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REGULAR ARTICLE

Functional characterization of three MATE genes in relation to aluminum-induced citrate efflux from soybean root

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Abstract
Background and aims Citrate transporters responsible for Aluminum (Al)-induced citrate efflux have not been identified in soybean.
Method Three soybean multi-drug and toxic compound extrusion (MATE) family genes were characterized by transcriptional expression, subcellular localization and overexpression experiments.
Results GmMATE75, GmMATE79 and GmMATE87 are localized to plasma membrane. Their overexpression respectively resulted in more citrate efflux and less Al contents in soybean hairy roots, alleviated root elongation inhibition in Arabidopsis and partially restored root growth in *atmate* mutant under Al stress. Al increased their transcriptional expression at either the root apex or the base zone. Cu^{2+}, Cd^{2+}, La^{3+} increased the expression of *GmMATE79* and *GmMATE87*. Ten day of –Fe culture increased the expression of *GmMATE75* and *GmMATE79*. Al treatment extended β-glucuronidase (GUS) staining from central cylinder to cortical and epidermis cells for *pGmMATE75::GUS* or *pGmMATE79::GUS* soybean hairy roots transformation. But GUS staining restricted within central cylinder for *pGmMATE87::GUS* transformation with or without Al treatment.
Conclusion GmMATE75, GmMATE79 and GmMATE87 are plasma-membrane-localized citrate transporters and have capabilities to increase citrate efflux. They played different role in Al-induced citrate secretion from soybean because their different tissue localization and expression patterns.

Keywords Aluminum toxicity · Citrate transporter · Iron deficiency · Multi-drug and toxic compound extrusion family · Resistance mechanism

Abbreviations

Al	Aluminum
CaMV	Cauliflower mosaic virus
GFP	Green Fluorescent protein
MATE	Multidrug and toxic compound extrusion

Introduction

Aluminum (Al)-induced organic acid excretion has been convincingly demonstrated as a major mechanism of Al resistance through Al exclusion in many plant species (Ma et al. 2001; Kochian et al. 2015). The first resistance gene in plants was cloned from wheat as

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TaALMT1 (aluminum-activated malate transporter) encoding an Al^{3+}-activated malate channel (Sasaki et al. 2004). Citrate transporters responsible for Al-activated citrate efflux were later identified by positional cloning in sorghum (*SbMATE*, multidrug and toxic compound extrusion, Magalhaes et al. 2007) and barley (*HvAACT1*,aluminum-activated citrate transported 1, Furukawa et al. 2007), respectively; these transporters are the members of the multi-drug and toxic compound extrusion (MATE) family. Transgenic Arabidopsis and tobacco showed enhanced Al resistance with the over-expression of *SbMATE* (Magalhaes et al. 2007) and *HvAACT1* (Furukawa et al. 2007), respectively. The transporter genes of the MATE and ALMT families can provide effective ways to enhance the Al resistance of plants. Unravelling additional genes encoding organic acid anion transporters will help more crops to adapt to Al-stresses under acidic soil environments.

MATE transporters are proposed to be involved in a wide range of biological events including xenobiotic efflux, accumulation of alkaloids and flavonoids, iron translocation, Al detoxification and plant hormone signalling (Takanashi et al. 2014). The identified citrate transporter genes in the MATE family have been suggested to function in Al resistance or iron uptake or both. First, SbMATE (Magalhaese et al. 2007), AtMATE (Liu et al. 2009), BoMATE (Wu et al. 2014), EcMATE1 and EcMATE3 (Sawaki et al. 2013), OsFRDL4 (ferric reductase defective like 4, Yokosho et al. 2011), OsFRDL1 (Yokosho et al. 2016a) and ScFRDL2 (Yokosho et al. 2010), VuMATE1 (Yang et al. 2011), VuMATE2 (Liu et al. 2018), ZmMATE1 (Maron et al. 2010; Maron et al. 2013), FeMATE1 (Lei et al. 2017) were reported to be responsible for Al-activated citrate efflux under Al stress. Second, members such as *AtFRD3* (Durrett et al. 2007), *GmFRD3b* (Rogers et al. 2009), *OsFRDL1* (Yokosho et al. 2009) and *ScFRDL1* (Yokosho et al. 2010), *LjMATE1* (Takanashi et al. 2013) were found to be localized to either the root pericycle cell or the nodules and were responsible for Fe supply by assisting the translocation of Fe by providing citrate. Thirdly, it was reported that *HvAACT1* and *TaMATE1b* were involved in both Fe translocation and Al^{3+} detoxification (Fujii et al. 2012; Tovkach et al. 2013; Takanashi et al. 2014).

Various regulatory systems were found to control citrate transporter expression and thus function. The first group is represented by *ZmMATE1*. Its gene duplications determined their expression and thus citrate efflux (Maron et al. 2013). The second group is represented by *HvAACT1* and *TaMATE1b*. HvAACT1, a citrate transporter in barley, provides primary transport of citrate into the root xylem for Fe translocation to the shoot as a Fe-citrate complex in the mature root zones (Fujii et al. 2012). The insertion of a 1 kb transposon in the 5′ upstream region of *HvAACT1* changed its distribution and density to facilitate citrate secretion from the root apex to the rhizosphere for Al detoxification (Fujii et al. 2012). Fujii et al. (2018) also found that retrotransposon insertion and DNA methylation in the upstream genomic region are involved in regulating the expression of *HvAACT1* then Al resistance in barley especially distributed in European areas with acid soil. A transposon-like element in *Triticum aestivum* increased citrate transporter *TaMATE1B* expression in root apex, where it facilitates citrate efflux and enhances Al tolerance (Tovkach et al. 2013). The third group is represented by *AtMATE* and *VuMATE1*. Their expression is regulated by Cys2His2 zinc finger-type transcription factor STOP1 (sensitive to proton rhizotoxicity1). The expression of *AtMATE* is regulated by the transcription factor *AtSTOP1* (Liu et al. 2009). Yeast one-hybrid examination has shown that there is an interaction between *VuSTOP1* and the promoter region of *VuMATE1and VuMATE2* (Yang et al. 2011; Fan et al. 2015; Liu et al. 2018). A 1.2 kb insertion in the *OsFRDL4* promoter region in the rice japonica subspecies increased the number of *cis*-acting elements of ART1 then enhanced *OsFRDL4* expression level, but did not alter its spatial expression or cellular localization (Yokosho et al. 2016b).

Multiple Al resistance mechanisms have been suggested to be involved in Al resistance in soybean (Nian et al. 2004). Correspondingly, multigenetic traits have been suggested to relate to mechanisms underlying Al resistance in soybean by genetic studies (Bianci-Hall et al. 1998, 2000). Compared with the progress made in other important crops and model plant species, little was known about the molecular mechanisms of Al resistance in soybean. Al-induced citrate efflux has been well established as an Al resistance mechanism in soybean (Yang et al. 2000, 2001; Silva et al. 2001). Liu et al. (2016) identified and named a total of 117 genes encoding MATE transporters in the whole soybean genome, which are unevenly distributed on the 20 soybean chromosomes. However, the citrate transporter responsible for citrate efflux from soybean under Al stress has not been identified until now.

Rogers et al. (2009) showed that *GmFRD3a* and *GmFRD3b* were induced by iron deficiency in an iron-efficient soybean cultivar. *GmFRD3b*, but not *GmFRD3a*, was expressed at higher levels in the iron-efficient cultivar than its iron-inefficient near-isogenic line, and thus *GmFRD3b* is suggested to function similarly to *AtFRD3* to mediate citrate efflux into the xylem. Their function has not been further characterized until now. Our previous microarray analysis found a MATE-family transcript (*GmMATE75*) was up-regulated by Al treatment in the Al-tolerant genotype Jiyu 70 (You et al. 2011) which was also predicated to be the candidate Al tolerance gene according to its gene expression pattern and *cis*-elements (Liu et al. 2016). The expression of *GmALCT1* (*GmMATE 79*) was also highly induced in soybean root apices under Al stress (Xu et al. 2010). In the present study, we compared three genes in the MATE family, *GmMATE75*, *GmMATE79* and *GmMATE87 (GmFRD3a)*, to reveal their functions in the process of Al-induced citrate efflux from soybean root apices.

Material and methods

Plant materials and growth conditions

Compared with Jiyu 62, soybean cultivar Jiyu 70 exhibited higher Al resistance and higher amount of citrate efflux under Al stress (Zhou et al. 2018a). They were used as plant materials in the present study. For hydroponic culture, soybean seeds were surface sterilized in 1.0% (*v/v*) sodium hypochlorite for 5 min, washed 3–4 times with tap water, and germinated in peat moss for 3 days at 25 °C in the dark. After germination, uniform seedlings were cultured in 1 L plastic pots filled with nutrient solution (pH 4.5) as described by Horst et al. (1992) containing 750 μM KNO_3, 250 μM $Ca(NO_3)_2$, 325 μM $MgSO_4$, 10 μM KH_2PO_4, 20 μM Fe-EDTA, 8 μM H_3BO_3, 0.2 μM $(NH_4)_6Mo_7O_{24}$. The solution was aerated and renewed every other day. Seven-day-old seedlings were transferred to 0.5 mM $CaCl_2$ solution overnight for the following treatments.

