficiency nor $LaCl_3$ stimulated the release of citrate (data not shown), indicating that the efflux was selective to Al stress. The efflux of citrate was largely confined to the apical 5 mm root zone (Fig. 2) where Al caused the greatest damage to root cells (Ryan *et al*., 1993), indicating the importance of citrate efflux in detoxifying Al. Although it is difficult to estimate how much the citrate efflux contributes to Al resistance in rice bean, there are ample examples to suggest that organic acids play an important role in improving Al resistance (for reviews, see Ma *et al*., 2001; Ryan *et al*., 2001; Kochian *et al*., 2004). The delayed efflux of citrate in response to Al stress is characteristic of a pattern II response (Ma *et al*., 2001), indicating that some inducible processes such as gene activation might be involved in the efflux of citrate. The same characteristic has been reported in a number of other plant species including *C. tora* (Yang *et al*., 2005*b*), rye (Li *et al*., 2000) and triticale (Ma *et al*., 2000).

Ma *et al*. (2001) postulated that the delayed efflux of organic acids in response to Al stress might be related to Al-activated gene expression and *de novo* protein synthesis. These genes may be involved in the metabolism (organic acids biosynthetic and/or turnover enzymes) of organic acids, the anion channel on the plasma membrane or transport of organic acids out of the mitochondria (Ma, 2000). However, the experimental evidence supporting the speculations is lacking, and most current work is focused on relating the organic acid efflux to changes in enzyme activities. For example, Al induced an increase of citrate content

in rye and soybean, and the increase was accompanied by an increase in citrate synthase (Li *et al*., 2000; Yang *et al*., 2001). In line with this, genetic manipulation of plants to overexpress enzymes involved in organic acid biosynthesis showed increased organic acid contents, efflux and Al resistance (de la Fuente *et al*., 1997; Tesfaye *et al*., 2001; Anoop *et al*., 2003). Others, however, have shown poor correlations between efflux and the activities of biosynthetic enzymes (Ryan *et al*., 1995; Delhaize *et al*., 2001; Hayes and Ma, 2003; Zhao *et al*., 2003; Yang *et al*., 2005*b*). In the present study, we found that Al treatment increased citrate content in root apices (Fig. 5B). However, it is not adequate to state that Al-regulated citrate metabolism plays an important role in Al-stimulated citrate efflux, and in the present study we even argue that citrate metabolism plays at most a minor role in the efflux of citrate. The reasons supporting this rely on the following two lines of evidence. One is that the Al-induced increase in the content of citrate in root apices preceded the efflux (Fig. 5B). The other is that a 6 h pulse with 50 μM Al also significantly stimulated citrate efflux after 3 h and this was decreased thereafter when roots were transferred to the Ca solution (Fig. 5A), but the citrate content in root apices remained steady (Fig. 5B). Furthermore, studies using the patch–clamp technique have demonstrated that anion-permeable channels allowing the passive flow of organic anions from the cytosolic side of the membrane to the apoplasm are involved in the efflux of organic anions (Ryan *et al*., 1997; Kollmeier *et al*., 2001; Piñeros and Kochian, 2001; Zhang *et al*., 2001). Although we did not investigate the relationship between enzyme activities and citrate biosynthesis, the present results clearly imply that the transport of citrate across the membrane might constitute the critical step in the efflux of citrate in roots of rice bean.

Citrate is produced in mitochondria through the tricarboxylic acid or Krebs cycle, and citrate carrier, an intrinsic protein of the inner mitochondrial membrane, plays a vital role in exporting citrate out of the mitochondria. Since the pH of the cell cytosol is near neutral, the concentration of the undissociated citric acid is very low. Thus the thermodynamically passive transport of citrate anions across the plasma membrane can be mediated by anion channels (Ryan *et al*., 2001). In the present study, although different inhibitors showed different effects on the efflux, either anion channel inhibitors or citrate carrier inhibitors inhibited the Al-stimulated citrate efflux from roots of rice bean (Figs 3 and 4), indicating the possible involvement of both the citrate carrier and anion channel in the efflux process. It has also been reported that anion channel inhibitors can effectively inhibit malate efflux in wheat (Ryan *et al*., 1995) and maize (Jorge and Menossi, 2005). Li *et al*. (2000) found that two citrate carrier inhibitors effectively inhibited citrate efflux in rye. In the present study, the fact that a 6 h pulse with 50 μM Al was sufficient to induce citrate efflux (Fig. 5A) indicated that the opening of transporters can be maintained at least for 3 h. However, the addition of either PI (a citrate carrier inhibitor) or A-9-C (an anion channel inhibitor) inhibited the efflux of citrate greatly, and efflux was almost completely inhibited when the roots were exposed to both inhibitors (Fig. 5A), further confirming the cooperative roles of both transporter proteins in the regulation of citrate efflux.

In a previous study, we demonstrated that a protein synthesis inhibitor, CHM, inhibited Al-induced citrate efflux in *C. tora* (reported as a pattern II plant), and concluded that *de novo* synthesis of the anion channel is involved in the Al-induced secretion of citrate in *C. tora* (Yang *et al*., 2005*b*). In the present study, we also found that the Al-stimulated citrate efflux was significantly inhibited by 20 μM CHM (Fig. 6), indicating that *de novo* protein synthesis is also required for citrate efflux in rice bean. Given that other proteins are not required for the activation of transporter proteins mediating citrate efflux, we proposed that Al first activated gene expression, then these genes encode both citrate carrier and anion channel protein synthesis. However, the possibility still remains that it is *de novo* synthesis of other proteins rather than the transporters themselves that is required for the activation of transporter proteins. For example, Osawa and Matsumoto (2001) demonstrated that a 48 kDa K-252a-sensitive protein kinase might be involved in Al-stimulated malate efflux in wheat. Shen *et al*. (2004) related the Al-stimulated citrate efflux in soybean to abscisic acid biosynthesis. Recently, they further demonstrated that upregulation of plasma membrane H^+-ATPase activity was associated with the citrate efflux in soybean roots (Shen *et al*., 2005).

In conclusion, these results suggest that Al-stimulated citrate efflux from roots of rice bean is mediated by both citrate carrier and anion channel. The possible involvement of other factors associated with the opening of transporter proteins has yet to be investigated.

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