

RESEARCH PAPER

The role of *VuMATE1* expression in aluminium-inducible citrate secretion in rice bean (*Vigna umbellata*) roots

Mei Ya Liu^{1*}, Wei Wei Chen^{2*}, Jia Meng Xu¹, Wei Fan¹, Jian Li Yang^{1†} and Shao Jian Zheng¹

¹ State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

² College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, PR China

* These authors contributed equally to this work.

† To whom correspondence should be addressed. Email: yangjianli@zju.edu.cn

Received 6 December 2012; Revised 21 January 2013; Accepted 24 January 2013

Abstract

Aluminium (Al)-activated citrate secretion plays an important role in Al resistance in a number of plant species, such as rice bean (*Vigna umbellata*). This study further characterized the regulation of *VuMATE1*, an aluminium-activated citrate transporter. Al stress induced *VuMATE1* expression, followed by the secretion of citrate. Citrate secretion was specific to Al stress, whereas *VuMATE1* expression was not, which could be explained by a combined regulation of *VuMATE1* expression and Al-specific activation of *VuMATE1* protein. Pre-treatment with a protein translation inhibitor suppressed *VuMATE1* expression, indicating that *de novo* biosynthesis of proteins is required for gene expression. Furthermore, post-treatment with a protein translation inhibitor inhibited citrate secretion, indicating that post-transcriptional regulation of *VuMATE1* is critical for citrate secretion. Protein kinase and phosphatase inhibitor studies showed that reversible phosphorylation was important not only for transcriptional regulation of *VuMATE1* expression but also for post-translational regulation of *VuMATE1* protein activity. These results suggest that citrate secretion is dependent on both transcriptional and post-transcriptional regulation of *VuMATE1*. Additionally, *VuMATE1* promoter- β -glucuronidase fusion lines revealed that *VuMATE1* expression was restricted to the root apex and was entirely Al induced, indicating the presence of *cis*-acting elements regulating root tip-specific and Al-inducible gene expression, which will be an important resource for genetic improvement of plant Al resistance.

Key words: aluminium toxicity, *cis*-acting element, promoter, reversible phosphorylation, signalling transduction, transcription factor.

Introduction

Aluminium (Al)-inducible organic acid (OA) secretion from plant roots is an important mechanism controlling the degree of resistance to Al toxicity in many plant species (Ryan *et al.*, 2001; Kochian *et al.*, 2004; Delhaize *et al.*, 2012). These plant species can be divided into two groups based on the timing of secretion (Ma *et al.*, 2001). In the pattern I type, roots secrete OAs immediately upon suffering Al stress; wheat (*Triticum aestivum*) and buckwheat (*Fygotyrum esculentum* Moench) are examples of this pattern. In plants of the pattern II

type, an induction period after the onset of Al stress is required before the roots secrete OAs; *Cassia tora* and sorghum (*Sorghum bicolor*) fit this pattern. The major difference between the two patterns lies in whether or not gene induction is required for OA secretion. The malate transporter gene *ALUMINIUM-ACTIVATED MALATE TRANSPORTER 1* (*ALMT1*) was first isolated in wheat (Sasaki *et al.*, 2004), and homologous genes have been isolated in *Arabidopsis* (Hoekenga *et al.*, 2006) and oilseed rape (*Brassica napus*)

Abbreviations: Al, aluminium; CHX: cycloheximide; GUS, β -glucuronidase; MATE: multidrug and toxic compound extrusion; OA: organic acid; SD, standard deviation.

© The Author(2) [2013].

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by-nc/3.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com.

(Ligaba *et al.*, 2006). Genes involved in Al-induced citrate secretion, which are members of the multidrug and toxic compound extrusion (MATE) family, have also been isolated in barley (*Hordeum vulgare*) (Furukawa *et al.*, 2007), sorghum (Magalhaes *et al.*, 2007), *Arabidopsis* (Liu *et al.*, 2009), maize (*Zea mays*) (Maron *et al.*, 2010), wheat (Ryan *et al.*, 2009), rye (*Secale cereale*) (Yokosho *et al.*, 2010), rice (*Oryza sativa*) (Yokosho *et al.*, 2011) and rice bean (*Vigna umbellata*) (Yang *et al.*, 2011). However, the mechanisms regulating the expression of these genes are not clear.

Protein phosphorylation has been implicated in Al-inducible OA secretion. For example, protein kinase inhibitors have been demonstrated to block Al-activated malate secretion from wheat roots (Osawa and Matsumoto, 2001) and citrate secretion from soybean (*Glycine max*) roots (Shen *et al.*, 2004). As *ALMT1* is expressed constitutively in wheat roots (Sasaki *et al.*, 2004), it is reasonable to deduce that these inhibitors may have been acting on the protein rather than on the pathways regulating expression. Ligaba *et al.* (2009) generated a series of mutations targeting possible phosphorylation sites on TaALMT1, and expression of some of these mutant proteins in *Xenopus* oocytes resulted in altered current events either in the absence or presence of Al. However, Kobayashi *et al.* (2007) demonstrated that malate secretion from *Arabidopsis* roots under Al stress requires protein phosphorylation, which acts on *AtALMT1* at both transcriptional and post-translational levels. These results indicated that protein phosphorylation could be important for OA secretion from plant roots with either the pattern I or pattern II response.

In *Arabidopsis*, both malate and citrate are secreted from roots in response to Al stress (Hoekenga *et al.*, 2003). Expression of both *AtMATE1* and *AtALMT1* requires SENSITIVE TO PROTON RHIZOTOXICITY 1 (STOP1), a putative Cys₂His₂-type zinc-finger transcription factor involved in low pH resistance and Al tolerance in *Arabidopsis* (Iuchi *et al.*, 2007; Liu *et al.*, 2009). Yamaji *et al.* (2009) identified the rice transcription factor ALUMINIUM RESISTANCE TRANSCRIPTION FACTOR 1 (ART1), which regulates 31 genes implicated in both internal and external detoxification of Al at different cellular levels. Among these, the expression of *OsFRDL4*, which mediates Al-activated citrate secretion from rice roots, requires ART1 (Yokosho *et al.*, 2011). Both STOP1 in *Arabidopsis* and ART1 in rice are expressed constitutively, indicating that Al may trigger the post-translational regulation of both transcription factors to affect downstream gene expression.

Rice bean is an Al-resistant species and is well adapted to grow on acid soils by means of secretion of citrate from root apices (Yang *et al.*, 2006). However, citrate secretion upon Al exposure is delayed by several hours, suggesting a pattern II response for this species. Recently, we isolated a gene responsible for Al-inducible citrate secretion from rice bean roots, *VuMATE1* (Yang *et al.*, 2011). In the absence of Al stress, *VuMATE1* is not expressed (Yang *et al.*, 2011).

This study sought to clarify the interplay between *VuMATE1* expression and citrate secretion in rice bean to elucidate the underlying regulatory mechanisms. The results

demonstrated that, although citrate secretion was specific to Al stress, *VuMATE1* expression was not, and it was found that citrate secretion comprised a complex regulation pathway involving both *de novo* protein biosynthesis and reversible protein phosphorylation. Furthermore, we characterized the 5' flanking sequence of the *VuMATE1* gene, which contains *cis*-acting elements regulating both root tip-specific and Al-inducible gene expression.