In the time course experiment, the seedlings were exposed to 30 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution. The root apices (0–1 cm) were excised at 0 h, 2 h, 4 h, 8 h, 12 h, and 24 h. For the other metal stresses, the seedlings were transferred into nutrient solution (pH 4.5) containing 25 μM $CdCl_2$, 10 μM $LaCl_3$, or 1 μM $CuCl_2$. Root apices (0–1 cm) were excised after 4 h metal exposure. For the iron deficiency experiment, the germinated seedlings were cultured in nutrient solution (Horst et al. 1992) (pH 4.5) without the addition of Fe-EDTA. Root apices (0–1 cm) were excised after 10 days of culture. For the root localization experiment, the seedlings were exposed to 0 or 30 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution for 4 h. Root segments of 0–1 cm, 1–2 cm, and 2–3 cm were excised respectively. All the excised root segments were put into liquid nitrogen instantly and stored in a − 80 °C freezer for RNA isolation. All hydroponic experiments were carried out in a controlled growth chamber at 25 °C day/22 °C night temperatures, 60% constant relative humidity, 14 h light/10 h dark cycles, and 300 μmol m^{-2} s^{-1} of light intensity during the day.

Gene cloning of *GmMATE75*, *GmMATE79* and *GmMATE87* and their bioinformatics analysis

With Affymetrix probe sequence, the CDS of *GmMATE75* was got by Blast in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Then, the encoding amino acid sequence of *GmMATE75* was searched against Phytozome (www.phytozome.net) to find its homologues. A phylogenetic tree was generated with TreeView 5.1 with GmMATE75, its homologues and other known citrate transporter. Primers were designed according to CDS sequences of *GmMATE79* (Glyma.13G339800), *GmMATE87* (Glyma.15G274600), *GmMATE75* (Glyma.13G203000) considering the enzyme cutting locus of BamHI in the modified vector pCAMBIA3301 (Table S1). Total RNA was isolated from 4 h Al-treated soybean root apices (Jiyu70 and Jiyu 62) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed with M-MLV reverse transcriptase (TaKaRa Bio Tokyo, Japan) according to the manufacturer's protocols. With the cDNA as template, the three genes were cloned by RT-PCR. The PCR products were purified by a TransGen Biotech kit according to the manufacturer's protocol and confirmed by sequencing. Jiyu 70 and Jiyu 62 displayed same sequences in the CDS region of *GmMATE79* (Glyma.13G339800), *GmMATE87* (Glyma.15G274600) or *GmMATE75* (Glyma.13G203000).

Transcriptional expression of putative citrate transporter genes

Quantitative real-time PCR (qRT-PCR) was performed to determine transcriptional expression of the three putative citrate transporter genes. Gene-specific primers were designed using Primer 3.0 online (http://primer3.ut.ee/) and listed in Table S1. The housekeeping gene *β-Tubulin* (GenBank ID: 100811275) was used as an internal standard. qRT-PCR was conducted in an Mx3005P machine (Stratagene, USA). The reaction system (25 μl) was as follows: 2 μl cDNA template (50–100 ng), 0.5 μl 10 mM specific forward primer, 0.5 μl 10 mM specific reverse primer, 12.5 μl 2× SYBR Premix Ex Taq (TaKaRa, Bio Inc.), and 9.5 μl double-distilled H_2O. The procedure was performed as follows: 1 cycle for 30 s at 95 °C, 30 cycles for 5 s at 95 °C and 20 s at 60 °C, and 1 cycle for 60 s at 95 °C, 30 s at 55 °C, and 30 s at 95 °C for the melting curve analysis. The relative expression level of each gene was computed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Subcellular localization of GmMATE75, GmMATE79 and GmMATE87

The CDSs of *GmMATE75*, *GmMATE79* and *GmMATE87* were cloned into the pENSG-N-GFP vector under the control of a CaMV 35S promoter. The transient expression of the translational in-frame fusion was achieved in Arabidopsis protoplast cells. Transformed Arabidopsis protoplast cells were stained by cell plasma membrane Marker stain(Cell Mask™ Orange plasma membrane stain,C10045,USA). The GFP fluorescence was observed by laser-scanning confocal microscopy (Leica TCS SP8X DLS, Wetzlar, Germany).

Overexpression of *GmMATE75*, *GmMATE79*, and *GmMATE87* in soybean hairy roots

The CDSs of *GmMATE75*, *GmMATE79*, *GmMATE87* were amplified and respectively cloned into the modified pCAMBIA3301 vector with CaMV 35S as promoter and labelled with Nanoluciferase. The transformed plasmids were sequenced then electroporated into the Agrobacterium strain K599. The transformation of soybean Jiyu 62 cotyledons was performed as described by Subramanian et al. (2005). Luciferase activity in hairy roots was measured by luminometer (Centro LB960 XS3, Bert-hold, Germany) with Coelenterazine (Prolume Ltd., Pinctop, USA) as substrate. The hairy roots with luciferase value greater than 3000 were considered as successful transformants. Hairy roots transformed by only K599 were considered untransformed type. Both transgenic and untransformed hairy roots were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) with or without 30 μM $AlCl_3$ within a 5-ml plastic tube. Root exudates were collected at 4 h for citrate efflux measurement. Citrate concentrations were measured by the enzymatic method according to Delhaize et al. (1993). Ten root apices (0–1 cm) were excised for Al content measurements. Al concentrations in hairy root apices were extracted by 2 M HCl and determined by an atomic adsorption spectrophotometer with a graphite furnace atomizer (PerkinElmer AAnalyst 700, USA).

Arabidopsis transformation of *GmMATE75*, *GmMATE79* and *GmMATE87*

The constructs of modified pCAMBIA3301 vector prompted by *CaMV 35S* and labelled by Nanoluciferase were introduced into an *Agrobacterium tumefaciens* strain (Agl0) and subsequently transformed into Arabidopsis ecotype (Col-4) or the *atmate* mutant (SALK_081671) by the floral dip method (Clough and Bent 1998). The transgenic seedlings were screened by spraying with Basta herbicide and confirmed by luciferase activity and reverse transcription-polymerase chain reaction (RT-PCR) measurements. Arabidopsis leaves with luciferase activity value greater than 5000 were considered as successful transformation. Homozygous T3 lines were cultured in Al-containing mediums for measuring relative root elongation according to Sun et al. (2014). The root growth of representative plants from two independent transgenic lines grown in MS medium for 5 d was recorded; uniform seedlings were grown on solid agar medium supplied with 4.3 mM $CaCl_2$ and 3% sucrose (with 0 or 100 μM $AlCl_3$ at pH 4.5 for 2 d).

Tissue-level localization of GmMATE expression via histochemical staining of GUS activity

The sequences from upstream of the start condon of *GmMATEs* respectively were isolated from Jiyu70 genomic DNA, then constructed to modified

pCAMBIA3301 vector with GUS label. The promoter sequences of *GmMATE75*, *GmMATE79*, *GmMATE87* were 1499 bp, 2022 bp and 2000 bp respectively. The resulting constructs were sequenced and electroplated into the Agrobacterium strain K599. The transformation of soybean Jiyu 62 cotyledons was performed according to Subramanian et al. (2005). After 4 h exposure to 0.5mM$CaCl_2$ solution (pH 4.5) containing 0 or 30 μM $AlCl_3$, hairy roots were rinsed within a X-gluc solution (RTU4032 Real-times Biotechnology, Beijing, China) for staining observation. The staining in roots were observed and photographed with microscope (Zeiss 2012 Observer A1, Göttingen, Germany). For transection observation, roots were dissected at 0–9 mm and immediately fixed in solution containing ethanol/ acetic acid /formaldehyde at a 9:1: 1 ratio. After 24 h fixation, the samples were rinsed by 70% ethanol for 3 times and dehydrated gradually in ethanol (70%, 85%, 95% and 100%), and embedded in paraffin wax. Slices with (10 μm) were dissected from the root apex part by a microtome (LEICA Biosystems RM2245, Wetzlar, Germany) and placed on a glass slides. The slides were covered with glycerol and observed by microscope.

Statistical analysis

Significant difference among treatments or transgenic lines were evaluated by Data Processing System (Tang and Zhang 2012).

Results

Bioinformatics analysis

GmMATE75, GmMATE79 and GmMATE87 highly conserved in including 12 transmembrane regions and a 97 amino acids loop between 2nd and 3rd transmembrane domains (Fig. S1). Much variation was present in the N-terminal and in this long loop between TM2 and TM3 among the three putative soybean citrate transporters (Fig. S1).

A sequence divergence of citrate transporters in the MATE family existed between monocots and dicots but was not strongly related to their functions in Al resistance or iron deficiency (Fig. S2). TaMATE1-4B, HvAACT1, ScFRDL1, OsFRDL1, SbMATE, OsFRDL4, ZmMATE1, and ZmMATE2 in monocots clustered closely and showed less similarity to EcMATE3, GmMATE87, AtMATE, BoMATE, EcMATE1, AtFRD3, GmMATE75, VuMATE1, LjMATE, GmMATE87 and GmFRD3b in dicots. GmMATE75 showed high amino acid sequence homology to VuMATE (with 78% identity) from *Vigna umbellata* and AtFRD3 (with 60% identity). GmMATE79 showed high similarity to AtMATE (65% identity) and BoMATE (65% identity). GmMATE87, encoding 553 amino acids, clustered closely with AtFRD3 (61%) and LjMATE (72%) (Fig. S2). The MATE proteins indicated with different colours represented different functions. Their varied distribution suggested their physiological role related less to their protein similarity.

