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Journal research area: Environmental Stress and Adaptation
Characterization of complex regulation of AtALMT1 expression in response to phytohormones and other inducers

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One sentence summary: Transcription of the gene encoding a malate transporter, AtALMT1, induced by aluminum stress, some phytohormones (auxin, abscisic acid) and some chemical inducers (H2O2, low pH, flg22), which could account for its contribution to pleiotropic traits.
Footnotes:

Financial support for this work was provided by the Japan Society for the Promotion of Science.

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Abstract

In Arabidopsis (*Arabidopsis thaliana*), malate released into the rhizosphere has various roles such as detoxifying rhizotoxic aluminum (Al) and recruiting beneficial rhizobacteria that induce plant immunity. *ALUMINUM ACTIVATED MALATE TRANSPORTER 1* (*AtALMT1*) is a critical gene in these responses, but its regulatory mechanisms remain unclear. To explore the mechanism of the multiple responses of *AtALMT1*, we profiled its expression patterns in wild-type, in transgenic plants harboring various deleted promoter constructs, and in mutants with defects in signal transduction in response to various inducers. *AtALMT1* transcription was clearly induced by indole-3-acetic acid (IAA), abscisic acid (ABA), low pH, and hydrogen peroxide, indicating that it was able to respond to multiple signals, while it was not induced by methyl jasmonate and salicylic acid. The IAA-signaling double mutant *nonphototropic hypocotyls 4-1; auxin responsive factor 19-1* and the ABA-signaling mutant *aba insensitive 1-1* did not respond to auxin and ABA, respectively, but both showed an Al-response comparable to that of wild-type. A synthetic microbe-associated molecular pattern peptide, flagellin22 (flg22), induced *AtALMT1* transcription, but did not induce transcriptions of IAA- and ABA-responsive biomarker genes, indicating that both Al- and flg22-responses of *AtALMT1* were independent of IAA and ABA signaling. An *in planta* GUS (β-glucuronidase) reporter assay identified that the ABA response was regulated by a region upstream (-317 bp) from the first ATG codon, but other stress responses may share critical regulatory element(s) located between -292 and -317 bp. These results illustrate the complex regulation of *AtALMT1* expression during adaptation to abiotic and biotic stresses.
Introduction

Organic acid (OA) excretion/uptake plays various roles in many plant tissues. For example, it regulates stomatal closure in the guard cells (Vahisalu et al., 2008), and in root tissues, it plays roles in nutrient uptake [e.g. iron (Fe) (Durrett et al., 2007) and phosphorus (P) (Neumann et al., 1999)] and in detoxification of toxic ions [e.g. aluminum (Al) (Pellet et al., 1995) and copper (Cu) (Murphy et al., 1999)] in the rhizosphere. Excretion of OA from the roots can also recruit beneficial bacteria that enhance defense mechanisms through induced systemic resistance (Rudrappa et al., 2008; Lakshmanan et al., 2012). These events are regulated in a complex manner by a system involving OA transporters and OA synthesis (Delhaize et al., 1993; Lopez-Bucio et al., 2000), at both transcriptional and post-translational levels [e.g. the SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) response to CO₂] (Negi et al., 2008). It is important to explore these mechanisms at the molecular level to understand the complex roles of OA acid transport in biological processes, and its contribution to pleiotropic traits.

Arabidopsis thaliana ALUMINUM ACTIVATED MALATE TRANSPORTER 1 (AtALMT1) (Hoekenga et al., 2006) was first identified as an ortholog of TaALMT1, which encodes a root-localized malate transporter in wheat (Triticum aestivum). This protein plays a critical role in Al tolerance by detoxifying Al rhizotoxicity (Sasaki et al., 2004). Excretion of malate via AtALMT1 is induced by infection of aerial tissues by pathogenic bacteria (Rudrappa et al., 2008). In such cases, malate recruits beneficial bacteria to form a biofilm at the root surface, activating induced systemic reactions to protect the plant against bacterial infection. Thus, AtALMT1 has pleiotropic effects in both abiotic (i.e. Al-resistance) and biotic (i.e. beneficial bacteria recruitment) stress resistance through its role in exuding malate from the roots. Its encoding gene, AtALMT1, is a good target for studies aimed at understanding the complex nature of regulation related to the multiple roles of OA transport.

