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

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ABSTRACT

Al-activated organic acid anion efflux from roots is an important Al resistance mechanism in plants. We have conducted homologous cloning and isolated Vigna umbellata multidrug and toxic compound extrusion (VuMATE), a gene encoding a de novo citrate transporter from rice bean. Al treatment up-regulated VuMATE expression in the root apex, but neither in the mature root region nor in the leaf. The degree of up-regulation of VuMATE was both partially Al concentration and time dependent, consistent with the delay in the onset of the Al-induced citrate efflux in rice bean roots. While La3+ moderately induced VuMATE expression, Cd2+ and Cu2+ did not induce the expression. Electrophysiological analysis of Xenopus oocytes expressing VuMATE indicated this transporter can mediate significant anion efflux across the plasma membrane. [14C]citrate efflux experiments in oocytes demonstrated that VuMATE is a H+–dependent citrate transporter. In addition, expression of VuMATE in transgenic tomato resulted in increased Al resistance, which correlated with an enhanced citrate efflux. Taken together, these findings suggest that VuMATE is a functional homolog of the known citrate transporters in sorghum, barley, maize and Arabidopsis. The similarities and differences of all the known citrate transporters associated with Al stress in the MATE family are also discussed.

Key-words: aluminium resistance; tomato; transgenic; Xenopus oocytes.

INTRODUCTION

Aluminium (Al) toxicity affects crop production on acid soils where Al complexed in aluminosilicate clays

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3–6 d in sorghum), prior to the substantial efflux of organic acid anions. The use of broad protein synthesis inhibitors (i.e. cycloheximide) allowed Yang et al. (2006b) to establish that while the transporter responsible for organic acid secretion pre-exists in plants exhibiting pattern I (i.e. buckwheat), Al-induced de novo synthesis of the organic acid anion transporter is required in pattern II plants (i.e. C. tora).

There are many genes potentially implicated in the overall organic acid anion efflux response, including those involved in the synthesis of organic acids, as well as transport across the plasma membrane, tonoplast membrane and mitochondrial membranes (Hill 1997). Heterologous expression of plasma membrane organic acid anion transporters has resulted in significant organic acid anion efflux both in transgenic plants and in Xenopus oocytes (e.g. Sasaki et al. 2004; Ligaba et al. 2006; Furukawa et al. 2007; Magalhaes et al. 2007), indicating transport across the plasma membrane is a crucial step in Al-induced organic acid anion secretion from the plant root. Following the cloning of the first Al-activated organic acid efflux transporter from wheat (i.e. TaALMT; Sasaki et al. 2004), which mediates malate efflux, TaALMT1 homologs have been cloned in rape (Ligaba et al. 2006) and Arabidopsis (Hoekenga et al. 2006). More recently, genes encoding citrate transporters that are members of a different transporter family, the multidrug and toxic compound extrusion (MATE) family, were isolated from barley (Furukawa et al. 2007) and sorghum (Magalhaes et al. 2007). MATE homolog genes have more recently been cloned in Arabidopsis (Liu et al. 2009), rye (Yokosho, Yamaji & Ma 2010) and maize (Maron et al. 2010).

With regard to members of these two families of organic acid anion transporter genes, TaALMT1 is found to be constitutively expressed in the root apices of Al-tolerant wheat genotypes (Sasaki et al. 2004). In the case of transporters from the MATE family, the expression level of HvMATE and SbMATE were found to have a constitutively higher expression in root tips of the Al-resistance genotypes (Furukawa et al. 2007; Magalhaes et al. 2007). However, sorghum also undergoes a significant Al-induced increase in SbMATE expression that takes 6 d of Al exposure to complete, while HvMATE expression is not enhanced by Al exposure. It is interesting to note that regardless of whether previously characterized plants were categorized as Pattern I (wheat, rape, barley) or Pattern II plant (sorghum, maize, rye), the organic acid efflux genes in all the these plant species exhibited some degree of significant constitutive expression prior to Al treatment. Therefore, the possibility of the existence of an organic acid anion transporter gene that is newly synthesized (i.e. de novo) in response to Al stress is interesting.

