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A promoter-swap strategy between the *AtALMT* and *AtMATE* genes increased Arabidopsis aluminum resistance and improved carbon-use efficiency for aluminum resistance

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SUMMARY

The primary mechanism of Arabidopsis aluminum (Al) resistance is based on root Al exclusion, resulting from Al-activated root exudation of the Al^{3+}-chelating organic acids, malate and citrate. Root malate exudation is the major contributor to Arabidopsis Al resistance, and is conferred by expression of *AtALMT1*, which encodes the root malate transporter. Root citrate exudation plays a smaller but still significant role in Arabidopsis Al resistance, and is conferred by expression of *AtMATE*, which encodes the root citrate transporter. In this study, we demonstrate that levels of Al-activated root organic acid exudation are closely correlated with expression of the organic acid transporter genes *AtALMT1* and *AtMATE*. We also found that the *AtALMT1* promoter confers a significantly higher level of gene expression than the *AtMATE* promoter. Analysis of *AtALMT1* and *AtMATE* tissue- and cell-specific expression based on stable expression of promoter–reporter gene constructs showed that the two genes are expressed in complementary root regions: *AtALMT1* is expressed in the root apices, while *AtMATE* is expressed in the mature portions of the roots. As citrate is a much more effective chelator of Al^{3+} than malate, we used a promoter-swap strategy to test whether root tip-localized expression of the *AtMATE* coding region driven by the stronger *AtALMT1* promoter (*$AtALMT1_P$::AtMATE*) resulted in increased Arabidopsis Al resistance. Our results indicate that expression of *$AtALMT1_P$::AtMATE* not only significantly increased Al resistance of the transgenic plants, but also enhanced carbon-use efficiency for Al resistance.

Keywords: aluminum resistance, transporter protein, organic acid exudation, promoter swap, carbon-use efficiency, *Arabidopsis thaliana*.

INTRODUCTION

Aluminum (Al) is ubiquitous in soils because the clay fraction of soils consists of aluminosilicate complexes. In acidic soils with pH values below 5.0, Al is solubilized as highly phytotoxic Al^{3+}, which inhibits root growth, damages root systems, and causes significant reductions in crop yields (Rao *et al.*, 1993; Kochian *et al.*, 2004). As approximately 30% of the world's total land area and over 50% of the world's potentially arable lands are acidic, Al toxicity is a major limitation to crop production worldwide (von Uexküll and Mutert, 1995; Matsumoto, 2000; Wood *et al.*, 2000; Yamamoto *et al.*, 2002; Sivaguru *et al.*, 2003; Kochian *et al.*, 2004; Horst *et al.*, 2010).

Many plant species have evolved Al resistance mechanisms based on exclusion of Al^{3+} from the root tip or sequestration of Al^{3+} that has entered the plant cells into subcellular compartments such as the vacuoles (Kochian

et al., 2004, 2005; Larson *et al.*, 2007; Ryan *et al.*, 2011). The Al exclusion mechanism is mediated by efflux of Al-chelating organic acids from root apices into the rhizosphere. The released organic acids detoxify Al^{3+} by forming stable, non-toxic complexes with Al^{3+} in the rhizosphere, limiting the uptake of toxic Al^{3+} into root cells (Kochian *et al.*, 2004, 2005; Ryan *et al.*, 2011).

Malate and citrate are the most common organic acids that are released from roots as part of the Al exclusion mechanism employed by a wide range of plant species (Kochian *et al.*, 2004, 2005). Therefore, it is not surprising that the first two Al resistance genes cloned from crop plants were those encoding an Al-activated malate transporter in wheat (*Triticum aestivum* L.) (Sasaki *et al.*, 2004), and Al-activated citrate transporters in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*) (Magalhaes *et al.*, 2007; Furukawa *et al.*, 2007).

In wheat, the *Alt1* locus is largely responsible for Al resistance. This locus is associated with Al-activated malate efflux from root apices of tolerant wheat genotypes (Delhaize *et al.*, 1993a,b). The *Alt1* locus was subsequently identified as the gene that encodes the wheat aluminum-activated malate transporter, TaALMT1 (Sasaki *et al.*, 2004). *TaALMT1* is constitutively and specifically expressed in the root apices and is responsible for Al-activated root malate exudation and Al resistance in wheat (Sasaki *et al.*, 2004; Raman *et al.*, 2005).

Similarly, the Al resistance locus Alt_{SB} in sorghum co-segregated with Al-activated citrate exudation from root tips (Magalhaes *et al.*, 2004). The gene underlying the Alt_{SB} locus is a member of the multi-drug and toxic compound extrusion (MATE) family of membrane transporters in sorghum (Magalhaes *et al.*, 2007). *SbMATE* encodes a citrate transporter that mediates Al-activated citrate efflux from the root apices of Al-tolerant sorghum lines (Magalhaes *et al.*, 2007). *HvMATE* (*HvAACT1*) has been identified as the barley Al resistance gene, encoding a root membrane transporter that facilitates Al-activated citrate efflux from root tips (Furukawa *et al.*, 2007).

Functional homologs of the ALMT1 and MATE family have been identified in other plant species, including rye (*Secale cereale*) (Collins *et al.*, 2008; Yokosho *et al.*, 2010), maize (*Zea mays*) (Maron *et al.*, 2010), wheat (Ryan *et al.*, 2009), Brassica (Ligaba *et al.*, 2006) and Arabidopsis (Hoekenga *et al.*, 2006; Liu *et al.*, 2009).

In Arabidopsis, Al-activated root malate release has been reported to be the major Al resistance mechanism (Hoekenga *et al.*, 2006). *AtALMT1*, a member of the Arabidopsis *ALMT* family, has been confirmed as a functional homolog of *TaALMT1* in Arabidopsis. Like *TaALMT1*, expression of *AtALMT1* is specifically localized to roots (Sasaki *et al.*, 2004; Raman *et al.*, 2005; Hoekenga *et al.*, 2006). However, unlike *TaALMT1*, which is constitutively expressed in roots of wheat Al-resistant genotypes, expression of *AtALMT1* is strongly induced by Al within 24 h of Al treatment (Hoekenga *et al.*, 2006; Liu *et al.*, 2009). The function of *AtALMT1* in Arabidopsis Al resistance has been verified by genetic and physiological studies. A homozygous T-DNA insertion in the first exon of the *AtALMT1* gene disrupted the function of AtALMT1, leading to the abolishment of Al-activated root malate efflux and a strongly hypersensitive phenotype for the *AtALMT1* knockout (*AtALMT1-KO*) mutant (Hoekenga *et al.*, 2006; Liu *et al.*, 2009).