The transcriptional patterns of *GmMATE75*, *GmMATE79* and *GmMATE87*

Jiyu70 has been reported to have a larger amount of Al-induced citrate efflux thus confers to higher Al resistance compared to Jiyu 62 (Zhou et al. 2018a).*GmMATE75*, *Gm MATE79* and *GmMATE87* all constitutively expressed in soybean roots (Fig. 1a-c). In the time course experiment, both genotypes displayed high transcriptional expression level of *GmMATE75* under Al stress. Nearly a 200-fold increase in *GmMATE75* expression was found in both genotypes after 4 h Al treatment (Fig. 1a). *GmMATE75* expression in Jiyu 70 was higher than that of Jiyu 62 starting from 8 h Al treatment (Fig. 1a). This result is consistent with our previous study, which showed that more citrate secretion from Jiyu 70 than Jiyu 62 was found after 8 h Al treatment (Zhou et al. 2018a). Almost 7-fold higher transcription expression of *GmMATE79* was induced in Jiyu 70 at 2 h, then decreased to about 2-fold within the following Al treatment duration (Fig. 1b). The expression of *GmMATE79* remained constant in Jiyu 62 over 24 h Al treatment (Fig. 1b). Higher expression of *GmMATE87* was also induced after 2 h Al treatment but then returned to basal level (Fig. 1c). The expression of *GmMATE87* sustained 2-fold increase from 4 h Al treatment duration for Jiyu62 (Fig. 1c). Spatial expression analysis showed that *GmMATE75*, *GmMATE79* and *GmMATE87* were distributed evenly in the root segments at 0–1 cm, 1–2 cm and 2–3 cm under control conditions (Fig. 1d-f). Four hours of Al treatment enhanced transcription levels of these three MATE-family genes in 0–3 cm root

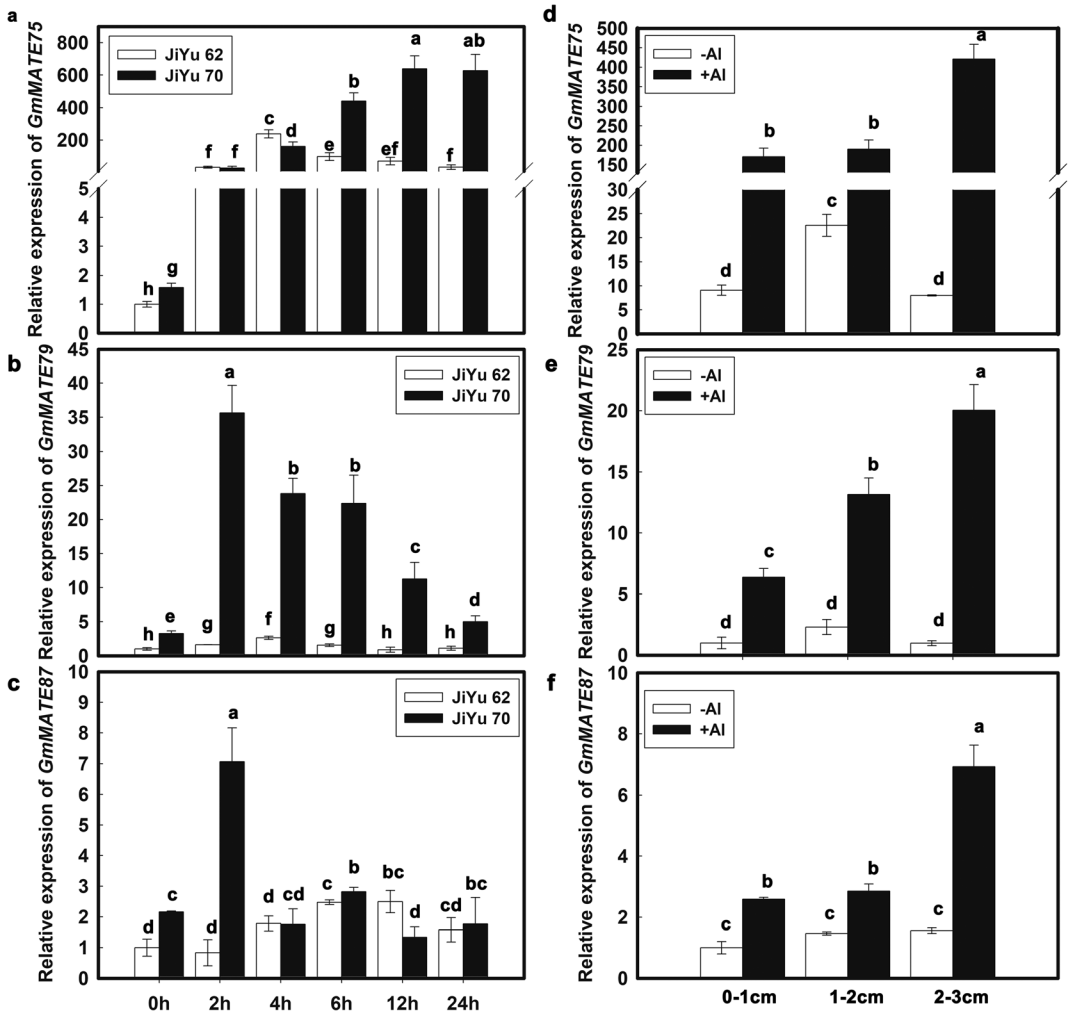


Fig. 1 Temporal and spatial expression of *GmMATE75* (ad), *GmMATE79* (be) and *GmMATE87* (cf) in soybean under Al stress. Seven-day-old soybean seedlings (Jiyu70 and Jiyu62) were exposed to 0.5 mM $CaCl_2$ solution containing 30 μM $AlCl_3$ (pH 4.5). The 0–1 cm root apices were excised after 0, 2, 4, 8, 12, and 24 h Al treatment to study temporal expression. The 0–1 cm, 1–2 cm and 2–3 cm root segments (Jiyu70) were also excised from 4 h Al-treated or control soybean roots to study spatial expression. The expression of *GmMATE75*, *GmMATE79* and *GmMATE87* were examined by qRT-PCR with *β*-tubulin as the reference gene. Data are represented as means±standard deviation (SD) of three biological replicates. Different letters above columns represented significantly different ($p < 0.05$)

segments, but the increase was more profound in mature root zones especially in the root segment of 2–3 cm (Fig. 1d-f).

The transcript levels of *GmMATE79* and *GmMATE87* (but not of *GmMATE75*) were also up-regulated under Cd^{2+}, La^{3+}, and Cu^{2+} stress (Fig. 2a-c). Ten days of -Fe treatment increased the transcript abundance about 2-fold for *GmMATE79* and approximately 30-fold for *GmMATE75*, but decreased the transcript level of *GmMATE87* by half compared with those in + Fe culture (Fig. 3a-c).

Subcellular localization of GmMATE75, GmMATE79 and GmMATE87

The cellular localization was characterized by a transient expression assay with translational fusions of the separate citrate transporter gene with *GFP* in Arabidopsis protoplast cells. In comparison of the non-specific fluorescence distribution of cell transformed with the empty GFP vector, transient expression of *GFP-GmMATE75*, *GFP-GmMATE79* and *GFP-GmMATE87* can respectively induce fluorescence

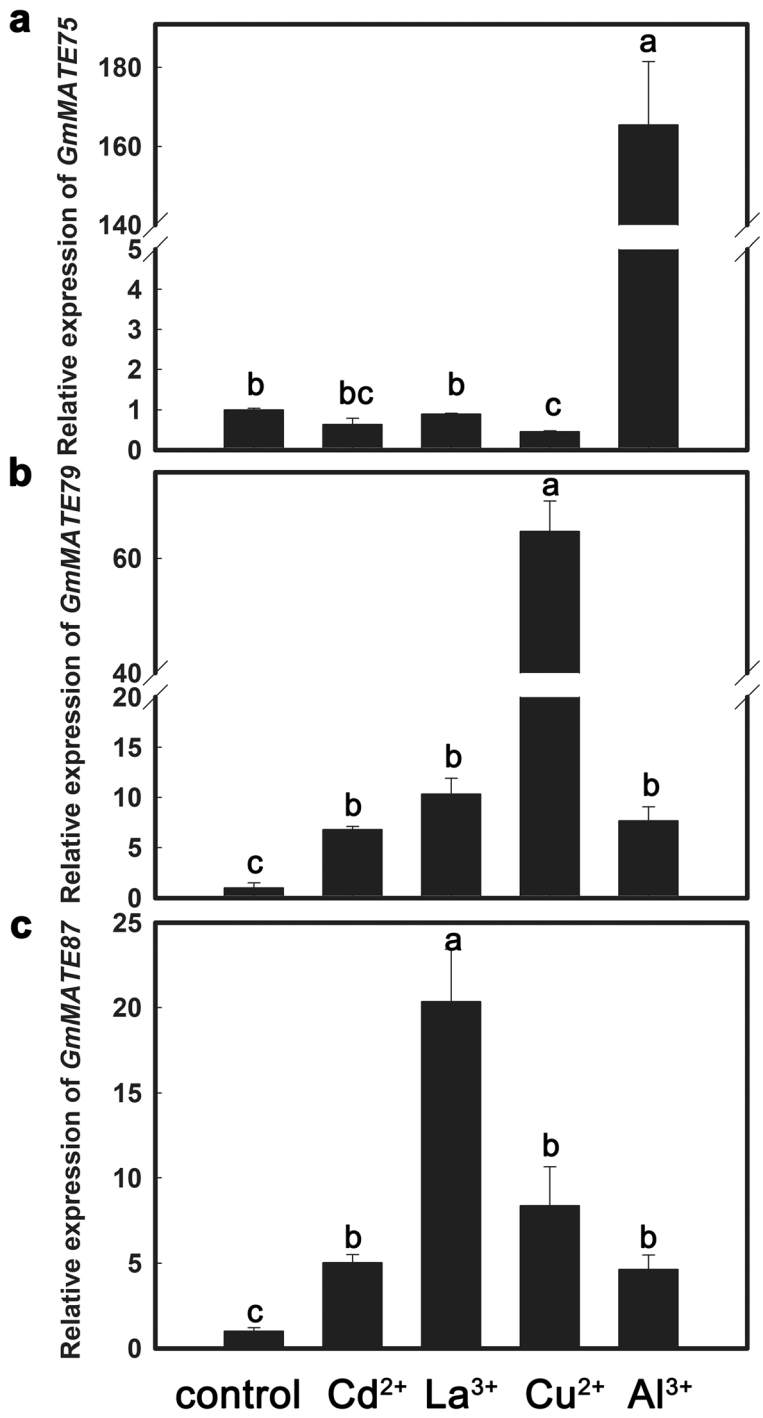


Fig. 2 Transcriptional expression of *GmMATE75* (a), *GmMATE79* (b) and *GmMATE87* (c) under Cd^{2+}, La^{3+}, Cu^{2+} and Al^{3+} stresses in soybean root apices. Seven-day-old soybean seedlings (Jiyu70) were exposed to 0.5 mM $CaCl_2$ solutions containing 25 μM Cd^{2+}, 10 μM La^{3+}, 1 μM Cu^{2+} and 30 μM Al^{3+} (pH 4.5). The 0–1 cm root apices were excised after 4 h Al treatment to study gene expression. Data are represented as means ±SD of three biological replicates. Different letters above columns represented significantly different ($p < 0.05$)

in the cell periphery of the Arabidopsis protoplasts that was overlapped with the plasma membrane marker staining (Fig. 4). Thus, all of GmMATE75, GmMATE79 and GmMATE87 are localized to the plasma membrane (Fig. 4).