The AtALMT1 protein transports malate into the rhizosphere, where it detoxifies Al³⁺ by converting it to the much less phytotoxic Al–malate chelating complex (Hoekenga et al., 2006). A knock-out mutation of the gene resulted in hyper-sensitivity to Al, suggesting that this protein is essential for survival of Arabidopsis in acid soils. In our previous study, we showed that malate excretion as a
mechanism of Al tolerance is likely to be optimized to minimize carbon loss via both transcriptional and post-translational regulation (Kobayashi et al., 2007). In that study, \textit{AtALMT1} expression was limited to the root tip, the tissue most sensitive to Al rhizotoxicity, under Al treatment, but it was barely expressed in response to other rhizotoxic ions. In addition, Al-activation was identified in the activation process of malate transport. Pharmacological analyses suggested that both processes involve protein phosphorylation/di-phosphorylation. Although the mechanisms of protein activation have not been clarified yet, bacterial infection of aerial tissues induced \textit{AtALMT1} transcription in the roots (Rudrappa et al., 2008). Treatment of aerial tissues with the elicitors coronatine and microbe-associated molecular patterns flg22 (QRLSTGSRINSKAADGIA) (Felix et al., 1999) also induced transcription of \textit{AtALMT1} in the roots (Lakshmanan et al., 2012). This finding indicated that the transcriptional regulation of \textit{AtALMT1} responds to multiple signals.

Multi-signal regulation has been reported for some transporters such as the inorganic anions transporter SLAC1, which regulates stomatal closure (Vahisalu et al., 2008). The SLAC1 malate transporter plays important roles in photosynthesis and the drought response (Geiger et al., 2009; Kusumi et al., 2012), both of which are regulated by complex systems that are responsive to multiple signal inducers such as abscisic acid (ABA) and reactive oxygen species (ROS). Some of the signaling pathways in those systems involve protein phosphorylation, such as type2C protein phosphatase (PP2C)/SNF1-related protein kinase 2 (SnRK2) in the ABA response (Umezawa et al., 2010). The multiple biological roles of \textit{AtALMT1} suggest that it is regulated by such a complex system. In the present study, we profiled \textit{AtALMT1} expression in response to various phytohormones and other chemicals. The aim of this study was to explore the complex transcriptional regulation of this gene, which encodes a protein that plays roles in various stress responses.
RESULTS

Profiling of \textit{ALMT1} transcription in response to rhizotoxins and chemical treatments

\emph{AtALMT1} transcription was profiled using transgenic plants carrying a \emph{AtALMT1\textit{promoter}}::GUS (-1110 from first ATG; the full promoter region) fusion construct after the short-term (6 h) treatments with phytohormones and chemical inducers [i.e. salicylic acid (SA) and methyl jasmonate (MeJA)]. Other than benzylaminopurine (BAP), all phytohormones and chemical inducers resulted in GUS activity (visualized as blue staining) in the root apices, but there were differences in the density and tissue localization of the staining among treatments (Fig. 1A). The indole-3-acetic acid- (IAA-) treated roots were stained in the elongation zone and vascular tissues in the upper parts. ABA treatment induced dense staining in the elongation zone, while the density depended on the ABA concentration. Roots treated with gibberellic acid (GA\textsubscript{3}) showed blue staining throughout the whole root apex, although the staining was much lighter than that in the IAA- and ABA-treated roots. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) resulted in slight staining at the root apex, which was similar to the root tips treated with SA and MeJA, which are major inducers of the biotic stress response (Fig 1A). It indicated that some of phytohormones can induce \emph{AtALMT1} expression within a short-term, but IAA and ABA have stronger activity to induce expression. Among the abiotic stressors, Al, low pH, and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) treatments induced stronger GUS staining than did by NaCl, Cu, and Cd treatments. Transcript analysis confirmed that there were higher transcript levels of \emph{AtALMT1} in densely stained treatments than in lightly stained treatments (Fig. 1B).

There were differences in GUS expression patterns in root tissues among the various treatments (Fig 1A). Only Al and H\textsubscript{2}O\textsubscript{2} induced GUS expression in the whole part of the root tip, including in root cap cells. Al, low pH, and IAA treatments induced GUS expression in the inner and upper parts of roots. Roots treated with ABA showed dense blue staining that was limited to the elongation zone and the meristem. These results confirmed that \emph{AtALMT1} transcription is induced by various signal inducers, while the position in the root tissues and the expression level are variable among the
AtALMT1 expression in IAA- and ABA-signaling mutants