Upon Al stress, Arabidopsis also releases a smaller amount of citrate from roots, and this is mediated by AtMATE, a functional homolog of SbMATE (Liu *et al.*, 2009). Like *AtALMT1*, expression of *AtMATE* is strongly and rapidly induced in root tissues by Al stress (Liu *et al.*, 2009). The role of AtMATE-mediated root citrate release in Al resistance in Arabidopsis has been demonstrated via reverse genetics studies. A homozygous T-DNA insertion in the *AtMATE* promoter disrupted Al-induced *AtMATE* expression and abolished Al-activated AtMATE-mediated root citrate exudation (Liu *et al.*, 2009). This *AtMATE* knockdown mutant (*AtMATE-KD*) showed moderate sensitivity to Al that was more clearly visualized in the *AtALMT1-KO* background: the *AtALMT1-KO/AtMATE-KD* double mutant is more sensitive to Al stress than the *AtALMT1-KO* single mutant (Liu *et al.*, 2009). It has been estimated that *AtALMT1*-mediated root malate exudation accounts for 70% of Arabidopsis Al resistance, while *AtMATE*-mediated root citrate exudation accounts for the other 30% (Liu *et al.*, 2009).

Organic acids are a valuable carbon resource and are essential for plant growth and development. This is especially true of citric acid, which, as an important intermediate in the Krebs (tricarboxylic acid) cycle, plays an essential role in the metabolism of virtually all living things (Berg *et al.*, 2002). Therefore, use of organic acid efflux as a resistance weapon against Al stress carries a significant energy cost for the plant, which may negatively affect plant yields. Therefore, well-balanced and finely controlled Al-activated root organic acid exudation is essential not only for Al resistance but also for maintaining sustainable growth and yields under Al stress.

Citrate has been demonstrated to be a better chelator for Al^{3+} than malate (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995; Ma, 2000; Ryan *et al.*, 2001), as the three carboxyl groups of citrate more effectively stabilize the citrate–Al complex than the two carboxyl groups in malate do. Because of this, and given the greater role that Al-activated root malate exudation plays in Arabidopsis Al resistance (Liu *et al.*, 2009), we used a promoter-swap strategy to evaluate the transcriptional regulation of Al-activated root malate and citrate exudation and the effectiveness of malate and citrate for Al resistance in Arabidopsis. Our results indicate that Arabidopsis Al resistance can be enhanced by combining the stronger *AtALMT1* promoter with the functionally more effective AtMATE citrate efflux transporter.

RESULTS

Transgenic *AtALMT1* and *AtMATE* promoters retain their Al inducibility, but are weaker than their corresponding native promoters

In a previous study, we demonstrated that transcription of *AtALMT1* and *AtMATE* is fully induced in the root but not in shoot tissues of Arabidopsis after 24 h Al treatment (Liu *et al.*, 2009). In the present study, to characterize the promoter strength of these two genes, we compared the relative expression levels of both genes in the roots of wild-type plants. Our results indicated that the transcript abundance for both *AtALMT1* and *AtMATE* increased approximately 2.6-fold after 24 h Al treatment (Figure 1a). The abundance of Al-induced *AtALMT1* transcripts was approximately 7–8-fold higher than that of Al-induced *AtMATE* transcripts, suggesting that the promoter of the *AtALMT1* gene is much stronger than the *AtMATE* promoter (Figure 1a). The expression patterns for *AtMATE* and *AtALMT1* were similar to those of the wild-type in the *AtALMT1-KO* and *AtMATE-KD* mutant backgrounds, respectively, indicating that *AtMATE* and *AtALMT1* expression are independent of each other, consistent with our previous findings (Figure 1) (Liu *et al.*, 2009).

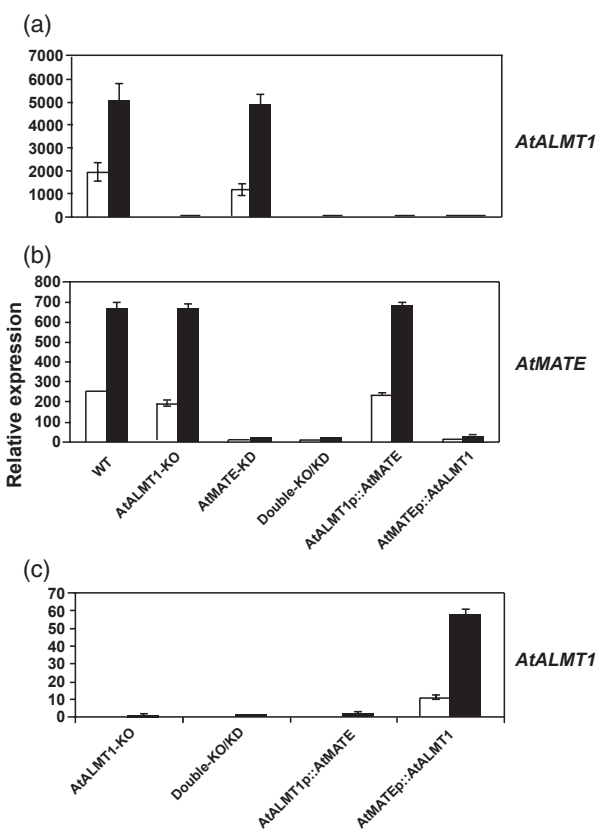

Figure 1. Relative expression patterns for *AtALMT1* (a, c) and *AtMATE* (b). The *18S* gene was used as an internal control. All of the data were normalized to a single calibrator. Open bars, without Al; closed bars, with Al. Note that, in (c), the scale on the *y* axis ranges from 0 to 70, whereas the *y* axis values range from 0 to 7000 in (a) and from 0 to 800 in (b), in order to accommodate the differences in transcript abundance for *AtALMT1* versus *AtMATE* in wild-type plants (a versus b), and the differences in transcript abundance for *AtALMT1p::AtMATE* versus *AtMATEp::AtALMT1* in the transgenic lines (b versus c).

To evaluate the role of transcriptional regulation of the *AtALMT1* and *AtMATE* genes and the effectiveness of malate and citrate in Al resistance, a promoter-swap strategy was used. A 2.1 kb promoter region was amplified by PCR for the *AtALMT1* and *AtMATE* genes, and the PCR fragments were fused to PCR-amplified genomic coding regions of the *AtMATE* and *AtALMT1* genes, respectively. The resulting promoter-swap constructs were individually transformed into the double mutant *AtALMT1-KO*/*AtMATE-KD* line (*double-KO/KD*). In the *double-KO/KD* line, the levels of Al-induced root *AtALMT1* and *AtMATE* transcripts (Figure 1a,b) and the rates of Al-activated root malate and citrate exudation (Figure 3) were greatly suppressed by the T-DNA insertions, leading to an Al hypersensitive phenotype for the double mutant (Figure 4). As a result, the *double-KO/KD* line provides a clear background for evaluating the relationships between expression of the transgenes, the levels of the organic acid exudation mediated by the transgenes, and Al resistance contributed by the transgenes.