Transgenic hairy roots with overexpression of *GmMATE75*, *GmMATE79* or *GmMATE87*

Promoter CaMV35S driven *GmMATE75*, *GmMATE79*, and *GmMATE87* were separately overexpressed in

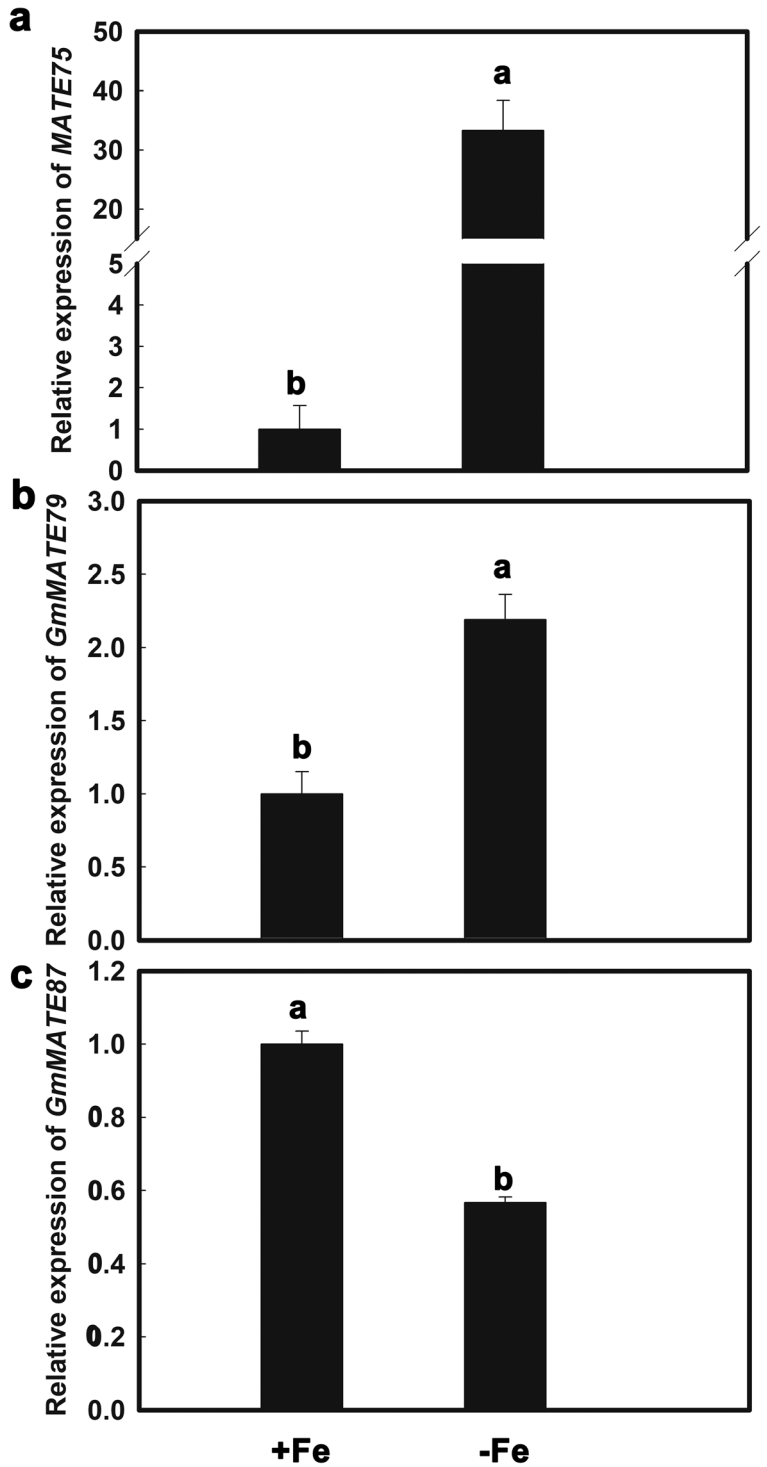


Fig. 3 Transcriptional expression of *GmMATE75* (a), *GmMATE79* (b) and *GmMATE87* (c) under Fe deficiency conditions. Germinated soybean seedlings (Jiyu 70) were cultured in nutrient solution containing Fe-EDTA or not. After 10 days of culture, soybean root apices (0–1 cm) were excised to extract RNA to study gene expression. Data are represented as means ±SD of three biological replicates. Different letters above columns represented significantly different ($p < 0.05$)

soybean hairy roots (Jiyu 62). *GmMATE75*-OE, *GmMATE79*-OE and *GmMATE87*-OE hairy roots increased their corresponding transcript levels (Fig. 5a-c), and citrate efflux under either –Al or + Al treatment (Fig. 5d-f). The Al-treated *GmMATE79*-OE, *GmMATE75*-OE, *GmMATE87*-OE hairy roots also contained less Al in their root apices than wild-type hairy roots (Fig. 5g). Consistent with its less citrate

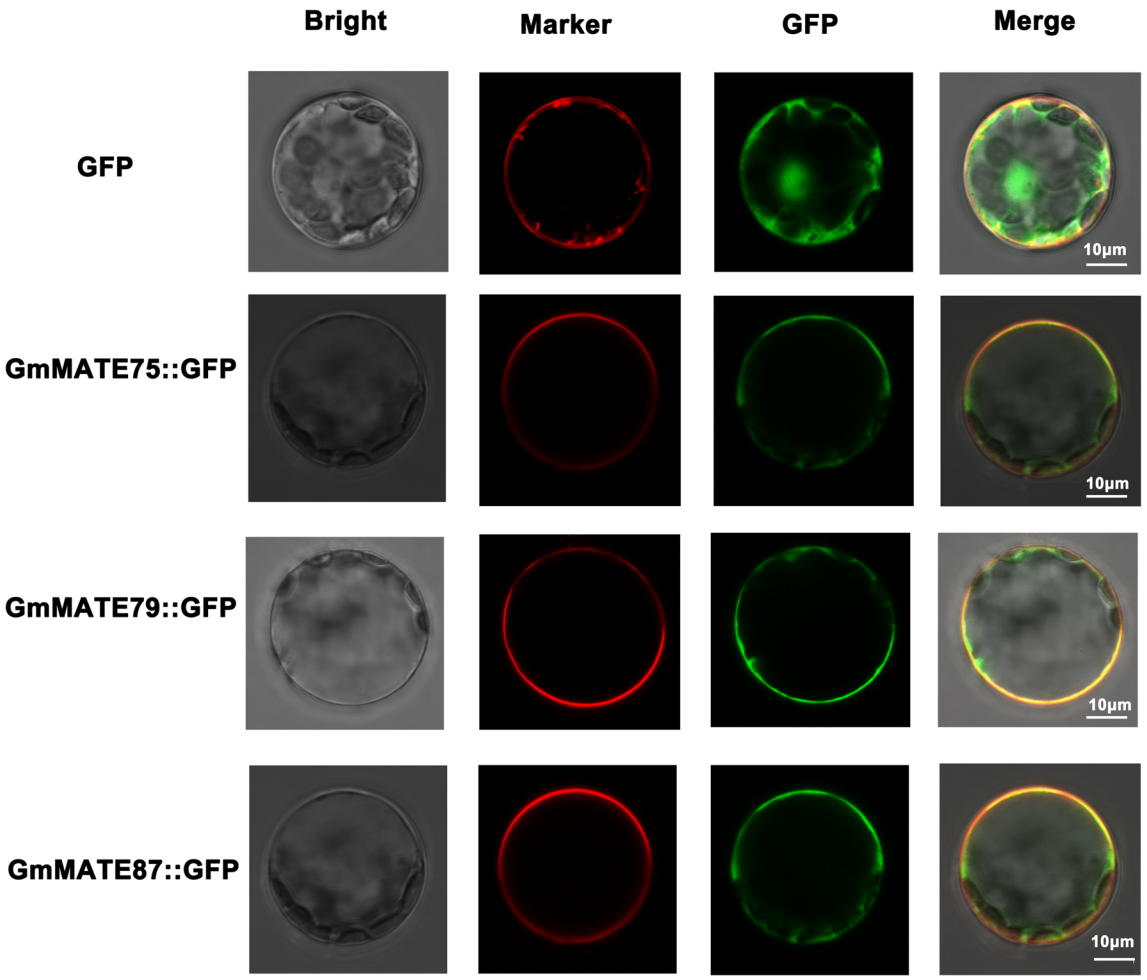


Fig. 4 Subcellular localization of proteins by transient expression of GFP-GmMATE75, GFP -GmMATE79 and GFP-GmMATE87 fusion proteins in Arabidopsis protoplast cells. The first-row images show GFP alone, and the second-, third- and fourth-row images are the fusion proteins GFP-GmMATE75, GFP -GmMATE79 and GFP-GmMATE87, respectively

efflux, *GmMATE87*-OE contained more Al in their root apices compared with *GmMATE79*-OE or *GmMATE75*-OE (Fig. 5g). The above results indicated that the overexpression of *GmMATE75*, *GmMATE79* and *GmMATE87* resulted in increased citrate efflux and decreased Al content in root apices, which underscores their contribution to Al resistance.

