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ORIGINAL ARTICLE

Characterization of Al-responsive citrate excretion and citrate-transporting MATEs in *Eucalyptus camaldulensis*

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Abstract Many plant species excrete organic acids into the rhizosphere in response to aluminum stress to protect sensitive cells from aluminum rhizotoxicity. When the roots of *Eucalyptus camaldulensis*, a major source of pulp production, were incubated in aluminum-toxic medium, citrate released into the solution increased as a function of time. Citrate excretion was inducible by aluminum, but not by copper or sodium chloride stresses. This indicated that citrate is the major responsive organic acid released from the roots of this plant species to protect the root tips from aluminum damage. Four genes highly homologs to known citrate-transporting multidrugs and toxic compounds exclusion proteins, named *EcMATE1–4*, were isolated using polymerase chain reaction-based cloning techniques. Their predicted proteins included 12 membrane spanning domains, a common structural feature of citrate-transporting MATE proteins, and consisted of 502–579 amino acids with >60 % homology to orthologous genes in other plant species. One of the homologs, designated *EcMATE1*, was expressed in the roots more abundantly than in the shoots and in response to both Al and low pH stresses. Ectopic expression of *EcMATE1* and *3* in tobacco hairy roots enhanced Al-responsive citrate excretion. Pharmacological characterization indicated that Al-responsive citrate excretion involved a protein phosphorylation/dephosphorylation process. These results indicate that citrate excretion through citrate-transporting multidrugs and toxic compounds exclusion proteins is one of the important aluminum-tolerance mechanisms in *Eucalyptus camaldulensis*.

Keywords Acid soil · Aluminum tolerance · Citrate excretion · *Eucalyptus camaldulensis*

Abbreviations

Al	Aluminum
ALMT1	Aluminum-activated malate transporter 1
ART1	Al-resistance transcription factor 1
AtFRD3	*Arabidopsis thaliana* ferric redictase defective3
HvAACT1	*Hordeum vulgare* aluminum-activated citrate transporter 1
GUS	β-Glucuronidase
MATE	Multidrugs and toxic compounds exclusion
MS	Murashige and Skoog
OAs	Organic acids
STOP1	Sensitive to proton rhizotoxicity1

Introduction

Eucalyptus sp., a typical fast growing tree species, is a major resource for pulp production (e.g., Greaves et al. 1997; Campinhos 1999). Over 187 million ha of plantations are currently operated throughout the world (FAO 2001). This species naturally adapts to various

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environmental conditions, but the established clones (i.e., elite clones) for plantation carrying suitable properties for pulp production such as long cellulose fiber length and high productivity of wood tips sometimes show poor growth at some sites. Molecular breeding such as marker-assisted selection and transgenic breeding would be one reasonable approach to confer tolerance to environment stresses to clones for plantation (Raymond and Apiolaza 2004). In fact, a genome sequence for establishing molecular markers is being developed by an international consortium (DOE Joint Genome Institute and the Eucalyptus Genome Network, EUCAGEN) and procedures for gene transformation have been established in some species (Ito et al. 1996). Identification of critical genes that regulate particular traits is important and remaining issues for realizing efficient breeding strategies.

Soil stress, such as salinity or acidity, is one of the major causes of poor growth in *Eucalyptus* plantations (White et al. 2009; Feikema and Baker 2011). Since acid soils are widely distributed in subtropical regions, identification of genes critical for resistance to acid soil stresses, such as aluminum (Al) stress, is important for developing molecular markers for breeding. Various resistance mechanisms, such as UDP-glucose-mediated resistance (Fukuda et al. 2007) and a lower negative charge of the plasma membrane surface (Wagatsuma et al. 1995), have been identified in various plant species, but organic acid (OA) excretion is the most common Al-resistance mechanism in many plant species (Ma et al. 2001; Kochian et al. 2004). OA excretion from the roots protects the sensitive cells in the root apex from aluminum (Al) rhizotoxicity (Delhaize et al. 1993). Inactivation of Al rhizotoxicity by phosphorus was observed in buckwheat (Zheng et al. 2005), but organic acids namely malate, citrate and oxalate excretion from the roots may be the most important mechanism of Al tolerance of this species (e.g. Ma et al. 1998; Zheng et al. 1998). Several studies have indicated that alterations in OA metabolism play a role in enhanced OA excretion in Al tolerance (e.g., canola, Anoop et al. 2003; alfalfa, Tesfaya et al. 2001), while transport capacity through the plasma membrane is also a regulating factor of this trait (Furukawa et al. 2007; Magalhaes et al. 2007). Similar mechanisms were identified in OA excretion enhanced by P-deprivation such as white lupin (Neumann et al. 1999; Kihara et al. 2003) and a mutant carrot cells (Takita et al. 1999; Ohno et al. 2003). Critical genes involved in these processes would be candidate molecular makers for marker-assisted selection to improve acid soil resistance.

An Al-responsive malate transporter, named Aluminum-activated malate transporter 1 (ALMT1), was identified in wheat (Sasaki et al. 2004) and other plant species such as *Arabidopsis thaliana* (Hoekenga et al. 2006). Citrate transporters, which are distinct from ALMT1 and belong to the multidrug and toxic compound extrusion (MATE) transporter family, have been identified in various plant species (Rogers and Guerinot 2002. Some citrate-transporting MATEs are Al responsive and determine the Al tolerance of crops such as sorghum (Magalhaes et al. 2007; Maron et al. 2010) and barley (HvAACT1: *Hordeum vulgare* aluminum-activated citrate transporter 1, Furukawa et al. 2007). Genetic analysis has revealed that the differences in Al tolerance of some varieties by OA excretion could be explained by differences in transcript levels (Sasaki et al. 2006; Furukawa et al. 2007) and protein transport capacity (Raman et al. 2008). This suggests that genes encoding these transporters would be reasonable targets for marker-assisted selection to improve acid soil resistance.