To characterize the induction of AtALMT1 mediated by IAA- and ABA-signaling pathways, we analyzed transcript levels of AtALMT1 in an IAA-signaling double mutant nonphototropic hypocotyls 4-1; auxin responsive factor 19-1 (nph4-1arf19-1) (Okushima et al., 2005; Okushima et al., 2007) and an ABA-signaling mutant aba insensitive 1-1 (abi1-1) (Leung et al., 1994; Meyer et al., 1994). Both mutants defect in the ability to activate transcription responded to ABA and IAA (see discussion). Transcript levels of AtALMT1 in the IAA- and the ABA-signaling mutants were analyzed by real time RT-PCR. After 6 h IAA treatment, AtALMT1 transcript levels were higher in wild-type than in nph4-1arf19-1 (Fig. 2A). Compared with that in wild-type, ABA-induced AtALMT1 transcription was significantly decreased in the ABA-signaling mutant abi1-1 (Fig. 2A). The fact that ABA- and IAA-induced AtALMT1 transcription was decreased in these mutants suggested that IAA and ABA signaling are involved in the activation of AtALMT1 transcription.

To evaluate cross-talk among these phytohormones and Al-inducible AtALMT1 expression, the transcript levels of AtALMT1 and IAA- and ABA-responsive genes were compared between the mutants and wild-type parental accessions, and between the control and an Al treatment in wild-type. In the Al treatment, transcript levels of AtALMT1 in the mutants were comparable to those in the parental accessions (Fig. 2A), suggesting that AtALMT1 transcription in response to Al treatment was not solely regulated by ABA and IAA. The transcript levels of some IAA-responsive and ABA-responsive genes were far lower in the Al treatment than in the IAA and ABA treatments (Fig. 3A, B). Such genes included the IAA-responsive genes ARF19, LATERAL ORGAN BOUNDARIES-DOMAIN 16 (LBD16), and GH3.5, and the ABA-responsive genes ABI1, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) and RESPONSIVE TO DESSICATION 29B (RD29B). Primary root growth of the IAA- and ABA-signaling mutants was comparable to that of the wild-type parental accessions in Al-toxic solution (Fig. 2B). These findings suggested that ABA and IAA signaling were not required to maintain the Al tolerance of the primary root via AtALMT1 expression.
Taken together, these results suggested that Al-responsive \textit{AtALMT1} transcription, at least after a short-term (< 6 h) Al treatment, is regulated by a pathway other than the ABA and IAA signaling pathways which may not have a critical role in Al tolerance (as measured by primary root growth) mediated by malate excretion.

**Promoter analysis to characterize \textit{AtALMT1} responses to inducers**

To characterize in detail the regulation of \textit{AtALMT1} transcription by Al and other inducers, we analyzed GUS expression in \textit{AtALMT1}::\textit{GUS} transgenic lines carrying different lengths of the 5’ region of the \textit{AtALMT1} promoter. Using the full promoter construct (-1110bp::\textit{GUS}) as the reference, we compared GUS expressions driven by deleted promoter regions [-317 bp (-317bp::\textit{GUS}) and -292 bp (-292bp::\textit{GUS})] in response to various signal inducers. GUS expression was lower in the -292bp-GUS line than in the -1110bp::\textit{GUS} line in response to Al, low pH, IAA, ABA, and H$_2$O$_2$. GUS expressions in response to the various inducers were similar between the -1110bp::\textit{GUS} line and the -317bp::\textit{GUS} line, except when ABA was used as the signal inducer, where the GUS expression in the -317bp::\textit{GUS} line was markedly lower than that in the -1110bp::\textit{GUS} line (Fig. 4). This result indicated that the major ABA-regulating element was localized further upstream of the \textit{AtALMT1} promoter than -317 bp. To find \textit{cis}-acting elements in the promoter, we searched the \textit{AtALMT1} promoter sequence between -317 and -1110 bp using the PLACE and PlantCARE databases (Higo et al., 1999; Lescot et al., 2002). Both databases predicted \textit{cis}-acting elements related to drought-inducible elements, CAAT-box (CCAAT) and MBS (CAACTG), in that region (Supplemental Table S1). In the Al, low pH, IAA, and H$_2$O$_2$ treatments, there were lower GUS expressions in the -292bp::\textit{GUS} line than in the -1110bp::\textit{GUS} line and the -317bp::\textit{GUS} line (Fig. 4). This indicated that the critical regulatory element(s) common to those treatments would be in the region from -292 to -317 bp, while no consensus sequence was predicted by the same databases.