Compared to the *double-KO/KD* line, the *AtALMT1*$_{Promoter}$*::AtMATE* transgenic line (*AtALMT1*$_P$*::AtMATE*) and the *AtMATE*$_{Promoter}$*::AtALMT1* transgenic line (*AtMATE*$_P$*::AtALMT1*) showed large increases in basal and Al-induced *AtMATE* and *AtALMT1* transcript abundance, respectively (Figure 1b,c). In addition, the two transgenes also showed similar degrees of Al inducibility to their corresponding native genes in the wild-type. As shown in Figure 1(b), in the *AtALMT1*$_P$*::AtMATE* line, Al induced a 2.8-fold increase in the level of *AtMATE* transcripts driven by the transgenic *AtALMT1* promoter, which is comparable to the 2.6-fold Al induction of *AtALMT1* transcript abundance driven by the native *AtALMT1* promoter in the wild-type background (Figure 1a). However, in contrast to the 2.6-fold increase in the level of *AtMATE* transcript driven by the native *AtMATE* promoter in the wild-type background (Figure 1b), Al induced an approximately 5.3-fold increase in the *AtALMT1* transcript abundance driven by the transgenic *AtMATE* promoter in the *AtMATE*$_P$*::AtALMT1* line (Figure 1c; note amended *y* axis scale). These results indicated that the transgenic *AtALMT1* and *AtMATE* promoters retain the *cis*-elements necessary for Al-induced transgene expression.

However, when the Al-induced *AtMATE* transcript abundance driven by the chimeric *AtALMT1* promoter in the *AtALMT1*$_P$*::AtMATE* line (Figure 1b) is compared to that of Al-induced *AtALMT1* driven by the native *AtALMT1* promoter in the wild-type background (Figure 1a), the latter was found to be approximately 7.4 times higher than the former. Similarly, the Al-induced *AtMATE* transcript abundance

driven by its native *AtMATE* promoter in the wild-type (Figure 1b) was approximately 11 times higher than that of Al-induced *AtALMT1* transcripts driven by the transgenic *AtMATE* promoter in the *AtMATE*$_P$*::AtALMT1* background (Figure 1c). These results indicate that the transgenic *AtALMT1* and *AtMATE* promoters are much weaker than the native *AtALMT1* and *AtMATE* promoters functioning in the wild-type background.

AtALMT1 and *AtMATE* are expressed in different and complementary regions of the Arabidopsis root

Our previous results indicated that expression of both *AtALMT1* and *AtMATE* genes is induced by Al in root tissues, but not in shoot tissues (Figure 1) (Liu *et al.*, 2009). To further characterize tissue-specific *AtALMT1* and *AtMATE* expression patterns *in planta*, promoter–β-glucuronidase (*AtALMT1*$_P$*::GUS* and *AtMATE*$_P$*::GUS*) fusion constructs were expressed in transgenic Col-0 plants. For both constructs, GUS expression was observed in the root but not in the shoot tissues of Al-treated transgenic seedlings, consistent with our real-time RT-PCR results (Figure 2). In the *AtALMT1*$_P$*::GUS* transgenic lines, GUS activity was localized to the root meristem, root elongation zone and root-hair regions (Figure 2a–d). In mature regions of the root from above the root-hair region to the root–shoot junction, little GUS staining was observed in the primary root, except in root hairs at the root–shoot junction region and in lateral roots (Figure 2e,f). In contrast, GUS expression in the *AtMATE*$_P$*::GUS* lines was predominantly localized to mature regions of the root, extending from above the root-hair region (Figure 2 g,h,k) to the root–shoot junction (Figure 2l). In the root-hair region of the primary root, *AtMATE* expression was observed in root hairs but not the rest of this root region (Figure 2j), in contrast to *AtALMT1* expression in this region, which occurred in both root hairs and the epidermis and outer cortex of this primary root region (Figure 2d). It therefore appears that root cell-specific expression of *AtALMT1* and *AtMATE* is complementary, with *AtALMT1* expression localized to the growing root tip (and root hairs), while *AtMATE* expression is localized to the mature root (and root hairs) (Figure 2).

Al-activated root citrate and malate exudation in transgenic promoter-swap lines

In the wild-type, Al stress induces a higher level of AtALMT1-mediated root malate exudation and a lower level of AtMATE-mediated citrate exudation (Figure 3) (Liu *et al.*, 2009). The rate of root malate exudation is approximately four times higher than that of root citrate exudation (Figure 3) (Liu *et al.*, 2009). The double *AtALMT1-KO/AtMATE-KD* mutant lacks both Al-activated root malate and citrate exudation and is very sensitive to Al stress (Figure 3). These results are consistent with our previous findings (Liu *et al.*, 2009).

