Characterization of *AtALMT1* Expression in Aluminum-Inducible Malate Release and Its Role for Rhizotoxic Stress Tolerance in Arabidopsis[1][W][OA]

Yuriko Kobayashi[2,3], Owen A. Hoekenga[2], Hirotaka Itoh, Midori Nakashima, Shoichiro Saito, Jon E. Shaff, Lyza G. Maron, Miguel A. Piñeros, Leon V. Kochian, and Hiroyuki Koyama*

Laboratory of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido Gifu 501–1193, Japan (Y.K., H.I., M.N., S.S., H.K.); and United States Plant, Soil, and Nutrition Laboratory, United States Department of Agriculture-Agricultural Research Service, Ithaca, New York 14853 (O.A.H., J.E.S., L.G.M., M.A.P., L.V.K.)

Malate transporters play a critical role in aluminum (Al) tolerance responses for some plant species, such as Arabidopsis (*Arabidopsis thaliana*). Here, we further characterize *AtALMT1*, an Arabidopsis aluminum-activated malate transporter, to clarify its specific role in malate release and Al stress responses. Malate excretion from the roots of accession Columbia was sharply induced by Al, which is concomitant with the induction of *AtALMT1* gene expression. The malate release was specific for Al among rhizotoxic stressors, namely cadmium, copper, erbium, lanthanum, sodium, and low pH, which accounts for the specific sensitivity of a null mutant to Al stress. Al-specific malate excretion can be explained by a combined regulation of *AtALMT1* expression and activation of AtALMT1 protein, which is specific for Al. Although low pH treatment slightly induced gene expression, other treatments did not. In addition, malate excretion in Al-activated seedlings was rapidly stopped by removing Al from the solution. Other rhizotoxic stressors were not effective in maintaining malate release. Protein kinase and phosphatase inhibitor studies indicated that reversible phosphorylation was important for the transcriptional and posttranslational regulation of *AtALMT1*. *AtALMT1* promoter-β-glucuronidase fusion lines revealed that *AtALMT1* has restricted expression within the root, such that unnecessary carbon loss is likely minimized. Lastly, a natural nonsense mutation allele of AtALMT1 was identified from the Al-hypersensitive natural accession Warschau-1.

Aluminum (Al) toxicity due to soil acidity inhibits root growth and function, which increases susceptibility to other stressors such as drought and mineral nutrient deficiency. Thus, improvement of Al tolerance is an especially important target for plant breeding, as it will have wide-ranging positive effects. Organic acid (OA) release from roots has been identified in several crop plants as a major component of Al tolerance, including wheat (*Triticum aestivum*; e.g. Delhaize et al. 1993), maize (*Zea mays*; Pellet et al., 1995; Piñeros et al., 2002), *Cassia tora* (Ma et al., 1997), snapbean (*Phaseolus vulgaris*; Miyasaka et al., 1991), and triticale (× *Triticosecale* Wittmack; Ma et al., 2000; for review, see Ma et al., 2001). Transgenic studies reveal overexpression of single key genes can dramatically enhance OA release and overall Al tolerance (Tesfaye et al., 2001; Delhaize et al., 2004). Gaining a strong, molecular understanding of this stress response should permit improvement of Al tolerance in crops by either molecular breeding and/or transgenic approaches (for review, see Kochian et al., 2004). With a firm grasp of the molecular mechanisms for Al-activated OA release, it may be possible to minimize possible negative consequences, such as excessive carbon loss or disturbance to primary metabolism, in transgenically modified crop plants.

OA release in response to Al can be categorized into two different types, designated by Ma et al. (2001) as pattern I and II. Pattern I is typified by immediate OA release in response to Al exposure of the root; wheat and buckwheat (*Fagopyrum esculentum*) are exemplars of pattern I (Delhaize et al., 1993; Zheng et al., 1998). Immediate activation of OA release in wheat is consistent with the molecular characterization of *ALMT1*, an aluminum-activated malate transporter, in wheat (hereafter *TaALMT1*), which is constitutively expressed in wheat roots, and the activation of OA transport activity can be followed at the biophysical level (Sasaki et al., 2004). *TaALMT1* gene expression is not induced by Al stress but is constitutive; expression levels correlate well with the amount of OA release and

[1] This work was supported by the Japan Society for the Promotion of Science-National Science Foundation research cooperative program and the Ministry of Economy, Trade and Industry for a joint research project between Gifu University and the Research Institute of Innovative Technology for the Earth.

[2] These authors contributed equally to the article.

[3] Present address: Forestry Research Institute, Oji Paper Company, 24-9 Nobono-cho Kameyama Mie 519–0212, Japan.

* Corresponding author; e-mail koyama@gifu-u.ac.jp.