In this study, we characterized OA release from *Eucalyptus* roots and identified genes encoding putative citrate-transporting MATE transporters by molecular cloning. Because citrate is the major OA released from *Eucalyptus* roots, genes encoding putative citrate-transporting MATEs were isolated. One of four isolates was inducible by Al treatment and had high homology to previously isolated genes for citrate-transporting MATEs in other plant species. Further, characterization confirmed that the gene encodes an Al-responsive citrate-transporting MATE in *Eucalyptus*.

Materials and methods

Plant materials and bacterial strains

Eucalyptus camaldulensis var. *obtuse* (Location: EMU CREEK PETFORD) obtained from the Australian Tree Seed Centre was used throughout the experiments. Tobacco (*Nicotiana tabacum*, BY-2 (Bright-Yellow 2)) and the hyper-virulent *Agrobacterium rhizogenes* strain ATCC15834 (American type culture collection, VA, USA) carrying a native Ri plasmid were used for hairy root development.

Rhizotoxic treatments

Eucalyptus seeds were surface sterilized with sodium hypochlorite (1 % available chlorine; 20 min at 10 °C) and then pre-cultured under aseptic conditions using the method that was originally developed for *Arabidopsis* in vitro culture by Kobayashi et al. (2007), with minor modifications. Four nylon mesh sheets (1.3 cm square of 50 mesh per inch; 20 seedlings per sheet) were placed on a floating plastic mesh (5 cm square) in a plastic pot containing 150 ml of pre-culture medium. The pre-culture medium contained modified Hoagland–Arnon nutrients [0.4 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$,

40 μM KCl, 5.4 μM EDTA-Fe, 1 μM $MnCl_2$, 4.6 μM H_3BO_3, 0.076 μM $ZnSO_4$, 0.032 μM $CuSO_4$, 0.001 μM $(NH_4)_6Mo_7O_{24}$] with 1 % sucrose at an initial pH of 5.6. The pots were kept at 23 °C with a 16-h photoperiod (20 μmol E m^{-2} s^{-1}).

After 6 days of pre-culture, the floating plastic meshes supporting seedlings were transferred to a new plastic pot containing control solution (with P_i removed from the pre-culture medium and the pH adjusted to 4.8) and pre-incubated for 1 h. For isolating RNA after rhizotoxic treatments, the pre-incubated seedlings were again transferred to new pots containing control, Cu-toxic (control solution + 1 μM $CuSO_4$, pH 4.8), NaCl-toxic (control solution + 30 mM NaCl, pH 4.8), Al-toxic (control solution + 50 μM $AlCl_3$, pH 4.6) or low pH (control solution at pH 4.0) solution. For collecting excreted organic acids from the roots, each nylon mesh growing pre-incubated seedlings on the floating plastic was transferred to a 6-well plate containing 3 ml of rhizotoxic and control solutions. To test the effect of inhibitors of protein phosphorylation and di-phosphorylation, calyculin, K-252a, staurosporin or cyclosporin were added to give a final concentration of 5 μM. All treatments were carried out under continuous light (20 μmol E m^{-2} s^{-1}) at 25 °C.

RNA isolation, reverse transcription, DNA sequencing and sequence analysis

Total RNA was isolated using the method developed by Suzuki et al. (2004). The total RNA was then reverse transcribed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Applied Science, Tokyo, Japan). The ABI PRISM3130xl DNA sequencer and ABI BigDye terminator system (ver3.1) were used for DNA sequencing analysis according to the manufacturer's recommended protocols. Sequence analyses, amino acid alignment and phylogenetic tree analysis were carried out in CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and prediction of membrane spanning domains was carried out using HMMTOP (http://www.enzim.hu/hmmtop/).

Isolation of *Eucalyptus* homologues of citrate-transporting MATE family genes

Partial cDNA fragments of putative citrate-transporting MATEs were obtained by nested PCR from the cDNA of Al-treated roots using degenerate primers. The degenerate primers were designed from the conserved domains of citrate-transporting MATEs from *Arabidopsis thaliana* (At3g08040), *Oryza sativa* (Os03g11734.1) and *Lupinus albus* (Q3T7F5) as follows; 1st PCR: forward, 5′-GCIGC IGAYCCIYTIGCI, reverse, 5′-RCARAAIGTIACIGCIAC IAC, 2nd PCR: forward, 5′-GAYACIGCITTYATHGGI reverse, 5′-RTCYTTRAAICCICKRAA. The resulting amplicons were sub-cloned and sequenced. After the sequencing analysis, the 3′ and 5′ ends of each gene were obtained according to the manufacturer's recommended protocols of the SMART RACE cDNA Amplification Kit (Takara-bio, Ohtsu, Japan) and then sequenced. The sequences of the isolated clones were deposited in the Genbank database (Accession numbers: *EcMATE1*, AB725912; *EcMATE2*, AB725913; *EcMATE3*, AB725914; *EcMATE4*, AB725915).