Rice bean [Vigna umbellata (Thunb.) Ohwi & Ohashi ‘Jiangnan’] cultivated in Quzhou (acid soil region, Zhejiang Province, China) is an Al-resistant species. In a previous study, we found that as in many other species, rice bean roots can specifically release citrate to alleviate Al toxicity. However, citrate efflux upon Al exposure was delayed by several hours (Yang et al. 2006a), suggesting a Pattern II-type response for this species. In the present study, we cloned VuMATE, which shares sequence similarities with SbMATE and other MATE-type organic acid transporters, and show here it is the citrate transporter in the root apex of rice bean. We found that although the transport properties of VuMATE are analogous to other plant MATE-type citrate transporters, VuMATE is the first citrate transporter gene from this family that is only expressed following Al treatment. Functional evidence for its role in Al resistance comes from its transgenic expression of VuMATE in tomato, which conferred citrate efflux and enhanced Al resistance.

MATERIALS AND METHODS

Plant cultivation and treatment

Seeds of rice bean (Vigna umbellata) were soaked in deionized 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 L solution of 0.5 mM CaCl2 (pH 4.5). The solution was renewed daily. On the third day, seedlings with similar size were transplanted into a 1 L plastic pot (eight seedlings per pot) containing aerated nutrient solution of the following composition (in 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-ethylenediaminetetraacetic acid (EDTA) (10) and (NH4)6Mo7O24 (1). The solution was adjusted to pH 4.5 by HCl and renewed daily. After 3 to 5 d of culture, the plants were subjected to the following treatments. The nutrient solution was used as basal treatment solution (Control), and different concentrations of AlCl3 were directly added into nutrient solution for Al treatment. For the time-course experiment, seedlings were subjected to 25 µM Al for 0, 3, 6, 9 or 24 h. For the Al concentration dependence experiment, seedlings were subjected to 0, 5, 10, 25 or 50 µM Al for 24 h. For the other metal treatments, the seedlings were subjected to the nutrient solution (pH 4.5) described earlier containing 25 µM CdCl2, 10 µM LaCl3, 0.5 µM CuCl2 either with or without 25 µM Al for 24 h. All of the experiments were carried out in an environmentally controlled growth room with a 14 h/26 °C day and a 10 h/22 °C night regime, a light intensity of 250 to 300 µmol photons m−2 s−1 and a relative humidity of 65%.

Cloning of VuMATE

VuMATE was cloned using homologous cloning and rapid amplification of cDNA ends (RACE) techniques. Total RNA was isolated from 25 µM Al-treated root tips (0–1 cm) of rice bean. Degenerated primers were designed from the conserved sequences of three known MATE-type citrate acid transporter genes (SbMATE, HvMATE and AtMATE), the Al-induced LnMATE homolog, and three other genes (GmA1Tsb1; accession no. EU586179; MtMATE; accession no. BT053089; Os10g0206800) found
by blast using LaMATE as query. Two fragments of about 900 and 300 bp were amplified using two pairs of degenerated primers: forward primer 5'-ACMACWCTNTTYG TNGCDGARGA-3', reverse primer 5'-HCCRTCNGCR AGAAGGDA-3' and forward primer 5'-TCCHCTTGY GCNGAYGG-3', reverse primer 5'-HCCRTCNGCRAG AAGDGA-3' respectively. The PCR products were purified by agarose gel DNA purification kit (BBI, Gwnet, UK), and cloned into the pMD20-T vector (Takara, Kyoto, Japan) for sequencing. The obtained two nucleotide sequences were aligned into one sequence. According to this sequence, two gene specific primers (5'-TGTTGAGTAGCGACCCAC AAACGGGATG-3' and 5'-AGCGTCTATTACGAGCAAGGCTTGGTTCA-3' for the 5' RACE and 5'-AGCGTCTATTACGAGCAAGGCTTGGTTCA-3' for the 3' RACE) were designed to clone 5' and 3' end of the cDNA using the SMART RACE cdNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. The PCR products were purified, sequenced and aligned as mentioned earlier. According to the 5'- and 3'-end sequences, the full-length cDNA of VuMATE was amplified with PCR forward primer 'TTAATGGAAGAGAATGG' and reverse primer 'CTAAGCCAATGAACAAC', cloned into pMD20-T vector (Takara) and fully sequenced using internal primers.