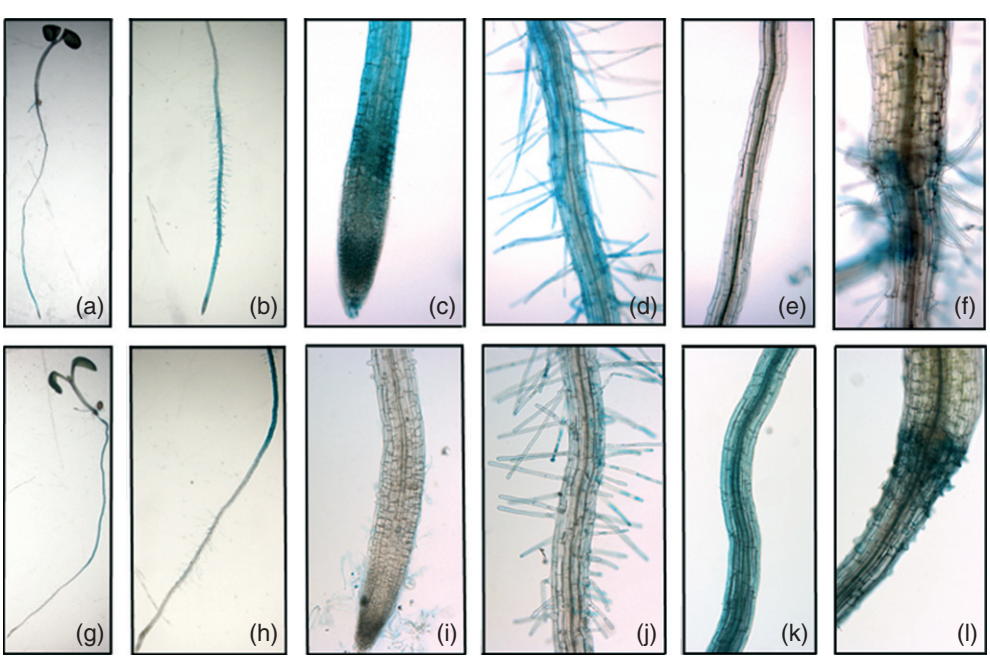

Figure 2. *AtALMT1*$_P$*::GUS* and *AtMATE*$_P$*::GUS* expression in transgenic plants.
(a–f) GUS staining in the *AtALMT1*$_P$*::GUS* line; (g–l), GUS staining in the *AtMATE*$_P$*::GUS* line. (a, g) Whole seedlings; (b, h) root meristem, zone of root elongation and root-hair regions; (c, i) higher magnification of the root tip showing the root meristem and zone of root elongation; (d, j) root-hair region; (e, k) mature root; (f, l) the root–shoot junction.

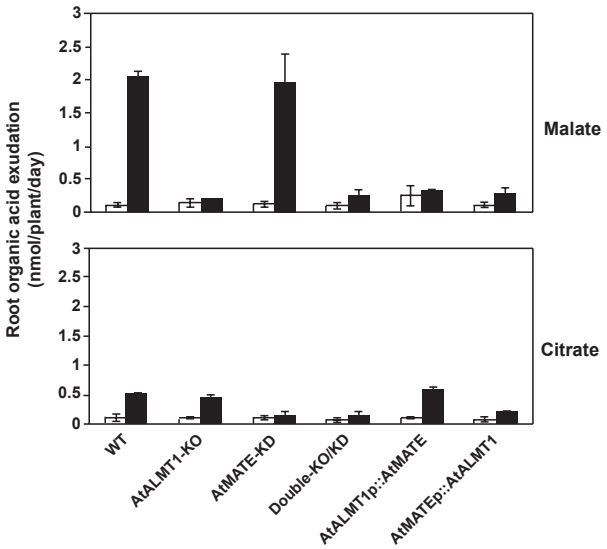

Figure 3. Root malate and citrate exudation in the presence and absence of Al in the various genotypes.
Open bars, without Al; closed bars, with Al.

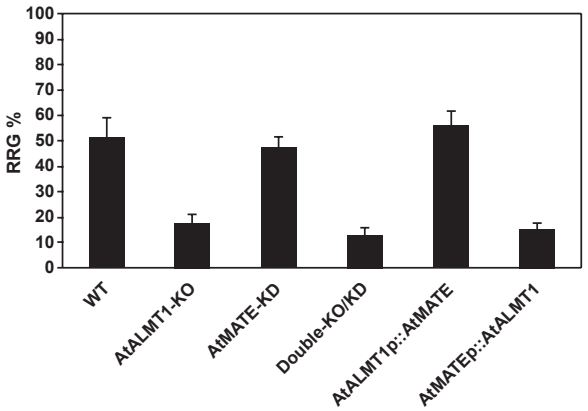

Figure 4. Aluminum resistance measured as a percentage of relative net root growth (%RRG = root growth in Al/control root growth ×100).

In the *AtALMT1*$_P$*::AtMATE* line, the rate of root citrate exudation increased 5.4-fold after 1 day of Al treatment compared to that in the absence of Al, indicating that the transgenic promoter-swap *AtALMT1*$_P$*::AtMATE* construct restored Al-activated root citrate exudation in the *double-KO/KD* background (Figure 3). However, the rate of root Al-activated citrate exudation mediated by the transgenic *AtMATE* gene driven by the *AtALMT1* promoter in this line was only approximately 28% of the root Al-activated malate exudation mediated by the native *AtALMT1* gene driven by its own promoter in the wild-type background (Figure 3). To examine whether the rates of root organic acid exudation are controlled at least in part by the levels of transcripts of the corresponding transporter genes, we compared the levels of Al-induced *AtALMT1* expression in the wild-type line, Al-induced *AtMATE* expression driven by the transgenic *AtALMT1* promoter in the *AtALMT1*$_P$*::AtMATE* line, and the corresponding Al-activated organic acid exudation in the same two lines (using the data in Figures 1 and 3). We found a strong correlation between the levels of gene expression and the rates of organic acid exudation (correlation coefficient = 0.89). Similarly, the levels of *AtMATE* expression in the wild-type background line and *AtALMT1* expression driven by the transgenic *AtMATE* promoter in the *AtMATE*$_P$*::AtALMT1* line were strongly correlated with the Al-activated root citrate exudation in these two lines (correlation coefficient = 0.84). These results suggest that the promoters of the *AtALMT1* and *AtMATE* genes play an important role in the rate of root organic acid efflux mediated by the transport proteins encoded by each gene in response to Al, presumably via increased abundance of each transport protein.

Recovery of Al resistance in the *AtALMT1*$_P$*::AtMATE* transgenic line

To evaluate the functions of the promoter-swapped transgenes, we compared the levels of Al resistance for the wild-type, *AtALMT1-KO*, *AtMATE-KD* and *double-KO/KD* mutants, and the two promoter-swap transgenic lines. We found that, after 6 days of Al exposure, root growth was inhibited by approximately 50% for the wild-type plants relative to control root growth in the absence of Al. Compared to the wild-type, the Al resistance measured as a percentage of relative net root growth (RRG%) of *AtALMT1-KO*, *AtMATE-KD* and *double-KO/KD* decreased by 66%, 7% and 75% respectively, confirming our previous findings that *AtALMT1* plays a major role and *AtMATE* plays a smaller role in Al resistance in Arabidopsis (Figure 4 and Table 1).

Transformation of the *AtALMT1*$_P$*::AtMATE* promoter-swap construct into the *double-KO/KD* background dramatically increased Al resistance for the transgenic plants. As seen in Figure 4, Al resistance (RRG%) of the *AtALMT1*$_P$*::AtMATE* line increased 4.5-fold compared to the *double-KO/KD* line, achieving a level of Al resistance comparable to wild-type plants. However, expression of the other promoter-swap construct, *AtMATE*$_P$*::AtALMT1*, in the same background led to only a small increase in Al resistance (Figure 4).

