Characterization of the Complex Regulation of AtALMT1 Expression in Response to Phytohormones and Other Inducers

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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 TRANSPORTER1 (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 plants, in transgenic plants harboring various deleted promoter constructs, and in mutant plants 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 hypocotyls4-1; auxin-responsive factor19-1 and the ABA-signaling mutant abi insensitive1-1 did not respond to auxin and ABA, respectively, but both showed an Al response comparable to that of the wild type. A synthetic microbe-associated molecular pattern peptide, flagellin22 (flg22), induced AtALMT1 transcription but did not induce the transcription 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 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 the adaptation to abiotic and biotic stresses.

Organic acid (OA) excretion/uptake plays various roles in many plant tissues. For example, it regulates stomatal closure in guard cells (Vahisalu et al., 2008), and in root tissues, it plays roles in nutrient uptake (e.g. iron [Durrett et al., 2007] and phosphorus [Neumann et al., 1999]) and in the detoxification of toxic ions (e.g. aluminum [Al; Pellet et al., 1995] and copper [Murphy et al., 1999]) in the rhizosphere. Excretion of OA from the roots can also recruit beneficial bacteria that enhance defense mechanisms through its role in inducing 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; López-Bucio et al., 2000) at both the transcriptional and posttranslational levels (e.g. the SLOW ANION CHANNEL-ASSOCIATED1 [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 transport in biological processes and its contribution to pleiotropic traits.

Arabidopsis (Arabidopsis thaliana) ALUMINUM-ACTIVATED MALATE TRANSPORTER1 (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.

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RESULTS

Profiling of AtALMT1 Transcription in Response to Rhizotoxins and Chemical Treatments

AtALMT1 transcription was profiled using transgenic plants carrying an AtALMT1promoter::GUS (~1,110 bp from the first ATG; the full promoter region) fusion construct after 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 GA3 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-aminoacyclopropane-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

Figure 1. GUS expression and AtALMT1 transcription in roots of Arabidopsis under various treatments. Transgenic Col-0 harboring AtALMT1promoter::GUS (the GUS reporter gene fused with the full promoter sequence of ~1,110 bp from the 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), H2O2 (300 μM; pH 5.5), or rhizotoxic ions (10 μM AlCl3, 1.6 μM CuSO4, 15 μM CdCl2, and 50 mM NaCl at pH 5.5 or pH 4.7 for low pH). Activation of the AtALMT1 promoter was observed by GUS staining (blue). A, GUS staining patterns in a transgenic line. Bar = 20 μM. B, AtALMT1 transcript levels in wild-type Col-0 in response to various inducers determined by real-time RT-PCR (using UBQ1 as an internal control). Values shown are means ± SD (n = 3). Different letters indicate statistically significant differences (P < 0.05, Tukey’s test). Seedlings were incubated to various solutions for 6 h before histochemical and transcripts analyses.

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 knockout mutation of the gene resulted in hypersensitivity to Al, suggesting that this protein is essential for the survival of Arabidopsis in acid soils. In our previous study, we showed that malate excretion as a mechanism of Al tolerance is 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. Some of the signaling pathways in those systems involve protein phosphorylation, such as type 2C protein phosphatase signaling pathways in those systems involve protein phosphorylation/dephosphorylation. Although the mechanisms of protein activation have not been clarified yet, bacterial infection of aerial tissues induced AtALMT1 transcription in the roots (Rudrappa et al., 2008). Treatment of aerial tissues with the elicitors coronatine and microbe-associated molecular patterns such as flagellin22 (flg22 [QRLSTGRIRN-SAKDDAAQLQIA]; Felix et al., 1999) also induced transcription of AtALMT1 in the roots (Lakshmanan et al., 2012). This finding indicated that the transcriptional regulation of AtALMT1 responds to multiple signals.