RNA isolation and gene expression analysis

Total RNA was isolated from eight root tips (0–1 or 1–2 cm) or 100 mg leaf tissue using the Plant RNA Out Kit (CAT#71203–50, TIANDZ) according to the manufacturer's instructions. One microgram of total RNA was synthesized into the first-strand cDNA using the Primerscript reverse transcriptase (Takara) following the manufacturer's instructions. Semi-quantitative RT-PCR was performed as described by Sun et al (2011) with some modifications. Harvesting of stages V and VI Xenopus laevis oocytes, cRNA microinjections and electrophysiological measurements were performed as described previously (Piñeros, Cancado & Kochian 2008; Maron et al. 2010). The ND96 (also referred to as ‘High NaCl’) bath solution contained (in mM): NaCl (96), KCl (1) and CaCl2 (1.8) with pH adjusted to 7.5 or 4.5. For the ‘Low NaCl’ bath solution, the NaCl concentration in the bath solution was reduced to 1 mM. The osmolarity of the later solution was reduced to 195 mOsm with sorbitol. The [14C]citrate efflux experiment was performed according to Maron et al. (2010) using ND96 bath solution adjusted to pH 4.5 or 7.5.

Electrophysiological assays in Xenopus oocytes

The coding regions (cDNA) of VuMATE were inserted between the 5' and 3' UTRs of the Xenopus ß-globin gene in a T7TS vector. After linearizing, cRNA was synthesized using a message machine in vitro transcription kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations. Harvesting of stages V and VI Xenopus laevis oocytes, cRNA microinjections and electrophysiological measurements were performed as described previously (Piñeros, Cancado & Kochian 2008; Maron et al. 2010). The ND96 (also referred to as ‘High NaCl’) bath solution contained (in mM): NaCl (96), KCl (1) and CaCl2 (1.8) with pH adjusted to 7.5 or 4.5. For the ‘Low NaCl’ bath solution, the NaCl concentration in the bath solution was reduced to 1 mM. The osmolarity of the later solution was reduced to 195 mOsm with sorbitol. The [14C]citrate efflux experiment was performed according to Maron et al. (2010) using ND96 bath solution adjusted to pH 4.5 or 7.5.

Heterologous expression of VuMATE in transgenic tomato

The coding region of the VuMATE was amplified using a forward primer with a KpnI restriction site at the 5' end (CGGGGTACCCCCTAATGGAAGATAAG) and a reverse primer with a BamHI restriction site (CGGG-GATCCAGCAATGGAACACCT). After treatment with KpnI and BamHI, the digested PCR product was cloned into the modified pCAMBIA2300 vector between the corresponding restriction sites under the control of the 35S promoter. The resulting construct was sequenced for its accuracy and electropropoteo into Agrobacterium tumefaciens strain EHA105. Genetic transformation of tomato cultivar Micro-Tom (Lycopersicon esculentum) was performed as described by Sun et al. (2006) with some epidermal cells with a 35S:VuMATE::green fluorescent protein (GFP) translational fusion cloned in the modified expression vector, pCAMBIA2300. The resulting construct was fully sequenced and checked for sequence accuracy. Transient expression of the VuMATE::GFP translational in-frame fusion was achieved by particle bombardment of onion (Allium cepa) epidermal cells using a biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) according to manufacturer’s instructions. In brief, 1.5 mg gold particles (diameter 1 μm) were coated with 2.5 μg of the VuMATE::GFP (or GFP only as a negative control) plasmid DNA. Epidermal onion peels were bombarded at a helium pressure of 27–28 MPa, and the tissue was incubated at MS medium in the dark at room temperature for 12 h following the bombardment. Documentation of GFP fluorescence was carried out using laser-scanning confocal microscopy (LSM510, Karl Zeiss, Jena, Germany) with a 488 nm excitation wavelength. Cell plasmolysis was induced by addition of 100 mM sucrose.

Subcellular localization of VuMATE protein

Subcellular localization of the VuMATE protein was determined via transient expression in onion (Allium cepa)
Root measurements

The Al resistance of the positive T1 transgenic lines identified above was based on root growth measurements performed in agar plates. Seeds from transgenic and wild type lines were first surface-sterilized and then soaked and shaken in deionized water to promote their germination. After germination, seeds were sown onto agar plates containing 0.5 mM CaCl2, pH 4.5 and 0.8% (w/v) agar. The seeds were held in a straight line across the agar plates and the plates were placed in a near-vertical position such that the line of seeds remained horizontal. When the length of the primary root was about 3 cm, the root elongated within 1 d was recorded. Subsequently, seedlings were transferred to identical agar plates supplemented with the same nutrient solution. Al treatment with the same nutrient solution. Al treatment or 6 h of Al exposure (Fig. 2b,d). To determine the Al-specificity of VuMATE up-regulation of expression, the effect of the other metals was also tested. As shown in Fig. 2c, while exposure to cadmium (Cd) or copper (Cu) did not induce VuMATE expression in response to La alone (Fig. 1b).