Transcript analysis of the EcMATE gene family

The expression levels of the *EcMATE* genes were analyzed by real-time PCR using the LightCycler 480 SYBR Green I Master kit (Roche) and a Light Cycler 480 (Roche) according to manufacturer's protocols with specific primer pairs; *EcMATE1*: forward 5′-AGTCTCCCTTATCAGCAT TGCTTCA, reverse 5′-TAAACGTTGTGGAAGAAGTC CTTCTCTAAT, *EcMATE2*: forward 5′-ATGCCAGAG GACAGTGTTCAGCATCT, reverse 5′-TGCAGTGTCAA TTAGGGAAGCAACAGGATC, *EcMATE3*: forward 5′-G CGTTGAATCTTTCTTGATTTTG, reverse 5′-CAGTCTC CCCACTTCAAGAATTA, *EcMATE4*: forward: 5′-CACA GGCGGCTTTGCTGCAA, reverse 5′-AGGCTGATAAC TATTGGCGCTG. *EcActin* (forward 5′-GTTGCA CCTCCTGAGAGAAAGT, reverse 5′-TAGCTCACCAA CAAAGACCTTGC) was used as an endogenous control.

Vector construction and *Agrobacterium rhizogenes*-mediated transformation

Mini-Ti plasmid vectors carrying *EcMATE1–4* were constructed using one of the special Gateway® vectors, pGWA2, for plant transformation (Nakagawa et al. 2009). Briefly, pGWA2 has a pBI121 backbone with a Gateway cloning site in which a gene can be inserted between the cauliflower mosaic virus 35s promoter (CaMV35s) and *Agrobacterium* nopaline synthase terminator (NOS-T). This cassette was in the T-DNA region and was flanked by gene cassettes for hygromycin resistance and kanamycin resistance. The genes for EcMATE1–4 were PCR amplified with primer pairs attaching the consensus clonase sequences and then inserted into the Gateway cloning site of the T-DNA region, resulting in pGWA2-EcMATE1–4 constructs for CaMV35s-driven overexpression of each gene in transformed hairy roots. All cloning procedures followed the standard protocols of the Gateway system (Invitrogen). Primer pairs used for directional TOPO cloning were as follows; *EcMATE1*: forward 5′-CACC ATGGCCGAGGACTCTGATGTTCGTG, reverse 5′-TCA TAAACGTTGTGGAAGAAGTCCTTCTCTAAT, *EcMATE2*: forward 5′-CACCATGCCAGAGGACAGTGTT

CAGCATCT, reverse 5′-TTATGAAGCCTGTGGTGTA CGTTGACCC, *EcMATE3*: forward 5′-CACCATGCCT CTGTCTATGTTCTTCAAGGA, reverse 5′-TTAGTT ATTTAGAAATCCCCAAGGTCCCATGCC, *EcMATE4*: forward: 5′-CACCATGGAGCCTCTGGAGGGTTCG, reverse 5′-TCAGCCCCAGAGAAAATTCCAAGGACCC.

pGWA2-EcMATE1 was introduced into *Agrobacterium rhizogenes* ATCC15834 by electroporation, and the strain was infected to aseptically grown tobacco leaves (BY-2) to produce transgenic hairy roots. Infected tobacco leaves (1 cm square) were kept on 1/4 strength B5 agar (1 % w/v) medium containing galactose (1 % w/v) and acetosyringone (20 μg/ml) for 2 days, and then washed in 6.25 mg/l of meropenem (Sumitomo pharmaceutical Co., Japan, Tokyo) solution. The washed leaves were further incubated on 1/4 strength B5 agar medium (1 % w/v agar) containing sucrose (1 % w/v), meropenem (6.25 mg/l), and 1 ppm of naphthylacetic acid (NAA). The medium was renewed every 2 weeks until hairy root formation. Transgenic hairy roots were then selected by further culture on the same agar containing 50 μg of hygromycin instead of meropenem. All cultures were kept at 22 °C with a 16-h photoperiod (20 μmol quanta m^{-2} s^{-1}).

Intracellular localization of EcMATE1 by *Agrobacterium*-mediated transient assay

Intracellular localization of EcMATE1 was analyzed by *Agrobacterium*-mediated transient assay in tobacco roots based on the method developed by Sparkes et al. (2006) that was used for transient assay in tobacco leaves using *Agrobacterium tumefaciens*. A cDNA encoding synthetic green fluorescent protein (sGFP; see Sawaki et al. 2009) was connected to the C-terminus of *EcMATE1* by PCR reactions. Each gene was first amplified separately using primer pairs for *EcMATE1* (forward 5′-TTAACCCGGGA TGGCCGAGGACTCTGATGT-3′, reverse 5′-TCGCCC TTGCTCACCATTAAACGTTGTGGAAGAAGT-3′) and *sGFP* (forward 5′-ACTTCTTCCACAACGTTTAATGGT GAGCAAGGGCGA-3′ and reverse 5′-CGAAGAGCTCT TACTTGTACAGCTCGTCCA-3′), then jointed *EcMATE1-sGFP* by PCR, using the *EcMATE1* forward and *sGFP* reverse primers. The joined DNA was replaced β-glucuronidase of pBI121 then transformed to a hypervirulence *Agrobacterium tumefaciens* EHA101. Root of tobacco, grown on a agar medium [1/2 strength MS medium (Murashige and Skoog 1962) containing 1 % (w/v) sucrose] for 4 weeks, were removed and rinsed in distilled water. Then immersed to the *Agrobacterium* buffer (pH 5.6 of 10 mM MES/KOH, 10 mM of $MgCl_2$, $OD_{660} = 0.2$) containing 150 μM of acetosyringone. The seedlings were kept 2 days on moistened filter paper, then the root cells were observed GFP by a fluorescent microscope (Axio, Zeiss-Japan, Tokyo).

Quantification of malate and citrate in root exudates

The citrate and malate in the solutions were measured enzymatically by the NAD^+/NADH cycling method (Hampp et al. 1984) as described previously (Kihara et al. 2003). Briefly, malate and citrate were stoichiometrically converted to NADH by malate dehydrogenase or citrate lyase and then the NADH was quantified by the cycling method.