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www.plantphysiol.org/cgi/doi/10.1104/pp.107.102335

overall Al tolerance (Sasaki et al., 2004; Raman et al., 2005). In this case, it was supposed that activation of the malate channel plays a critical role in the quick response of malate release to Al treatment (for review, see Ryan and Delhaize, 2001; Delhaize et al., 2007). In fact, protein kinase-inhibiting agents can block OA release from intact wheat roots, suggesting that reversible phosphorylation of *TaALMT1* may be involved in its transport activity (Osawa and Matsumoto, 2001). However, heterologous expression of *TaALMT1* in *Xenopus* oocytes demonstrates that TaALMT1 protein has the capacity to respond to Al ions itself (Sasaki et al., 2004). Pattern II OA release requires an induction period for OA release after Al treatment (Ma et al., 2001). Citrate release from *C. tora* is a typical example of pattern II, which requires 4 h for OA release after the Al exposure (Ma et al., 1997). Sorghum (*Sorghum bicolor*) also exhibits pattern II and requires days to reach full OA release (Magalhães et al., 2007). In sorghum, it is clear that induction of the SbMATE OA transporter protein, a sorghum homolog of the multidrug and toxic compound extrusion transporter, occurs during the lag between onset of Al stress and OA release (Magalhães et al., 2007). Induction of Al-activated OA transporters has also been reported in oilseed rape (*Brassica napus*), which is consistent with the observation that rape has pattern II OA release (Ligaba et al., 2006). The delay in OA release may also be due to a lag in OA production; altered OA metabolism has been observed in response to phosphorus deficiency in cluster roots of white lupin (*Lupinus albus*; Johnson et al., 1994) and carrot (*Daucus carota*) cell culture (Koyama et al., 1992). Al stress does not affect OA metabolism in triticale while pattern II OA release is observed there (Hayes and Ma, 2003).

Al-activated OA release, specifically malate, was first associated with Al tolerance in Arabidopsis (*Arabidopsis thaliana*) via a series of quantitative trait locus (QTL) studies (Hoekenga et al., 2003; Kobayashi et al., 2005). In both Landsberg *erecta* (L*er*) × Columbia (Col) and L*er* × Cape Verde Islands populations, the major Al tolerance QTLs were consistent with the position of an Arabidopsis homolog of the *TaALMT1* (Kobayashi and Koyama, 2002; Hoekenga et al., 2003; Sasaki et al., 2004; Kobayashi et al., 2005). Analysis of a knockout mutant for the *AtALMT1* locus confirmed the importance and uniqueness for this member of the *AtALMT* gene family (Hoekenga et al., 2006). However, further comparison of the L*er* × Col QTL studies from the two different labs, which feature dramatically different nutrient solutions and pH conditions, revealed that the Al tolerance QTLs on chromosome 1 do not in fact overlap with each other (Kobayashi and Koyama, 2002; Hoekenga et al., 2003). Fine-scale mapping of the Al tolerance QTL detected in the pH 4.2, high ionic strength solution established that *AtALMT1* is not consistent with this QTL (Hoekenga et al., 2006). Biophysical and biochemical analyses of the *AtALMT1* transporter indicated this protein possesses substrate selectivity similar to wheat *TaALMT1* (Sasaki et al., 2004; Hoekenga et al., 2006). These results indicate that Al tolerance by enhanced malate release is shared by a wide variety of plant species and utilizes the same type of membrane transporter (Magalhães, 2006). At this time, it is unclear whether Arabidopsis follows pattern I or II, but the importance of Al-activated OA release for Arabidopsis Al tolerance is clear. Given the physiological genomic resources available, a more detailed characterization of Al-activated OA release in Arabidopsis would be useful.

In previous studies, we developed growth conditions that separate Al toxicity from proton toxicity (Koyama et al., 2001; Kobayashi and Koyama, 2002). This schema is also applicable for characterization of other rhizotoxic metals, some of which also elicit OA release (Murphy et al., 1999; Toda et al., 1999). In addition, we developed a sensitive method for detecting malate release from the roots (Hoekenga et al., 2003). In this study, we sought to clarify the interplay between *AtALMT1* expression, malate release, and root responses to abiotic stressors so as to better understand OA release from Arabidopsis roots. We show the specificity of malate release with Al stress responses, identify that regulation of malate release exists on at least two levels (transcriptional and posttranslational), establish that Arabidopsis fits the pattern II of OA release, and identify a natural *AtALMT1* mutant that will be an important resource for future structure/function research on AtALMT1.

RESULTS

Time Course of Malate Release and *AtALMT1* Expression in Hydroponic Culture

To establish whether Arabidopsis utilizes pattern I or II OA release in response to Al stress (10 μM), we performed a time course analysis of malate release and *AtALMT1* expression. Malate release rates were equivalent in both Al-treated and controls at the first 2-h time point (Fig. 1A). Malate release rate gradually decreased in control treatment as a function of time, but rates increased with Al treatment (Fig. 1A). Malate release rates increased with time, reaching a steady state after the 4- to 6-h time point. In parallel, *AtALMT1* mRNA levels were determined by reverse transcription (RT)-PCR (Fig. 1B). *AtALMT1* had very low levels of expression in the absence of Al treatment; however, within 1 h, *AtALMT1* mRNA levels approximately doubled (Fig. 1B). The number of transcripts continued to rise through time, gaining an approximately 70-fold increase by 12 h (Fig. 1B). Thus, *AtALMT1* expression is induced by Al treatment within 1 h then followed by induction of malate release.