Materials and methods

Plant materials and culture conditions

Seeds of rice bean (*V. umbellata*) were soaked in deionized water overnight, and germinated at 26 °C in the dark. After germination, the seeds were transferred to a net floating on a 0.5 mM CaCl₂ solution (pH 4.5). The solution was renewed daily. On d 3, seedlings were transferred to a 1.2 l plastic pot (eight seedlings per pot) containing aerated nutrient solution. The nutrient solution contained the following macro- and micronutrients (μM): CaSO₄ (200), CaCl₂ (200), MgSO₄ (100), KNO₃ (400), NH₄NO₃ (300), NaH₂PO₄ (5), H₃BO₃ (3), MnCl₂ (0.5), ZnSO₄ (0.4), CuSO₄ (0.2), Fe-EDTA (10), and (NH₄)₆Mo₇O₂₄ (1). The solution was adjusted to pH 4.5 with HCl and renewed daily. The plants, which were grown for an additional 3–5 d in an environmentally controlled growth room with a 14 h/26 °C day (light intensity of 300 μmol photons m⁻² s⁻¹) and a 10 h/22 °C night regime, were used for the following experiments.

Treatments

Root apices (apical 10 mm) were excised with a razor blade, and ten root apices were incubated at 26 °C in a 5 ml centrifuge tube containing 3 ml of 0.5 mM CaCl₂ solution (pH 4.5; Ca solution). Root apices were rinsed with the Ca solution three times (10 min each) to diminish ion leakage derived from cutting damage. During all incubation periods, root apices were shaken at 80 rpm. For the time-course study, root apices were exposed to Ca solution containing either 25 μM AlCl₃ (Al solution) or 25 μM LaCl₃ every 3 h, and the old solutions and roots were collected at each time point. The protein translation inhibitor cycloheximide (CHX) and the transcription inhibitor actinomycin D were dissolved in deionized water and applied at a final concentration of 20 and 10 μM, respectively. Protein kinase and protein phosphatase inhibitors K252a and cyclosporin A were applied at a final concentration of 5 μM. They were first dissolved in a small amount of dimethyl sulfoxide and then added to treatment solutions. The effects of various inhibitors on gene expression and citrate secretion were determined in two ways. The first set of experiments was carried out by incubating the root apices in inhibitors for 1 h, rinsing the roots with fresh Ca solution, and then transferring them to either Ca solution or Al solution for various periods. The second set of experiments was carried out by incubating the root apices in Al solution for 6 h, rinsing the roots with fresh Ca solution, and then treating them with various inhibitors for 3 h.

Citrate measurement

Citrate concentrations were determined according to the enzymatic method described by Zhao *et al.* (2003). Briefly, to 1 ml of treatment solution, 120 μl 1 M Tris/HCl buffer (pH 7.8) and 15 μl 10 mM NADH were added. After incubation at 25 °C for 40 min, 2 μl enzyme mixture (containing 1.25 U lactate dehydrogenase and 0.5 U malate dehydrogenase) was added and the reaction mixture was incubated for a further 40 min. The change in A₃₄₀ was monitored after the reaction was initiated with citrate lyase (0.5 U). Standard measurements were performed using the same procedure.

RNA extraction and quantitative real-time RT-PCR

Plant material was ground in liquid nitrogen and total RNA was extracted using an RNAplant_plus kit (Tiangen, Shanghai, China). First-strand cDNAs were synthesized from 1 μ g total RNA using a PrimeScriptTM RT-PCR Kit (Takara Bio, Japan), and diluted to 50–100 ng μ l⁻¹. Quantitative real-time PCR was performed on a LightCycler480 machine (Roche Diagnostics, Basel, Switzerland) using a SYBR PremixEx Taq kit (Takara Bio). The primer pairs used have been described previously (Yang *et al.*, 2011). Three biological replicate RNA/cDNA samples were generated, and each cDNA sample was tested with three technical replicates, from which the relative expression was calculated against that of the internal control 18S rRNA gene using the formula $2^{-\Delta\Delta C_p}$.

Construction of genomic libraries and isolation of the *VuMATE1* promoter

The 1.55 kb *VuMATE1* promoter was obtained according to the user manual of the GenomeWalkerTM Universal Kit (Clontech, CA, USA). In brief, four genome walker libraries were constructed by digesting separate aliquots of DNA with four different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, and *Stu*I), followed by ligation to a genome walker adaptor. Nested PCR was performed using the outer/inner adaptor primer provided by the kit and two *VuMATE1* gene-specific primers (5'-TTGGTGCTGAAAGATGACTGGAGAAGTGAT-3' and 5'-GATCTTCTACATTGGGTGCCTAACT-3'). After the nested PCR, amplified fragments were cloned into the pMD18-T vector (Takara Bio). The sequences that extended upstream of the cDNA clones were isolated as the 5'-upstream regions of the gene.

Construction of the *VuMATE1*- β -glucuronidase (*GUS*) chimaeric gene and histochemical staining of *VuMATE1*-*GUS* plants

The 1.55 kb *VuMATE1* promoter was amplified from genomic DNA (primers MATE1GUSfor 5'-GGTACCTAAGGCTCGCA CAAGCTTCA-3' and MATE1GUSrev 5'-CCATGGTAATGAATT TGGATGAACTC-3') and cloned into the pMD18-T vector (Takara Bio). The correct nucleotide sequence was verified and subcloned into the multicloning site between *Kpn*I and *Nco*I of pCAMBIA1301. The generated *VuMATE1p::GUS* construct was introduced into the *Agrobacterium tumefaciens* strain EHA105, and *A. tumefaciens*-mediated transformation of *Arabidopsis* plants (Col-0) was accomplished according to the floral dip protocol (Clough and Bent, 1998). T1 seeds were surface sterilized and plated on one-half strength MS medium supplemented with hygromycin. The resistant plants were transferred to soil and allowed to set seed (T2). Transgenic lines that displayed a 3:1 ratio for hygromycin resistance in the T3 generation were selected for further analysis. All experiments were performed using plants corresponding to the T4 generation. *GUS* staining was performed according to the method of Jefferson *et al.* (1987) after exposure to Al (1.0 μ M activity) at pH 5.0. Seedlings were observed and photographed with a Nikon AZ100 microscope.

Results

Time course of Al-induced *VuMATE1* expression and citrate secretion

There is a lag phase for secretion of citrate from the intact roots of rice bean in response to Al treatment (Yang *et al.*, 2006), which is typical of pattern II OA release. To establish whether gene induction was required for this pattern II response, we performed a time-course analysis of citrate secretion and *VuMATE1* expression in the root apex. Citrate secretion rates were equivalent in both Al-treated and control

plants at the first 3 h time point (Fig. 1A). Citrate secretion rates remained unchanged for the duration of the treatment in the absence of Al, but increased with Al treatment, reaching a steady state after 6–9 h of treatment. In parallel, no signals could be detected for *VuMATE1* mRNA in the absence of Al treatment, and only very low levels of expression were observed within 3 h of Al treatment. However, the transcript abundance of *VuMATE1* greatly increased after 6 h of Al treatment and then decreased to relatively low levels thereafter (Fig. 1B). Our previous study demonstrated that *VuMATE1* expression remained constant after reaching a maximum by 6 h of Al exposure (Yang *et al.*, 2011). This discrepancy might arise from the different experimental conditions used, i.e. whole roots in the previous study and excised root apices in the present study. From the above results, it was clear that, in addition to the lag between the onset of Al stress and *VuMATE1* expression, there was a further 3 h lag between gene induction and citrate secretion.