Detection of root organic acid exudation

Root organic acid exudation both in rice bean and transgenic tomato was detected. For rice bean, after two weeks culture in the nutrient solution with low ionic strength and P concentration described above, the plants were subjected to Al treatment with the same nutrient solution. Al treatment experiment included exposing the seedlings in 25 µM Al-containing solutions for 0, 3, 6, 9 or 24 h, and in 0, 5, 10, 25 or 50 µM Al-containing solutions for 24 h. After that, root exudates from individual treatments were collected. For transgenic tomato, T1 transgenic lines carrying VuMATE were grown for 6 weeks in aerated nutrient solution containing the following macronutrients (in mM): KNO3 (1.0); Ca(NO3)2 (1.0); MgSO4 (0.4); and (NH4)H2PO4 (0.2), and the micronutrients in µM: NaFeEDTA (20); H3BO3 (3); MnCl2 (0.5); CuSO4 (0.2); ZnSO4 (0.4); and (NH4)6Mo7O24 (1). The pH of the nutrient solution was adjusted to pH 4.5 with HCl. Plant growth conditions were identical to those described above for rice bean. Root exudates from individual seedlings were collected after exposing them to 0.5 mM CaCl2 solution for a 3 h period. Subsequently the solution was replaced by the same CaCl2 solution containing 25 µM Al, and root exudates were collected after 3 h of Al exposure. Root exudate samples were passed through cationic and anionic chromatography columns (Econo-Pac columns, Bio-Rad) filled with 5 g Amerlite IR-120B resin (H+ form, Muromachi Chemical, Tokyo, Japan) or 1.5 g Dowex 1 × 8 resin (100–200 mesh, formate form) separately. The organic acid anions absorbed in the Dowex 1 × 8 resin were desorbed with 15 mL of 1 M HCl, and the eluate was concentrated to dryness using a rotary evaporator at 40 °C. The residue was re-dissolved in 800 µL of Milli-Q water. Citrate concentrations were then measured according to the enzymatic method described in Delhaize et al. (1993).

RESULTS

Cloning of VuMATE in rice bean

Implementation of homologous cloning and RACE resulted in the isolation of VuMATE, a full-length cDNA from rice bean. The coding region of VuMATE consists of 1698 bp encoding 565 amino acids in the protein. VuMATE showed high amino acid sequence homology to other plant MATE transporters such as LaMATE (with 64% identity and 75% similarity) from white lupin, HvMATE (with 52% identity and 68% similarity) from barley and SbMATE (with 51% identity and 66% similarity) from sorghum. Consistent with the highly conserved secondary structure predictions for this group of membrane transport proteins, VuMATE encodes a membrane protein with 12 membrane-spanning domains and a characteristic highly conserved amino acid sequence in the loop between the second and third transmembrane domains (Fig. 1a). Phylogenetic relationship analysis indicated that VuMATE was most closely clustered with LaMATE from white lupin (Fig. 1b).

Expression pattern of VuMATE

VuMATE mRNA transcription was not detected in neither root tip nor leaf tissue in the absence of Al treatment. Al treatment induced VuMATE expression only in the first centimetre of the root, while still no expression could be detected in the 1–2 cm root region and nor in leaf tissue (Fig. 2a). Al induction of VuMATE expression was both partially Al concentration and time dependent, increasing at higher Al levels and durations of Al exposure, but nearly maintained constant after reaching the maximum at 25 µM Al treatment or 6 h of Al exposure (Fig. 2b, d). To determine the Al-specificity of VuMATE up-regulation of expression, the effect of the other metals was also tested. As shown in Fig. 2c, while exposure to cadmium (Cd) or copper (Cu) did not induce VuMATE expression in response to La + Al was higher than in response to La alone (Fig. 2c).