Organic acid excretion from transgenic tobacco hairy roots

Transgenic tobacco hairy roots were pre-grown for 1 week in 25 % strength MS solution (pH 5.6) with 2 % sucrose. The excised root tips (3 cm; 100 root tips) were pre-treated for 2 h in 30 ml of control treatment solution in 100 ml flasks containing 1/20 strength MS solution with 1 % (w/v) sucrose at pH 4.8 but lacking phosphate. After pre-treatment, the roots were each transferred to 30 ml of control or Al-toxic solution (control-treatment solution + 25 μM $AlCl_3$). The medium was renewed at 24 h then incubation continued until 48 h. All flasks were kept on a rotary shaker (50 rpm) in dark room at 25 °C. The medium after 24-h treatment was used for organic acids analysis. Growth of the transgenic hairy roots was examined by root elongation for 3 days in control and Al-toxic solutions used for organic excretion assay.

Statistical analysis and reproducibility

All experiments were repeated at least twice, and confirmed reproducibility. Statistical analysis such as student *t* test was performed using a software Microsoft excel.

Results

Characteristics of organic acid excretion from *Eucalyptus*

Various plants excrete organic acids from the roots in response to Al, but there are differences between species in the organic acids excreted and the patterns of excretion in terms of induction period. In preliminary experiments, we detected both malate and citrate excretion, but not oxalate from the roots of *E. camaldulensis* among the major organic acids that detoxify Al toxicity. It was thus both malate and citrate in the culture solutions were quantified in various conditions.

To determine whether malate or citrate was the major organic acid in the root exudates, the roots of 1-week-old *E. camaldulensis* seedlings (root length 5–10 mm) were exposed to Al solutions. Citrate excretion was increased

when the Al concentration was greater than 25 μM, while malate excretion showed no response to external Al (Fig. 1a). The citrate excretion in Al solution was time-dependent (Fig. 1b). The excretion was determined every 3 h and increased for at least the first 12 h. These results suggested that *E. camaldulensis* possesses Al-inducible citrate excretion capacity.

To further compare citrate and malate excretion from the roots, both organic acids were quantified after incubation in other rhizotoxic treatments, including 50 μM Al, low pH (pH 4.0), 1.0 μM Cu or 30 mM NaCl. Each stress treatment caused 50 % inhibition of root elongation compared with controls (Fig. 1c). Similar levels of malate excretion were detected in all treatments, but citrate excretion was only detected in the Al treatment, in which it was greater than malate excretion (Fig. 1d). These results indicated that citrate excretion is specific to Al stress.

Isolation of putative *EcMATE* genes by PCR-based cloning

Using degenerate primers designed from the conserved amino acid sequence shared by the reported citrate-transporting MATE genes, about 40 partial cDNA fragments encoding *EcMATE* family genes were obtained. From subsequent 3′ and 5′ RACE using specific primers designed from the partial fragments, four different genes encoding putative EcMATE proteins were isolated. The cDNA clones showed conserved amino acid sequences at the positions of the degenerate primers, and encoded proteins of 502 (EcMATE3) to 579 (EcMATE1) amino acids (Table 1). These genes contained both 5′ and 3′ UTR regions.

The amino acid sequences of the EcMATEs shared typical features of citrate-transporting MATE proteins in other plant species (Fig. 2a). All of the EcMATE proteins had 12 predicted trans-membrane domains, which was identical to other citrate-transporting MATE proteins. Phylogenetic tree analysis grouped the EcMATE1 and two proteins in the same group, while EcMATE 3 and 4 fell into another group (Fig. 2b). All of these proteins were closer to the dicotyledonous plants (*Arabidopsis* and white lupin) than to homologs in monocotyledonous plants. AtFRD3 (*Arabidopsis thaliana* ferric redictase defective3) a citrate transporter involved in iron-acquisition, was closest to EcMATE1 and 2, while AtMATE, an Al-responsive citrate-transporting *Arabidopsis* MATE, was closest to EcMATE4.

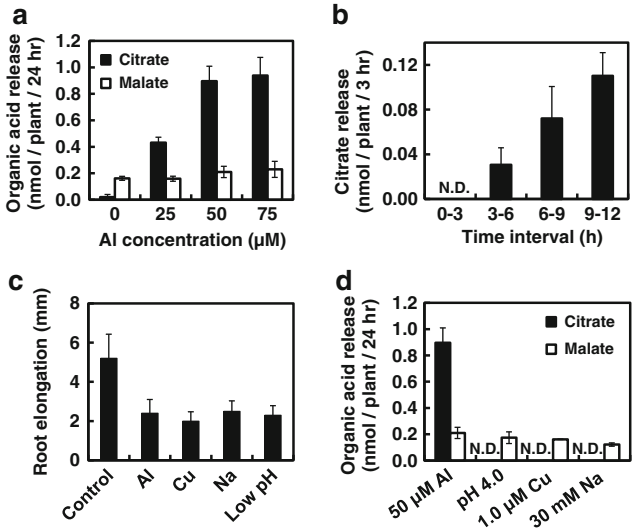

Fig. 1 Citrate and malate release profile in *Eucalyptus camaldulensis*. **a** Response to Al concentration. **b** Time-course response to Al (50 μM) stress. **c** Growth of roots with various stressors (50 μM $AlCl_3$, low pH (pH 4.0), 1.0 μM $CuSO_4$ and 30 mM NaCl) for 3 days. **d** Citrate release with various stressors (identical to conditions used for **c**). Each solution was adjusted to pH 4.6 except the low pH treatment. Each experiment was replicated three times and mean ± SD are shown