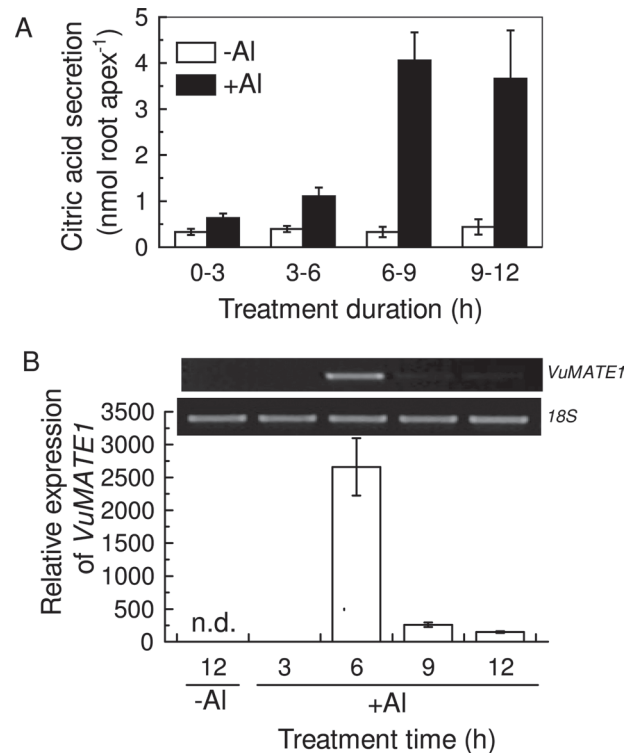


Fig. 1. Citrate release and *VuMATE1* expression time series. (A) Induction of citrate secretion from root apices of rice bean by Al treatment. Excised root apices (10 mm in length) were placed in 0.5 mM CaCl₂ solution containing 0 or 25 μ M Al. Data are means \pm standard deviation (SD) of three independent experiments ($n=3$ for each experiment). (B) Time course of *VuMATE1* expression in the roots of rice bean in response to Al treatment. Expression levels were normalized to that of the 18S rRNA gene and then to the expression of *VuMATE1* in root apices at 3 h of Al treatment, which was arbitrarily fixed at 1. *VuMATE1* expression in root apices treated with 0.5 mM CaCl₂ solution for 12 h was included. Results are shown as mean expression \pm SD of three independent experiments. n.d. indicates that no signal was detected. The inset shows semi-quantitative RT-PCR of *VuMATE1* expression.

Effect of La on *VuMATE1* expression and citrate secretion

Several lanthanides such as La^{3+} and Yb^{3+} have ionic properties similar to those of Al^{3+} in an aqueous solution. In the Al-resistant wheat genotype ET8, these lanthanides can induce malate secretion (Kataoka *et al.*, 2002). However, whether *TaALMT1* induction is involved in lanthanide-induced malate secretion from wheat roots is not clear. In our previous study, we demonstrated that rice bean roots did not respond to La stress in terms of citrate secretion, indicating that citrate secretion is specific to Al stress in rice bean (Yang *et al.*, 2011). To determine whether *VuMATE1* expression was specific to Al treatment as well, we exposed root apices to La stress treatment. Citrate secretion rates were equivalent in both La-stressed roots and controls during the entire 12 h of treatment (Fig. 2A). However, *VuMATE1* expression was induced by La stress and the timing of expression was similar to that under Al stress, although the expression level was

lower in La-stressed roots than in Al-stressed roots (Figs 1B and 2B).

De novo protein biosynthesis is involved in Al-induced gene expression and citrate secretion

We demonstrated previously that application of CHX, a broad translation inhibitor, effectively inhibited Al-triggered citrate secretion from the intact roots of rice bean (Yang *et al.*, 2006). To examine whether *de novo* biosynthesis of proteins was required for *VuMATE1* induction, CHX was applied in two sets of experiments. First, excised root apices were treated with or without CHX for 1 h and then moved to Ca solution with or without Al. CHX pre-treatment completely blocked Al-inducible citrate secretion during the following 9 h of Al exposure. CHX pre-treatment also resulted in inhibition of *VuMATE1* expression by 65% (Fig. 3). The time course analysis of *VuMATE1* expression with CHX pre-treatment showed that the transcript abundance was very low in the first 6 h of Al exposure (data not shown), indicating that

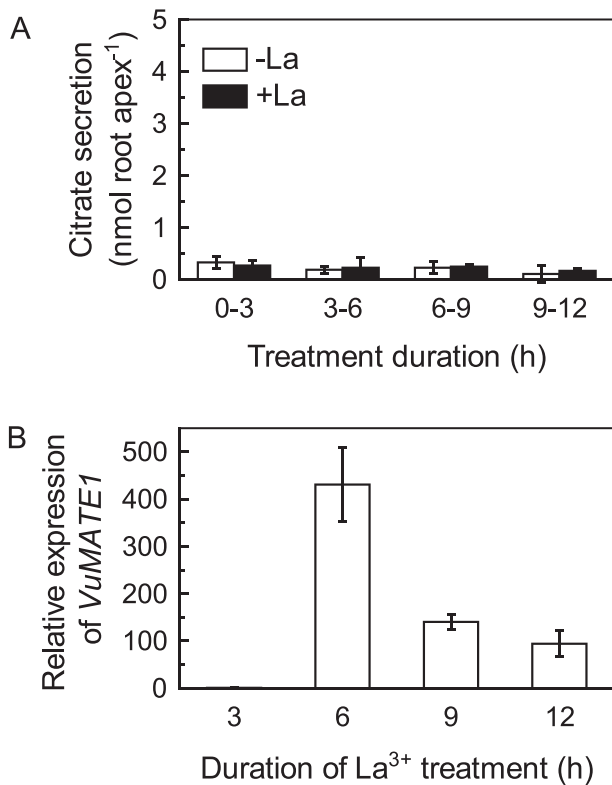


Fig. 2. Time course of citrate release and *VuMATE1* expression in the roots of rice bean in response to LaCl_3 treatment. (A) Induction of citrate secretion from root apices of rice bean by La treatment. Excised root apices (10 mm in length) were placed in 0.5 mM CaCl_2 solution containing 0 or 25 μM La. Data are means \pm SD of three independent experiments ($n=3$ for each experiment). (B) Time course of *VuMATE1* expression in the roots of rice bean in response to La treatment. Expression levels were normalized to that of the 18S rRNA gene and then to *VuMATE1* expression in the root apices at 3 h of La treatment, which was arbitrarily fixed at 1. Results are shown as mean expression \pm SD of three independent experiments.