The *AtALMT1*$_P$*::AtMATE* transgenic line uses less carbon than the wild-type to achieve the same level of Al resistance

To estimate the carbon-use efficiency for Al resistance in the various genotypes, we normalized Al resistance measured as RRG% to the total carbon released by the roots under Al stress due to malate and citrate efflux. As shown in Table 1 and Figure 5, after Al treatment, the roots of wild-type plants released a mean of 0.52 nmol citrate and 2.05 nmol malate per plant per day. This is equivalent to a release of 3.12 nmol

Table 1 Al resistance-associated carbon use efficiency for wild type and transgenic lines

	WT	*AtALMT1- KO*	*AtMATE- KD*	*Double- KO/KD*	*AtALMT1p:: AtMATE*	*AtMATEp::AtALMT1*
RRG%	51.5 ± 7.4	17.3 ± 3.5	47.7 ± 4.1	12.7 ± 3.4	55.7 ± 6.4	15.0 ± 3.0
Al-activated citrate efflux[a]	0.5 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.0
Al-activated malate efflux[a]	2.1 ± 0.1	0.2 ± 0.0	2.0 ± 0.5	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
Al-activated citrate + malate efflux[a]	2.6	0.6	2.2	0.4	0.9	0.5
Total Al-activated carbon efflux[a]	11.3	3.4	9.2	1.9	4.7	2.4
Carbon use efficiency[b]	4.6	5.1	5.2	6.8	11.9	6.3

[a]The units are nmol/plant/day.
[b]Carbon use efficiency is the Al resistance (RRG%) normalized by the amount of carbon lost (C) from the roots due to Al resistance (malate + citrate efflux) = RRG%/Total Al-activated carbon efflux. The unit for carbon use efficiency is %/nmol C/plant/day.

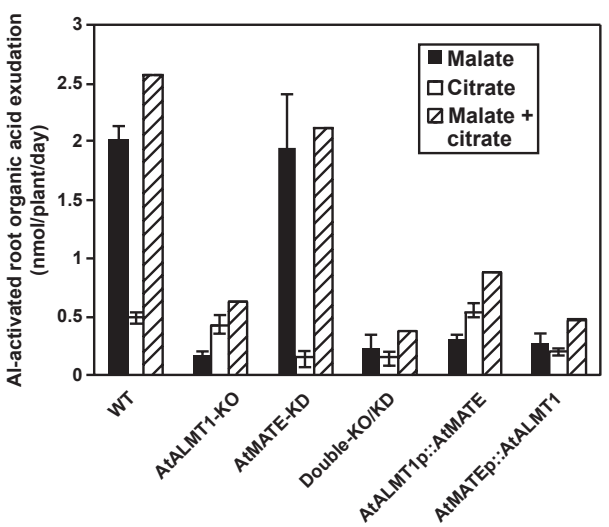

Figure 5. Carbon-use efficiency for the various genotypes during Al resistance mediated by Al-activated root malate and citrate efflux.
Carbon-use efficiency is calculated as Al resistance (RRG%) normalized to the amount of carbon lost from the roots due to Al resistance (malate + citrate efflux), i.e. RRG%/total Al-activated root carbon efflux.

carbon per plant per day from citrate, which has six carbon atoms, and 8.2 nmol carbon per plant per day originating from malate, which has four carbon atoms, giving a total of 11.3 nmol carbon released per plant per day (Table 1). Thus, the carbon-use efficiency for wild-type plants was estimated as 4.6 units of Al resistance (RRG%) per nmol carbon lost from roots per plant per day (Table 1).

Carbon-use efficiency was also calculated for all of the transgenic lines used in this study, including *AtALMT1-KO*, *AtMATE-KD*, *double-KO/KD*, *AtALMT1$_P$::AtMATE* and *AtMATE$_P$::AtALMT1*. As shown in Table 1, compared to the *AtALMT1-KO*, *AtMATE-KD* and *double-KO/KD* lines, the wild-type line was the most Al-tolerant. However, there was a significant carbon cost for this higher Al resistance, and the carbon use efficiency in wild-type plants was as much as 50% lower than that calculated for the *AtALMT1-KO* and *AtMATE-KD* lines (4.6 versus 6.8 in wild-type and *double-KO/KD* lines, respectively) (Table 1).

Even more interesting, in the *AtALMT1$_P$::AtMATE* line, a significant increase in carbon-use efficiency was seen, almost threefold higher than in wild-type (11.9 versus 4.6) and almost twofold higher than the *AtMATE$_P$::AtALMT1* line (11.9 versus 6.3). As the difference between the *double-KO/KD* and *AtALMT1$_P$::AtMATE* lines is due to the presence of the transgenic promoter-swap construct in the latter, the increase in Al resistance in the *AtALMT1$_P$::AtMATE* line is the result of the 3.0-fold increase in Al-activated root citrate exudation mediated by the transgenic AtMATE citrate transporter whose gene expression is driven by the stronger *AtALMT1* promoter (Figure 3 and Table 1).

The much higher carbon-use efficiency in the *AtALMT1$_P$::AtMATE* line was due to the fact that, although Al resistance and the rate of root citrate exudation for this transgenic line are comparable to that of wild-type, the level of root malate exudation in this *AtALMT1$_P$::AtMATE* line is negligible compared with that in wild-type plants, leading to a 58% decrease in total root carbon release in the *AtALMT1$_P$::AtMATE* line compared to the wild-type (Table 1).

As described above, the root malate exudation resulting from expression of the *AtALMT1* gene driven by its own native *AtALMT1* promoter in wild-type plants, and the root citrate exudation resulting from *AtMATE* expression driven by the transgenic *AtALMT1* promoter in the *AtALMT1$_P$::AtMATE* line, are the major determinants for Al resistance in each line (Table 1). Due to the differences in promoter strength of the native and transgenic *AtALMT1* promoters (Figure 1), the rate of root malate exudation in the wild-type was 3.6 times higher than that of root citrate exudation in the *AtALMT1$_P$::AtMATE* line (Figure 5 and Table 1). Both lines exhibited similar levels of root citrate exudation; however, both lines displayed comparable levels of Al resistance (Table 1). This is quite interesting, and, as discussed below, is due to the efflux of citrate, a much more effective chelator than malate, being more localized to the growing root tip in the *AtALMT1$_P$::AtMATE* line and not expressed more diffusely throughout the mature root region as it is in wild-type plants. These findings also suggest that the carbon loss due to the large malate efflux in wild-type plants is a significant carbon cost on root growth, and the same level of Al

resistance in the *AtALMT1*$_P$*::AtMATE* line with similar citrate and much less malate exudation than wild-type may be due in part to a reduction in this carbon cost.