Expression profile of the *EcMATE* genes in response to rhizotoxic treatments

To characterize *EcMATE* gene expression, transcripts levels of the genes were analyzed in both shoots and roots under various rhizotoxic treatments. The *EcMATE4* transcript was expressed in both shoots and roots, while the transcript levels of *EcMATE1*, *2* and *3* were greater in the roots under various treatments (Suppl Fig. S1). Among the root-abundant *EcMATE* homologs, *EcMATE1* showed Al- and low pH-responsive expression, when expression levels were compared among stress treatments causing 50 % growth inhibition (Fig. 1c). *EcMATE1* expression was induced by Al and low pH, while both NaCl (30 mM) and Cu (1.0 μM) did not induce (Fig. 3a). The greatest fold change (i.e., Al/control) of *EcMATE1* was 6.4 after 4-h Al treatment, when the roots began citrate excretion (Figs. 1b, 3b). *EcMATE1* expression was induced by Al and low pH stress in a dose-dependent manner (Fig. 3c, d). In addition, *EcMATE1* expression was greater in the root tip than the mature part (Fig. 3e), which was the site of OA excretion (Suppl Fig. S2). These results suggested that *EcMATE1* plays an important role in Al-inducible citrate excretion in *E. camaldulensis*.

Table 1 Basal information of MATE family genes in *E. camaldulensis*

Gene name	ORF (bp)	Amino acid (AA)	Transmembrane helices
EcMATE1	1740	579	12
EcMATE2	1641	546	12
EcMATE3	1509	502	12
EcMATE4	1608	534	12

The transmembrane helices were predicted by the HMMTOP program

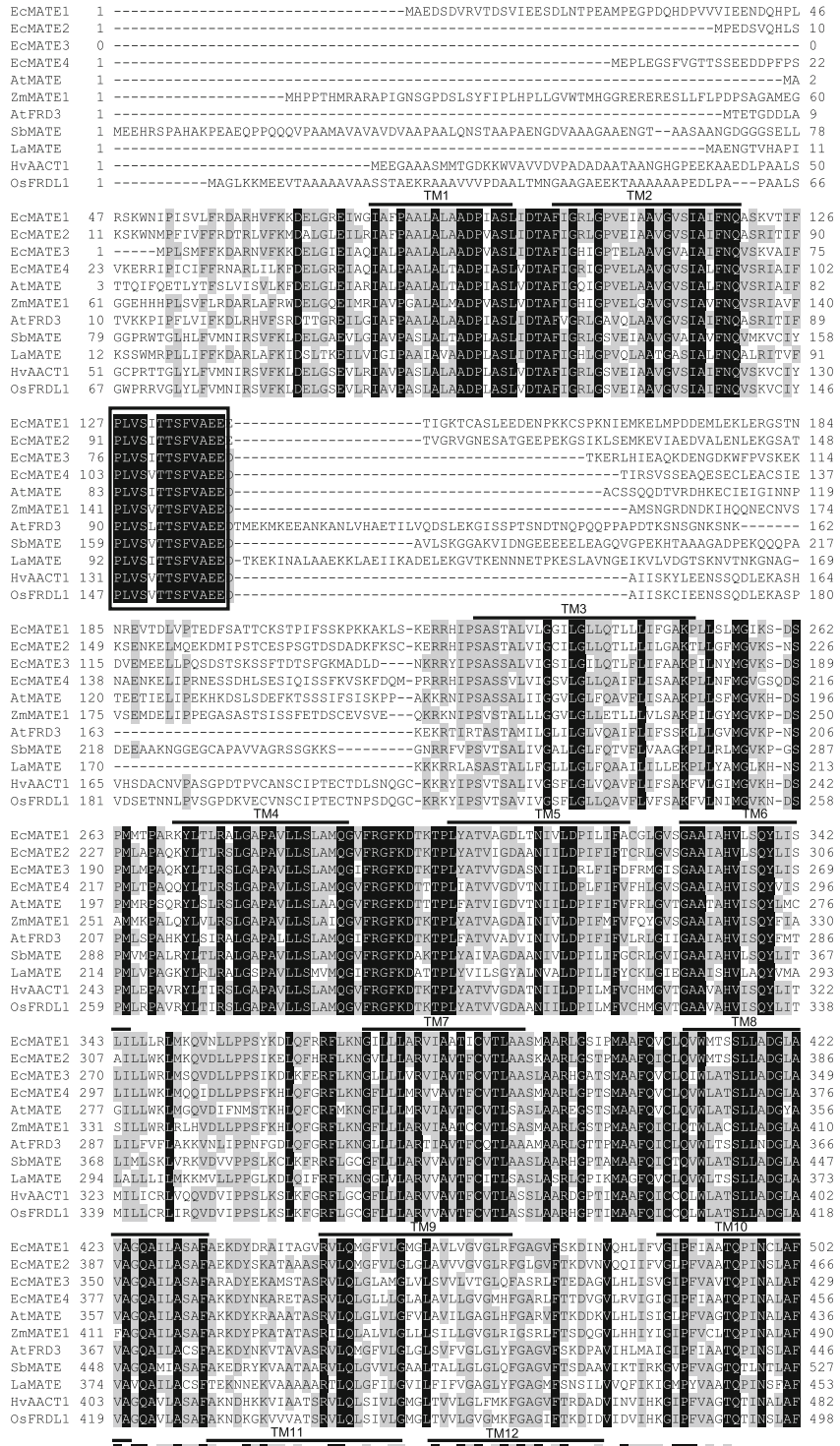

Fig. 2 Amino acid alignment (**a**) and phylogenetic tree (**b**) of citrate-transporting MATE proteins from *Eucalyptus* and other plant species. EcMATE1–4, *Eucalyptus camaldulensis*; AtMATE (At1g51340) and AtFRD3 (At3g08040), *Arabidopsis thaliana*; OsFRDL1 (Os03g0216700), *Oryza sativa*; HvAACT1 (BAF75822), *Hordeum vulgare*; SbMATE (ABS89149), *Sorghum bicolor*; LaMATE (AAW30733), *Lupinus albus*; ZmMATE1 (FJ015156.1), *Zea maize*. TM1–12 in **a** indicates transmembrane regions predicted by HMMTOP, and the box indicates the highly conserved region. The *scale bar* in **b** indicates amino acid substitutions per site. The actual value depends on the branch lengths in the tree

