ZmMATE6 From Maize Encodes a Citrate Transporter That Enhances Aluminum Tolerance in Transgenic Arabidopsis Thaliana

Hanmei Du
Sichuan Agricultural University
Peter Ryan
Commonwealth Scientific and Industrial Research Organisation
Chan Liu
Sichuan Agricultural University
Hongjie Li
Sichuan Agricultural University
Wanpeng Hu
Sichuan Agricultural University
Weina Yan
Sichuan Agricultural University
Ying Huang
Sichuan Agricultural University
Wenzhu He
Sichuan Academy of Agricultural Sciences
Bowen Luo
Sichuan Agricultural University
Xiao Zhang
Sichuan Agricultural University
Shibin Gao
Sichuan Agricultural University
Shufeng Zhou
Sichuan Agricultural University
Suzhi Zhang (suzhi1026@163.com)
Key Laboratory of Biology and Genetic Improvement of Maize in Southwest China of Agricultural Department, Ministry of Agriculture, Maize Research Institute, Sichuan Agricultural University, Wenjiang 611130, China

Research Article

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Abstract

Background: The yields of cereal crops grown on acidic soils are often reduced by aluminum (Al) toxicity because the prevalence of toxic Al^{3+}cations increases as pH falls below 5.0. The Al-activated release from the roots of simple organic acids such as malate and citrate confers resistance to Al in a many plant species including maize (*Zea mays* L.). The Al-dependent release of citrate from resistant lines of maize is controlled by *ZmMATE1* which encodes a multidrug and toxic compound extrusion (MATE) transporter protein. *ZmMATE1* resides on chromosome 6 and its expression in roots is induced up to four-fold by Al treatment. *ZmMATE6* is another member of this family in maize whose expression is also increased by Al treatment. We investigated the function of this gene in more detail to determine whether it also contributes to Al resistance.

Results: Quantitative RT-PCR measurements found that in the absence of Al *ZmMATE6* was expressed in the roots and leaves of Al-resistant and sensitive in-bred lines, and that expression levels in the root tips were lower than in the rest of the root tissue. Treatment with Al induced *ZmMATE6* expression in all tissues but several other divalent or trivalent cations tested had no effect on expression. This expression pattern and the induction by Al treatment was confirmed in Arabidopsis lines transformed with β-glucuronidase where the *ZmMATE6* promoter was used to drive expression. While subcellular localization of the ZmMATE6 protein could not be determined with confidence transgenic Arabidopsis lines expressing *ZmMATE6* displayed a greater Al-activated release of citrate from the roots and were significantly more resistant to Al toxicity than controls. This was associated with reduced accumulation of Al in the root tissues.

Conclusions: Our results demonstrated that *ZmMATE6* expression is induced by Al and functions as a citrate transporter. While some findings are consistent with *ZmMATE6* contributing to Al resistance in maize, further research is required to confirm this result.

Background

Approximately 30 % of arable soil and 50% of the potential farmland globally has a pH of 5.0 or less [1]. These acid soils present many challenges to crop growth but the most important is aluminum (Al) stress. The highly toxic Al^{3+} cations becomes more prevalent in the soil solution at low pH and can inhibit root elongation at low concentrations [2–4]. Smaller root systems reduce the uptake of water and nutrients and increase the risk of lodging which comprise yields [5, 6].

Some plant species and even genotypes within species are able to resist Al stress better than others. Many mechanisms have been proposed to account for this resistance including those that exclude Al from the root tissues and others that sequester or otherwise detoxify any Al that is taken up by the roots [7, 8]. An important mechanism of Al exclusion that occurs in a wide range of species relies on the release of low molecular weight organic anions from the roots, especially malate and citrate [9–12]. The Al-dependent release of these anions protects the roots, and especially the root tips, by binding with the Al^{3+} cations in the apoplast to form complexes that are less harmful and less easily absorbed by the roots [13, 14]. The Al-dependent release of organic anions from roots can be divided into two types of responses depending on how rapidly after the release begins to occur after Al is added. In some species the release is rapid (Type I pattern) because Al interacts with transporter proteins in the plasma membrane of the root cells that are expressed constitutively. In other species, organic anion release increases gradually over several hours (Type II pattern) because Al first induces the expression of the genes and proteins encoding the transporters. Type I responses have been described in wheat (*Triticum aestivum* L.) [14], buckwheat (*Fygopyrum esculentum* Moench) [15] and rape (*Brassica napus*) [16] while Type II responses have been described in rye (*Secale cereale* L.) [17], *Arabidopsis* [18], rice bean (*Vigna umbellata*) [19] and common bean [20]. Since these two responses are independent processes they can occur together. For instance, rye displays a Type I pattern for malate release and a Type II pattern for citrate release [17, 21].

The Al-dependent release of malate is controlled by members of the aluminum-activated malate transporter (ALMT) gene family which encode anion channels in the plasma membrane of root cells [22]. While ALMTs have been shown to contribute to Al resistance in a wide range of species [11, 23] most members of this family perform other functions unrelated to Al resistance [24]. The Al-dependent release of citrate, by contrast, is mediated by members of the multidrug and toxic compound extrusion (MATE) family of transporter proteins. This family is widely distributed in prokaryotes and eukaryotes [25] and forms a very large family in plants. MATEs transport a diverse range of compounds and function in the export of secondary metabolites and toxins, development, nutrition and stress responses [26–29] .The SbMATE transporter from sorghum (*Sorghum bicolor* L.) and HvAACT1 (HvMATE) transporter from barley (*Hordeum vulgare* L.) were the first MATEs linked with Al resistance [30–32], but others perform similar roles in many other species [19, 33–40].

Al tolerance in maize (*Zea mays* L.) is a quantitative trait [41–43] with at least five QTLs on chromosomes 2, 6 and 8 in one biparental population examined [44]. The *ZmMATE1* gene co-locates with a resistance QTL on chromosome 6 and explains ~ 16 % of the phenotypic variation in resistance [33]. *ZmMATE1* encodes plasma membrane-localized protein that mediates the Al-dependent release of citrate from cells and shares significant amino acid identity with SbMATE (52%) from sorghum and AtMATE (64%) from Arabidopsis [33]. *ZmMATE1* expression was induced by Al, particularly in the root tips, and Al-tolerant lines show a greater increase in expression than Al-sensitive lines due to a higher copy number of the *ZmMATE1* gene [45].

The maize genome contains ~ 49 *MATE* genes which separate into seven phylogenetic categories (I-VII) [46]. Only a few of these genes have been examined in detail but researchers have performed transcript analyses and showed that the expression of several *MATE* genes, in addition to *ZmMATE1*, was responsiveness to Al treatment [46]. Among these responsive genes *ZmMATE6* (*GRMZM2G080450*) (denoted as *ZmMATE23* in Zhu et al [46]) was rapidly induced by three-fold following Al treatment. This study provides the first functional analysis of *ZmMATE6* and begins to investigate whether it could also be contributing to Al resistance in maize in the same way as *ZmMATE1*.

Results

Cloning and molecular characteristics of the ZmMATE6 sequence

The complete coding sequences of *ZmMATE6* was amplified and sequenced from the Al-resistant inbred line 178 using gene-specific PCR primers (**Fig. S1**). The gene is 1596 bp long with 13 exons and 12 introns (Fig. 1a) and encodes a protein of 531 amino acids. Six residues were different between lines 178 and B73 (**Fig. S2**). The protein has an estimated molecular mass of 55.96 kDa and pI 9.59 and likely to contain 11 transmembrane spanning regions (Table 1). A multiple sequence alignment found that ZmMATE6 shared more than 30% identity at the amino acid level with ZmMATE1, SbMATE, HvAACT1 and OsFRDL4 (Fig. 1b). The 50 amino acid domain between A114 to I163 forms the citrate exuding motif (CEM) and the smaller region between P150 and I163 is a cytoplasmic loop (Fig. 1). Both of these domains have been linked with the subset of MATE proteins involved with citrate transport [27, 47].