Heterologous overexpression of *GmMATE75*, *GmMATE79* and *GmMATE87* in Arabidopsis

RT-PCR has shown that the transcript levels of *GmMATE75*, *GmMATE79* and *GmMATE87* were separately increased in their corresponding Arabidopsis overexpression lines (Fig. S3). Without Al, transgenic and Col-4 Arabidopsis showed similar root growth (Fig. 6). Root elongation was inhibited by 55% by 100 μM Al stress in Col-4 Arabidopsis (WT) or vector transgenic line (Vector). *GmMATE75*-OE lines showed very similar root elongation inhibition at approximately 53% (*GmMATE75*-OE1, *GmMATE75*-OE2) (Fig. 6ad. Two *GmMATE79*-OE lines showed 51% and 55% root elongation inhibition, respectively, under Al stress (*GmMATE79*-OE1, *GmMATE79*-OE2) (Fig. 6b, e). Root elongation inhibition was approximately 55% and 58% in two *GmMATE87*-OE lines under Al stress (*GmMATE87*-OE1, *GmMATE87*-OE2) (Fig. 6c, f). Increases in root growth were found in the *GmMATE79*-OE, *GmMATE75*-OE and *GmMATE87*-OE lines comparing with Col-4 or vector transformation line under Al

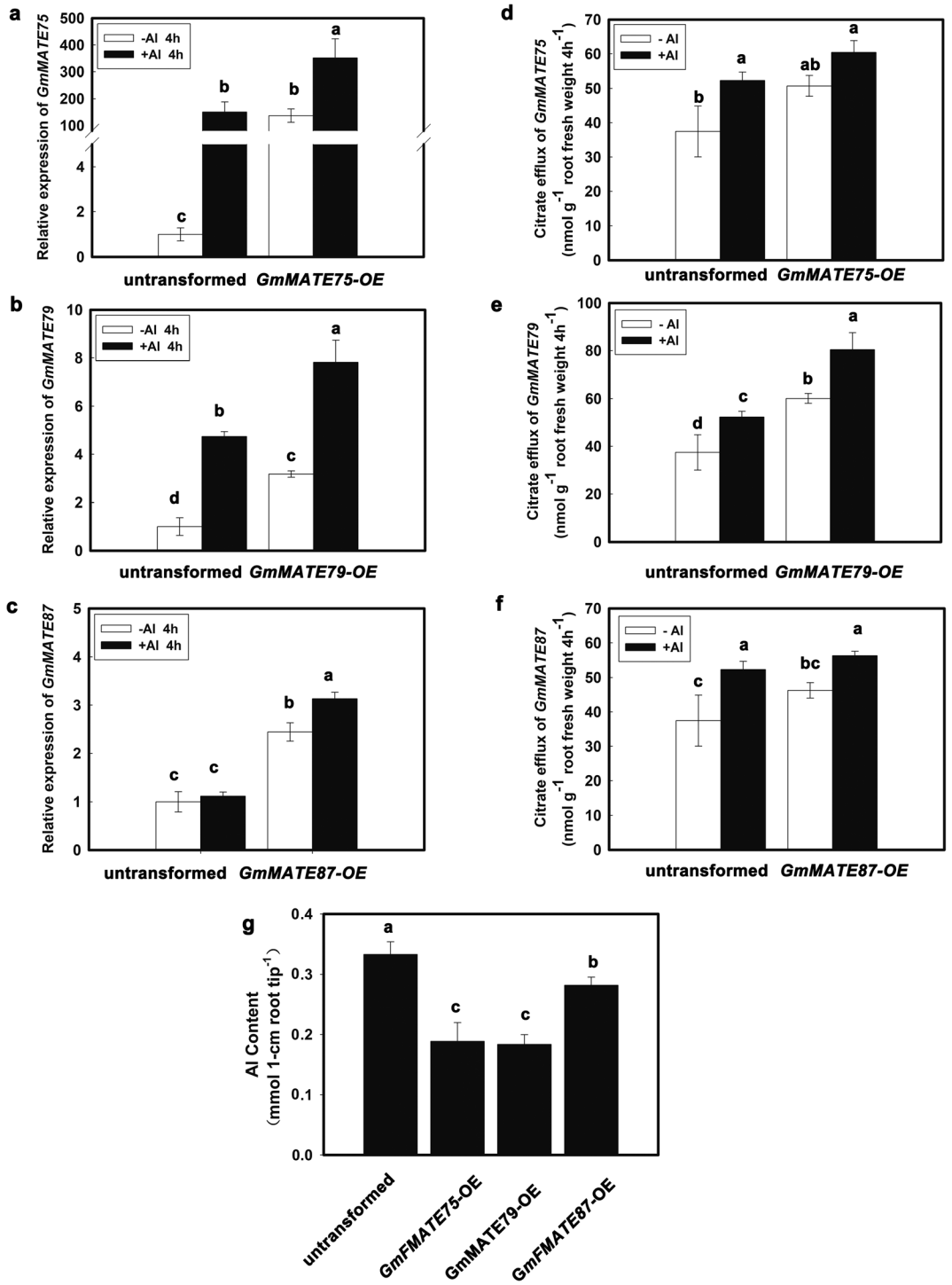


Fig. 5 Phenotype of Al tolerance in soybean hairy roots over-expressing *GmMATE75*, *GmMATE79* and *GmMATE87*. Transcriptional expression of *GmMATE75* in *GmMATE75*-OE soybean hairy roots (**a**), *GmMATE79* in *GmMATE79*-OE soybean hairy roots (**b**) and *GmMATE87* in *GmMATE87*-OE soybean hairy roots (**c**). Citrate efflux from soybean hairy roots of *GmMATE75*-OE (**d**), *GmMATE79*-OE (**e**) and *GmMATE87*-OE (**f**). (**g**)Al content in the root apices of soybean hairy roots of *GmMATE75*-OE, *GmMATE79*-OE and *GmMATE87*-OE. The induction of soybean hairy roots and treatment procedure was described in Material and Methods. Al content in root apices of *GmMATE75*-OE, *GmMATE79*-OE and *GmMATE87*-OE(**g**). Data are represented as means ±SD of three biological replicates. Different letters above columns represented significantly different ($p < 0.05$)

stress (Fig. 6a-f). Consistently, more citrate efflux were found in the *GmMATE79*-OE, *GmMATE75*-OE and *GmMATE87*-OE lines compared with the wild type under Al stress (Fig. 6g).

RT-PCR monitor has shown that *GmMATE75*, *GmMATE79* and *GmMATE87* were separately complementally expressed in Arabidopsis *atmate* mutant (Fig. S3). Root elongation inhibition of 75% was found in the *atmate* mutant or *atmate*-vector line under Al stress (Fig. 7). The Al-induced root elongation inhibition was approximately 62% and 70%, respectively, in the two complementary lines of *GmMATE75* (*GmMATE75*-CE1, *GmMATE75* -CE2) (Fig. 7a, d). Complementary expression of *GmMATE79* decreased the root elongation inhibition to 62% and 69%, respectively, in two Arabidopsis lines (*GmMATE79*-CE1, *GmMATE79*-CE2) (Fig. 7b, e). Root elongation inhibition under complementary expression of *GmMATE87* was 70% and 71% under Al stress (*GmMATE87*-CE1, *GmMATE87*-CE2) (Fig. 7c, f). Thus, each of Gm*MATE75*, *GmMATE79* and *GmMATE87* can partially restore root elongation in the *atmate* mutant under Al stress (Fig. 7). The amounts of citrate efflux from *GmMATE75*-CE, *GmMATE79*-CE and *GmMATE87*-CE lines was also higher than those in the control (Fig.7g).