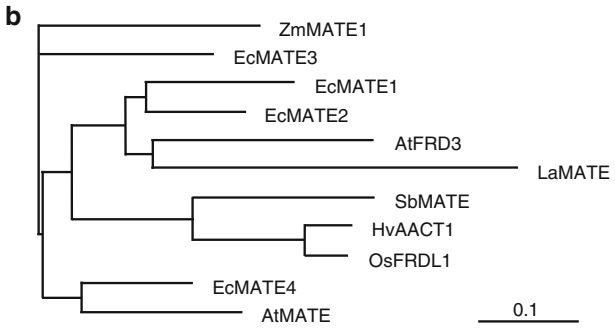

Fig. 2 continued

Subcellular localization of *EcMATE1*

To visualize subcellular localization of *EcMATE1*, we analyzed localization of EcMATE1:sGFP by *Agrobacterium*-mediated transient expression assay. When cytosolic sGFP was transformed to the tobacco roots, all part of cells fluoresced brightly (Fig. 4a, c). On the other hand, plant expressing EcMATE1:sGFP fusion protein carried many cells that showed green fluoresce at the edge of the cell (Fig. 4b, d). It suggested that EcMATE1 protein localized at the plasma membrane.

Ectopic expression of *EcMATE1* in tobacco hairy roots

To characterize the function of *EcMATE1* in Al-responsive citrate efflux, transgenic hairy roots of tobacco were obtained using a hyper-virulent *A. rhizogenes* strain carrying native Ri plasmids and Ti plasmids containing *EcMATE* and Km^R genes in the T-DNA region. Transgenic hairy roots carrying CaMV35s:: *EcMATE1*, which was selected as Km^R, excreted citrate into the Al medium, at levels greater than the GUS (β-glucuronidase)-transformed control. The *EcMATE1* transgenic did not show enhanced citrate excretion in the absence of Al (Fig. 5a). In addition, the transgenic hairy roots carrying *EcMATE1* grew better than the control transgenic carrying GUS (Fig. 5b). Among the other homologs, *EcMATE3* showed Al-responsive citrate excretion (Suppl Fig. S3), while it was not inducible by Al (Fig. 1c). These results indicated that both *EcMATE1* encodes Al-responsive citrate-transporting MATE in *E. camaldurensis*.

Response of *EcMATE1* expression to protein kinase and phosphatase inhibitors

To determine whether the citrate release in *Eucalyptus* is regulated by a protein phosphorylation/dephosphorylation process, Al-responsive citrate excretion was characterized in the presence of various protein kinase and phosphatase inhibitors (Fig. 6). Al-responsive citrate excretion was affected by both protein kinase (K-252a and staurosporine) and phosphatase inhibitors (calyculin A and cyclosporin A). Cyclosporin A inhibited excretion by 40 %, while the others inhibited excretion by over 70 % (Fig. 6a). These effective inhibitors, K-252A, staurosporine and calyculin, repressed the expression of *EcMATE1* (Fig. 6b), while a