Multisignal regulation has been reported for some transporters such as the inorganic anion 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. Some of the signaling pathways in those systems involve protein phosphorylation, such as type 2C protein phosphatase (PP2C)/SNF1-related protein kinase2 (SnRK2) in the ABA response (Umezawa et al., 2010). The multiple biological roles of AtALMT1 suggest that it is regulated by such a complex system. In this study, we profiled 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.
inducers of the biotic stress response (Fig. 1A). This indicated that some phytohormones can induce AtALMT1 expression within the short term, but IAA and ABA have stronger activity to induce expression. Among the abiotic stressors, Al, low pH, and hydrogen peroxide (H$_2$O$_2$) treatments induced stronger GUS staining than did NaCl, copper, and cadmium treatments. Transcript analysis confirmed that there were higher transcript levels of 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$_2$O$_2$ induced GUS expression in the whole 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 AtALMT1 transcription is induced by various signal inducers, while the position in the root tissues and the expression level are variable among the treatments.

**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 hypocotyls4-1 (atia4-arf19-1; Okushima et al., 2005, 2007), and an ABA-signaling mutant, aba insensitive1-1 (abi1-1; Leung et al., 1994; Meyer et al., 1994). Both mutants defective in the ability to activate transcription responded to ABA and IAA (see “Discussion”). Transcript levels of AtALMT1 in the IAA- and ABA-signaling mutants were analyzed by real-time reverse transcription (RT)-PCR. After 6 h of IAA treatment, AtALMT1 transcript levels were higher in the wild type than in nph4-arf19-1 (Fig. 2A). Compared with that in the 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 the 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. 3). Such genes included the IAA-responsive genes

**Figure 2.** AtALMT1 expression and Al tolerance in ABA- and IAA-signaling mutants. A, Roots of the IAA-signaling mutant nph4-1arf19-1 (Col-0 background) and the 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 AlCl$_3$) 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. Asterisks indicate significant differences 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 the primary root after 7 d in 4 μM AlCl$_3$ solution, pH 5.0). Values are means ± SD (n = 5).

ARF19, LATERAL ORGAN BOUNDARIES DOMAIN16 (LBD16), and GH3.5 and the ABA-responsive genes ABI1, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3 (NCED3), and RESPONSIVE TO DESSIONATION29B (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 AtALMT1 transcription, at least after a short-term (less than 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 AtALMT1 Responses to Inducers**

To characterize in detail the regulation of AtALMT1 transcription by Al and other inducers, we analyzed GUS expression in AtALMT1 promoter::GUS transgenic lines carrying different lengths of the 5’ region of the AtALMT1 promoter. Using the full promoter construct (~1,110bp::GUS) as the reference, we compared GUS expression driven by deleted promoter regions (~317 bp [-317bp::GUS] and ~292 bp [-292bp::GUS])
and then roots were incubated in 10 treatments. Seedlings were grown for 6 d 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 differences compared with the control (P < 0.05, Tukey’s test).

in response to various signal inducers. GUS expression was lower in the −292bp-GUS line than in the −1,110bp::GUS line in response to Al, low pH, IAA, ABA, and H₂O₂. GUS expression in response to the various inducers was similar between the −1,110bp::GUS line and the −317bp::GUS line, except when ABA was used as the signal inducer, where the GUS expression in the −317bp::GUS line was markedly lower than that in the −1,110bp::GUS line (Fig. 4). This result indicated that the major ABA-regulating element was localized farther upstream of the AtALMT1 promoter than −317 bp. To find cis-acting elements in the promoter, we searched the AtALMT1 promoter sequence between −317 and −1,110 bp using the PLACE and PlantCARE databases (Higo et al., 1999; Lescot et al., 2002). Both databases predicted cis-acting elements related to drought-inducible elements, CAAT box (CCAAT) and Myb binding site (CAACTG), in that region (Supplemental Table S1). In the Al, low-pH, IAA, and H₂O₂ treatments, there was lower GUS expression in the −292bp::GUS line than in the −1110bp::GUS line and the −317bp::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 AtALMT1 in Roots of Arabidopsis by flg22**