**Activation of \textit{AtALMT1} in roots of \textit{Arabidopsis} by flg22**

Some bacteria induce \textit{AtALMT1} expression through shoot–root signaling (Lakshmanan et al., 2012); however, the direct activation of \textit{AtALMT1} transcription in the roots had not been analyzed. To determine whether the \textit{AtALMT1} expression could be induced by
bacterial challenge to the roots, we treated the roots with flagellin22 (flg22), a biological inducer of the plant-bacterial response, and performed histochemical analysis and expression analysis of GUS expression from AtALMT1promoter::GUS fusion constructs. In the -1110bp::GUS line, GUS was expressed throughout the whole root tip in response to flg22, similar to the pattern of GUS transcription in response to Al treatment (Fig. 5A) Compared with that in wild-type, flg22-induced AtALMT1 transcription was significantly decreased in the flg22-signaling mutant flagellin-sensitive 2 (fls2), which defect in the flg22 receptor for activating transcription in response to flg22 (Fig. 5B). The fls2 showed AtALMT1 expression by Al treatment, which was comparable to Col-0 (Fig. 5B). It suggests that flg22 signaling is involved in the activation of AtALMT1 expression, but not affected Al-responsive induction. In addition, real-time RT-PCR analyses showed that flg22-induced GUS transcription in the -317bp::GUS line was comparable to that in the -1110bp::GUS line. However, the fold-change in GUS transcript levels was significantly reduced in the -292bp::GUS line compared with those in the other two lines (Fig. 5C), the same pattern as that observed in response to other inducers. Using inducible biomarker genes, we evaluated IAA- and ABA-signaling in flg22-induced AtALMT1 expression. All of the IAA-responsive genes (ARF19, LBD16 and GH3.5) and ABA-responsive genes (ABI1, NCED3 and RD29B) did not respond to flg22 treatment of the roots (Fig. 6), suggesting that the short-term response of AtALMT1 expression induced by flg22 differs from that induced by IAA and ABA, and it is independent from Al response.

Transcript profiling of AtMATE and ALS3

We analyzed the transcript levels of some other Al-tolerance genes in response to inducers that activate AtALMT1 expression. AtMATE encodes an Al-responsive citrate transporter that belongs to multi-drug and toxic compound extrusion (MATE) efflux family protein (Liu et al., 2009), while ALUMINUM SENSITIVE 3 (ALS3) (Larsen et al., 2005) encodes a putative homolog of rice (Oryza sativa) STAR2 (for SENSITIVE TO ALUMINUM RHIZOTOXICITY 2), which encodes a subunit of a UDP-glucose transporter in rice (Huang et al., 2009). The transcription pattern of AtMATE was similar to that of AtALMT1. Although there were lower transcript levels of AtMATE (Fig 7A) than AtALMT1 (Fig 1B), all of the inducers of AtALMT1 transcription also induced
AtMATE transcription (Fig 7A). However, only the Al treatment was able to induce ALS3 transcription (Fig 7B). This indicated that similar complex regulations of transcription would be shared by some other Al tolerance genes.

**DISCUSSION**

In this study, we identified that some signal inducers can trigger AtALMT1 expression within the short-term (< 6 h). Phytohormones (IAA and ABA) and other inducers (H₂O₂, low pH, and flg22 peptide) (Fig. 5) all induced AtALMT1 transcription. This finding showed that AtALMT1 is regulated by multiple stressors that result in production of these signal inducers. Our analyses using IAA- and ABA-signaling mutants, nph4-1arf19-1 and abi1-1, respectively, identified that AtALMT1 expression in response to each inducer is mediated by particular pathways linked to their genotypes. In the nph4-1arf19-1 double mutant, IAA signaling regulated by ARF-Aux/IAA proteins and SCF^{TIR1/AFB}-mediated Aux/IAA proteolysis is blocked (Quint and Gray, 2006). The ABA-insensitive mutant abi1-1 shows a block in ABA signaling through the PP2C/SnRK2 pathway (Umezawa et al., 2009). In our ongoing research, we have not found any experimental evidence that ABA- and IAA-signaling are directly involved in the short-term activation of AtALMT1 expression by Al. Al treatments induced AtALMT1 transcription in these mutants (Fig. 2A), but did not induce ABA- and IAA-inducible biomarker genes (Fig. 3). This finding confirmed that Al-activation of AtALMT1 transcription is not simply regulated by one of these phytohormones.

ABA- and IAA-signaling pathways did not directly contribute to the short-term expression of AtALMT1 induced by flg22 (Figs. 2 and 6). In addition, SA and MeJA, which are major inducers of the biotic stress response, could not induce AtALMT1 transcription (Fig. 1). This finding showed that these signal inducers do not directly contribute to AtALMT1 expression in response to flg22, similar to the response to Al. However, H₂O₂ and low pH stress could be involved in flg22- and Al-activation of AtALMT1 transcription. A transcriptome analysis showed that gene expression patterns during the bacterial response resembled those under low-pH stress in Arabidopsis (Lager et al., 2010), while the microbe-associated molecular patterns response results in H₂O₂ production, which is coupled with flg22 recognition (Torres et al., 2006). In this study, H₂O₂ and low pH activated AtALMT1 transcription. Consistent with this, Al
treatments trigger H₂O₂ accumulation in the root tip (Kobayashi et al., 2005) and decrease cellular pH (Moseyko and Feldman, 2001). Further research is required to clarify the interactions among these factors in Al- and flg22-responsive AtALMT1 transcription.