β-Glucuronidase (GUS) staining

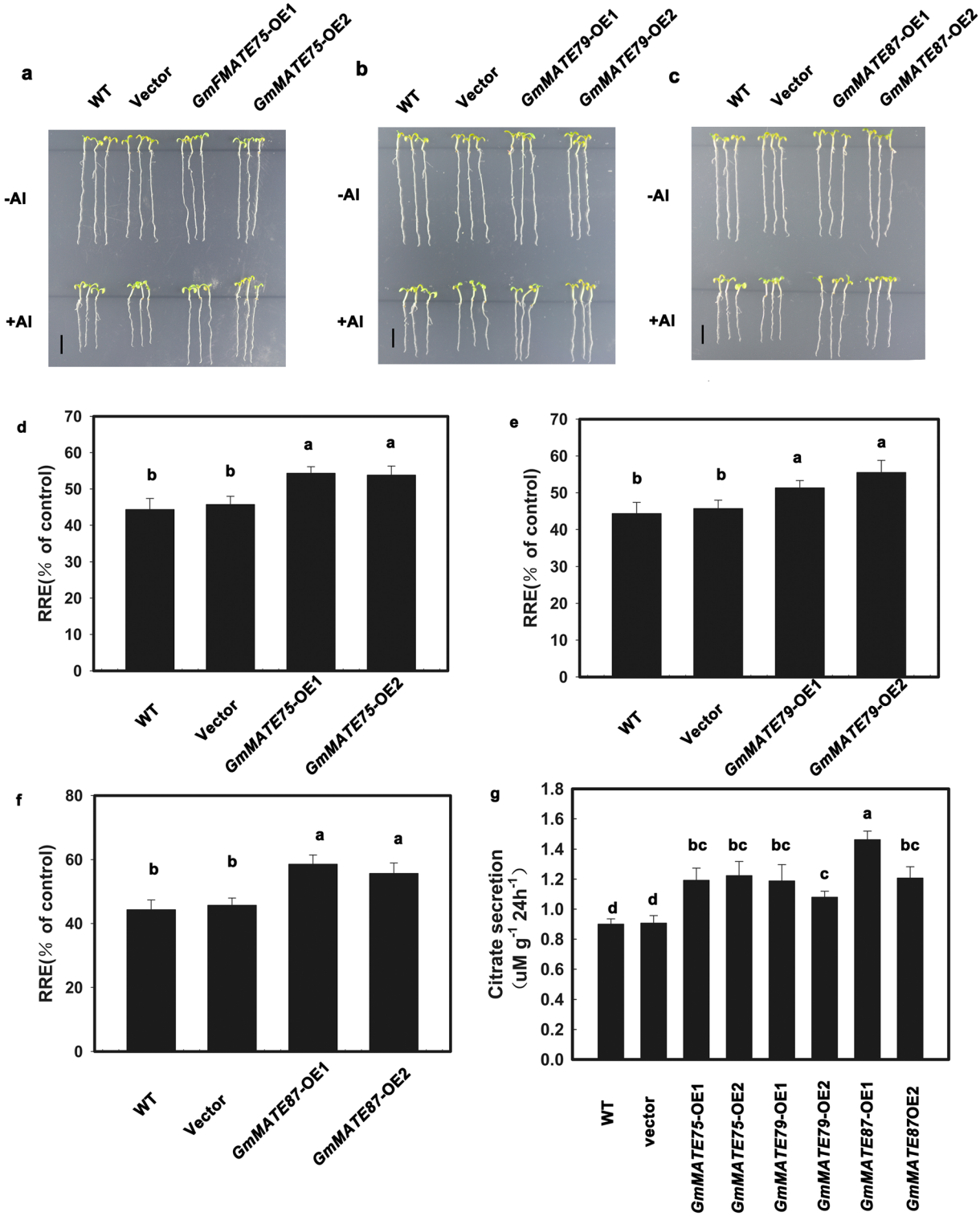
Jiyu 62 exhibited different sequences in the promoter region of three GmMATEs from Jiyu 70. We successfully cloned 1499 bp, 2022 bp, and 2000 bp DNA sequences respectively from the upstream of start condon of *GmMATE75*, *GmMATE79* and *GmMATE87* from Jiyu 70. But we failed to clone those sequences from Jiyu 62. As a transcription factor, GmSTOP1a was found to regulate Al and proton resistance in soybean (Zhou et al. 2018b). Jiyu 70 and Jiyu 62 displayed same *GmSTOP1a* sequence and very similar expression pattern under Al stress (Data not shown). It is easy to induce strong hairy roots from cytocledon of Jiyu 62. Thus, each promoter fragment fused with a *GUS* reporter gene from Jiyu 70 was introduced into Jiyu 62 hairy roots with Agrobacterium transformation. In the absence of Al stress, GUS staining was restricted in the central cylinder zone of root apex for each of *pGmMATE75::GUS*, *pGmMATE79::GUS* and *pGmMATE87::GUS* transformation (Fig. 8a-d, i-l, q-t). After 4 h Al exposure, GUS staining extended from central cylinder to cortical and epidermis cells for *pGmMATE75::GUS* or *pGmMATE79::GUS* transformation (Fig.8e-h, m-p). But for *pGmMATE87::GUS* transformation, Al exposure strengthened the GUS staining, but still restricted within central cylinder region for the three root segments within 0–9 mm root apical zone (Fig. 8u-x).

Discussion

Al-induced citrate efflux has been well-proven as one of the most important Al resistance mechanism in soybean (Yang et al. 2000, 2001; Silva et al. 2001). In this report, GmMATE75, GmMATE79 and GmMATE87 in the MATE family are proposed as plasma-membrane-localized citrate transporters (Fig. 4) conferring Al-induced citrate efflux in soybean (Figs. 5, 6, and 7).The transcript levels of *GmMATEs* displayed almost evenly within root apices and base regions (Fig. 1d-f). GUS staining of transgenic hairy roots indicated that three *GmMATEs* mainly expressed in the central cylinder under –Al stress but the expression of *GmMATE75* and *GmMATE79* was strengthen and expanded to the cortex and epidermal cells in root apical region under Al stress (Fig. 8). The three MATE genes exhibited distinct expression patterns under Al stress (Figs. 1, 2, and 3), which might result from various regulation methods that contribute differently to Al-induced citrate efflux in soybean.

GmMATE75, GmMATE79 and GmMATE87 are all citrate transporters conferring citrate efflux from soybean roots

The 22 reported citrate transporters in the MATE family were conserved between dicots and monocots, but loosely correlated with their known biological functions, such as Al resistance or Fe acquisition (Fig. S1, Fig. S2). GmMATE79, GmMATE75 and GmMATE87 showed the highest similarities to AtMATE (Liu et al. 2009), VuMATE1 (Liu et al. 2013) and LjMATE (Takanashi et al. 2013) respectively (Fig. S2). The former two are Al-activated citrate transporters that conferred Al-induced citrate efflux in Arabidopsis andrice bean, respectively (Liu et al. 2009; Yang et al. 2011), and the latter was a citrate transporter responsible for iron supply to the nodule infection zone of *Lotus japonicus* (Takanashi et al. 2013).


a
WT
Vector
GmFMATE75-OE1
GmMATE75-OE2
-Al
+Al
b
WT
Vector
GmMATE79-OE1
GmMATE79-OE2
-Al
+Al
c
WT
Vector
GmMATE87-OE1
GmMATE87-OE2
-Al
+Al
d
RRE(% of control)
b
b
a
a
WT
Vector
GmMATE75-OE1
GmMATE75-OE2
e
RRE(% of control)
b
b
a
a
WT
Vector
GmMATE79-OE1
GmMATE79-OE2
f
RRE(% of control)
b
b
a
a
WT
Vector
GmMATE87-OE1
GmMATE87-OE2
g
Citrate secretion (uM g-1 24h-1)
d
d
bc
bc
bc
c
a
bc
WT
vector
GmMATE75-OE1
GmMATE75-OE2
GmMATE79-OE1
GmMATE79-OE2
GmMATE87-OE1
GmMATE87OE2

◀ **Fig. 6 Phenotype of Al resistance in transgenic Arabidopsis overexpressing *GmMATE75*, *GmMATE79* or *GmMATE87*.** Phenotype analysis of Arabidopsis Col-4, *GmMATE75*-OE (**a**), *GmMATE79*-OE(**b**) and *GmMATE87*-OE (**c**). Relative root elongation of Arabidopsis Col-4, *GmMATE75*-OE (**d**), *GmMATE79*-OE (**e**), and *GmMATE87*-OE (**f**) under Al stress. Scale bar = 5 mm. Citrate efflux from Arabidopsis Col-4 and transgenic Arabidopsis overexpressing roots of *GmMATE75*-OE, *GmMATE79*-OE and *GmMATE87*-OE (**g**).The gene transformation and treatment procedure was described in Material and Methods. Data are represented as means ±SD (n = 10 for root elongation measurement, n = 3 for the citrate efflux measurement). Different letters above columns represented significantly different ($p < 0.05$)