Table 1
Predictions for the number of transmembrane regions (TMR) in ZmMATE6 and its subcellular localization.

Predictions for the transmembrane regions of ZmMATE6		
Program	**TMRs**	**Online linkage**
TMHMM	9	http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?jobid=5F686608000011CF37D23A6D&wait=20
TCDB	11	http://www.tcdb.org/progs/TMS.php
TMpred	11	https://embnet.vital-it.ch/cgi-bin/TMPRED_form_parser
SOSUI	11	http://harrier.nagahama-i-bio.ac.jp/sosui/cgi-bin/adv_sosui.cgi
HMMTOP	11	http://www.enzim.hu/hmmtop/server/hmmtop.cgi
Predictions for the subcellular location of ZmMATE6		
Program	**Location**	**Online linkage**
YLoc	Chlorop	https://abi-services.informatik.uni-tuebingen.de/yloc/webloc.cgi
Cell-PLoc2.0	PM	http://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi
WoLFPSORT	Chlorop, PM	https://www.genscript.com/tools/wolf-psort/detail?file=2020/09/06/htdocs/results/159944442328764.detailed1.html#159944442328764
BUSCA	Mitochon	http://busca.biocomp.unibo.it/33ebe09c-e5fd-48e8-8bda-140d40b3d1cc/showresult/

A phylogenetic analysis was performed to compare *ZmMATE6* with a range of other *MATE* genes including some that are known to transport citrate and linked with either aluminum resistance or iron nutrition (**Table S2**). Figure 2 shows that ZmMATE6 is included in the major clade that contains all members possessing the CEM domain, including ZmMATE1. Interestingly, within this larger clade, ZmMATE6 and another protein from rye, ScMATE3, separate early from the main cluster and become distinct lineages.

Distribution of pattern of ZmMATE6 expression in maize

A previous genome wide analysis showed that *ZmMATE6* expression was induced in roots by Al treatment [46]. To confirm this result *ZmMATE6* expression was first measured in the root tips of the Al-resistant inbred line 178 using quantitative RT-PCR. In the control treatment (-Al), *ZmMATE6* expression was low but detectable and was increased by $AlCl_3$ treatment (pH 4.2) (Fig. 3a). Expression was induced up to 20 μM Al but then saturated at higher concentrations at levels ~ 2.5-fold greater than controls.

We then investigated whether this response was specific for the Al^{3+} cation or whether different forms of Al or other cations can also induce *ZmMATE6* expression in the same way. Al ions hydrolyse in solution and the molar fraction of several different soluble ions change as pH changes. Below pH 4.5, most Al exists as Al^{3+} which is largely responsible for the toxicity to plants in acidic soils. At values above pH 4.5, the molar fraction of Al^{3+} declines rapidly and the $AlOH^{2+}$ and $Al(OH)_2^+$ species become more prevalent [48]. We measured *ZmMATE6* expression in the root tips of plants treated with 60 μM $AlCl_3$ at pH 4.2 and pH 5.6. At pH 5.6, no induction of *ZmMATE6* expression was detected whereas at pH 4.2 treatment with 60 μM $AlCl_3$ significantly induced expression by 50% and 60 μM $AlCl_3$ induced expression by 350% (Fig. 3b). These results indicate that Al^{3+} could induce *ZmMATE6* expression but $AlOH^{2+}$ and $Al(OH)_2^+$ could not. We then tested whether a range of other divalent and trivalent cations including cadmium (Cd), lanthanum (La), zinc (Zn),

copper (Cu), manganese (Mn) or iron (Fe) could induce expression. None of these cations were able to induce *ZmMATE6* expression in the same way as Al^{3+}.

The time-dependence of *ZmMATE6* expression in Al-resistant and Al-sensitive genotype was measured through time in different plant tissues. The Al-resistant line 178 and the Al-sensitive line B73 were treated with 60 μM $AlCl_3$ (pH 4.2) and expression was measured through time in the root tips (RT, apical 10 mm), the rest of roots (ROR) and the leaves (L). *ZmMATE6* expression was detected in the roots and leaves of both genotypes and Al treatment steadily increased expression levels over approximately 12 h before declining again (Fig. 4a and 4b). While some variation in expression was detected between different tissues through time, we conclude that the expression in the Al-resistant and sensitive maize lines generally displayed similar patterns.

Examining ZmMATE6 expression with promoter–GUS fusions

To confirm these expression patterns, a 1.5 kb gDNA sequence upstream of the *ZmMATE6* start codon was isolated and fused with the *GUS* reporter gene in an expression plasmid. The construct was transformed into *Arabidopsis thaliana* using the *Agrobacterium*-mediated method. Figures 5a–5c show that, in the absence of Al stress, GUS staining was low but detectable in the root tips, mature roots and leaves. After exposure to 60 μM $AlCl_3$ (pH 4.5) for 9 h, GUS staining clearly increased in all tissues with stronger staining occurring in the mature root tissue than the root tips (Fig. 5d-5f). These results confirm the qRT-PCR results and suggest that the 1.5 kb region of sequence upstream of *ZmMATE6* contains all the promoter information required to mimic the expression patterns observed in maize plants.

Subcellular localization of ZmMATE6

The ZmMATE6 protein is predicted to have multiple transmembrane regions and therefore is likely to localize to a membrane. Several predictive algorithms were utilized to determine the sub-cellular localization of the protein but the results were inconsistent. Some algorithms predicted localization of ZmMATE6 to the plasma membrane while others targeted the mitochondria or chloroplast (Table 1). In an attempt to determine the subcellular localization experimentally, we fused the *GFP* reporter gene to the 3'-terminus of the *ZmMATE6* coding region and ligated it into the pCAMBIA2300 plasmid with the CaMV*35S* promoter to drive expression (35S:*ZmMATE6*::*GFP*). A control construct consisted of CaMV*35S* promoter driving expression of GFP alone (pCAMBIA2300-*eGFP*). Both constructs were transiently expressed in *Nicotiana benthamiana* leaves and protoplasts prepared from maize leaves. Fluorescence signals showed that the soluble GFP control localized to the cytoplasm and the nucleus in both cell types as expected (**Fig. S3**). Fluorescence from 35S:*ZmMATE6:GFP* constructs, however, was again inconclusive. Fluorescent signals were detected in the periphery of the tobacco cells and maize protoplasts and on some internal structures. These signals were not typical of the soluble GFP control and they could not be clearly linked with another membrane or organelle (**Fig. S3**). Neither transient expression system could confidently determine the sub-cellular localization of ZmMATE6 protein in these experiments.

Expression of ZmMATE6 in Arabidopsis increases citrate release and Al resistance

To investigate the function of *ZmMATE6*, the coding region was expressed in the *Arabidopsis thaliana* Columbia-0 (Col-0) using the CaMV*35S* promoter (**Fig. S4**). Seven independent T_0 plants were selected and presence of the transgene was confirmed using PCR (Fig. 6a). The *ZmMATE6* expression in leaf of these lines was four to 12-fold greater than the wild type control (Fig. 6b). Three T_0 lines (#1, #4 and #6) were selected to generate homozygous T_3 lines for further experiments.