Pattern of citrate secretion

To examine whether the expression pattern of VuMATE was consistent with the organic acid exudation pattern, we characterized citrate exudation from rice bean roots. In contrast to a number of previous studies where organic acid anions secretion was measured in a simple CaCl2 solution, we used a nutrient solution with low ionic strength and P concentration to mimic acid soil condition. Citrate exudation from rice bean roots could not be detected under
normal growth conditions. Exposure to Al induced citrate exudation within 3 h, being significantly induced after 9 h exposure to 25 μM Al (Fig. 3a). This time-dependent citrate exudation was same as our previous report (Yang et al. 2006a), which chose a simple CaCl2 solution to measure the citrate efflux in rice bean. Therefore, although the basal solution used for Al treatment was different, the pattern of citrate secretion was not affected. External Al concentration as low as 5 μM induced the exudation, with the magnitude of citrate exudation being dependent on the external Al concentrations (Fig. 3b). These results are consistent with rice bean as a typical pattern II plant (Ma 2000).

Subcellular localization of VuMATE

The cellular localization of the VuMATE protein was investigated in a transient expression assays with a VuMATE::GFP translational fusion in onion epidermal cell using particle bombardment. The fluorescence of the VuMATE::GFP chimera was associated with the cell periphery of the onion epidermal cells, suggesting a plasma membrane localization (Fig. 4). Cell plasmolysis confirmed that the fluorescence in cells expressing VuMATE::GFP was localized to the plasma membrane. In contrast, the fluorescence of cells transformed with the empty GFP containing vector was associated with the nucleus and cytosol. Together, these results strongly suggest that as with other citrate permeable plant MATE homologs, VuMATE is localized to the plasma membrane.

Functional expression of VuMATE in Xenopus oocytes

We examined the transport characteristics of VuMATE via two-electrode voltage clamp (TEVC) of Xenopus oocytes expressing the protein. In bath solution containing high NaCl, VuMATE-expressing cells showed large, slowly activating time-dependent inward currents, significantly larger than those elicited in control cells using identical voltage. VuMATE-mediated inward currents were also pH dependent, such that increasing the pH of the bath medium from 4.5 to 7.5 resulted in a significant decrease in the magnitude of the VuMATE-mediated inward currents. Minimizing the extracellular cation influx driving force by lowering the NaCl concentration in the bath medium resulted in a reduction of the VuMATE mediated inward current (Fig. 5) accompanied by a significant positive shift in the reversal potential (Erev, the holding potential at which the current changes sign) relative to that experienced by control cells. Under this set of ionic conditions, VuMATE-mediated inward currents were also pH sensitive. Interestingly, addition of Al3+ to the bath solution significantly decreased the inward currents both at high and low NaCl condition (Fig. 5). By convention, net inward currents are the product of net positive charge influx (e.g. Na+ and/or H+) and/or net negative charge efflux (e.g. organic or inorganic anions). The observed pH dependence suggested VuMATE-mediated inward currents reflected proton-coupled transport. To further elucidate the identity of the electrogenic transport recorded in VuMATE expressing cells, we examined radioactively labelled citrate efflux from oocytes loaded with [14C]citrate. 14C efflux from VuMATE-expressing cells was at least double than that observed in control cells (i.e. not expressing VuMATE) (Fig. 6). Furthermore, 14C efflux from

Figure 1. Sequence (a) and phylogenetic (b) analysis of Vigna umbellata multidrug and toxic compound extrusion (VuMATE) and other known plant citrate transporters (LaMATE, AtFRD3, AtMATE, ZmMATE1, SbMATE, OsFRDL1 and HvMATE). (a) Multiple sequence amino acid alignment (clustalW) of deduced amino acid sequences indicating high sequence similarity through the 12 span transmembrane proteins as predicted by HMMTOP. The highly conserved amino acid sequences located in the intracellular loop between TM2 and TM3 were indicated by the boxed region. (b) Phylogenetic relationship of VuMATE and other known citrate permeable MATE transporters. The unrooted NJ tree was generated with MEGA 3.1. Bootstrap values from 1000 replicates were indicated at each branch.

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cells expressing VuMATE was also pH-dependent, being significantly larger under acidic conditions, consistent with the pH dependence described for the VuMATE electrogenic transport described above.

**VuMATE expression in tomato confers citrate efflux and Al resistance**

The coding region of *VuMATE*, driven by the CaMV 35S promoter, was introduced and stably expressed into the tomato cultivar, Micro-Tom. Transgenic plants (TG2 and TG3) expressing *VuMATE* exhibited significantly higher citrate exudation rates, in contrast to wild type Micro-Tom, which lack citrate efflux, or were below detectable levels (Fig. 7). Interestingly, although citrate efflux in *VuMATE*-expressing transgenic plants was observed both in the presence and absence of Al, the efflux rates were significantly lower in Al-treated plants. The root growth of transgenic plants overexpressing *VuMATE* was inhibited by about 20% for transgenic line TG2 and nearly not affected for TG3 after a 100 μM Al exposure for 24 h, whereas root growth of wide type control plants was inhibited by about 40% (Fig. 7c). These findings clearly indicated that overexpression of *VuMATE* in Micro-Tom increased citrate efflux in roots, which underlies the increase in Al resistance.