DISCUSSION

Many plant species have adopted a physiological mechanism for Al resistance that involves efflux of Al-chelating organic acids from roots upon Al stress, among which malate and citrate are the most commonly used by various plant species (Kochian *et al.*, 2004, 2005; Ma, 2007; Ryan *et al.*, 2011). Due to differences in chemical structure, particularly three carboxyl groups compared with two in malate, citrate has better Al^{3+}-detoxifying capacity than malate (Hue *et al.*, 1986). It has been shown that, to detoxify a particular amount of Al^{3+}, an equimolar amount of citric acid is sufficient (Ma *et al.*, 1997); however, 6–8 times more malic acid are required to detoxify the same amount of Al^{3+} (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995). It is not clear why evolution has resulted in different organic acids being used by different plant species for this Al resistance mechanism. In Arabidopsis, a larger amount of malate and a small amount of citrate are released from the roots in response to Al stress (Magalhaes *et al.*, 2007; Liu *et al.*, 2009). The Al-activated root malate and citrate exudation are mediated by *AtALMT1*, a functional homolog of *TaALMT*, and *AtMATE*, a functional homolog of *SbMATE*, respectively (Liu *et al.*, 2009). It has been estimated that, in Arabidopsis, Al-activated *AtALMT1*-mediated root malate exudation accounts for approximately 70% of Al resistance, and Al-activated *AtMATE*-mediated root citrate exudation accounts for 30% (Liu *et al.*, 2009).

In this study, we further characterized the relationship between *AtALMT1* and *AtMATE* expression and root malate and citrate exudation in Arabidopsis. We found that the levels of Al-induced *AtALMT1* and *AtMATE* transcripts were highly correlated with the levels of Al-activated root malate and citrate exudation, respectively, and the degree of Al resistance in Arabidopsis (Figures 1, 3 and 4). We recognize that the regulation and extent of root malate and citrate efflux most likely involves more than simply alterations in the level of AtALMT1 and AtMATE transcripts and proteins as measured by quantitative RT-PCR assays and expression of promoter::GUS reporter constructs in transgenic plants. *Cis*-elements and *trans*-acting factors that regulate *AtALMT1* and *AtMATE* expression, as well as post-transcriptional and post-translational regulation, most likely also play roles in determination of the abundance and localization of the AtALMT1 and AtMATE transcripts and proteins. However, the research presented here focusing on the transcript abundance of these two genes determined by quantitative RT-PCR and the tissue localization and strength of gene expression via promoter::GUS reporter constructs expressed in transgenic plants represents the first steps in a broader future examination of expression of AtALMT1 and AtMATE genes and proteins and the relationship of these processes with root malate and citrate exudation and Arabidopsis Al resistance.

Interestingly, the findings shown in Figure 2 for tissue localization of expression of the two genes suggest that, although both *AtALMT1* and *AtMATE* transcription are induced by Al stress, expression of *AtALMT1* is predominantly localized to the root apices, including the root meristem, the zone of root elongation and the region of root-hair growth, whereas *AtMATE* expression is more diffuse, being localized to the root mature region starting just above the root region where root hairs are seen and extending up the primary root to the root–shoot junction. These results indicate that Al-activated AtALMT1-mediated malate release occurs in the root apical region and Al-activated AtMATE-mediated citrate efflux occurs in the mature part of the roots. It has been well documented that the root apical region is the root region that is most sensitive to Al stress, and the root mature region is less sensitive to such stress, at least with regard to root growth (Ryan *et al.*, 1993; Sivaguru *et al.*, 1999; Sasaki *et al.*, 2004; Rangel *et al.*, 2007). In Arabidopsis, the higher amount of Al-activated malate exudation more densely localized to the root apex, and the lower level of Al-activated citrate release more widely distributed along the mature root regions, explain why malate efflux plays a more important role in Al resistance in Arabidopsis. It is interesting that citrate exudation from the mature root plays a moderate role in Arabidopsis root growth in response to Al. Possibly one aspect of this is to help exclude Al from the rhizosphere next to root regions where new lateral roots are emerging. Overall, our findings indicate that promoter strength, localization of gene expression and the identity of the organic acid released all play important roles in determining the level of Al resistance conferred by organic acid exudation.

To test whether Al resistance and the carbon cost associated with this Al resistance mechanism can be improved by combining a better Al chelator efflux gene with a stronger and more Al-inducible promoter, we used a promoter-swap strategy. Two promoter-swap constructs were made. In the first case, the strong *AtALMT1* promoter was used to drive the coding region of the *AtMATE* gene, whose product mediates the release of citrate, a more effective Al^{3+} chelator. In the second case, the weaker *AtMATE* promoter was fused to the *AtALMT1* coding sequence, whose product mediates the efflux of malate, a less effective Al^{3+} chelator. Both constructs were individually transformed into the *AtALMT1-KO/AtMATE-KD* double mutant background, which lacks both Al-activated root malate and citrate exudation, and thus provides a clear background for evaluating the phenotypic effects of the transgenes.

Our results indicated that, in the *double-KO/KD* background, the transgenic *AtALMT1* and *AtMATE* promoters mediated Al-induced expression of the transgenic *AtMATE*

and *AtAMLT1* genes, respectively, and the level of Al-induced transgenic *AtMATE* transcripts was approximately 12-fold higher than that of Al-induced transgenic *AtALMT1* transcripts (Figure 1). These results indicate that the 2.1 kb transgenic promoters retained the necessary *cis*-elements for Al-inducible gene expression, and that the transgenic *AtALMT1* promoter is much stronger than the *AtMATE* promoter. However, the levels of Al-induced transgenic *AtMATE* and *AtALMT1* transcripts in the *AtALMT1$_P$::AtMATE* and *AtMATE$_P$::AtALMT1* lines, respectively, were lower than found in wild-type plants (Figure 1). These results suggest that either some *cis*-elements necessary for enhancing expression of the transgenes are located in regions other than the 2.1 kb transgenic promoters, and/or the positions of the transgenes in the genome affect their expression (position effects).

Transformation of the *AtALMT1$_P$::AtMATE* construct into the *double-KO/KD* background greatly enhanced its Al resistance; the Al resistance (RRG%) of the *AtALMT1$_P$::AtMATE* line increased 4.4-fold compared with that in the *double-KO/KD* line to a level comparable with that of wild-type plants (Figure 4). However, no significant differences in Al resistance between the *AtMATE$_P$::AtALMT1* line and the *double-KO/KD* line were found, indicating that the weak promoter in combination with efflux of the weaker Al^{3+} -chelating organic acid, malate, which is more diffusely localized to the mature root, did not improve Al resistance in the Arabidopsis mutant (Figure 4).