MATE genes from other species whose expression is induced by Al often function as citrate transporters (see Introduction). We therefore measured the release of organic acids from the roots of 14 d old transgenic *Arabidopsis*

seedlings with and without Al treatment. In the control treatment (0.5 mM $CaCl_2$, pH 4.5) only malate and citrate were detected. Malate release was 0.02 pmol plant^{-1}12h^{-1} for all genotypes (data not shown) and citrate release was 0.3 nmol plant^{-1}12h^{-1} for all genotypes (Fig. 6c). When plants were treated with 60 μM $AlCl_3$ (pH 4.5) malate release remained unchanged whereas citrate release was significantly increased in all genotypes but the increases were significantly larger in the transgenic lines than wild-type. Wild-type plants increased citrate release by four-fold to 1.3 nmol plant^{-1}12 h^{-1} whereas the two transgenic lines tested increased release by ten-fold to approximately 2.8 nmol plant^{-1}12 h^{-1} (Fig. 6c).

To determine whether the increase in citrate release affected Al resistance of the transgenic *Arabidopsis*, seedlings were transferred to plates containing 0 or 100 μM $AlCl_3$ and grown for a further four days (Fig. 7a). Root growth of the wild-type and transgenic *Arabidopsis* lines was similar in the absence of Al. In the presence of 100 μM Al, net root growth was inhibited in all genotypes but the inhibition was significantly greater in the wild-type control than the transgenic lines (Fig. 7a, b). Relative root elongation (RRE) was less than 10% for wild-type plants compared to 46%, 58% and 86% for the three transgenic lines (Fig. 7c).

We tested whether the enhanced resistance to Al toxicity observed in the transgenic lines was associated with a reduction accumulation of Al in the roots and shoots. The first experiment estimated Al uptake by staining roots with hematoxylin. This compound turns a blue-purple colour when it chelates Al, so a darker stain indicates more Al is present in the tissue. Seedlings were treated with 0 or 60 μM $AlCl_3$ for 12 h and the roots stained with hematoxylin (Fig. 8a). In control solution, the staining was very faint but it became darker in the root tips and mature roots after treatment with Al. The staining was less intense in the three transgenic lines than the wild-type plants, especially at the root tips (Fig. 8a). These results suggest that less Al accumulates in the roots of the transgenic lines than the wild-type controls. We quantified these differences by measuring the Al concentration in the roots before and after Al treatment using ICP-MS. In the control treatment, the transgenic lines and wild-type plants contained similar concentrations of Al of approximately 2.5 mg gDW^{-1} (Fig. 8b). These concentrations increased in all lines after exposure to Al but the transgenic lines accumulated less than 30% of the Al that the wild-type plants accumulated. Al content in leaves was similar in all lines and did not change with Al treatment (**Fig. S5**). These results suggest that overexpression of *ZmMATE6* increased Al resistance by reducing Al accumulation in the roots.

Discussion

This study investigated the function of *ZmMATE6* in maize. We established that *ZmMATE6* is widely expressed in maize tissues including in the root tips, mature roots and leaves. Expression was induced by Al^{3+} but not by other cations tested. The distribution of expression and the induction by Al^{3+} were confirmed using transgenic *Arabidopsis* plants expressing GUS using the *ZmMATE6* promoter. Induction peaked after 12 h of Al treatment which differs from Zhu et al [46] who found maximum expression after 6 h. This difference is likely related to experimental conditions since Zhu et al [46] used a more severe treatment consisting of 222 μM $AlK(SO_4)_2$ at pH 4.0. The finding that *ZmMATE6* is widely expressed throughout the plant, and not just at the root tips, does not preclude it from being involved in Al resistance. Several *MATEs* that confer Al resistance in other species are expressed throughout the roots and even in the leaves. Examples of these include *ZmMATE1* in maize [33], *HvAACT1* in barley [49], *TaMATE1B* in wheat [37], *BoMATE* in *Brassica oleracea* [38] and *OsFRDL2* in rice [50].

The subcellular location of ZmMATE6 could not be established with confidence in this study despite expressing GFP-tagged proteins in two experimental systems. Even the online algorithms were inconsistent in their predictions which may suggest ZmMATE6 localizes to several different membranes. Nevertheless, *Arabidopsis* plants expressing *ZmMATE6* under the CaMV*35S* promoter showed significantly greater citrate release which suggests some protein is

localizing to the plasma membrane. Citrate release from these transgenic lines was low in control conditions (-Al) and similar to wild-type plants. Al treatment enhanced citrate release from the transgenic and control plants but the magnitude of increase from the transgenic lines was two-fold greater than from wild-type plants. By contrast, malate release was not affected by Al treatments which demonstrates that citrate exudation was a regulated transport process and not a result of general leakage from damaged tissues.

The finding that ZmMATE6 transports citrate is consistent with it possessing the citrate exuding motif (CEM) and a specific region forming a cytoplastic loop because both domains have been associated with the subset of MATE proteins that function as citrate cotransporters [27, 47]. The fifth aspartate (D) residue in the CEM plays an important role in organic cation transport activity because the transport function was lost when this residue was substituted for an asparagine in SbMATE [27, 51]. Interestingly, the fifth residue in the CEM domain of ZmMATE6 is a glutamate (E) which indicates that this more conservation substitution (aspartate and glutamateare both anionic residues) does not inhibit function. Phylogenetic analysis placed ZmMATE6 in the large cluster with other MATEs known to confer Al resistance (e.g. SbMATE, HvAACT1, TaMATE1B and OsFRDL4) but ZmMATE6 was soon separated from the others and placed on a branch by itself. This suggests that ZmMATE6 has a different evolutionary lineage and perhaps additional functions from the others.

The finding that Al treatment increased citrate release from the transgenic *Arabidopsis* lines constitutively expressing *ZmMATE6* indicates that Al not only induces *ZmMATE6* expression in maize but also triggers the activity of the ZmMATE6 transporter. In this respect, ZmMATE6 shows similarities with SbMATE in sorghum because Al induces the expression of *SbMATE* and activates the transport function of that protein [30, 52]. ZmMATE6 differs from ZmMATE1 [33] and VuMATE in rice bean [19] because, although Al induces the expression of their genes, it is not required to activate protein function. ZmMATE6 also differs from TaMATE1B in wheat which is neither induced nor activated by Al [37, 53]. We should be mindful that determining whether Al induces *MATE* expression and/or activates protein function cannot be done *in situ* because the two processes cannot be distinguished. Instead, the genes need to be expressed in a heterologous system like *Xenoupus* oocytes or other model species like *Arabidopsis* using a constitutive promoter and then the effect of Al treatment assessed. Therefore, it is possible that the function of these transporters in other cell types do not reflect their function *in situ* due to differences in post-translational modifications, missing cofactors or signaling pathways [54].