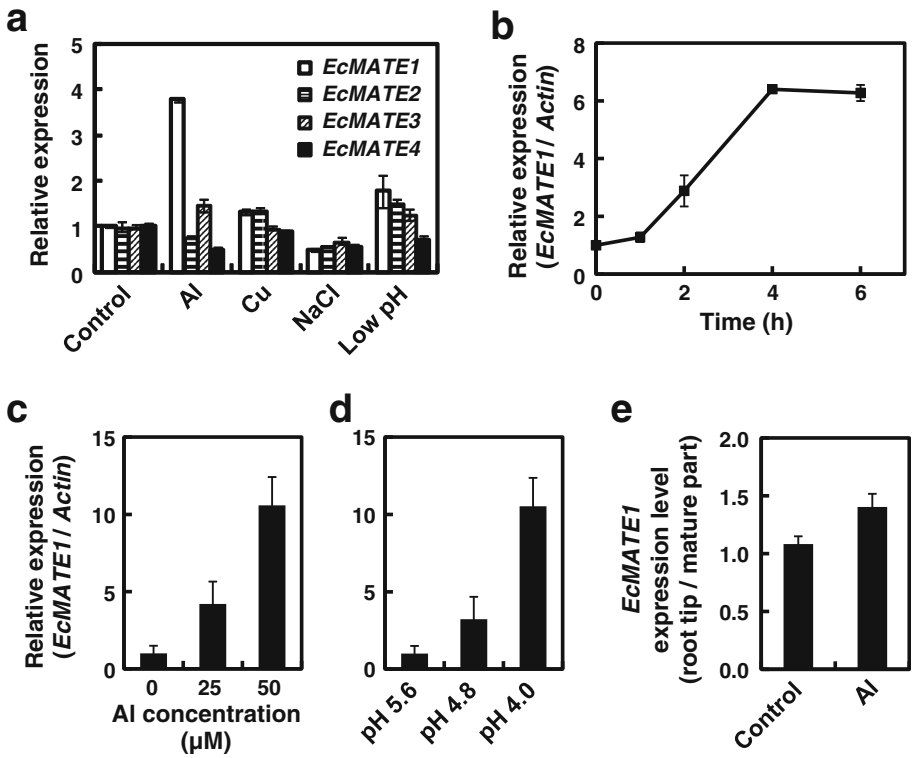

Fig. 3 MATE gene expression analysis of *E. camaldulensis* roots treated with ion stress. **a** Expression of MATE1–4 to various stressors for 24 h (see conditions in Fig. 1c), **b** time-course analysis of *EcMATE1* expression treated with 50 μM Al by real-time PCR. Dose response analysis of Al (**c**) and low pH (**d**). **e** Relative expression of EcMATE1 in root tip (0–5 mm) and mature part of roots (>5 mm) with or without 50 μM Al for 24 h. Expression level was quantified by real-time PCR. Means of three replicates and the error bar ± SD are indicated

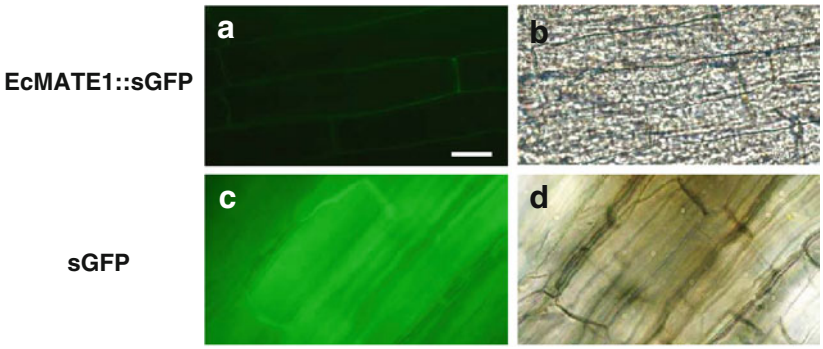

Fig. 4 Subcellular localization of EcMATE1:: sGFP fusion protein in tobacco root cells. Genes for fusion protein of EcMATE1:: sGFP (**a**, **b**) and cytosol-localizing sGFP (**c**, **d**) were introduced to tobacco by *Agrobacterium* transformation then visualized by a fluorescent microscope at 3 days after infection (**a**, **c**). *Bright field images* (**b**, **d**) are also shown. *Bar* indicates 50 μm

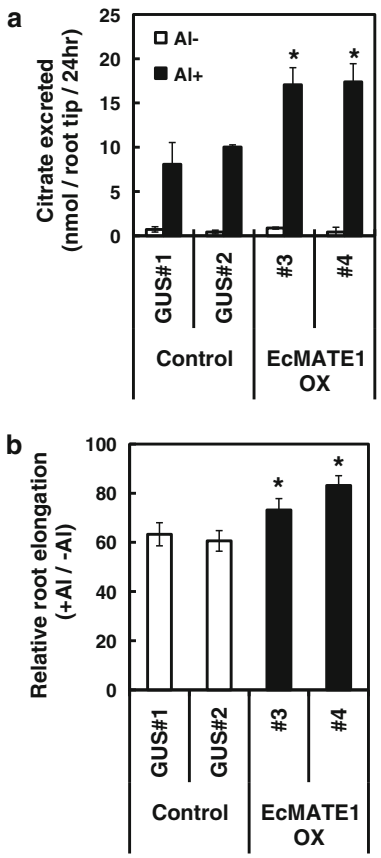

Fig. 5 Citrate release and growth of transgenic tobacco hairy roots carrying *EcMATE1*. **a** Citrate release at 24 h in Al (25 μM at pH 4.8) and control (pH 4.8) media. **b** Relative root elongation (25 μM Al at pH 4.8–0 Al control at pH 4.8). GUS transgenic lines were used as a control experiment. Mean ± SD are shown ($n = 3$). *Asterisks* indicate significant difference compared with each control (GUS) line by student *t* test ($P < 0.05$)

constitutively expressing *EcMATE2* maintained expression (Suppl Fig. S4). These results suggested that Al-responsive citrate excretion through *EcMATE1* involves a protein phosphorylation/dephosphorylation process for inducing citrate excretion.

Discussion

Many plant species release OA from the roots to protect Al-sensitive cells in the apex (Ma et al. 2001; Kochian et al. 2004). This strategy is common in herbaceous plants in both monocot and dicots. In this study, we determined that *Eucalyptus* releases citric acids in response to Al (Fig. 1a). This indicates that OA excretion from the roots also plays a role in the Al tolerance of some woody plant species. *Eucalyptus* excreted malate and citrate into the rhizosphere under control conditions (Al stress free conditions), but Al toxicity only enhanced the citrate excretion (Fig. 1d). This can be explained by the Al-inducible expression of *EcMATE* transporter(s) closely homologous to known plant citrate-transporting MATE proteins.

It has been reported that MATEs have 12 transmembrane domains and one conserved region linked to citrate-transporting capacity (Yang et al. 2011). Four MATE homologs were isolated from *E. camaldulensis*. All genes shared the consensus features of citrate-transporting MATEs, namely the 12 transmembrane domains and the conserved region among the known citrate-transporting MATEs (Fig. 2). Among the homologs, *EcMATE1* was uniquely up-regulated during Al treatment and nearly specific to Al and low pH (Fig. 3), which is a typical expression pattern of Al-responsive OA transporters. For example, *AtALMT1* regulating Al-responsive malate excretion in *Arabidopsis thaliana* is inducible by Al in an ion-specific manner (Kobayashi et al. 2007). Although the contribution to Al tolerance was less than *AtALMT1*, a citrate-transporting MATE (*AtMATE*) showed a similar Al-induced pattern in *Arabidopsis* (Liu et al. 2009).