In the wild-type, Al stress activated the release of a large amount of malate (2.05 $\pm$ 0.09 nmol per plant per day) from root apices and a smaller amount of citrate (0.52 $\pm$ 0.01 nmol per plant per day) from the mature root regions (Figures 2 and 3; Table 1). The *AtALMT1-KO* mutant showed a 91% decrease in Al-activated root malate exudation and a 66% decrease in Al resistance (RRG%) (Table 1), and the *AtMATE-KD* line showed a 54% decrease in Al-activated root citrate exudation and a 7% decrease in Al resistance (Table 1). Compared to the wild-type, the rates of Al-activated root citrate and malate exudation in the *double-KO/KD* line were suppressed by 60% and 90%, respectively (Table 1). As a result, the double mutant was more sensitive to Al than the single *AtALMT1-KO* mutant (Figure 4 and Table 1).

Although the level of Al resistance was similar between the wild-type and the *AtALMT1$_P$::AtMATE* lines, the rate of Al-activated root malate exudation in wild-type was 3.5-fold higher than the rate of Al-activated root citrate efflux in the *AtALMT1$_P$::AtMATE* line (Table 1). Thus, using the stronger *AtALMT1* promoter to drive expression of the citrate efflux transporter results in much less carbon being lost to achieve the same level of Al resistance. In fact, it may be possible that the major reason for the better root growth in response to Al in the *AtALMT1$_P$::AtMATE* line may be the reduced carbon cost, if this cost actually results in somewhat lower root growth in wild-type plants.

There is a lack of quantitative information on the carbon cost to the plant imposed by root exudation of organic acids. The best-studied system for this is the proteoid (cluster) roots of species such as *Lupinus alba*. In response to P deficiency, the root systems of lupins and related species produce densely packed lateral roots (Johnson *et al.*, 1996a). Associated with this morphological alteration is stimulation of Krebs cycle enzymes associated with citrate and malate synthesis, resulting in large increases in root citrate and malate content and exudation of citrate, and to a lesser degree malate, from the roots (Johnson *et al.*, 1994, 1996b). The exuded citrate and malate chelate Fe and Al oxides on soil particle surfaces, releasing P fixed at these sites and making it available for root uptake. It has been estimated that more than 20% of the total fixed carbon in these lupin plants is released primarily as citrate in response to low P status (Dinkelaker *et al.*, 1989; Johnson *et al.*, 1996b). There has been much less work to quantify the proportion of total C in the plant that is released from cereal roots. Barber and Martin (1976) studied barley and wheat plants grown in either sterile or non-sterile soil for a 3-week period in constant environment chambers with air containing ^{14}C-labeled CO_2. They then quantified amount of ^{14}C-labeled soluble C released by the roots into the soil, and estimated that between 7 and 12% of the dry matter during the 3-week period was released as soluble C into sterile soil, increasing to 18–25% for roots grown in non-sterile soil. Thus, even plant roots of agronomic crop species release a significant proportion of their total C as soluble C from roots, and the soluble C released from roots is primarily organic acids, sugars and amino acids (Jones and Darrah, 1995).

Although there is little information in the literature regarding how much soluble C must be excreted from roots before plant growth is negatively affected, in our own studies with transgenic Arabidopsis plants over-expressing *AtALMT1* or *AtMATE*, the extent of root citrate and malate exudation required to confer Arabidopsis Al resistance appears to impose a measurable cost on the growth of the plant. In transgenic Arabidopsis lines expressing *AtALMT1* or *AtMATE*, there is higher constitutive citrate and malate release in the absence of Al in the transgenic lines compared to wild-type plants. The magnitude of this higher malate or citrate release in the transgenic plants is comparable to the increase in the rate of root citrate and malate release in response to Al in wild-type plants. In wild-type plants, the much lower constitutive malate and citrate release is increased 6–10-fold for malate exudation and 2–4-fold for citrate exudation in response to Al (Magalhaes *et al.*, 2007). This increase in citrate and malate release in response to Al in wild-type is due to a combination of Al-induced increases in *AtALMT1* and *AtMATE* gene expression, and Al-activated citrate or malate transport at the level of protein function. When root growth under control conditions (without Al) in the transgenic plants was compared with that in

wild-type plants, we observed a 20–30% reduction in root growth in the transgenic lines. The finding of a moderate decrease in root growth in transgenic lines that exhibit a level of malate and citrate exudation in the absence of Al that is similar to the rates of malate/citrate exudation in wild-type plants in the presence of Al is consistent with this level of C loss from roots due to organic acid exudation that exerts a measurable C cost to the plant.

Additionally, the role of microbial decomposition of root-released malate and citrate in the soil needs to be considered with regard to possibly decreasing the effectiveness of this Al resistance mechanism and thus increasing the carbon cost. We have previously shown that malate exuded from roots into soil is readily degraded by soil microbes (Jones *et al.*, 1996b). This also occurs for citrate released from roots into the soil (Jones and Edwards, 1998). Thus, it has been speculated that soil microbial decomposition of organic acids released from roots may have a negative impact on this Al resistance mechanism (Ryan *et al.*, 2001). However, the potential negative impact of microbial degradation on this Al resistance mechanism may be ameliorated by the following processes. First, citrate can be sorbed onto soil surfaces relatively strongly (Jones *et al.*, 1996a), as can malate, albeit less strongly (Jones *et al.*, 1996b). Geelhoed *et al.* (1999) showed that, when citrate is sorbed onto soil surfaces, it is more resistant to microbial degradation, and thus sorbed citrate could represent a 'protected' pool of citrate in the soil in equilibrium with soluble citrate that chelates Al^{3+}. Second, citrate and malate chelation of Al^{3+} only needs to occur right at the surface of the root apex (in the unstirred layer just a few microns from the root), and, as the root grows through the soil fairly rapidly and new organic acid is being exuded as the root grows, these processes can keep ahead of microbial degradation.

When comparing the Al resistance and root organic acid exudation profiles of the *AtALMT1-KO* and *AtALMT1*$_P$*::AtMATE* lines, we found that the levels of Al-activated malate exudation were negligible in both lines compared to wild-type plants. Furthermore, although the levels of Al-activated citrate exudation were similar between these two lines,the Al resistance of the *AtALMT1*$_P$*::AtMATE* line was much higher than that of the *AtALMT1-KO* line (Figure 4 and Table 1). This can be explained by the fact that, in the *AtALMT1-KO* line, Al-activated citrate efflux was driven by the native *AtMATE* promoter, whose expression is diffusely distributed along the mature regions of the roots (Figure 2), while in the *AtALMT1*$_P$*::AtMATE* line, the Al-activated citrate exudation is primarily driven by the transgenic *AtALMT1* promoter, whose expression is more discretely localized to the root apex, the primary site of Al toxicity (Figure 2). Thus, although these two lines released similar amounts of citrate upon Al stress (Table 1, $P = 0.14$), the localization of citrate release in the roots of each line contributed to the significantly different levels of Al resistance.

