The role of \textit{VuMATE1} expression in aluminium-inducible citrate secretion in rice bean (\textit{Vigna umbellata}) roots

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Abstract

Aluminium (Al)-activated citrate secretion plays an important role in Al resistance in a number of plant species, such as rice bean (\textit{Vigna umbellata}). This study further characterized the regulation of \textit{VuMATE1}, an aluminium-activated citrate transporter. Al stress induced \textit{VuMATE1} expression, followed by the secretion of citrate. Citrate secretion was specific to Al stress, whereas \textit{VuMATE1} expression was not, which could be explained by a combined regulation of \textit{VuMATE1} expression and Al-specific activation of \textit{VuMATE1} protein. Pre-treatment with a protein translation inhibitor suppressed \textit{VuMATE1} expression, indicating that \textit{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 \textit{VuMATE1} is critical for citrate secretion. Protein kinase and phosphatase inhibitor studies showed that reversible phosphorylation was important not only for transcriptional regulation of \textit{VuMATE1} expression but also for post-translational regulation of \textit{VuMATE1} protein activity. These results suggest that citrate secretion is dependent on both transcriptional and post-transcriptional regulation of \textit{VuMATE1} expression. Additionally, \textit{VuMATE1} promoter-\textit{β}-glucuronidase fusion lines revealed that \textit{VuMATE1} expression was restricted to the root apex and was entirely Al induced, indicating the presence of \textit{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, \textit{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 \textit{et al.}, 2001; Kochian \textit{et al.}, 2004; Delhaize \textit{et al.}, 2012). These plant species can be divided into two groups based on the timing of secretion (Ma \textit{et al.}, 2001). In the pattern I type, roots secrete OAs immediately upon suffering Al stress; wheat (\textit{Triticum aestivum}) and buckwheat (\textit{Fragopyrum 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; \textit{Cassia tora} and sorghum (\textit{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 \textit{ALUMINIUM-ACTIVATED MALATE TRANSPORTER 1 (ALMT1)} was first isolated in wheat (Sasaki \textit{et al.}, 2004), and homologous genes have been isolated in \textit{Arabidopsis} (Hoekenga \textit{et al.}, 2006) and oilseed rape (\textit{Brassica napus}).
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)6MoO4·2H2O(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 14h/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 5ml 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 A440 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 Trizol Plant Plus kit (Tiangen, Shanghai, China). First-strand cDNAs were synthesized from 1 μg total RNA using a PrimeScript™ RT-PCR Kit (Takara Bio, Japan), and diluted to 50–100 ng μl⁻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⁻ΔΔCₚ.

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 GenomeWalker™ Universal Kit (Clontech, CA, USA). In brief, four genome walker libraries were constructed by digesting separate aliquots of DNA with four different restriction enzymes (DraI, EcoRV, PvuII, and ScaI), 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'-GATCTTCTACATTGGGTGCTA-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 MATE1prom5'-GGTACCTAAGGCTCGCA CAAGGTTCA-3' and MATE1promrev5'-CCATGGAATGAATT CAAGCTTCA-3') and cloned into the pMD18-T vector (Takara Bio). The correct nucleotide sequence was verified and subcloned into the multicloning site between KpnI and NcoI of pCAMBIA1301. The generated VuMATE1prom: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 μM 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.
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
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 rule out the contribution of *VuAMTE1* transcription 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 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 9 h.
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 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
Regulation of \textit{Vu-MATE1} expression by Al

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

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

\textbf{Fig. 7.} \textit{VuMATE1p::GUS} expression in transgenic plants. Ten-d-old seedlings were treated with modified one-fifth-strength Hoagland solution (pH 5.0) containing 7 \( \mu \text{M} \text{Al}^{3+} \) (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.
other activating factors. Thirdly, actinomycin D was able to completely inhibit \textit{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 \textit{VuMATE1} expression levels (Fig. 6).

\textit{VuMATE1} is upregulated by Al stress, suggesting the involvement of upstream sensing and/or signalling components. Recent studies on rice and \textit{Arabidopsis} have identified ART1 (Yamaji et al., 2009) and STOP1 (Liu et al., 2009) as the core components regulating \textit{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 \textit{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 \textit{VuMATE1} transcription (Fig. 3), implying that regulator(s) involved in \textit{VuMATE1} expression need to be synthesized \textit{de novo}.

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

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\item It is reasonable to infer that a functional homologue of STOP1 or ART1 also exists in rice bean.
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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. Pretreatment 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 mature root 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 QA 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 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.

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