Ectopic expression of *EcMATE1* in tobacco by *Agrobacterium rhizogenesis*-mediated transformation enhanced Al-responsive citrate excretion in the hairy roots (Fig. 3). This indicated that the citrate-transporting *EcMATE1* could function in tobacco cells. This was similar to results where transgenic *Arabidopsis* carrying SbMATE displayed Al

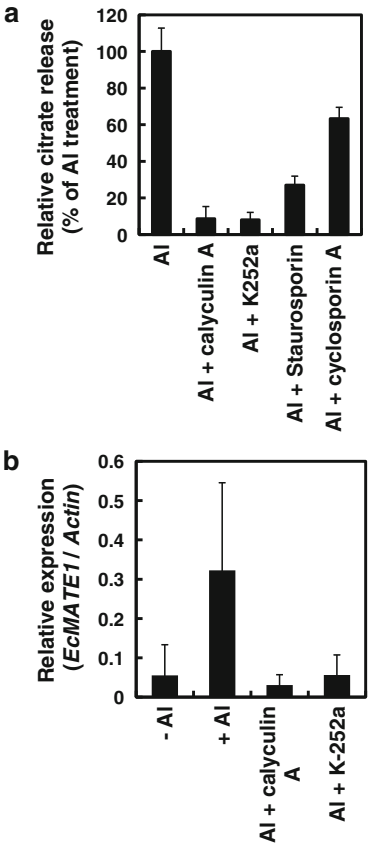

Fig. 6 Effect of protein kinase and phosphatase inhibitors on citrate release and *EcMATE1* expression. **a** Citrate release to Al solution (50 μM, pH 4.6) from *E. camaldulensis* roots in the presence of protein kinase inhibitors (K-252a and staurosporin A) and protein phosphatase inhibitors (calyculin A and cyclosporin A) or the absence of protein phosphorylation/dephosphorylation inhibitors. **b** Transcripts of *EcMATE1* in roots treated with Al solution (50 μM, pH 4.6) alone or supplemented with calyculin A or K-252a. Citrate released into the medium and the transcript levels of *EcMATE1* were analyzed after 24-h treatment. Each experiment was replicated three times and mean ± SD are shown. Calyculin A and K-252a greatly repressed Al-responsive citrate release from the roots

tolerance by enhanced citrate excretion (Magalhaes et al. 2007). The citrate transport functionality of these ectopically expressed MATE proteins could be explained by the activation of the protein by Al. Oocyte studies have indicated that MATE proteins could be activated by direct interaction with Al (e.g., Magalhaes et al. 2007). The involvement of protein phosphorylation in this Al-activating process was suggested by the Al-responsive malate excretion of an ALMT1-type malate transporter in *Arabidopsis* (Kobayashi et al. 2007). Similar protein phosphorylation might have activated EcMATE1 in the *Eucalyptus*. In fact, inhibitors of protein phosphorylation/di-phosphorylation affected Al-responsive citrate excretion.

Organic acid excretion plays a role in various plant stress tolerances, including Al tolerance, efficient P_i (Neumann et al. 1999) and Fe acquisition (Miethke and Marahiel 2007), and rhizo-bacterium-mediated pathogen resistance (e.g., Rudrappa et al. 2008). Molecular studies of OA excretion have revealed that transporters and their regulation are critical factors for determining organic acid species and stress responses. Recently, promoter evolution was proposed as the mechanism by which barley has obtained Al-responsive HvAACT1-mediated citrate excretion. Promoter analysis of HvAACT1 indicated that evolutionary changes in the promoter of a citrate-transporting MATE, possibly for Fe-acquisition, resulted in altered expression patterns of the gene to protect the sensitive root tip from Al toxicity (Fujii et al. 2012). *EcMATE1* expression was slightly greater in the root tip than mature root tissue (Fig. 3e) and would localize plasma membrane (Fig. 4) similar to HvAACT1. Although genetic association of EcMATE1 and Al tolerance has not been clarified yet, further analysis of *EcMATE1* would be interesting to understand the evolution of Al-responsive citrate excretion in woody plant species.

The direction of further studies will be towards enhanced Al tolerance of *Eucalyptus*. Overexpression of Al-tolerance genes such as *EcMATE1* would be one approach. In this case, the combination of *EcMATE1* overexpression and the alteration of OA metabolism (e.g., over-expression of citrate synthase; in canola by Anoop et al. 2003, in *Nicotiana benthamiana* by Deng et al. 2009) might be useful to efficiently enhance Al tolerance via citrate excretion. Recently, key transcription factors for Al tolerance were isolated in *Arabidopsis* (STOP1: sensitive to proton rhizotoxicity1, Iuchi et al. 2007) and rice (ART1: Al-resistance transcription factor 1, Yamaji et al. 2009). These proteins have high homology to Cys2–His2 zinc finger proteins and regulate multiple genes controlling Al tolerance. Although regulation of EcMATE1 by a STOP1 homolog has not been investigated in *Eucalyptus*, co-regulation of multiple Al-tolerance genes, including AtMATE (Liu et al. 2009), was associated with *Arabidopsis* STOP1 (Sawaki et al. 2009) and rice ART1 (Yamaji et al. 2009). Further analysis to identify STOP1-homolog regulatory genes would be an interesting approach for identifying co-regulated Al-tolerance genes in *Eucalyptus*. Such genes could also be utilized for developing molecular markers in *Eucalyptus* for efficient molecular breeding of Al tolerance.

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