Although the IAA- and ABA-signaling mutants did not show enhanced Al sensitivity in our experimental conditions (Fig. 2B) the responses involving IAA and ABA might have roles in Al tolerance in the natural environment. We used the length of the primary roots in hydroponic culture as the indicator of Al tolerance. The architecture of whole roots affects water acquisition and drought resistance in plants in the natural environment (Xiong et al., 2006). For example, IAA accumulation is an essential step for lateral root development, and thus nph4-1arf19-1 cannot form lateral roots (Okushima et al., 2007). We observed that the transgenic line carrying AtALMT1promoter::GFP expressed GFP at that site without any exogenous inducer treatments (Supplemental Fig. S1). Inducible expression mediated by IAA could result in accumulation of AtALMT1 proteins in the tip of lateral roots before they come into contact with rhizotoxic Al. Dehydrated roots in dried soils produce ABA, which can induce stomatal closure via long-distance signaling, thus increasing drought tolerance (Zhang et al., 2006). We observed that ABA treatment induced AtALMT1 transcription. In a drought situation, it is reasonable to expect that AtALMT1 transcription induced by ABA (i.e. like that in the root tip in Fig. 1A) could protect the roots from Al rhizotoxicity, which, if unchecked, could further exacerbate drought sensitivity by inhibiting root development. In addition, ABA accumulation in response to Al occurs in some crop plants such as barley and soybean (Kasai et al., 1993; Hou et al., 2010). Together, these findings suggest that responses involving IAA and ABA play a role in Al tolerance in Arabidopsis in the natural soil environment.

Lakshmanan et al. (2012) reported that application of flg22 to the aerial parts induced AtALMT1 expression in the roots, which is concomitant with ABA accumulation and stomatal closure in the shoots. This suggested that AtALMT1 expression mediated by ABA signaling would have a role in biotic stress resistance, in particular, in the shoot–root interaction. On the other hand, in the present study, flg22 treatment of the roots induced AtALMT1 transcription within 6 h (Fig. 5). This pattern of AtALMT1 expression could be reasonable to recruit beneficial rhizobacteria similar to
Bacillus subtilis FB17 (Rudrappa et al., 2008). FB17 was attracted to the root surface by malate-chemotaxis, and then it induced systemic resistance to protect aerial tissues from infection by pathogenic bacteria. AtALMT1-mediated malate excretion is a critical step for attraction of the beneficial bacteria to the root surface. Interestingly, FB17-derived elicitors (i.e. autoclaved cells) did not induce AtALMT1, possibly because of difference of its flagella that is not highly conserved the structure of flg22 (Lakshmanan et al., 2012). The significant induction of AtALMT1 by flg22 to the roots suggests that other members of the rhizobacterial community conserving flg22 structure may support the recruitment of beneficial bacteria that require AtALMT1-dependent malate excretion.

Promoter-GUS reporter analyses revealed some of the mechanisms underlying the complex regulation of AtALMT1 expression in response to various inducers. These analyses indicated that an ABA-responsive element is located in the upstream (-317 bp to -1110 bp) promoter region, while the responsive element for other signal inducers (Fig. 4) was located closer to the start codon, between -317 bp and -292 bp. Several drought responsive cis-element motifs were predicted by the PlantCARE and PLACE databases (see Supplemental Table S1). A common region (-317 bp and -292 bp) contained a putative cis-acting site GGN(T/g/a/C)V(C/A/g)S(C/G) (Tsutsui et al., 2011) that binds the ALUMINUM RESISTANCE TRANSCRIPTION FACTOR 1 (ART1) zinc finger transcription factor (Yamaji et al., 2009), while we cannot show further experimental evidence confirming the regulatory element(s) in the region. AtALMT1 expression requires Arabidopsis thaliana SENSITIVE TO PROTON RHIZOTOXICITY 1 (AtSTOP1) (Iuchi et al., 2007), which contains sequences highly homologous to those of the zinc-finger domains of ART1. It is possible that an interaction with AtSTOP1 is critical for regulating AtALMT1 expression via the common region. This possibility requires further research, but AtMATE, which is regulated by AtSTOP1, showed multiple responses to the same signal inducers, suggesting that some of the genes regulated by AtSTOP1 may share similar complex regulatory mechanisms allowing responses to various stressors.