**DISCUSSION**

Rice bean can specifically secrete citrate from the root apex as a mechanism of Al resistance (Yang et al. 2006a). The
limited genetic and genomic information for rice bean has hindered and challenged the isolation and cloning of genes from this leguminous plant species. Recently, the Al-induced citrate transporter genes ShMATE from sorghum (Magalhaes et al. 2007), HvMATE from barley (Furukawa et al. 2007) and AtMATE from Arabidopsis (Liu et al. 2009) were isolated and characterized. These citrate transporters are members of the MATE family. This recent information in combination with RACE technology enabled us to conduct homologous cloning aimed at isolating the gene encoding the citrate transporter in rice bean. The resulting full-length cDNA was designated VuMATE. VuMATE has a high amino acid sequence identity to LaMATE, HvMATE, ShMATE and the other known citrate transporters, sharing a conserved secondary structure consisting of 12 transmembrane domains and a unique cytoplasmic loop between TM2 and TM3 (Fig. 1a). This cytoplasmic loop is unique to those members of the MATE family implicated in citrate transport (Liu et al. 2009). VuMATE is localized to the plasma membrane (Fig. 4), and resembles those reported for other citrate-permeable MATEs in barley (Furukawa et al. 2007), sorghum (Magalhaes et al. 2007), maize (Maron et al. 2010) and Arabidopsis (Liu et al. 2009). VuMATE also has some unique characteristics that differ from the other MATEs. The overall characteristics of the citrate-permeable MATEs reported in the literature are summarized in Table 1.

The uniqueness of VuMATE consist of the following: Firstly, HvMATE from barley, ShMATE from sorghum, ZmMATE1 from maize and AtMATE from Arabidopsis exhibit varying degrees of constitutive expression (i.e. in the absence of Al), and their expression is up-regulated by Al treatment (except for HvMATE). In contrast, VuMATE expression is entirely Al induced. No VuMATE expression was detected in the absence of Al stress, and expression was quickly induced within 3 h of Al treatment, remaining enhanced at 6 h, and then maintaining constant (Fig. 2d). This is consistent with the 3 h lag phase observed for citrate exudation (Fig. 3). Therefore, among all the MATEs encoding citrate transporters associated with Al stress reported so far, VuMATE is the first one that is entirely induced by Al treatment.

Because of the unique transcriptional regulation of VuMATE expression, it may be enlightening to transcription factors that regulate its expression. The transcription factor STOP1 has been reported to be involved in AtMATE expression and Al-activated citrate exudation in Arabidopsis (Liu et al. 2009). Yamaji et al. (2009) also reported that in rice, a zinc finger transcription factor ART1 regulates multiple genes implicated in Al resistance in rice, including members from the MATE transporter family; however, organic acid exudation does not explain the Al resistance differences across various rice cultivars (Yang et al. 2008; Famoso et al. 2010). Current efforts using proteomic and cDNA cloning approaches are underway to identify the transcription factors regulating VuMATE expression.

![Figure 4. Subcellular localization of Vigna umbellata multidrug and toxic compound extrusion (VuMATE). The fusion protein VuMATE::GFP (left columns) and GFP alone (right columns) were transiently expressed in onion epidermal cells. The images were acquired before (first panels) and after (second and third panels) plasmolysing the cells with 100 mM sucrose. First and third panels show the overlay images of the bright-field and fluorescence images. Scale bar = 100 μm. PM, plasma membrane, CW, cell wall.](image-url)
The restricted spatial distribution of VuMATE to the apical root (i.e. expression was only detected in the 0–1 cm from root apex) and is tightly associated with the spatial location where citrate exudation take place (Fig. 2a). Al-activated citrate secretion in rice bean has been shown to be mostly confined to the 0–5 mm of root apex (Yang et al. 2006a). In contrast, the MATE homologs from barley (Furukawa et al. 2007), sorghum (Magalhaes et al. 2007) and maize (Maron et al. 2010) exhibit expression both in root apex and mature regions, with the expression in mature regions being much lower than root tip MATE expression. Physiological studies have indicated that when present, organic acid anion exudation acts at the root apex, the primary and immediate target of Al toxicity, to protect the roots from Al phytotoxicity (Ryan, Ditomaso & Kochian 1993; Ryan, Delhaize & Randall 1995; Zheng et al. 1998). The difference in the tissue specificity of VuMATE in comparison with its homologs suggests that the citrate exudation in rice bean may be under slightly different tissue-specific regulation. Given that efflux of organic acid anions represents a significant carbon cost to plants, limiting the amount of citrate confined to the most critical site, the root apex, is the most economic strategy. Thus, dissection of the regulatory regions of the VuMATE gene targeting its expression to a confined root apex region should prove useful.