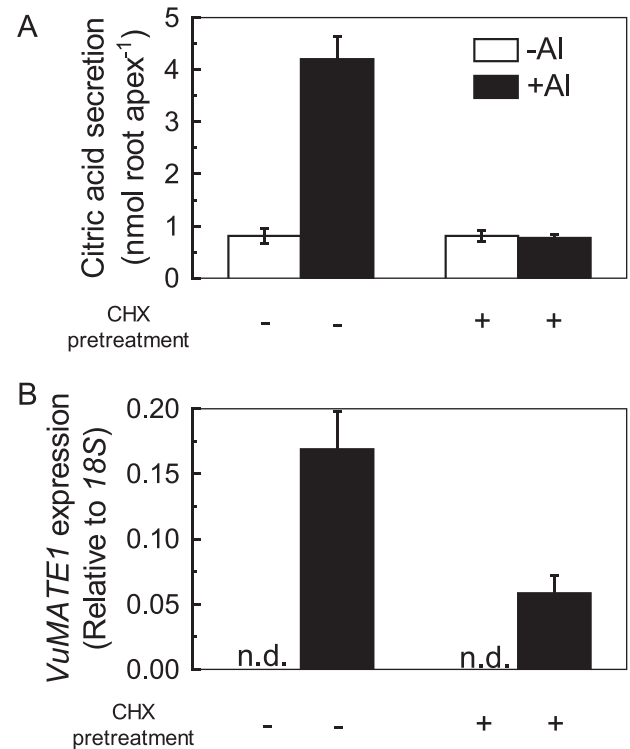


Fig. 3. Effect of protein-synthesis inhibitor (CHX, 20 μM) pre-treatment on the activation of citrate secretion from rice bean roots by Al treatment. Root apices pre-incubated for 1 h in 0.5 mM CaCl_2 solution with or without CHX were transferred to solutions with or without Al for 9 h. (A) Root exudates were collected for measurement of citrate. Results are shown as means \pm SD of three independent experiments ($n=3$ for each experiment). (B) Root apices were used to analyse gene expression. Expression of *VuMATE1* was analysed by quantitative RT-PCR, using the 18S rRNA gene as a reference. Results are shown as mean expression \pm SD of three independent experiments. n.d. indicates that no signal was detected.

the *VuMATE1* expression detected at the 9 h time point was probably due to diminishing CHX action. In the absence of Al stress, *VuMATE1* expression was not detected, irrespective of treatment with CHX or not. These results implied that *de novo* protein biosynthesis is required for *VuMATE1* expression, and rule out the possible involvement of a repressor protein suppressing *VuMATE1* transcription in the absence of Al.

Secondly, root apices were treated with Al for 6 h and then moved to Al-containing solution (as a control) or Al-free solution with or without CHX for 3 h (CHX post-treatment; Fig. 4A). In comparison with continued Al stress, withdrawal of Al had no effect on citrate efflux during the following 3 h of treatment (Fig. 4B), indicating either that Al is not required for citrate efflux after gene induction or that the residual Al was sufficient to maintain secretion. However, when CHX was included in the medium, secretion of citrate was almost completely blocked (Fig. 4B). In parallel, there were no significant differences in *VuMATE1* transcript abundance between continuous Al stress and withdrawal of Al, and the expression under both conditions was significantly lower than that after 6 h of Al stress (Fig. 4C). The *VuMATE1* mRNA decreased to a very low level with CHX post-treatment (Fig. 4C). These results indicated that the translation of *VuMATE1* into protein after gene induction is the critical step for citrate secretion to citrate efflux in this experiment, we used the transcription inhibitor actinomycin D. Application of actinomycin D to pre-activated root apices completely inhibited *VuMATE1* expression but had no effect on citrate secretion (Fig. 4).

Protein phosphorylation is involved in Al-induced gene expression and citrate secretion

Protein phosphorylation has been implicated in Al-induced malate and citrate secretion from plant roots (Osawa and Matsumoto, 2001; Shen *et al.*, 2004; Ligaba *et al.*, 2009). We examined whether reversible protein phosphorylation affected Al-inducible citrate secretion in rice bean roots by perturbing protein kinase and phosphatase function using the protein kinase inhibitor K252a and the protein phosphatase inhibitor cyclosporin A. These inhibitors were applied in two sets of experiments. First, inhibitors were used to pre-treat root apices before Al exposure. Pre-treatment with either K252a or cyclosporin A completely blocked Al-induced citrate secretion from root apices during the following 9 h (Fig. 5A). There are various levels at which reversible protein phosphorylation could regulate Al-inducible citrate secretion: acting on *VuMATE1* protein to modulate transport activity, influencing *VuMATE1* expression, or both. After 6 h of Al exposure, *VuMATE1* expression was not detected in root apices pre-treated by the inhibitors, and after 9 h of Al exposure, it was only beginning to be expressed (Fig. 5B), indicating that protein phosphorylation is required for transcriptional regulation of *VuMATE1* expression.

Secondly, these inhibitors were used to treat root apices already stressed by Al in order to examine the effect of these inhibitors on transcribed *VuMATE1*. Root apices were

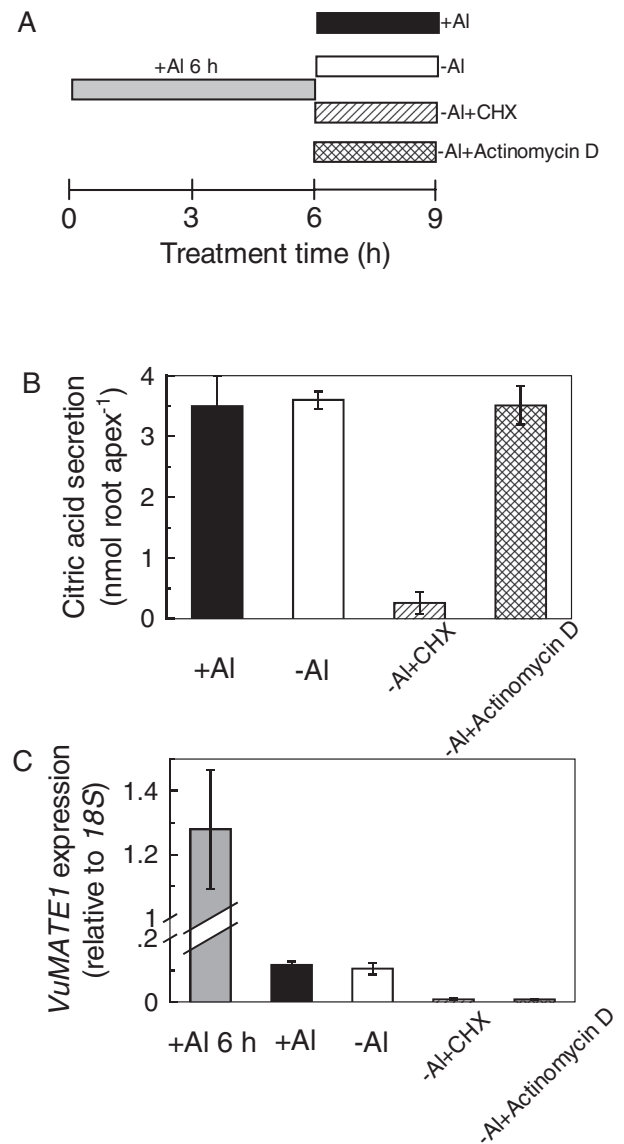


Fig. 4. Effect of a translation inhibitor (CHX, 20 μ M) or a transcription inhibitor (actinomycin D) on pre-activated citrate secretion from rice bean roots. (A) Summary of treatment conditions. Root apices were pre-incubated in Al (25 μ M) for 6 h (shaded bar), and transferred to Al solution (+Al; filled bar) or to Ca solution with CHX (-Al+CHX; striped bar), actinomycin D (-Al+actinomycin D; hatched bar), or neither (-Al; open bar) for 3 h. (B) Root exudates were collected for citrate measurement. Results are shown as means \pm SD of three independent experiments ($n=3$ for each experiment). (C) Root apices were used to analyse gene expression. For comparison, *VuMATE1* expression in pre-activated roots (Al 6 h) was included. Expression of *VuMATE1* was analysed by quantitative RT-PCR, using the 18S rRNA gene as a reference. Results are shown as mean expression \pm SD of three independent experiments.