Some results presented here support a role for *ZmMATE6* in the Al resistance of maize. These include the findings that *ZmMATE6* is induced by Al treatment and that the ZmMATE6 protein is activated by Al to transport citrate out of cells. Additionally, transgenic plants expressing *ZmMATE6* accumulate less Al in their roots and are more resistance to Al stress. Other results are not consistent with *ZmMATE6* performing a major role in Al resistance. *ZmMATE6* is located on chromosome 3 and therefore is not associated with the five QTL identified by Ninamango-Cárdenas [44]. However, *ZmMATE6* could still have linked been with Al resistance if it represented an additional resistance gene that was not segregating in the biparental population examined by Ninamango-Cárdenas [44]. The parents in that population were the Al-sensitive inbred line L53 and the Al-resistant inbred line L1327 and Al resistance phenotype linked with *ZmMATE6* (if it is a resistance gene) would not be detected if neither parent possessed the resistant allele for *ZmMATE6* or if both parents possessed the same allele of *ZmMATE6*. Another result that is initially inconsistent with *ZmMATE6* contributing to resistance was the finding that *ZmMATE6* expression was induced by Al in an Al-resistant inbred line 178, and an Al-sensitive inbred line B73, in a generally similar pattern. However, since the predicted amino acid sequences of ZmMATE6 in 178 and B73 differ by six residues it is possible that ZmMATE6 functions differently in these two lines, such that it is less effective at releasing citrate in B73. This question will be resolved in future studies by expressing the *ZmMATE6* gene from B73 and other sensitive and resistant lines in a heterologous expression system and comparing their transport functions directly. It is also important to determine whether other major resistance mechanisms are operating in these lines and obscuring more subtle contributions from *ZmMATE6*. This could be achieved by generating a segregating

population between 178 and B73 and assessing whether Al resistance can be linked with *ZmMATE6* on chromosome 3. Those results will help determine whether or not *ZmMATE6* contributes to the baseline level of Al resistance or maize or even accounts for some of the genotypic variation in this trait.

Conclusion

ZmMATE6 is another *MATE* gene which is involved in Al-induced secretion of citrate from the roots after *ZmMATE1*. Overexpression of *ZmMATE6* enhances the resistance to Al toxicity in transgenic *Arabidopsis* plants by mediating root citrate efflux.

Materials And Methods

Plant materials and culture conditions

The Al-tolerant maize inbred line 178 and the Al-sensitive inbred line B73 were used in this study [55]. Seedling cultivation were carried out as described [56].

For measuring *ZmMATE6* expression during different cation treatments, the seedlings of 178 with two leaves were subjected to the nutrient solution (pH 4.2) with 30 μM $CdCl_2$, 2.0 μM $CuCl_2$, 10 μM $LaCl_3$, 100 μM $ZnCl_2$, 200 μM $MnSO_4$, 20 μM $FeCl_3$ or 60 μM $AlCl_3$ for 6 h. For the time-course treatment, seedlings were subjected to 60 μM $AlCl_3$ (pH 4.2) for 0, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. For the Al concentration dependence experiment, seedlings were treated to 0, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM $AlCl_3$ (pH 4.2) for 6 h. The roots or/and the shoots from all the treated seedlings were immediately frozen in liquid nitrogen, and stored at -80 ^oC for RNA extraction.

For root growth assays of the transgenic *Arabidopsis thaliana* (Columbia ecotype, Col-0), sterilized seeds were first grown on 1/2 MS medium (pH 4.5), after the roots grew to about 1 cm, the seedlings were transplanted into 1/8 MS medium plates (pH 4.5) with or without 100 μM $AlCl_3$.

Gene cloning and sequencing

Based on the reference sequence of B73 cDNA, the *ZmMATE6* gene was amplified by specific primers (**Table S1**). The multiple sequence alignment of previously reported MATEs from other plants amino acid sequences was performed by DNAMAN software. The phylogenetic analysis was inferred using MEGA 6 by the Neighbor-Joining (NJ) method with a bootstrap of 1000 replicates [57].

RNA extraction and quantitative real-time RT-PCR

Total RNA extraction and quantitative RT-PCR were performed as described before [56]. The primers were listed in **Table S1**. *ZmGAPDH* and *AtACT2* were used as the reference gene. The relative expression level was calculated with the $2^{-\triangle\triangle CT}$ method [58]. Three biological replicate samples were generated, and each sample was tested at least three technical replicates.

Subcellular localization

ZmMATE6 sequence without terminator was fused to pCAMBIA2300 to establish the 35S:*ZmMATE6*::*GFP* vector. The subcellular localization was determined via transient expression of translation fusions with GFP in tobacco leaves and maize leaf protoplasts. Details were performed as the previous study [33].

Expression of ZmMATE6 **in** Arabidopsis

The p35S:*ZmMATE6* vector was transformed into *Arabidopsis* ecotype Columbia-0 (Col-0) via floral dip method mediated by *Agrobacterium tumefacien* strain *EHA105* [59]. The positive homozygous seeds of T_3 generation were used for follow experiments.

Isolation of the ZmMATE6 **promoter and GUS staining**

The 1.5 kb promoter of *ZmMATE6* was amplified from the genomic DNA of 178 and inserted into the vector of pCAMBIA1305. The constructed *ZmMATE6p*::GUS vector was transformed into *Arabidopsis* as described above [59]. Finally, positive homozygous T_3 seedlings were selected for 2.7 *GUS staining*

GUS staining was performed according to the previous study with a little modifications [60] after exposure to 60 μM $AlCl_3$ at pH 4.5.

Hematoxylin staining to assess Al accumulation in roots

The hematoxylin method [61] was used in this study to compare the Al accumulation in transgenic *Arabidopsis* plants and control plants after Al treatment for 12 h.

Quantitative determination of Al content

Two-week-old WT plants and transgenic lines grown in 2% MGRL solution (pH 4.5) were pre-treatment with 0.5 mM $CaCl_2$ for 30 min and then treated with or without 60 μM $AlCl_3$ for 12 h. Root and shoot samples were collected, separately. Al concentrations were measured by ICP-MS (PerkinElmer, NexION 2000).

Detection of organic acid exudation from roots

Root exudates were collected after exposing the plants to the 0.5 mM $CaCl_2$ (pH 4.5) and 0.5 mM $CaCl_2$ with 60 μM $AlCl_3$ (pH 4.5) for 12 h. Organic acid exudates were measured by Thermo Fisher U3000 HPLC (Syncronis C18, Dim 250 mm × 4.6 mm, 5 μm) utilizing a mobile phase of 0.05 M potassium dihydrogen orthophosphate, buffered to pH 2.68 with orthophosphoric aicd, and a UV detector at 210 nm.

Statistical analysis and reproducibility

All experiments included at least three samples to confirm reproducibility. And all treatments were repeated 2 or 3 times. Statistical analysis including student's *t* test was performed using the SPSS software.

Abbreviations

Al
Aluminum
MATE
Multidrug and toxic compound extrusion
PCR
Polymerase chain reaction
B73 and 178
Two maize inbred lines
CEM
The citrate exuding motif
#1/#4/#6
Overexpression transgenic lines of *ZmMATE6*
RRE

Relative root elongation
WT
Wide type of *Arabidopsis* (Col-0)

Declarations

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Author information

Affiliations

Key Laboratory of Biology and Genetic Improvement of Maize in Southwest China of Agricultural Department, Ministry of Agriculture, Maize Research Institute, Sichuan Agricultural University, Wenjiang 611130, China

Han-mei Du, Chan Liu, Hong-jie Li, Wan-peng Hu, Wei-na Yan, Ying Huang, Bo-wen Luo, Xiao Zhang, Shi-bin Gao, Shu-feng Zhou & Su-zhi Zhang

CSIRO Agriculture and Food, GPO Box 1700, Canberra, ACT 2601, Australia

Peter R. Ryan

Crop Research Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China

Wen-zhu He

Contributions

Han-mei Du carried out the experiments, analyzed the data, and drafted the manuscript; Chan Liu, Hong-jie Li, Wan-peng Hu, Wei-na Yan, Ying Huang, Wen-zhu He, Bo-wen Luo, Xiao Zhang, Shu-feng Zhou and Shi-bin Gao contributed with consultation; Su-zhi Zhang designed the experiment; Su-zhi Zhang and Peter R Ryan revised the manuscript.