In conclusion, our results illustrated the complex patterns of AtALMT1 transcription in response to various signal inducers. However, for successful malate excretion, the AtALMT1 protein must be activated. This would be also regulated in a complex manner, and would be another critical factor in regulating malate excretion.
from the roots. Previous research on Al tolerance indicated that protein
phosphorylation/di-phosphorylation is involved in Al-activation of AtALMT1
(Kobayashi et al., 2007). Although this process is yet to be characterized in detail, the
increase in malate excretion induced by beneficial bacteria (Rudrappa et al., 2008)
would require a similar mechanism. Among the tested signal inducers, ABA and H2O2
induced malate excretion during 24 hours treatment (Supplemental Fig. S2). It suggests
that some of signal inducers may activate the AtALMT1 protein. Further research on the
activation process of protein is required to understand how plants regulate malate
excretion from the roots for acquiring stress resistance.

MATERIALS AND METHODS

Plant materials
Arabidopsis thaliana accessions Columbia (Col-0) and Landsberg erecta (Ler-0) were
obtained from the Riken BioResource Center (RIKEN BRC) and the Nottingham
Arabidopsis Stock Center (NASC). The IAA-signaling double mutant nph4-larf19-1
was kindly provided by Dr. Hidehiro Fukaki (Kobe University, Japan). An
ABA-signaling mutant abi1-1 was obtained from the RIKEN BRC. A flg22-signaling
mutant fls2 was kindly provided by Dr. Ken Shirasu (RIKEN Plant Science Center,
Japan). The mutant abi1-1 was in the Ler-0 background, and other lines/mutants were in
the Col-0 background.

Construction of transgenic lines for AtALMT1 promoter analysis
A series of transgenic plants carrying AtALMT1promoter::GUS (promoter-GUS) fusion
constructs in Col-0 was prepared by Agrobacterium tumefaciens-mediated
transformation using the following procedures. All PCRs were carried out using
PrimeSTAR max (Takara, Ohtsu, Japan) high fidelity taq polymerase and the sequences
of the amplified products were checked with an ABI PRISM 3100 Genetic Analyzer
using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster
City, CA, USA) according to the manufacturer’s protocol. Details of primer sequences
are shown in Supplemental table S2. A series of 5’ deleted promoters, -1110, -317 and
-292 bp from A of the start codon of AtALMT1, were amplified by PCR. Each sequence
was attached to the 5’ end of the ORF of the GUS gene connected to the nopaline synthase (NOS) terminator by overlapping extension PCR (Horton et al., 1989). The amplicon was digested with \( \textit{SfiI} \) and then introduced into the T-DNA region of pBE2113 (Mitsuhara et al., 1996), which contains a kanamycin resistance cassette as the selection marker. We used the hyper-virulent \textit{Agrobacterium} strain GV3101 to transform plant tissues using the floral-dip method (Clough and Bent, 1998). The transgenic seeds that were obtained were screened using kanamycin (50 g/ml in MS medium containing 1% agar) (Murashige and Skoog, 1962) as the selection marker.

**Growth conditions and root growth test**

\textit{Arabidopsis} seedlings were grown hydroponically according to the method of Kobayashi et al. (2007) in modified 1/50 MGRL solution (+Pi and pH 5.6 for transcript analyses; -P and pH 5.0 for growth tests to determine Al tolerance). Seedlings were grown at 24 ± 2°C under a 12-h light/12-h dark photoperiod, with light supplied at a photosynthetic photon flux density of 37 \( \mu \text{mol E m}^{-2} \text{s}^{-1} \). The culture solutions were renewed every 2 days. Seedlings were grown for 6 days for transcript analyses and GUS staining, and for 7 days for the root growth test to assess Al tolerance, otherwise it is mentioned. Root length was measured at the 7th day in control and Al-toxic solutions (4 \( \mu \text{M AlCl}_3 \)). Five of the 10 seedlings with the longest roots in Al-toxic solution were used to calculate mean values and SD to assess the Al tolerance of lines, as described previously (Kobayashi et al., 2007).