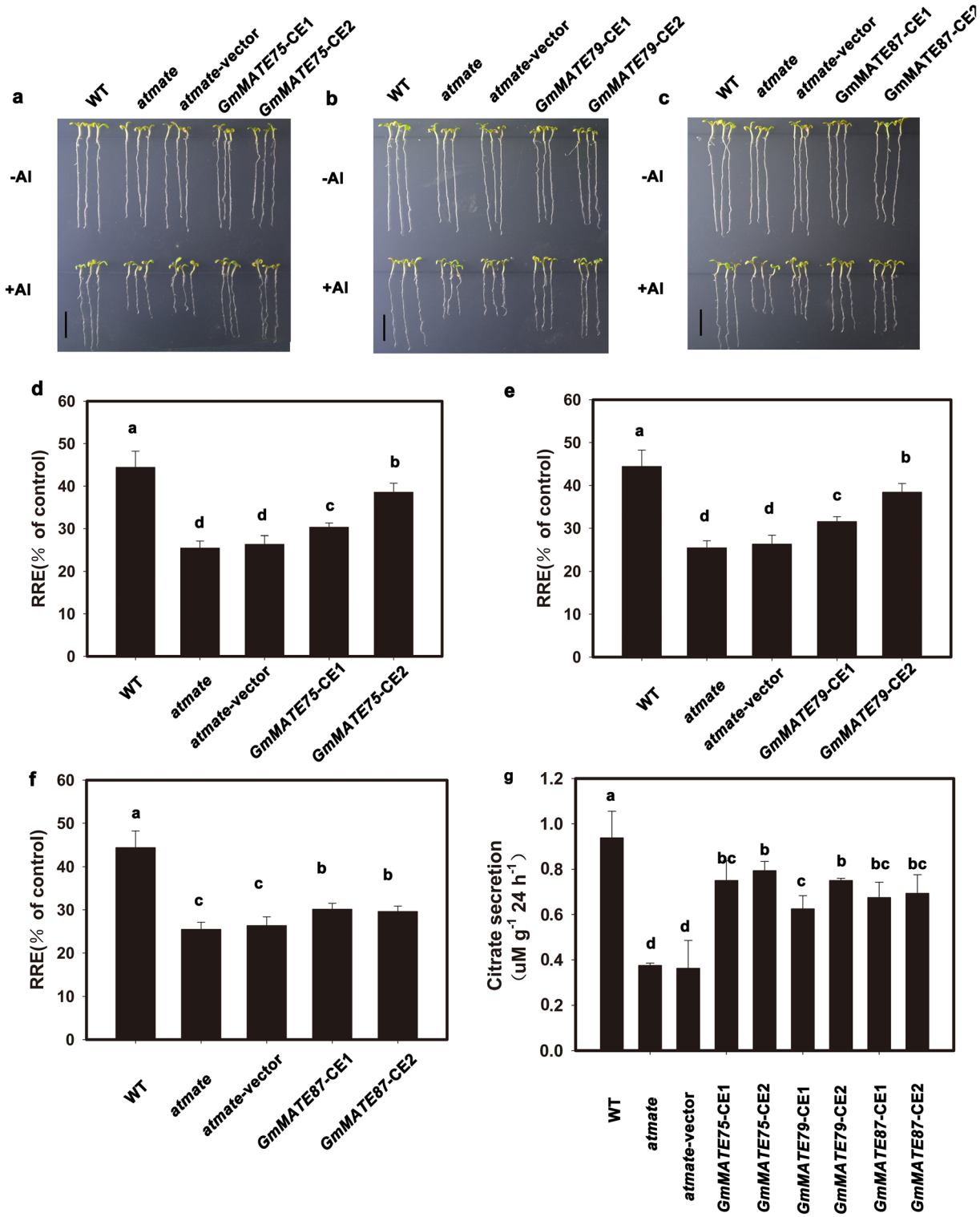
Consistent with the above multiple sequence alignment and phylogenetic tree, subcellular localization analysis in Arabidopsis protoplast verified that GmMATE79, GmMATE75 and GmMATE87 are all localized to the plasma membrane (Fig. 4). CaMV 35S promoter-driven overexpression of *GmMATE75*, *GmMATE79* or *GmMATE87* in soybean hairy roots, Arabidopsis or Arabidopsis mutant all resulted in increased citrate efflux under either –Al or + Al stress (Figs. 5, 6, and 7). Thus, we speculated each of GmMATE79, GmMATE75 or GmMATE87 have the capability to release citrate. The lower Al content in *GmMATE75*-OE, *GmMATE79*-OE or *GmMATE87*-OE soybean hairy roots (Fig. 5g), and higher relative root elongation in Arabidopsis *GmMATE75*-OE, *GmMATE79*-OE, *GmMATE87*-OE (Fig. 6) or *GmMATE75*-CE, *GmMATE79*-CE, *GmMATE87*-CE (Fig. 7) also indicated their acquirement of Al exclusion and Al resistance.

There existed an approximately 4 h lag phase preceding a large amount of citrate efflux and Al exposure in soybean (Yang et al. 2001), which was clearly classified in pattern II (Ma et al. 2001). Citrate exudation was non-detectable by HPLC when soybean was grown in the absence of Al (Silva et al. 2001; Yang et al. 2001). A small amount of citrate efflux from soybean roots can be detected after 2 h or 3 h Al treatment (Silva et al. 2001; Yang et al. 2001). However, in the present study, the more sensitive enzymatic assay detected some citrate efflux from both wild-type and transgenic hairy roots without Al treatment. *GmMATE75*-OE, *GmMATE79*-OE or *GmMATE87*-OE hairy roots each with higher transcript levels of *GmMATEs* (Fig. 5a-c), secreted more citrate without Al treatment compared to the untransformed hairy roots (Fig. 5d-f). *GmMATE75*, *GmMATE79* and *GmMATE87* constitutively contribute to citrate efflux and do not require Al activation, which is similar to *AtFRD3* overexpression in barley but different from *HvAACT1* or *SbMATE* with necessarity of Al to activate citrate efflux (Zhou et al. 2014).

CaMV 35S was used to constitutively express the MATE proteins, thus abrogating the tissue specific localization factor, which has been regarded as critical in determining their biological functions in Fe acquisition or Al detoxification (Yokosho et al. 2011; Maron et al. 2010; Fujii et al. 2012). For example, AtFRD3 functions as a citrate transporter mainly facilitating Fe acquisition (Durrett et al. 2007), but its CaMV 35S -driven overexpression can enhance Al resistance in Arabidopsis (Durrett et al. 2007) and barley (Zhou et al. 2014). VuMATE1 was proven as citrate transporter conferring Al resistance (Fan et al. 2015). But, complementation of *frd3–1* with CaMV 35S mediated *VuMATE1* expression could restore its Fe deficiency phenotype (Liu et al. 2016). Thus, tissue expression or *Cis*-regualtion of citrate transporters seemed to play more important role in determining their biological function.

The expression of *GmMATE75*, *GmMATE79* and *GmMATE87*

Tissue localization of citrate transporter was pivotal for its Al detoxification capability. Typically, Fujii et al. (2012, 2018) indicated that barley accessions in East Asia and Europe have developed independent strategies including 1 kb transponson insertion or 15.3 kb multi-retrotransposon-like sequence insertion to regulate *HvAACT1* spatial expression to adapt to acid soils. qRT-PCR revealed that three *GmMATEs* display similar spatial expression patterns with distinct level in response to Al stress (Fig. 1a-c). They were constitutively expressed in soybean roots with slightly higher expression in the more mature region (2–3 cm) (Fig. 1d-f). Their spatial expression and distribution were different from those of other MATE citrate transporter homologues, such as *SbMATE* (Magalhaes et al. 2007), *HvAACT1* (Fuji et al. 2012) and *OsFRDL4* (Yokosho et al. 2011), whose expression levels were found to be higher at the root apices than that in the mature zones. The *VuMATE1* in rice bean even showed restricted expression in only the root apex zone (Yang et al. 2011). The distinct spatial expression features of the soybean *MATE* genes might relate to the following two factors. (1) Soybean Al accumulation characteristics: In soybean, Al accumulation was detected in all of the root segments with significantly higher Al


a
WT
atmate
atmate-vector
GmMATE75-CE1
GmMATE75-CE2
-Al
+Al
b
WT
atmate
atmate-vector
GmMATE79-CE1
GmMATE79-CE2
-Al
+Al
c
WT
atmate
atmate-vector
GmMATE87-CE1
GmMATE87-CE2
-Al
+Al
d
RRE(% of control)
0
10
20
30
40
50
60
a
d
d
c
b
WT
atmate
atmate-vector
GmMATE75-CE1
GmMATE75-CE2
e
RRE(% of control)
0
10
20
30
40
50
60
a
d
d
c
b
WT
atmate
atmate-vector
GmMATE79-CE1
GmMATE79-CE2
f
RRE(% of control)
0
10
20
30
40
50
60
a
c
c
b
b
WT
atmate
atmate-vector
GmMATE87-CE1
GmMATE87-CE2
g
Citrate secretion
(uM g-1 24 h-1)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
a
d
d
bc
b
c
b
bc
bc
WT
atmate
atmate-vector
GmMATE75-CE1
GmMATE75-CE2
GmMATE79-CE1
GmMATE79-CE2
GmMATE87-CE1
GmMATE87-CE2

◀ **Fig. 7 Phenotype of Al resistance of Arabidopsis *atmate* mutants complementarily expressing *GmMATE75*, *GmMATE79* or *GmMATE87*.** Phenotypic analysis of WT, *atmate*, *GmMATE75*-CE(**a**), *GmMATE79*-CE (**b**) and *GmMATE87*-CE(**c**). Scale bar = 5 mm. Relative root elongation of *atmate*, WT, *GmMATE75*-CE(**d**), *GmMATE79*-CE(**e**) and *GmMATE87*-CE(**f**) under Al stress. The bars represent the means ± SE, $n = 10$. Citrate efflux from Arabidopsis Col-4, Arabidopsis atmate mutants and atmate mutants complementarily expressing of *GmMATE75*-CE, *GmMATE79*-CE and *GmMATE87*-CE (**g**).The gene transformation and treatment procedure was described in Material and Methods. Data are represented as means ±SD ($n = 10$ for root elongation measurement, $n = 3$ for the citrate efflux measurement). Different letters above columns represented significantly different ($p < 0.05$)