Corresponding author

Correspondence to Su-zhi Zhang

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Availability Of Data And Materials

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

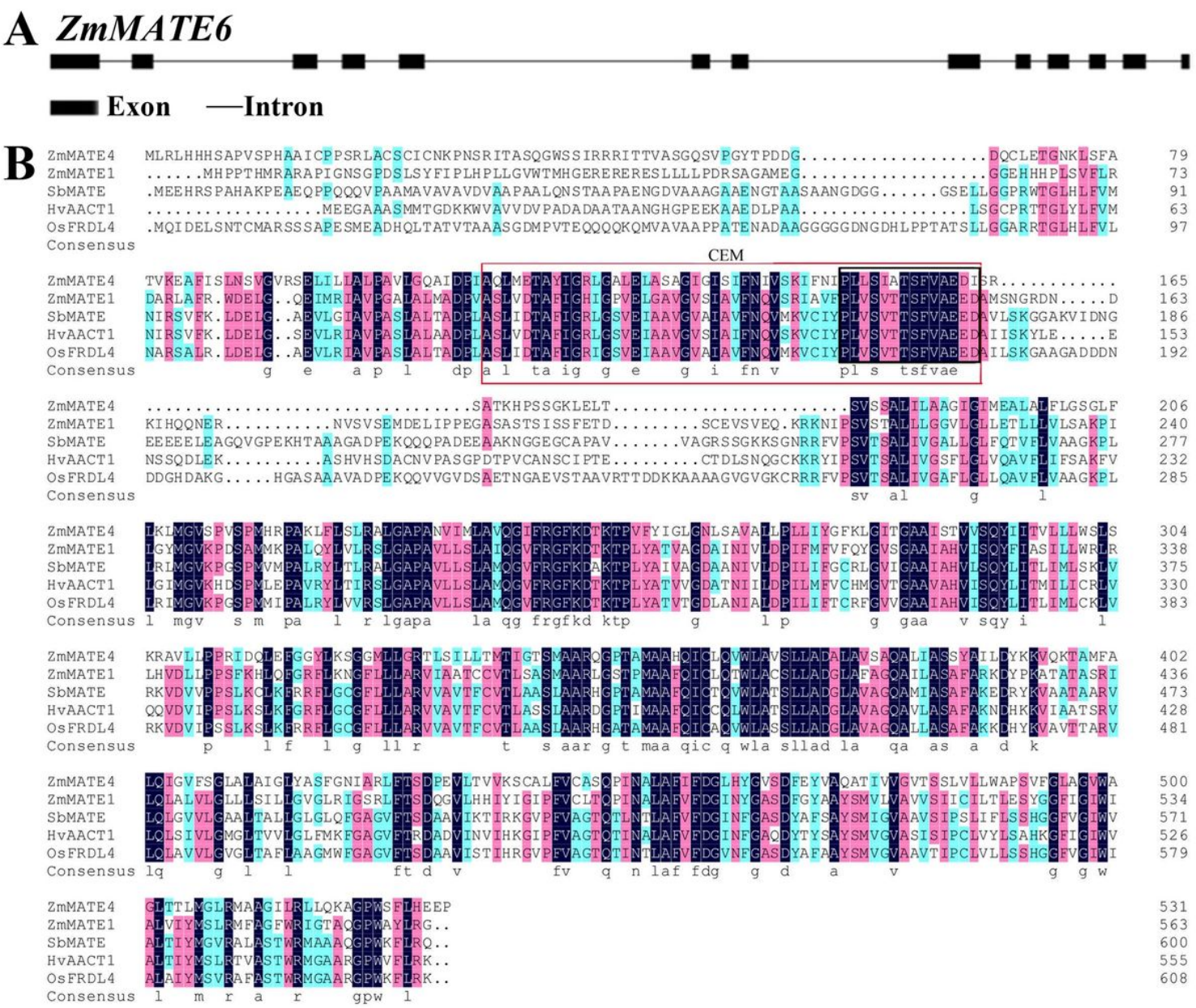

Figure 1

Sequence alignment of ZmMATE6 with other citrate transporters The citrate transporters were ZmMATE1 (ACM47309) from maize, SbMATE (XP_021311565) from sorghum, HvAACT1 (ANN88344) from barley and OsFRDL4 (BAS75911) from rice. Identical (100%), conservative (75%–99%) and blocks (50%–74%) of similar amino acid residues are shaded in deep blue, cherry red, and cyan, respectively. The conserved CEM and REF motifs are indicated by red and black boxes, respectively.