treated with Al for 6 h and then transferred to Ca solution with or without the inhibitors (Fig. 6A). In comparison with Ca solution, application of cyclosporin A had no effect on citrate secretion, whilst K252a decreased citrate secretion by

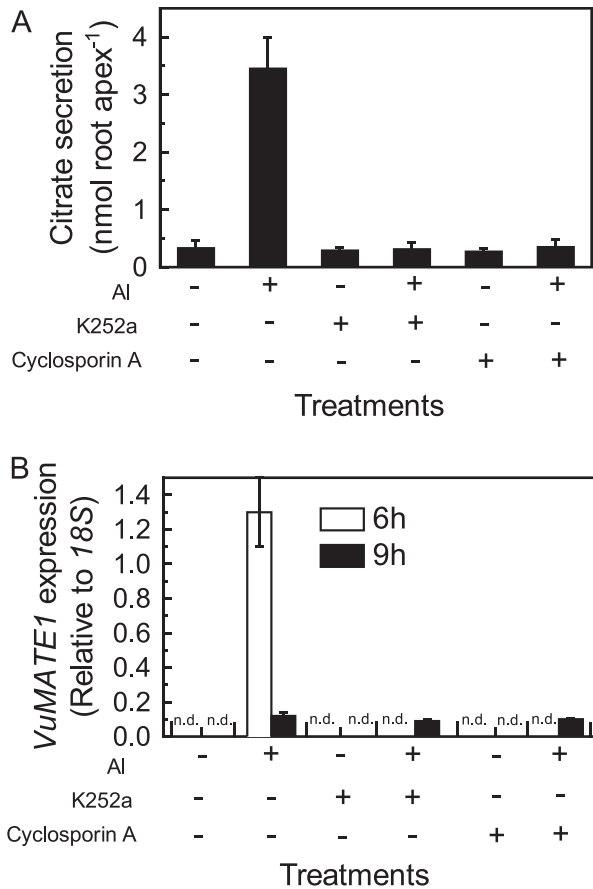


Fig. 5. Effect of protein kinase inhibitor or protein phosphatase inhibitor pre-treatment on the activation of citrate secretion from rice bean roots. Root apices pre-incubated for 1 h in 0.5 mM CaCl₂ solution containing 5 μ M protein kinase inhibitor K252a, 5 μ M protein phosphatase inhibitor cyclosporin A, or neither were transferred to solutions with or without Al for 9 h. (A) Root exudates were collected for measurement of citrate. Results are shown as means \pm SD of three independent experiments ($n=3$ for each experiment). (B) Root apices were used to analyse gene expression. Expression of *VuMATE1* was analysed by quantitative RT-PCR, using the 18S rRNA gene as a reference. Results are shown as mean expression \pm SD of three independent experiments. n.d. indicates that no signal was detected.

47% (Fig. 6B). In parallel, these inhibitors had no effect on *VuMATE1* expression (Fig. 6C). The results indicated that a K252a-sensitive protein kinase is involved in post-transcriptional regulation of *VuMATE1* activity.

Tissue-level localization of *VuMATE1* expression via promoter-GUS fusions

To analyse further the expression pattern and location of *VuMATE1*, a 1.55 kb DNA sequence upstream of the start codon was isolated. This promoter fragment was fused to a *GUS* reporter gene and transformed into wild-type *Arabidopsis* by *Agrobacterium*-mediated transformation. In the absence of Al stress, *GUS* staining was not observed in the root apex (Fig. 7A). After a 3 h exposure of the roots to

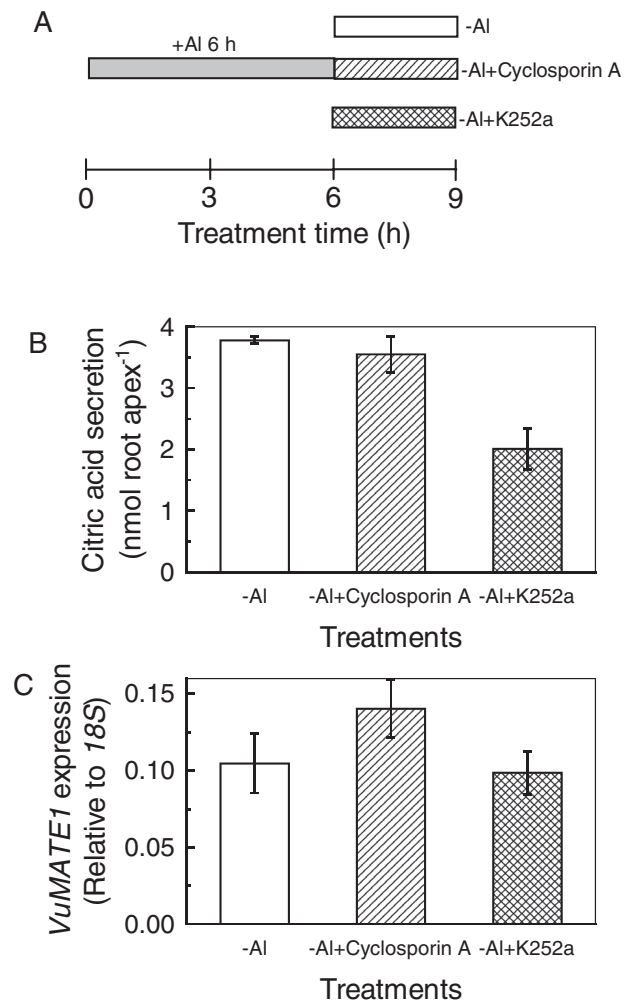


Fig. 6. Effect of protein kinase inhibitor (K252a, 5 μ M) or protein phosphatase inhibitor (cyclosporin A, 5 μ M) on pre-activated citrate secretion from rice bean roots. (A) Treatment conditions. Root apices were pre-incubated in Al (25 μ M) for 6 h, and transferred to Ca solution with cyclosporin A (-Al+cyclosporin A; striped bar), K252a (-Al+K252a; hatched bar) or neither (-Al; open bar) for 3 h. (B) Root exudates were collected for citrate measurement. Results are shown as means \pm SD of three independent experiments ($n=3$ for each experiment). (C) Root apices were used to analyse gene expression. Expression of *VuMATE1* was analysed by quantitative RT-PCR, using the 18S rRNA gene as a reference. Results are shown as mean expression \pm SD of three independent experiments.

Al, *GUS* staining appeared from the central cylinder, and extended to the entire root apex after prolonged exposure (Fig. 7B–D). In the mature root, the vascular cylinder was the site of *GUS* activity independent of Al stress, whilst the cortical and epidermis cells did not show expression (Fig. 7E–H).