Heterologous expression of VuMATE in Xenopus oocytes showed that when the bath solution contained high NaCl and the bath pH was varied between 4.5 and 7.5, the anion currents were pH dependent, increasing as the pH is decreased (Fig. 5). The pH-dependent inward currents suggests that VuMATE mediates a H⁺-coupled transport, which presumably is a proton-citrate antiport. A similar pH dependency for anion efflux has been observed for ShMATE (Kochian, unpublished results) and ZmMATE1 (Maron et al. 2010). Lowering the NaCl concentration in the bath solution significantly reduced the magnitude of the inward currents (Fig. 5), suggesting that Na⁺ might also

![Figure 5. Electrophysiological characterization of Vigna umbellata multidrug and toxic compound extrusion (VuMATE) expressed in Xenopus oocytes via two electrode voltage clamp. (a) Examples of families of currents from VuMATE-expressing (top row) and control (bottom row) cells in response to different voltage pulses ranging from −160 to +120 mV (in high NaCl) or −140 to +140 mV (in low NaCl) in 20 mV steps. Cells were bathed in a solution containing (left panel) or lacking (right panel) 96 mM NaCl at the pH indicated at the top of each set of traces. Where noted the solution also contained 100 μM AlCl₃. The arrows on the left of each set of traces indicate the zero current level. Time and current scales are indicated on the right of the high and low set of traces. Note the difference in current scales between high and low NaCl recordings. (b) Mean current/voltage (I/V) relationship from recordings as those shown in (a). Symbols correspond to the ones above the set of traces in part (b). Note the difference in the current y-axis scale between the I/V curves. The arrow above the x-axis indicates the reversal potential (Erev).](image-url)
support the driving force for VuMATE-mediated transport. 

NorM, is the first MATE gene isolated, from a slightly halo-
philic marine bacterium, Vibrio parahaemolyticus, and
confers resistance to norfloxacin and other antimicrobial
agents via Na+/drug antiport, a mechanism highly conserved
through the MATE family of transporters (Morita et al.
1998, 2000). Interestingly, although multiple sequence
alignments indicated the high protein similarity among
MATE-type plant citrate transporters (Fig. 1a), the NorM
domain is only present in VuMATE, but not in HvMATE,
SbMATE, AtMATE, ZmMATE1, LaMATE, OsFRDL1 or
AtFRD3. Therefore, we propose that VuMATE maybe
function as a Na+ and H+ (and potentially other cations)
coupled secondary antiporter for citrate efflux, which under
physiologically relevant ionic apoplastic environments, will
predominantly use H+ as the transport driving force.

Table 1. Characteristics of citrate transporters associated with Al stress in the multidrug and toxic compound extrusion (MATE) family, including information about source plant, gene name, gene expression before and after Al treatment, spatial distribution, NorM domain and citrate efflux under Al stress