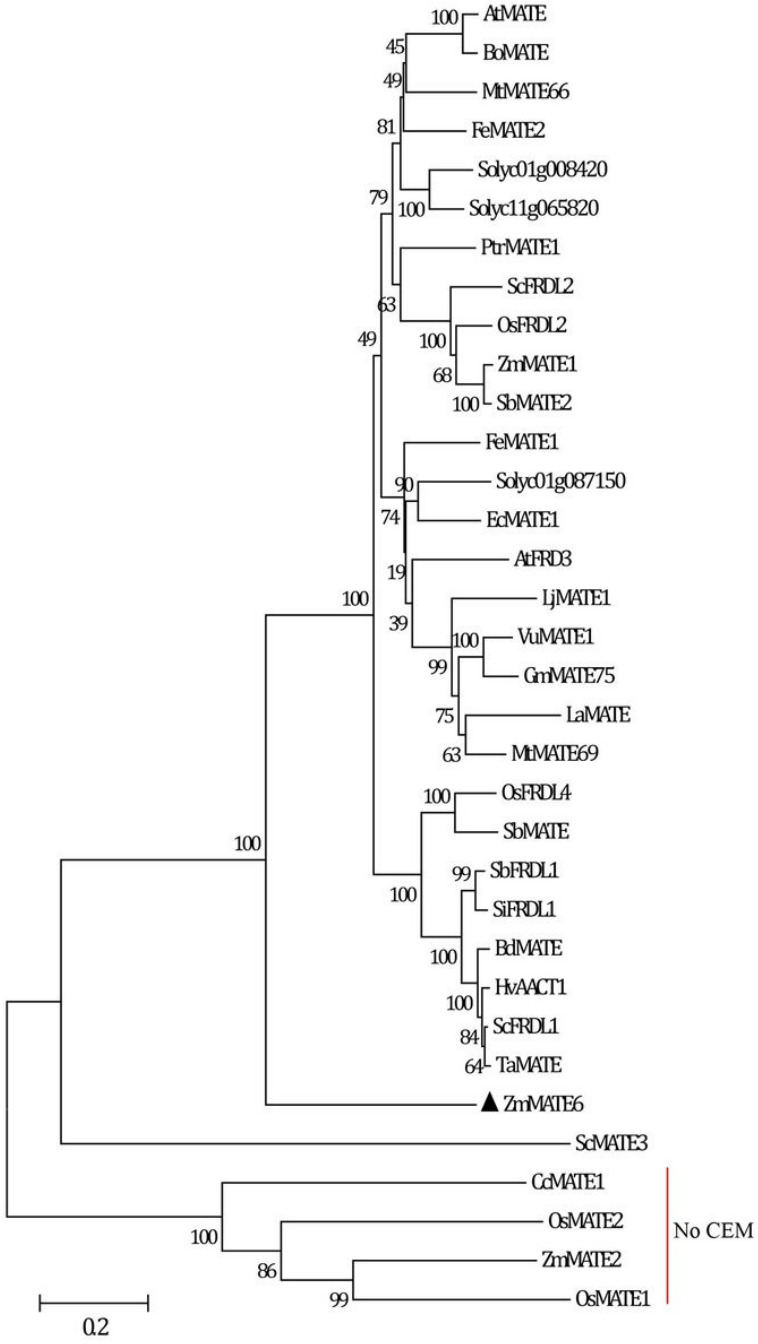

Figure 2

Phylogenetic analysis of ZmMATE6

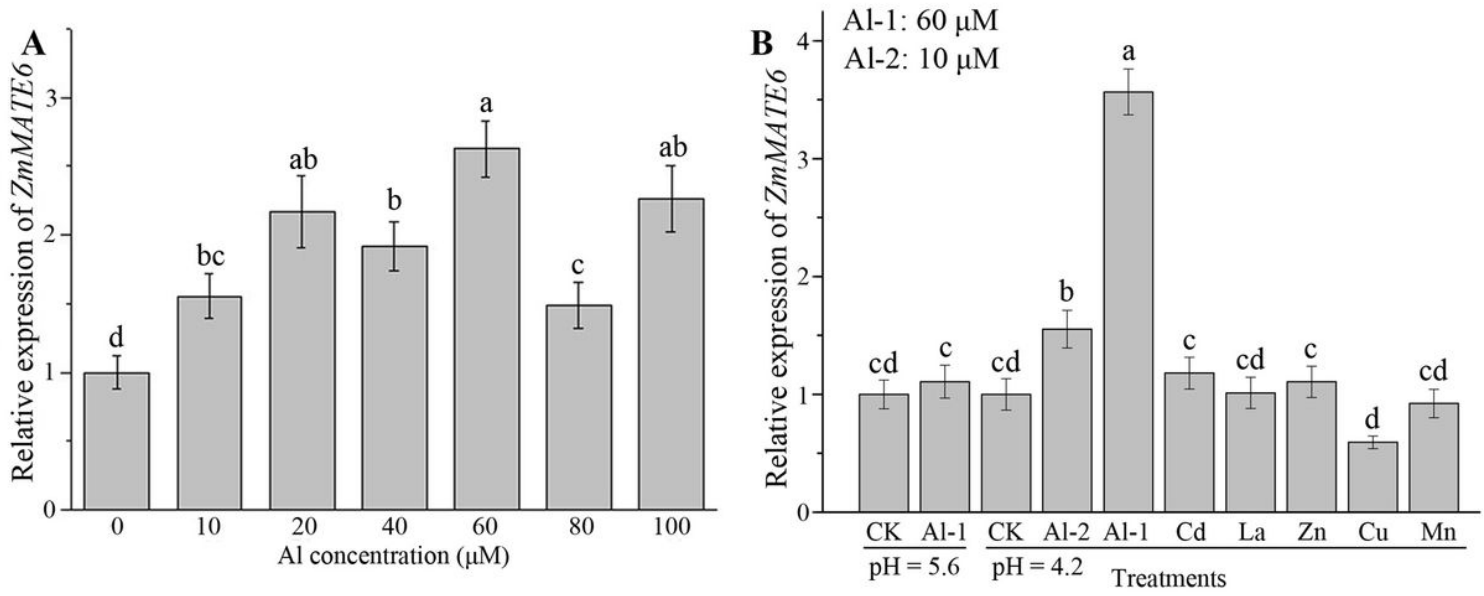

Figure 3

Expression pattern of ZmMATE6 gene in maize roots (A) Concentration effect of Al treatment on ZmMATE6 expression. Seedlings of the maize inbred line 178 were treated for 6 h by 0, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM Al. (B) Effect of other cations on ZmMATE6 expression. Seedlings were exposed for 6 h to a 0.5 mM CaCl2 solution (pH 4.2) containing 30 μM CdCl2, 2.0 μM CuCl2, 10 μM LaCl3, 100 μM ZnCl2, 200 μM MnSO4, 20 μM FeCl3 and 60 μM AlCl3, respectively. ZmGAPDH was used as internal control. The values were presented as mean ± SD (n = 3) and marked with different letters to indicate statistic significant difference at $P < 0.05$ (student's t test).

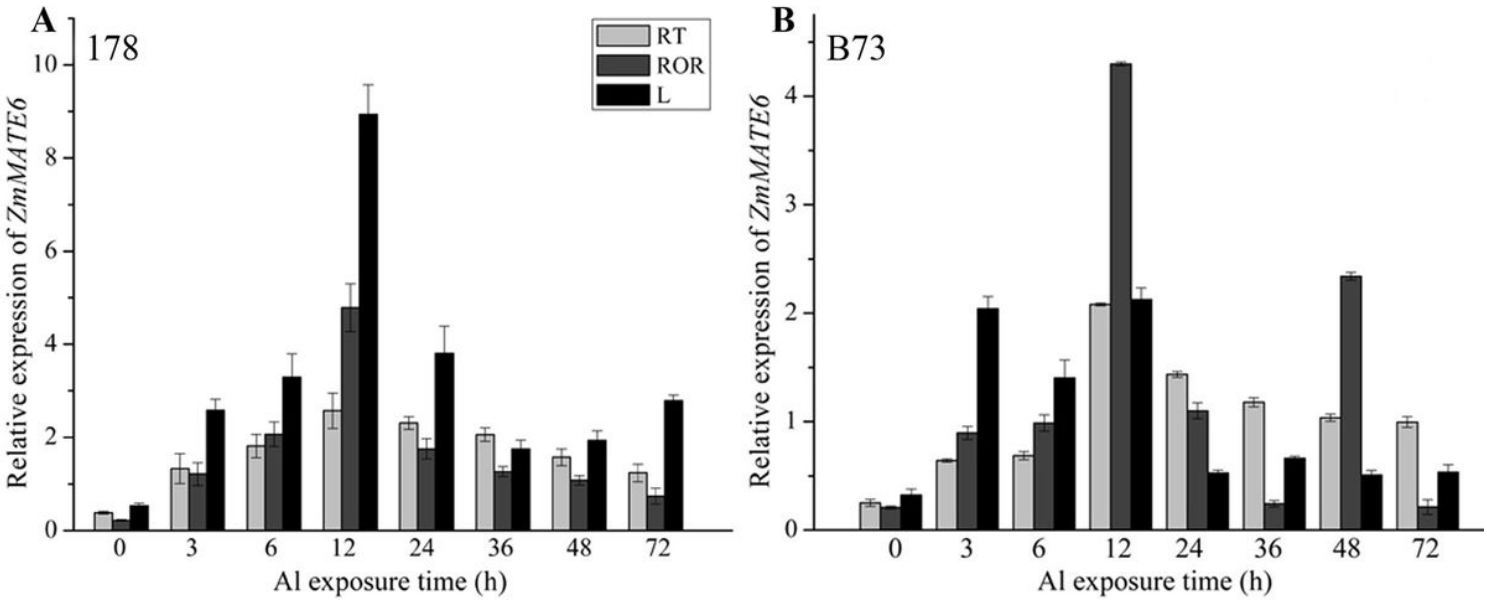

Figure 4

Tissue specificity of expression of ZmMATE6 in different genotype Transcription of ZmMATE6 in maize inbred line (A) 178 (Al-tolerance) and (B) B73 (Al-sensitive) seedlings, including root tips (R), the rest of root (ROR) and leaves (L) were quantified at 0, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h after exposure to 60 μM AlCl3 (pH 4.2). ZmGAPDH was used as internal control. The values were presented as mean ± SD (n = 3).