**Stress, phytohormone, and chemical treatments**

The roots of the seedlings pre-grown as described above were transferred to solutions containing various rhizotoxins (10 \( \mu \text{M AlCl}_3 \), 1.6 \( \mu \text{M CuSO}_4 \), 15 \( \mu \text{M CdCl}_2 \), or 50 mM NaCl) or chemicals [10 or 100 \( \mu \text{M IAA, ABA, ACC, BAP, GA}_3 \), methyl jasmonic acid (MJ), salicylic acid (SA), or 300 mM H\(_2\)O\(_2\)], and then incubated for 6 h. All rhizotoxic ions and chemicals were added to solution containing 1/50 MGRL nutrients with extra CaCl\(_2\) added to make a final concentration of 200 \( \mu \text{M}, \text{pH 5.5} \) (solution did not contain P).
**Histochemical analysis of reporter expression**

GUS staining was performed as described previously (Kobayashi et al., 2007) with -1110bp AtALMT1promoter::GUS transgenic lines. Briefly, the roots of seedlings were treated with rhizotoxins and chemicals as described above and stained with staining solution (1.0 mM X-glucuronide, 0.1 M sodium phosphate buffer [pH 7.0], 10 mM EDTA [pH 8.0], 0.5 mM potassium ferricyanide [pH 7.0], 0.5 mM potassium ferrocyanide [pH 7.0], 0.3% Triton X-100, and 20% methanol) for 15 min at 37°C. The samples were observed and photographed under an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 Camera system (Olympus).

**RNA extraction and expression analysis**

Total RNA was extracted as described by (Suzuki et al., 2003). First-strand cDNA was synthesized from total RNA with ReverTra Ace (Toyobo, Japan) and Oligo dT(18) primers. Quantitative real time RT-PCR was performed using gene-specific primer pairs (Supplemental Table S3) with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) and the Thermal Cycler Dice Real Time System II (Takara Bio Inc.) following the manufacturer’s instructions. Reactions were performed with three biological replicates for each sample. Transcript levels of the target gene were normalized to that of UBQ1.

**Promoter motif search**

PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE/single scan (http://www.dna.affrc.go.jp/PLACE/) software were used to scan for cis-elements in the AtALMT1 promoter sequence (-1110 bp from A of first ATG). The cis-elements identified using PlantCARE were evaluated by scanning at the PLACE database. Only cis-elements related to the drought response in Arabidopsis are shown in Supplemental table S3.

**SUPPLEMENTAL DATA**

**Supplemental Figure S1.** Histochemical analysis of AtALMT1 expression during lateral root formation in Arabidopsis
**Supplemental Figure S2.** Malate excretion from Arabidopsis roots after the treatments of Al and other signal inducers, inducing *AtALMT1* expression.

**Supplemental Table S1.** The list of *cis*-acting elements of the *AtALMT1* promoter.

**Supplemental Table S2.** The list of primer sequence for *AtALMT1Promoter::GUS* construct of the transgenic plant.

**Supplemental Table S3.** Sequences of primers used for quantitative RT-PCR.

**ACKNOWLEDGMENTS**

We are grateful for the technical support provided by Atsuko Iuchi, Fumie Mori, and Setsuko Kawamura of the RIKEN BioResource Center (RIKEN BRC). We thank the RIKEN BRC, the *Arabidopsis* Biological Resource Center, and the Nottingham *Arabidopsis* Stock Center for providing *Arabidopsis* seeds. We are grateful to Dr. Hidehiro Fukaki (Kobe University) and Dr. Ken Shirasu (RIKEN Plant Science Center) for providing *Arabidopsis* mutants.
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Figure 1. GUS expression and AtALMT1 transcription in roots of Arabidopsis under various treatments

Transgenic Col-0 harboring AtALMT1promoter::GUS (GUS reporter gene fused with full promoter sequence of -1110 bp from first ATG) and wild-type Col-0 were analyzed after exposure to various phytohormones (10 or 100 μM IAA, ABA, ACC, MeJA, SA, GA3, and BAP), hydrogen peroxide (300 μM H2O2, pH 5.5), or rhizotoxic ions (10 μM AlCl3, 1.6 μM CuSO4, 15 μM CdCl2, 50 mM NaCl at pH 5.5, and pH 4.7 for low pH). Activation of the AtALMT1 promoter was observed by GUS staining (blue). (A) GUS staining patterns in transgenic line. Bar = 20 μm. (B) AtALMT1 transcript levels in wild-type Col-0 in response to various inducers were determined by real time RT-PCR (using UBQ1 as an internal control). Values shown in (B) are means ± SD (n=3). Different letters indicate statistically significant difference (P < 0.05, Tukey’s test). Seedlings were incubated to various solutions for 6 hours before histochemical and transcripts analyses.