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Source plant</th>
<th>Gene name (accession no.)</th>
<th>Gene expression before Al treatment</th>
<th>Gene expression after Al treatment</th>
<th>Spatial distribution</th>
<th>NorM domain</th>
<th>Citrate efflux under Al stress (in Xenopus oocytes/transgenic plant)</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>Monocots</td>
<td>Sorghum</td>
<td>ShMATE (EF611342)</td>
<td>+</td>
<td>Enhanced</td>
<td>Root apex; mature region</td>
<td>–</td>
<td>↓↓(Arabidopsis)</td>
<td>Magalhaes et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Barley</td>
<td>HvMATE (AB302223)</td>
<td>+</td>
<td>Not enhanced</td>
<td>Root apex; mature region</td>
<td>–</td>
<td>↑/? (Tobacco)</td>
<td>Furukawa et al. 2007</td>
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<tr>
<td></td>
<td>Maize</td>
<td>ZmMATE1 (NM_001170581)</td>
<td>+</td>
<td>Enhanced</td>
<td>Root apex; mature region</td>
<td>–</td>
<td>↓↑(Arabidopsis)</td>
<td>Maron et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Rice bean</td>
<td>VuMATE ()</td>
<td>–</td>
<td>Induced</td>
<td>Root apex</td>
<td>+</td>
<td>↓↓(Tomato)</td>
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Figure 6. [14C]citrate efflux in Xenopus oocytes. Control and 
Vigna umbellata multidrug and toxic compound extrusion 
(VuMATE)-expressing oocytes injected with [14C] citrate were 
kept in ND96 solution. The radioactivity in the bathing solution 
was measured at the indicated time points; values are expressed 
as a percentage of the total radioactivity injected. Data are 
means ± standard deviation (n = 6).

Figure 7. Overexpression of Vigna umbellata multidrug and 
toxic compound extrusion (VuMATE) in tomato results in 
increased citrate release and Al tolerance. The wild type (WT) was 
used as control. (a) Expression of VuMATE in selected T1 
positive plants from transgenic lines TG2 and TG3 verified by 
reverse-transcriptional PCR. (b) Root citrate secretion from T1 
lines expressing VuMATE (TG2 and TG3) in the absence or 
presence of 25 μM Al for 3 h. Data are means ± standard 
deviation (SD) (n = 3). (c) Al tolerance in transgenic lines TG2 
and TG3. Percent relative root elongation (%RRE) was 
calculated by dividing root growth values in +Al (100 μM) agar 
for 24 h by root growth values in control (–Al) for 24 h × 100. 
Data are means ± SD (n = 3–10).
It is interesting to note that extracellular Al³⁺ inhibits VuMATE-mediated citrate transport in transgenic tomatoes (Fig. 7b). In previous studies, citrate exudation in transgenic plants overexpressing the MATE genes encoding citrate efflux transporters including SbMATE and ZmMATE1 in Arabidopsis (Magalhaes et al. 2007; Maron et al. 2010) and HvMATE in tobacco (Furukawa et al. 2007), was significantly increased upon exposure to Al stress. In contrast, extracellular Al had an inhibitory effect on the VuMATE transport activity in oocytes (Fig. 5), similar to that reported for ZmMATE1 expressing Xenopus oocytes (Maron et al. 2010). These findings, along with the results in Fig. 7b showing that Al inhibits the root citrate efflux in transgenic tomato mediated by VuMATE, suggest that Al activation and regulation of this citrate transporter in planta may involve additional elements such as regulatory proteins that are not present in oocytes or the transgenic tomato. As mentioned earlier, both STOP1 and ART1 are positive regulators for the Al-activated citrate exudation (Liu et al. 2009; Yamaji et al. 2009). It is possible that some similar positive regulators do not exist in the tomato cultivar Micro-Tom, especially that which lacks the ability of Al-induced citrate secretion (Fig. 7b), whereas some unknown repressive regulators for citrate exudation do respond to Al stress in transgenic tomato. In our study with SbMATE, we really found that the activity of MATE is still dependent on its interaction with other membrane bound protein (Kochian, unpublished data). However, it is not clear from our data whether Al is needed to activate the VuMATE protein after the expression is induced by Al. But the finding that citrate efflux from the transgenic tomato plants is constitutive may indicate that the protein is not activated by Al in rice bean plants. In this respect rice bean shows some similarities with the constitutive citrate efflux mediated by TaMATE1 in wheat (Ryan et al. 2009). Both MATEs are not activated by Al. To our knowledge, these are the only examples of this type of response/phenotype. However, little is known about the specific regulation model of the known MATEs at protein level, which needs to be studied in the further work. The total induction of VuMATE expression by Al might function as a specific regulator to avoid unnecessary citrate loss when Al is not around.

In conclusion, in this study we have described a de novo synthesized citrate transporter, VuMATE, from rice bean, a dicot plant. VuMATE is the first plant MATE-type citrate transporter gene shown to be completely induced by Al. Functional analysis in Xenopus oocytes and transgenic tomato indicates that VuMATE is a rice bean Al-resistant protein mediating root citrate efflux that possibly is a H⁺ (and possibly Na⁺) coupled citrate efflux transporter, ultimately resulting in Al-detoxification at the root tip surface.

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