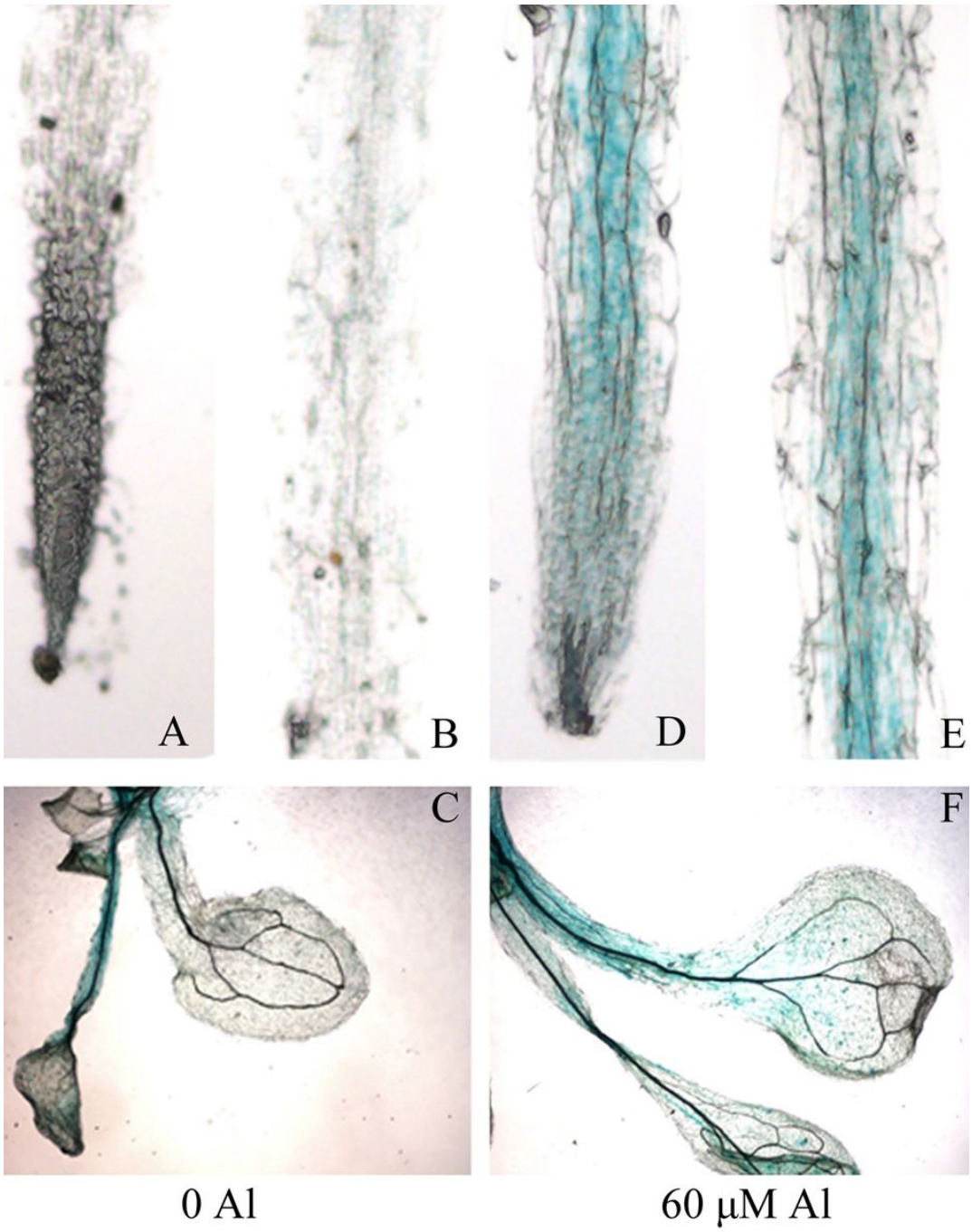

Figure 5

ZmMATE6p::GUS expression in transgenic plants The transgenic Arabidopsis was cultured with 2% MGRL (pH 5.6) nutrient solution for 7 d, and then treated with or without 60 μM AlCl3 for 0 h (A, B, C) and 9 h (D, E, F). A and D showed the root apex, B and E show the mature zone. C and F showed the leaves.

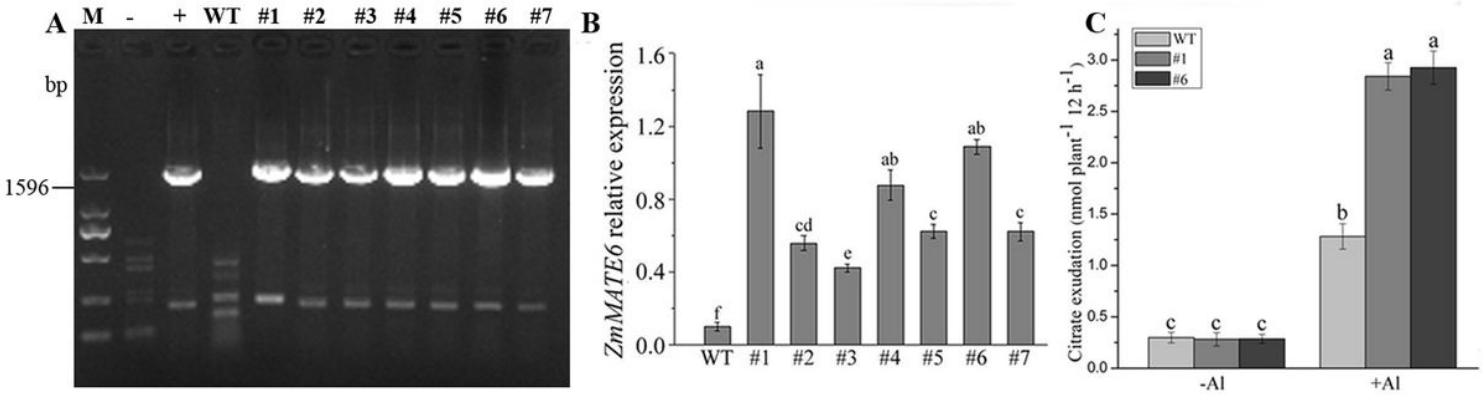

Figure 6

Transgenic Arabidopsis expressing ZmMATE6 release citrate Arabidopsis was transformed with ZmMATE6 using the CaMV35S promoter. (A) PCR analyses of seven transgenic Arabidopsis events (T0), [M-2000 bp DNA ladder; -: negative control (water), +: positive control (binary plasmid), WT: wild type Arabidopsis]; (B) Relative expression of ZmMATE6 in the T0 plants using RT-PCR where AtACT2 was the internal reference control; (C) Organic anion release from roots of transgenic Arabidopsis with and without 60 μM AlCl3 (pH 4.5). Values represent mean ± SD (n ≥ 20). Different letters indicate significant differences (P < 0.01) (multiple comparison).