Discussion

Al-induced citrate secretion is a major Al tolerance mechanism in both dicots and monocots, mediated by members of the MATE family (Delhaize *et al.*, 2012). *VuMATE1* is

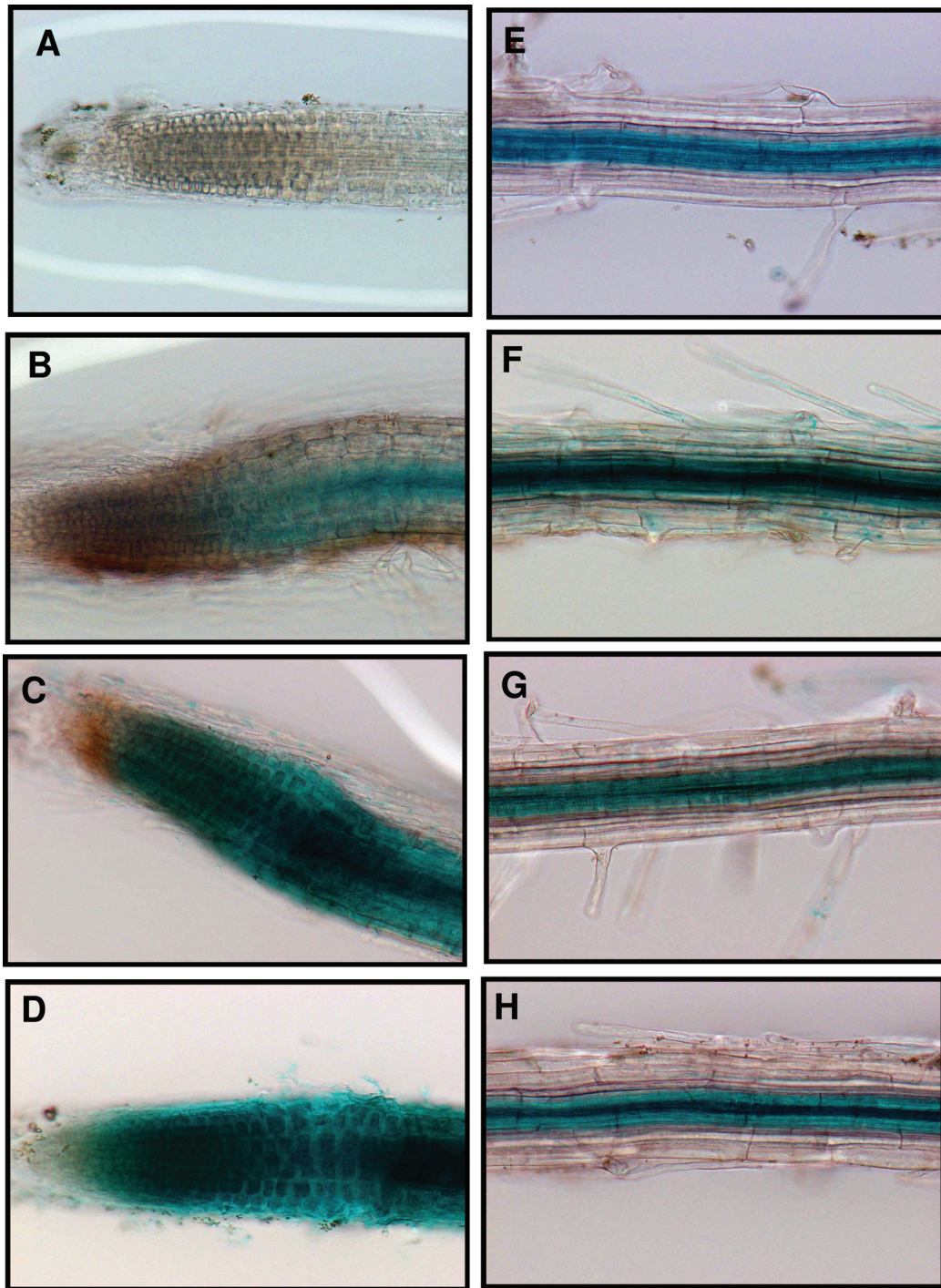


Fig. 7. *VuMATE1p::GUS* expression in transgenic plants. Ten-d-old seedlings were treated with modified one-fifth-strength Hoagland solution (pH 5.0) containing $7 \mu\text{M Al}^{3+}$ ($1 \mu\text{M}$ activity) for 0 (A, E), 3 (B, F), 6 (C, G), or 9 (D, H) h. (A)–(D) show the root apex, and (E)–(H) show the mature zone.

responsible for Al-activated citrate secretion in rice bean roots (Yang *et al.*, 2011). We have shown here that the function of *VuMATE1* under Al stress is subject to both transcriptional and post-transcriptional regulation, and that *VuMATE1* expression is necessary but not sufficient for citrate secretion in rice bean.

Citrate secretion was more tightly regulated by *VuMATE1* protein activity than by the pattern of gene expression. First,

whereas La treatment enhanced accumulation of *VuMATE1* mRNA, it did not increase the rate of citrate secretion from the root apex (Fig. 2). Secondly, *VuMATE1* expression was maximal after 6 h of Al exposure, but citrate secretion rates took until 9 h of Al exposure to reach their maximum (Fig. 1). This inconsistency suggests either that it takes about 3 h to translate *VuMATE1* mRNA into protein or that regulation of the transport activity of *VuMATE1* depends on some