In conclusion, the results presented here demonstrate that the nature of the organic acids involved in the Al detoxification process affect the efficiency of Al resistance. Furthermore, a previously unexplored aspect of this mechanism of Al resistance is the carbon cost associated with citrate and malate efflux. Plants have evolved mechanisms to reduce unnecessary cost. Thus malate and citrate release primarily occur only when Al stress occurs, to minimize organic acid efflux in the absence of Al. Also, many plant species restrict the organic acid released to the region of the root that most needs to be protected from Al toxicity, i.e. the root apex. Our results suggest that science can improve upon what plants normally do by employing a combination of a strong Al-inducible promoter that is expressed in the most critical root region with efflux of the organic acid that is the most effective Al^{3+} chelator, to generate genotypes with enhanced Al resistance and carbon-use efficiency. These findings may be useful in future generation of highly carbon use-efficient Al-tolerant crop cultivars through genetic engineering and possibly molecular breeding.

EXPERIMENTAL PROCEDURES

Plant materials

The Arabidopsis T-DNA insertion mutants *AtALMT1-KO* (SALK_009629) and *AtMATE-KD* (SALK_081671) were acquired from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). The homozygous double *AtALMT1-KO/AtMATE-KD* mutant was generated as described by Liu *et al.* (2009).

Cloning promoter-swap constructs and generation of promoter-swapped transgenic plants

The *AtALMT1* and *AtMATE* promoters were amplified by PCR from wild-type (Col-0) genomic DNA as 2.1 kb fragments upstream of their translational start codon. The forward and reverse primers for the *AtALMT1* promoter were 5′-AATAGGTACCGGTGGTGGTTTAG TAGTGAT-3′ (underlined bases indicate the *Kpn*I site) and 5′-AATACCCGGGACACCTTTTGATGGTCACTCAGCT-3′ (underlined bases indicate the *Xma*I site) respectively, and the forward and reverse primers for the *AtMATE* promoter were 5′-AATAGAG CTCCCAGCTTATGTGAACTACCA-3′ (underlined bases indicate the *Sac*I site) and 5′-AATACCCGGGCTTCAGACATCATTAAGAGATGT TACTG-3′ (underlined bases indicate the *Xma*I site), respectively. Genomic fragments of 2.3 and 2.5 kb spanning the complete coding sequence for the *AtALMT1* and *AtMATE* genes, respectively, were obtained by PCR using forward primers 5′-AATACCCGGG ATGGAGAAAGTGAGAGAGAT-3′ (underlined bases indicate the *Xma*I site) and 5′-AATACCCGGGATGATGTCTGAAGATGGCTAC-3′ (underlined bases indicate the *Xma*I site) for *AtALMT* and *AtMATE*, respectively, and reverse primers 5′-AATAGGTGACCTTACTGA AGATGCCCATTAC-3′ (underlined bases indicate the *Bst*EII site) and 5′-AATAGGTGACCTCAGCTCCTAAGAAAAGACCA-3′ (underlined bases indicate the *Bst*EII site) for *AtALMT* and *AtMATE*, respectively.

To generate the *AtALMT1*$_P$*::AtMATE* promoter-swap construct, the amplified *AtALMT1* promoter and *AtMATE* coding sequence fragments were digested with *Kpn*I/*Xma*I and *Xma*I/*Bst*EII,

respectively, followed by three-way ligation of the digested PCR fragments with the pCAMBIA1305.1 vector (http://www.cambia.org/daisy/cambialabs/3703.html) cut with *Kpn*I/*Bst*EII. Similarly, the *AtMATE*$_P$*::AtALMT1* promoter-swap construct was generated by three-way ligation of the *AtMATE* promoter fragment, the *AtALMT1* coding sequence fragment and the pCAMBIA1305.1 vector. All constructs were confirmed by sequencing.

The promoter-swap constructs were individually transformed into the *Agrobacterium tumefaciens* strain GV3101 (Invitrogen, http://www.invitrogen.com/) by electroporation, followed by transformation into the *double-KO/KD* mutant plants.

Approximately 100 independent T_2 lines transformed with either of the promoter-swap constructs were tested for root growth in hydroponics as described by Hoekenga *et al.* (2006). Representative homozygous T_3 transgenic lines were used for subsequent experiments.

GUS staining assays

The 2.1 kb *AtALMT1* and *AtMATE* promoters were PCR-amplified as described above. The promoter fragments were then cut with *Pst*I and *Nco*I and ligated into the *Pst*I/*Nco*I double-digested pCAMBIA1305.1 vector. The resulting *AtALMT1*$_P$*::GUS* and *AtMATE*$_P$*::GUS* constructs were individually transformed into *Agrobacterium tumefaciens* strain GV3101, followed by transformation into the wild-type (Col-0) as described above.

The forward and reverse primers for the *AtALMT1* promoter were: 5′-aatactgcagGGTGGTGGTTTAGTAGTGAT-3′ (lower-case letters indicate the adaptor of the primer, underlined bases indicate the *Pst*I site) and 5′-aataccatggACACCTTTTGATGGTCACTCAGCT-3′ (underlined bases indicate the *Nco*I site), respectively. The forward and reverse primers for the *AtMATE1* promoter were: 5′-aatactgcaggcTTAGAGGTGCATATCGGA-3′ (underlined bases indicate the *Pst1* site) and 5′-aataccatggCTTCAGACATCATTAAGAGATGTTACTG-3′ (underlined bases indicate the *Nco1* site), respectively.

RNA isolation and quantitative real-time RT-PCR

Plants were germinated and grown in hydroponic culture as described by Hoekenga *et al.* (2006). On the 6th day, seedlings were transferred to new hydroponic growth solutions supplemented with or without 1.5 μM Al^{3+} activity for 1 day. Then, root samples were collected and immediately frozen in liquid nitrogen. Total RNA was extracted as described by Liu *et al.* (2009) except that random primers were used instead of the oligo(dT)$_{16}$ primer.

Quantitative real-time RT-PCR was performed as described by Liu *et al.* (2009), except that, for direct comparison of the *AtMATE* and *AtALMT1* gene expression, the relative expression levels of both genes were normalized against a common calibrator in a single real-time PCR experiment.

Analysis of organic acid exudation from roots

Arabidopsis lines were grown in hydroponic culture (pH 4.2) for 6 days, followed by 1 day of treatment with or without Al as described by Liu *et al.* (2009). Seedlings were then transferred to exudation solution (pH 4.2) supplemented with or without Al for 1 day, followed by measurement of organic acid content as described by Liu *et al.* (2009).

Root growth and measurement

Root growth experiments were performed in hydroponic culture with or without Al treatment as described by Hoekenga *et al.* (2006). Root length measurements were performed after 6 days of growth in hydroponic culture.

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