Some bacteria induce AtALMT1 expression through shoot-root signaling (Lakshmanan et al., 2012); however, the direct activation of AtALMT1 transcription in the roots had not been analyzed. To determine whether AtALMT1 expression could be induced by bacterial challenge to the roots, we treated the roots with flg22, a biological inducer of the plant-bacteria response, and performed histochemical analysis and expression analysis of GUS expression from AtALMT1 promoter::GUS fusion constructs. In the −1,110bp::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 the wild type, flg22-induced AtALMT1 transcription was significantly decreased in the flg22-signaling mutant flagellin-sensitive2 (fls2), which is defective in the flg22 receptor for activating transcription in response to flg22 (Fig. 5B). The fls2 mutant showed AtALMT1 expression by Al treatment, which was comparable to Columbia (Col-0; Fig. 5B). It suggests that flg22 signaling is involved in the activation of AtALMT1 expression but is not affected by 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 −1,110bp::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 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 the 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 that it is independent from the 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 the multidrug and toxic compound extrusion (MATE) efflux protein family (Liu et al., 2009), while ALUMINUM SENSITIVE3 (ALS3; Larsen et al., 2005) encodes a putative homolog of rice (Oryza sativa) SENSITIVE TO ALUMINUM RHIZOTOXICITY2, which encodes a subunit of a UDP-Glc 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 regulation 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 (less than 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 the 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...
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 expression (treatment/control) was calculated. Values are means ± SD (n = 3). Different letters indicate significant differences from the transcript level in the control (P < 0.05, Tukey’s test).

**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 expression (treatment/control) was calculated. Values are means ± SD (n = 3). Different letters indicate significant differences from the transcript level in the control (P < 0.05, Tukey’s test).

ARF-Aux/IAA proteins and SCF<sup>TR1/AFB</sup>-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 (Umehara 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 pattern 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-larf19-1* cannot form lateral roots (Okushima et al., 2007). We observed that the transgenic line carrying *AtALMT1 promoter::GFP* expressed GFP at that site without any exogenous inducer treatments (Supplemental Fig. S1). Inducible expression mediated by IAA could result in an 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 several crop plants such as barley (*Hordeum vulgare*) and soybean (*Glycine max*; 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 this study, flg22 treatment of the roots induced *AtALMT1* transcription within 6 h (Fig. 5). This pattern of *AtALMT1* expression could be reasonable for recruiting 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 the...
attraction of beneficial bacteria to the root surface. Interestingly, FB17-derived elicitors (i.e. autoclaved cells) did not induce *AtALMT1*, possibly because of differences of its flagella that are not highly conserved in 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 to −1,110 bp) promoter region, while the responsive element for other signal inducers (Fig. 4) was located closer to the start codon, between −317 and −292 bp. Several drought responsive cis-element motifs were predicted by the PlantCARE and PLACE databases (Supplemental Table S1). A common region (−317 to −292 bp) contained a putative cis-acting site, GGG(T/g/a/C)V(C/A/g)S(C/G) (Tsutsumi et al., 2011), that binds the ALUMINUM RESISTANCE TRANSCRIPTION FACTOR1 (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 SENSITIVE TO PROTON RHIZOTOXICITY1 (*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 illustrate 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 also be 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/dephosphorylation is involved in Al activation of AtALMT1 (Kobayashi et al., 2007). Although this process has 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 h of treatment (Supplemental Fig. S2). This suggests that some signal inducers may activate the AtALMT1 protein. Further research on the activation process of the protein is required to understand how plants regulate malate excretion from the roots for acquiring stress resistance.