Figure 2. AtALMT1 expression and Al tolerance in ABA- and IAA-signaling mutants

(A) Roots of IAA-signaling mutant nph4-1arf19-1 (Col-0 background) and ABA-signaling mutant abi1-1 (Ler-0 background) were immersed in solutions containing 10 μM IAA and 100 μM ABA, or in Al (10 μM AlCl3) rhizotoxic solution, for 6 h. AtALMT1 transcripts were quantified by real time RT-PCR using UBQ1 as an internal control. Transcript levels in parental accessions, Col-0 and Ler-0, were also quantified. Asterisk indicates significant difference from Col-0 (P < 0.05, Student’s t-test). (B) Al tolerance of mutants and parental accessions, as determined by root growth assay (length of primary root after 7 d in 4 μM AlCl3 solution, pH 5.0). Values are mean ± SD (n = 5).

Figure 3. Transcription of IAA- and ABA-responsive genes under Al treatment

Transcript levels of IAA-responsive genes (ARF19, LBD16 and GH3.5; A) and ABA-responsive genes (ABI1, NCED3 and RD29B; B) in wild-type (Col-0) under Al and phytohormone treatments. Seedlings were grown for 6 days in control nutrient
solution, and then roots were incubated in 10 μM IAA, 100 μM ABA, or 10 μM AlCl₃ at pH 5.5 for 6 h. Transcript levels were analyzed by real time RT-PCR. Different letters indicate significant difference compared with control (P < 0.05, Tukey’s test).

**Figure 4.** GUS transcription in transgenic plants transformed with different AtALMT1 promoter deletion constructs in response to various signaling factors

Transcript levels of GUS in -1110, -317, and -292 bp AtALMT1 promoter:GUS transgenic plants were analyzed by real-time quantitative RT-PCR. Seedling roots were treated with 10 μM AlCl₃, 300 μM H₂O₂, 10 μM IAA, or 100 μM ABA at pH 5.5 and pH 4.7 (low pH) for 6 h. Transcript levels of GUS were normalized to that of UBQ1, and then fold-change (treatment/control) was calculated for each line. Relative fold-change (relative value of each line to mean fold-change of -1110 bp AtALMT1 promoter construct) was calculated. Values are mean ± SE (n = 3). Asterisks in each treatment represent significant difference compared with -1110 bp promoter construct (P < 0.05, Student’s t-test).

**Figure 5.** AtALMT1 transcription and GUS expression in roots treated with flg22

(A) Induction of AtALMT1 by flg22 in transgenic Arabidopsis carrying AtALMT1 promoter::GUS constructs. Images of GUS staining in root after 6 h treatment with flg22 (10 μM) and AlCl₃ (10 μM). Bar = 20 μm. (B) AtALMT1 transcript levels in roots of flg22 signaling mutant fls2 and wild-type (Col-0) incubated for 6 h with or without flg22 (10 μM) or AlCl₃ (10 μM). Values are mean ± SD (n = 3). Asterisk indicates significant difference from Col-0 transcript level (P < 0.05, Student’s t-test).

(C) Transcript levels of GUS in -1110, -317 and -292 bp AtALMT1 promoter:GUS transgenic plants were analyzed by real-time quantitative RT-PCR. Roots of seedlings were treated with 10 μM flg22 for 6 h. GUS transcript levels were normalized to that of UBQ1, and then fold-change (treatment/control) was calculated for each line. Relative fold-change (relative value of each line to mean fold-change of -1110 bp AtALMT1 promoter construct) was calculated. Values are mean ± SE (n = 3). Asterisks represent significant difference from -1110 bp promoter construct (P < 0.05, Student’s t-test).
Figure 6. Transcription of IAA- and ABA-responsive genes in response to flg22 treatment to roots

Transcript levels of IAA-responsive genes (ARF19, LBD16 and GH3.5) and ABA-responsive genes (ABI1, NCED3 and RD29B) in roots of wild-type (Col-0) treated with flg22. All seedlings were grown for 6 days in control nutrient solution, and then roots were incubated in 10 μM flg22 for 6 h. Transcript levels were analyzed by real time RT-PCR and normalized to that of UBQ1.

Figure 7. Transcription of AtMATE and ALS3 in Arabidopsis roots in response to various treatments

Transcript levels of Al-responsive genes (AtMATE; A and ALS3; B) were determined by real-time PCR in wild-type (Col-0) seedling roots after 6 h treatments with IAA (10 μM), ABA (100 μM), H2O2 (300 μM), AlCl3 (10 μM), flg22 (10 μM), or low pH (pH 4.7). Transcript levels were normalized to that of UBQ1, and relative expressions (treatment/control) were calculated. Values are mean ± SD (n = 3). Different letters indicate significant difference from transcript level in control (P < 0.05, Tukey’s test).
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