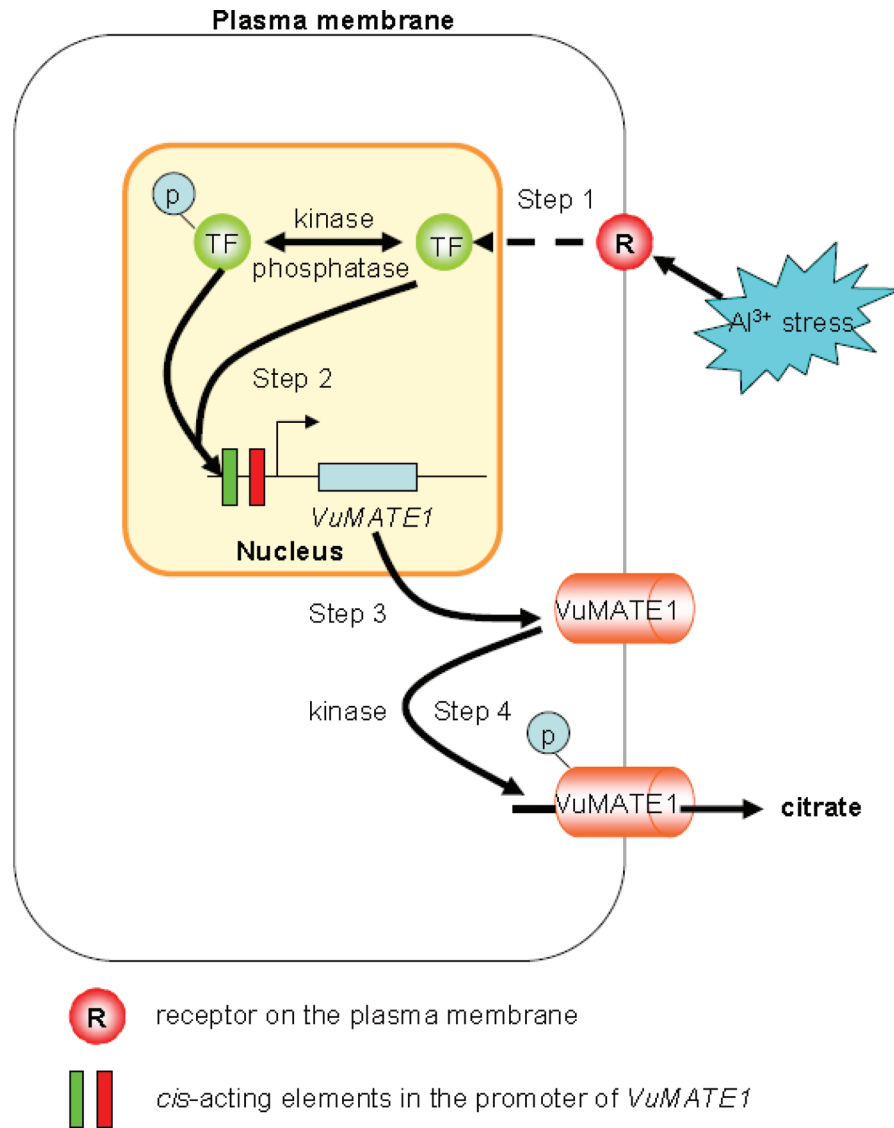


Fig. 8. Hypothetical model for Al-induced citrate secretion from roots of a typical pattern II-type rice bean plant. At least four steps are involved in the process. Step 1: Al^{3+} interacts with a receptor (R) on the plasma membrane to initiate a signal transduction pathway, which triggers the *de novo* biosynthesis of an unidentified transcription factor (TF). Step 2: reversible protein phosphorylation is required for the TF to interact with sequences in the promoter of *VuMATE1* and induce its expression. Step 3: translation of *VuMATE1* mRNA into protein. Step 4: a K252a-sensitive protein kinase is involved in the phosphorylation of *VuMATE1* for activation of *VuMATE1* to transport citrate out of the cell through the plasma membrane. (This figure is available in colour at *JXB* online.)

other activating factors. Thirdly, actinomycin D was able to completely inhibit *VuMATE1* expression but had no effect on citrate secretion once the root apex had been pre-activated by Al stress (Fig. 4). Finally, the protein kinase inhibitor K252a could block citrate secretion without reducing *VuMATE1* expression levels (Fig. 6).

VuMATE1 is upregulated by Al stress, suggesting the involvement of upstream sensing and/or signalling components. Recent studies on rice and *Arabidopsis* have identified ART1 (Yamaji *et al.*, 2009) and STOP1 (Liu *et al.*, 2009) as the core components regulating *MATE* and *ALMT* genes involved in OA secretion. It is reasonable to infer that a functional homologue of STOP1 or ART1 also exists in rice bean. As neither *STOP1* in *Arabidopsis* nor *ART1* in rice expression

is induced by Al, the signal transduction pathway probably involves post-transcriptional and post-translational regulation of both regulators. It can be assumed that STOP1 and ART1 proteins accumulate upon Al stress and activate gene transcription, or that protein abundance is not affected but instead the proteins are activated directly by the Al signal. In rice bean, pre-treatment with CHX for 1 h resulted in inhibition of *VuMATE1* transcription (Fig. 3), implying that regulator(s) involved in *VuMATE1* expression need to be synthesized *de novo*.

We have demonstrated that reversible protein phosphorylation is necessary for *VuMATE1* expression and citrate secretion. Reversible protein phosphorylation is also implicated in Al-induced malate secretion from the wheat root apex and

citrate secretion from soybean roots (Osawa and Matsumoto, 2001; Shen *et al.*, 2004). However, the question arises as to whether protein kinases and phosphatases act upstream or downstream of transporters to regulate OA secretion. Pre-treatment with the protein kinase inhibitor K252a or protein phosphatase inhibitor cyclosporin A blocked citrate secretion, which closely followed the blocking of gene expression (Fig. 5). This indicated that both protein kinases and phosphatases are required to activate regulators acting upstream of *VuMATE1*. This is consistent with similar experiments in *Arabidopsis*, where both a protein kinase inhibitor (staurosporine) and a phosphatase inhibitor (calyculin A) reduce Al-induced malate secretion, probably due to a reduction in *AtALMT1* expression (Kobayashi *et al.*, 2007). On the other hand, we further demonstrated that protein kinases are also involved in post-transcriptional regulation of *VuMATE1* activity. When Al was withdrawn from the solution bathing the root apex, citrate secretion rates remained unchanged (Figs 4B and 6B). Cyclosporin A application did not interfere with secretion rates; however, K252a treatment reduced citrate secretion rates by 47% (Fig. 6B). Because the non-inhibitory or inhibitory effects of cyclosporin A or K252a, respectively, on citrate secretion were not related to the pattern of gene expression (Fig. 6C), it is reasonable to speculate that K252a-sensitive protein kinases are involved in activating or maintaining the activity of the *VuMATE1* transporter.

We demonstrated previously that the expression of *VuMATE1* is entirely Al-induced and restricted to the root apex (Yang *et al.*, 2011). In the present study, the reporter gene (*GUS*), when driven by the 5' flanking sequence of the *VuMATE1* gene, exhibited temporal and spatial expression patterns similar to those observed by RT-PCR analysis of gene expression (Fig. 7; Yang *et al.*, 2011). However, we also observed constitutive expression of reporter activity in the vascular cylinder of the mature root region. Fujii *et al.* (2012) reported that barley *HvAACT1* is typically expressed in the pericycle cells of the root mature zone, functioning as a citrate transporter required for Fe nutrition. However, in Al-tolerant barley cultivars, a 1 kb insertion upstream of the *HvAACT1* coding region changes the location of expression to the root tip, thereby protecting the root tip from Al toxicity (Fujii *et al.*, 2012). Whether *VuMATE1* plays two physiological roles in both detoxifying Al toxicity and regulating Fe translocation has yet to be investigated. The root tip is the major site for Al toxicity (Ryan *et al.*, 1993). Only when the expression of Al tolerance genes is confined to this critical area is detoxification capacity maximized. For instance, although a moderate amount of citrate is secreted from *Arabidopsis* roots under Al stress, the role of *AtMATE1* in Al resistance is diminished, as its expression is localized to the mature root regions (Liu *et al.*, 2012). The restricted spatial distribution of Al-induced expression of *VuMATE1* and *GUS* to the root apex suggested that *cis*-acting elements involved in root tip-specific expression are present in the 5' flanking sequence of the *VuMATE1* gene.

In addition to the presence of root tip-specific elements, there are Al-inducible sequences within the promoter of *VuMATE1*. To date, *MATE* genes involved in Al-mediated

citrate secretion have been isolated from at least nine plant species (Delhaize *et al.*, 2012). However, unlike *VuMATE1*, which is not transcribed in the root apex in the absence of Al (Fig. 7A), other *MATE* genes exhibit varying degrees of constitutive expression, e.g. *HvAACT1* expression in barley is not affected by Al and *SbMATE* expression in sorghum is further increased by Al stress (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007). Although the transgenic approach has been proven to be a useful strategy for genetic improvement of plant performance in acidic soils, most of the transgenes are driven by constitutive promoters. For example, overexpression of *VuMATE1* under the control of the CaMV35S promoter in tomato results in citrate secretion in the absence of Al (Yang *et al.*, 2011). Considering that the efflux of OA anions constitutes a significant carbon cost to plants and may affect plant growth, and that transcription of genes consumes additional energy, limiting the expression of Al tolerance genes to only when Al toxicity occurs constitutes an efficient resource-saving strategy. Thus, dissection of *cis*-acting elements that regulate Al-inducible gene expression will be useful for genetic improvement of Al resistance through promoter reconstruction. However, the *cis*-acting sequences regulating root tip-specific gene expression and responsible for Al-inducible gene expression need further characterization.