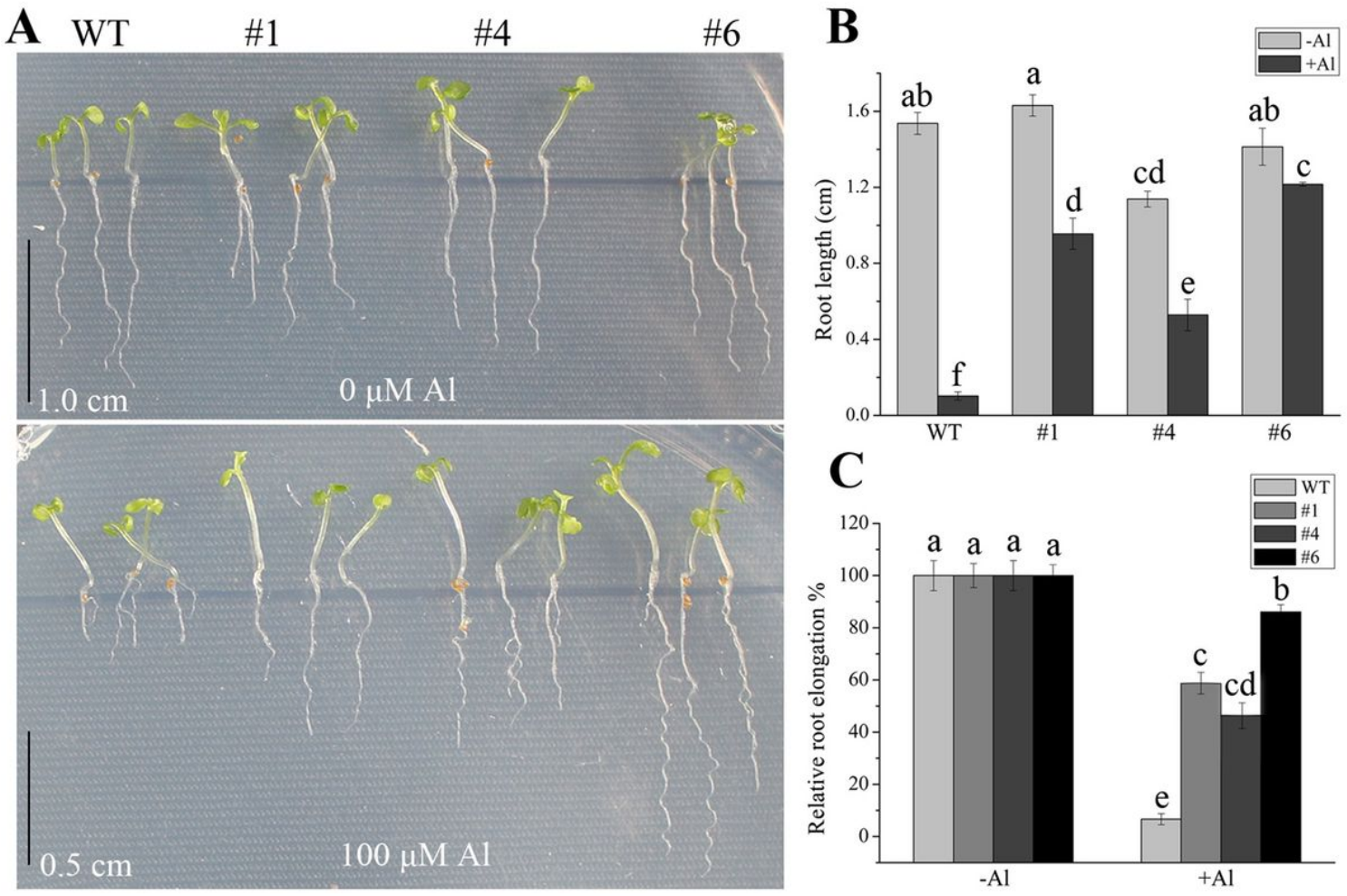

Figure 7

Transgenic Arabidopsis expressing ZmMATE6 are more Al resistant (A-C) Phenotype of plants (A), root length (B) and relative root elongation (C). Seedlings of wild type Arabidopsis and transgenic lines (#1, #4 and #6) were grown on a plate containing 0 or 100 μM AlCl3. Scale bar: 1.0 cm. Values represent mean ± SD (n ≥ 3). Different letters indicate significant differences (P < 0.01) (multiple comparison).

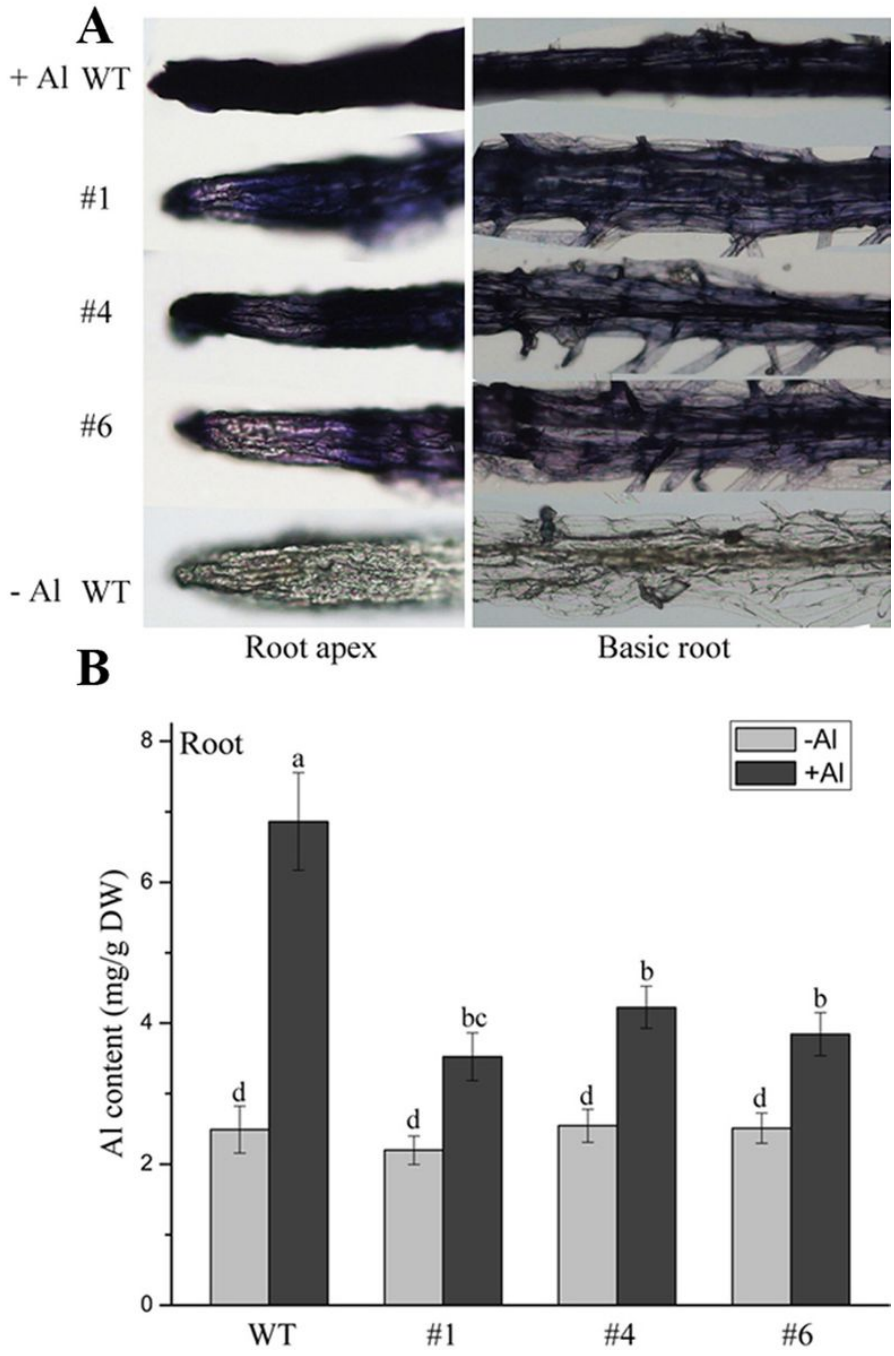

Figure 8

Overexpression of ZmMATE6 reduces Al accumulation in roots (A) Hematoxylin staining after 12 h exposure to 0 and 60 μM AlCl3 in roots of the transgenic lines overexpressing ZmMATE6 and WT plants. (B) Al contents of lines (as in (A)) under Al stress for 12 h. Values represent mean ± SD (n ≥ 10). Different letters indicate significant differences (P < 0.01) (multiple comparison).

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