concentrations in the 2–3 and 3–4 cm segments (Nian et al. 2004). The hematoxylin-stained region was confined to the mature root zone and extended to include the root apices with increasing Al treatment concentrations (Nian et al. 2004); (2) The three MATE genes might also be required for both characterized and still-uncharacterized functions in the absence of Al stress. Small amounts of citrate and malate efflux were detected from soybean roots under Cu^{2+}, Cd^{2+}, or La^{3+} treatment (Nian et al. 2004; Silva et al. 2001) Consistently, the expression of *GmMATE79* and *GmMATE87* was less specific to Al stress and responded notably to Cu^{2+}, Cd^{2+} or La^{3+} stress (Fig. 2b, c). It has been known that homologues of citrate transporters are involved in either Fe translocation or Al tolerance depending on individual genes (Yokosho et al. 2011; Maron et al. 2010). After 10 days -Fe culture resulted in 2 folds increase of *GmMATE79* (Fig. 3b) and almost 30-folds increase in *GmMATE75* expression (Fig. 3a), implying their putative roles in Fe acquisition. However, the transcript abundance of *GmMATE87*, which clustered more closely with *AtFRD3a* and *LjMATE*, known citrate transporters functioning in Fe acquisition (Durret et al. 2007; Takanashi et al. 2013), was decreased after 10 days of Fe deficiency. GUS-staining observation indicated Al treatment strengthened and extended *pGmMATE75::GUS* or *pGmMATE79::GUS* from central cylinder to outer tissues including cortex and epidermis cells (Fig.8). Considering that citrate need to release to rhizosphere to protect Al injury, *GmMATE75* and *GmMATE79* might be more important in Al detoxifying for soybean. But the capabilities of *GmMATEs* were probably underestimated with GUS staining observation after promoter of Jiyu 70 transformation into Jiyu 62. (1) The regulatory systems of MATE family citrate transporters are complex. STOP1/ART1 type transcription was reported to regulate citrate transporter genes such as *AtMATE* (Liu et al. 2009), *VuMATE1and VuMATE2* (Yang et al. 2011; Fan et al. 2015; Liu et al. 2018) and *OsFRDL4* (Yokosho et al. 2016b). Its regulation was affected by other factors. WRKY22 was found to function together with ART1 in regulation of OsFRDL4 (Li et al. 2018). An F-box protein-encoding gene *regulation of Atalmt1 expression 1* (RAE1) was reported to interact with and promote the degradation of AtSTOP1 via the ubiquitin-26S proteasome pathway (Zhang et al. 2018). Even Jiyu 70 and Jiyu 62 displayed same *GmSTOP1a* sequence and very similar expression pattern under Al stress (Data not shown). We can't ensure the homologies of *GmSTOP1a* or other genes such as *WRKY* or *RAE1* displayed same sequence and expression pattern between Jiyu 70 and Jiyu 62; (2) The capacity of *GmMATEs* regulation also depended on promoter length. Typically, very important regulatory elements including transponson insertion or DNA methylation localized at more than 5000 bp upstream of 5' UTR of HvAACT1 (Fujii et al. 2012, 2018). More effective regulatory elements were expected to reveal in promotor regions of *GmMATEs.*

Fine-tuning root citrate secretion with two separate root citrate transport systems was reported in rice bean to regulate biphasic Al-induced citrate efflux (Liu et al. 2018). The constitutively expressed *VuMATE2* and Al-increased expressed *VuMATE1* were respectively suggested to be responsible for the early phase of minor citrate secretion and late phase of large amount citrate efflux under Al stress (Liu et al. 2018). In present study, the rapid and strong induction of *GmMATE75* is similar to or even greater than that of *OsFRDL4* (Yokosho et al. 2011). Its expression was greatly induced at 2 h Al treatment (near 100-fold) and reached approximately 500-fold at 8 h (Fig. 1a). *GmMATE79* (Fig. 1b) was also induced after 2 h Al treatment with lower level as *AtMATE* (Liu et al. 2009), *ZmMATE1* (Maron et al. 2010) and *ScFRDL2* (Yokosho et al. 2010), but decreased its expression in the following Al treatment duration. According to their expression patterns, we propose as follows: the constitutive expression and smaller increase of *GmMATE79* and *GmMATE75* expression resulted in minor citrate efflux from soybean roots under no Al or less than 4 h Al treatment. However, the large amount of Al-induced citrate efflux after 4 h might result from the strong induction of *GmMATE75*. The higher expression of *GmMATE75* in

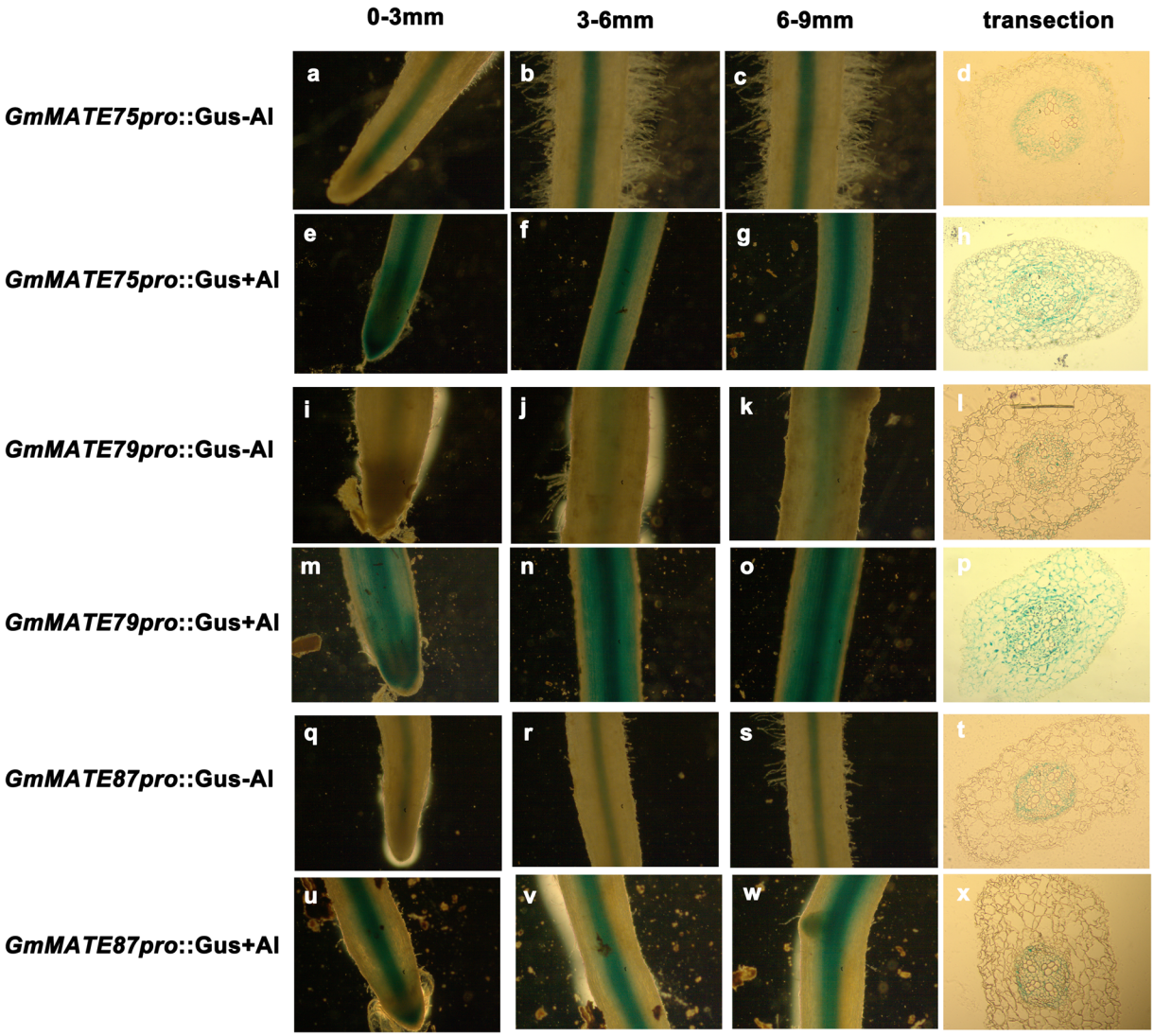


Fig. 8 Tissue-level localization of *GmMATE75*, *GmMATE79* and *GmMATE87* expression via promoter-Gus fusion. Transgenic soybean hairy roots were exposed to 0.5 mM $CaCl_2$ solution with or without 30 μM $AlCl_3$ for 4 h. Activation of the *GmMATEs* promoter was observed by Gus staining in intact root (0–3 mm, 3–6 mm and 6–9 mm) or its cross section slices (within 0–9 mm). **a-d** showed promoter of *GmMATE75* under no Al stress. **e-h** showed promoter of *GmMATE75* under stress. **i-l** showed promoter of *GmMATE79* under no Al stress. **m-p** showed promoter of *GmMATE79* under Al stress. **q-t** showed promoter of *GmMATE87*under no Al stress. **u-x** showed promoter of *GmMATE87* under Al stress

Jiyu70 (Fig. 1) could also partially explain the genotypic difference that Jiyu 70 secreted more citrate than Jiyu 62 after 8 h Al treatment (Zhou et al. 2018a).

The origin of the Al-induced citrate efflux pattern in soybean roots is complex. (1) The contribution of citrate metabolism to citrate efflux can not be neglected. Our previous study has shown that cytosolic malic enzyme (GmME1) contributed to internal citrate content then Al-induced citrate efflux from soybean (Zhou et al. 2018a). *GmME1* displayed higher expression in Jiyu70 in comparison to Jiyu62 under Al stress (Zhou et al. 2018a) (2) The contribution of the other putative soybean citrate transporters has not been clarified; (3) More effective regulatory elements might exist in 5'UTR-uptream of *GmMATEs* to promote the high expression of *GmMATEs* under Al stress. Jiyu 70 was bred from Jilin province of China with less acidic soils and exhibited moderate Al resistance. Efficient regulation of *GmMATEs* mechanisms deserved to be revealed in soybean cultivars with higher Al resistance capability. The genetics studies have

revealed the complexity of Al-induced citrate efflux mechanisms in soybean. Some quantitative trait loci analyses have indicated that Al tolerance in soybean is a multigene trait (Abdelhaleem et al. 2014; Sharma et al. 2010; Qi et al. 2008). Using recombinant inbred lines (RILs) derived from the cross of 'Essex' with 'Forrest' identified two major loci encompassing genes implicated in citrate metabolism (Sharma et al. 2010). The population originating from Young × PI 416937 identified sixteen single nucleotide polymorphisms in the citrate synthase homologue on chromosome Gm08 (Abdelhaleem et al. 2014). However, no genetic report has stated the loci or genes conferring citrate transport implicated in Al resistance in soybean until now.

In summary, GmMATE75, GmMATE79 and GmMATE87 are proposed as plasma-membrane-localized citrate transporters, having capability to release citrate efflux under –Al or + Al stress. Their additive effects might contribute to Al-induced citrate efflux from soybean. Their expression levels were also affected under iron deficiency conditions, but their functions in iron acquisition are not discussed here.

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