In summary, we have demonstrated that the regulation of citrate secretion in response to Al stress operates on multiple levels. As illustrated in Fig. 8, at least four steps are involved in the process. In step 1, transcription factor(s) or upstream control elements are synthesized *de novo* after sensing of the Al stress signal. In step 2, reversible protein phosphorylation is directly or indirectly involved in the interaction of transcription factors with the promoter of *VuMATE1* to initiate the transcription of *VuMATE1*. As the accumulation of *VuMATE1* mRNA and the appearance of *GUS* activity driven by the promoter of *VuMATE1* began after 3 h of Al exposure (Figs 1 and 7), it takes about 3 h to complete the first two steps. In step 3, the full induction of *VuMATE1* mRNA requires about 3 h; at this time point, a portion of the mRNA has been translated into functional protein, as citrate secretion has begun. In step 4, translation of the majority of *VuMATE1* mRNA into protein and translational modification are required for maximal citrate secretion, and this step may take about 3 h, as the maximum secretion of citrate occurred after 9 h of exposure to Al stress (Fig. 1). We also demonstrated that the promoter of *VuMATE1* contains *cis*-acting elements regulating both root tip-specific and Al-inducible gene expression, which provides an important resource for generation of crop varieties more suitable for growth on acidic soils.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (31071849 and 31222049), the Zhejiang Education Department (20070170), and the Changjiang Scholarship and Innovative Research Team (IRT1185).

References

- Clough SJ, Bent AF.** 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.
- Delhaize E, Ma JF, Ryan PR.** 2012. Transcriptional regulation of aluminium tolerance genes. *Trends in Plant Science* **17**, 341–348.
- Fujii M, Yokosho K, Yamaji N, Saisho D, Yanane M, Takahashi H, Sato K, Nakazono M, Ma JF.** 2012. Acquisition of aluminium tolerance by modification of a single gene in barley. *Nature Communications* **3**, 713.
- Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF.** 2007. An aluminum-activated citrate transporter in barley. *Plant & Cell Physiology* **48**, 1081–1091.
- Hoekenga OA, Maron LG, Piñeros MA, et al.** 2006. *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in *Arabidopsis*. *Proceedings of the National Academy of Sciences, U S A* **103**, 9738–9743.
- Hoekenga OA, Vision TJ, Shaff JE, Monforte AJ, Lee GP, Howell SH, Kochian LV.** 2003. Identification and characterization of aluminum tolerance loci in *Arabidopsis* (*Landsberg erecta* × *Columbia*) by quantitative trait locus mapping: a physiologically simple but genetically complex trait. *Plant Physiology* **132**, 936–948.
- Iuchi S, Koyama H, Iuchi A, Kobayashi Y, Kitabayashi S, Kobayashi Y, Ikka T, Hirayama T, Shinozaki K, Kobayashi M.** 2007. Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and co-regulates a key gene in aluminum tolerance. *Proceedings of the National Academy of Sciences, U S A* **104**, 9900–9905.
- Jefferson RA, Kavanagh TA, Benan MW.** 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901–3907.
- Kataoka T, Stekelenburg A, Nakanishi TM, Delhaize E, Ryan PR.** 2002. Several lanthanides activate malate efflux from roots of aluminium-tolerant wheat. *Plant Cell & Environment* **25**, 453–460.
- Kobayashi Y, Hoekenga OA, Itoh H, Nakashima M, Saito S, Shaff JE, Maron LG, Piñeros MA, Kochian LV, Koyama H.** 2007. Characterization of *AtALMT1* expression in aluminum-inducible malate release and its role for rhizotoxic stress tolerance in *Arabidopsis*. *Plant Physiology* **145**, 843–852.
- Kochian LV, Hoekenga OA, Piñeros MA.** 2004. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorus efficiency. *Annual Review of Plant Biology* **55**, 459–493.
- Ligaba A, Katsuhara M, Ryan PR, Shibasaka M, Matsumoto H.** 2006. The *BnALMT1* and *BnALMT2* genes from rape encode aluminum-activated malate transporters that enhance the aluminum resistance of plant cells. *Plant Physiology* **142**, 1294–1303.
- Ligaba A, Kochian LV, Piñeros M.** 2009. Phosphorylation at S384 regulates the activity of the TaALMT1 malate transporter that underlies aluminum resistance in wheat. *The Plant Journal* **60**, 411–423.
- Liu J, Luo X, Shaff J, Liang C, Jia X, Li Z, Magalhaes J, Kochian LV.** 2012. A promoter-swap strategy between the *AtALMT* and *AtMATE* genes increased *Arabidopsis* aluminum resistance and improved carbon-use efficiency for aluminum resistance. *The Plant Journal* **71**, 327–337.
- Liu J, Magalhaes JV, Shaff JE, Kochian LV.** 2009. Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer *Arabidopsis* aluminum tolerance. *The Plant Journal* **57**, 389–399.
- Ma JF, Ryan PR, Delhaize E.** 2001. Aluminum tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* **6**, 273–278.
- Magalhaes JV, Liu J, Guimaraes CT, et al.** 2007. A member of the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. *Nature Genetics* **39**, 1156–1161.
- Maron LG, Piñeros MA, Guimaraes CT, Magalhaes JV, Pleiman JK, Mao CZ, Shaff J, Belicuas SNJ, Kochian LV.** 2010. Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *The Plant Journal* **61**, 728–740.
- Osawa H, Matsumoto H.** 2001. Possible involvement of protein phosphorylation in aluminum-responsive malate efflux from wheat roots apex. *Plant Physiology* **126**, 411–420.
- Ryan PR, Delhaize E, Jones DL.** 2001. Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 527–560.
- Ryan PR, DiTomaso JM, Kochian LV.** 1993. Aluminum toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. *Journal of Experimental Botany* **44**, 437–446.
- Ryan PR, Raman H, Gupta S, Horst WJ, Delhaize E.** 2009. A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. *Plant Physiology* **149**, 340–351.
- Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H.** 2004. A wheat gene encoding an aluminum-activated malate transporter. *The Plant Journal* **37**, 645–653.
- Shen H, Ligaba A, Yamaguchi M, Osawa H, Shibata K, Yan XL, Matsumoto H.** 2004. Effect of K-252a and abscisic acid on the efflux of citrate from soybean roots. *Journal of Experimental Botany* **55**, 663–671.
- Yamaji N, Huang CF, Ngao S, Yano M, Sato Y, Nagamura Y, Ma JF.** 2009. A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *The Plant Cell* **21**, 3339–3349.
- Yang JL, Zhang L, Li YY, You JF, Wu P, Zheng SJ.** 2006. Citrate transporters play a critical role in aluminium-stimulated citrate efflux in rice bean (*Vigna umbellata*) roots. *Annals of Botany* **97**, 579–584.
- Yang XY, Yang JL, Zhou Y, Piñeros MA, Kochian LV, Li GX, Zheng SJ.** 2011. A *de novo* synthesis citrate transporter, *Vigna umbellata* multidrug and toxic compound extrusion, implicates in Al-activated citrate efflux in rice bean (*Vigna umbellata*) root apex. *Plant Cell & Environment* **34**, 2138–2148.
- Yokosho K, Yamaji N, Ma JF.** 2010. Isolation and characterization of two MATE genes in rye. *Functional Plant Biology* **37**, 296–303.
- Yokosho K, Yamaji N, Ma JF.** 2011. An Al-inducible MATE gene is involved in external detoxification of Al in rice. *The Plant Journal* **68**, 1061–1069.
- Zhao Z, Ma JF, Sato K, Takeda K.** 2003. Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). *Planta* **217**, 794–800.