**MATERIALS AND METHODS**

**Plant Materials**

Arabidopsis (Arabidopsis thaliana) accession Col-0 and Landsberg erecta (Ler-0) were obtained from the RIKEN BioResource Center (BRC) and the Nottingham Arabidopsis Stock Centre. The IAA-signaling double mutant nph4-larf19-1 was kindly provided by Dr. Hidehiro Fukaki (Kobe University). The ABA-signaling mutant abt1-1 was obtained from the RIKEN BRC. The flg22-signaling mutant fls2 was kindly provided by Dr. Ken Shirasu (RIKEN Plant Science Center). The mutant abt1-1 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 fusion constructs in Col-0 were prepared by Agrobacterium tumefaciens-mediated transformation using the following procedures. All PCRs were carried out using PrimeSTAR max (Takara) high-fidelity Taq polymerase, and the sequences of the amplified products were checked with an ABI PRISM 3100 Genetic Analyzer using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s protocol. Details of primer sequences are shown in Supplemental Table S2. A series of 5’ deleted promoters, −1,110, −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 open reading frame of the GUS gene connected to the nopaline synthase terminator by overlapping extension PCR (Horton et al., 1989). The amplicon was digested with SfiI and then introduced into the transfer DNA region of pBE2113 (Mitsukura et al., 1996), which contains a kanamycin resistance cassette as the selection marker. We used the hypervirulent *A. tumefaciens* 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⁻¹ in Murashige and Skoog medium containing 1% agar; Murashige and Skoog, 1962) as the selection marker.

**Growth Conditions and Root Growth Test**

Arabidopsis seedlings were grown hydroponically according to the method of Kobayashi et al. (2007) in modified MGRL solution diluted to 1:50 (with inorganic phosphate and pH 5.6 for transcript analyses; without phosphorus and pH 5.0 for growth tests to determine Al tolerance). Seedlings were grown at 24°C ± 2°C under a 12-h-light/12-h-dark photoperiod, with light supplied at a photosynthetic photon flux density of 37 µmol m⁻² s⁻¹. The culture solutions were renewed every 2 d. Seedlings were grown for 6 d for transcript analyses and GUS staining and for 7 d for the root growth test to assess Al tolerance, unless mentioned otherwise. Root length was measured at day 7 in control and Al-toxic solutions (4 µM AlCl₃). Five of the 10 seedlings with the longest roots in Al-toxic solution were used to calculate mean values and to assess the Al tolerance of the lines, as described previously (Kobayashi et al., 2007).

**Stress, Phytohormone, and Chemical Treatments**

The roots of seedlings pregrown as described above were transferred to solutions containing various rhizotoxins (10 µM AlCl₃, 1.6 µM CuSO₄·5 H₂O, 15 µM CdCl₂, or 50 mM NaCl) or chemicals (10 or 100 µM IAA, ABA, ACC, BAP, GA₃, MeJA, or SA, or 300 mM H₂O₂) and then incubated for 6 h. All rhizotoxic ions and chemicals were added to solution containing 1/50 MGRL nutrients with extra CaCl₂ added to make a final concentration of 200 µM, pH 5.5 (solution did not contain phosphorus).

**Histochemical Analysis of Reporter Expression**

GUS staining was performed as described previously (Kobayashi et al., 2007) with −1,110bp *AtALMT1promoter::GUS* transgenic lines. Briefly, the roots of seedlings were treated with rhizotoxic ions/chemicals as described above and stained with staining solution (1.0 mM X-glucuronide, 0.1 mM 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 with an Olympus BX51 microscope equipped with an Olympus DP70 camera system.

RNA Extraction and Expression Analysis
Total RNA was extracted as described (Suzuki et al., 2003). First-strand complementary DNA was synthesized from total RNA with ReverTra Ace (Toyobo) 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) and the Thermal Cycler Dice Real Time System II (Takara Bio) 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 (~1,110 bp from A of the 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
The following materials are available in the online version of this article.

Supplemental Figure S1. Histochemical analysis of AtALMT1 expression during lateral root formation in Arabidopsis.

Supplemental Figure S2. Malate excretion from Arabidopsis roots after treatment with Al and other signal inducers, inducing AtALMT1 expression.

Supplemental Table S1. List of cis-acting elements of the AtALMT1 promoter.

Supplemental Table S2. List of primer sequences for the AtALMT1 promoter::GUS construct of the transgenic plant.

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

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LITERATURE CITED

AtALMT1 Expression during Stress Responses