Specificity of Malate Release and *AtALMT1* Expression to Abiotic Stresses

To determine whether *AtALMT1* expression and malate release are specific to Al, we exposed roots to

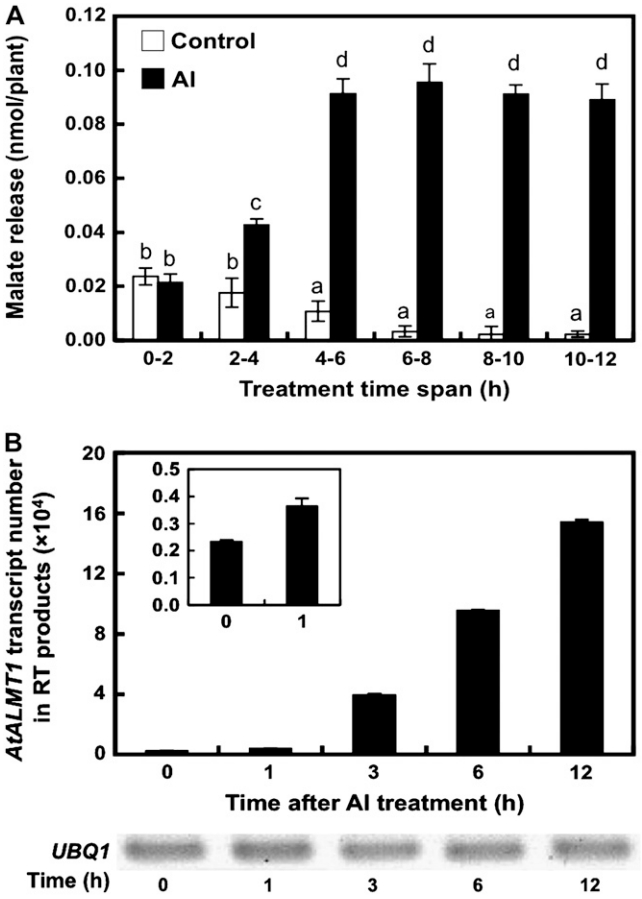

Figure 1. Malate release and *AtALMT1* expression time series. A, Induction of malate release from the roots of Arabidopsis by Al treatment. The roots of aseptically grown seedlings were incubated in Al medium containing 10 μM Al (black bars) or control medium (no Al; white bars). Means and SE values are shown ($n = 3$). Different letters indicate significant difference by LSD test ($P < 0.05$). B, Time course of *AtALMT1* expression in the roots of Arabidopsis in response to Al treatment, which were quantified by real-time PCR. Means and SE values from three samples are shown ($n = 3$). Inset is a magnified image for 0 and 1-h time points. The bottom segment shows the results of the control amplification (ubiquitin).

a range of abiotic stresses that induce rhizotoxicity: cadmium (Cd), copper (Cu), erbium (Er), lanthanum (La), sodium (Na), and low pH (4.4). Each of these stresses induces a similar level of rhizotoxicity as 10 μM Al does at pH 5.0 in the low ionic strength nutrient solution, which is an approximately 90% inhibition of root growth after 7-d stress treatment. Al treatment was the only rhizotoxic treatment to induce malate release above the level seen in the control treatment in 24-h root exudation experiments (Fig. 2A). *AtALMT1* expression was significantly induced by three rhizotoxic treatments; Er induced a small change relative to control, while the low pH stress induced a larger increase in *AtALMT1* expression. By far the largest increase came with Al treatment, while ubiquitin expression remained relatively constant throughout the treatments (Fig. 2B). Malate release appears to stop in relatively rapid response to changing growth environments. Plants acclimated to Al were moved to fresh nutrient solutions with new rhizotoxic ions or no stress. All treatments led to significant (60%–80%) reductions in malate release after 2 h (Fig. 3A). However, *AtALMT1* expression was not dramatically altered during these treatments, suggesting that inactivation of malate release does not involve changes in *AtALMT1* mRNA levels within the 2-h time frame (Fig. 3B).

To further verify that *AtALMT1* is not involved in diverse abiotic stress responses, we compared root growth responses of wild-type Col and an *AtALMT1* knockout mutant variety from the Salk Institute T-DNA collection (Alonso et al., 2003). As seen with the pH 4.2, high ionic strength nutrient culture conditions in our previous study (Hoekenga et al., 2006), the

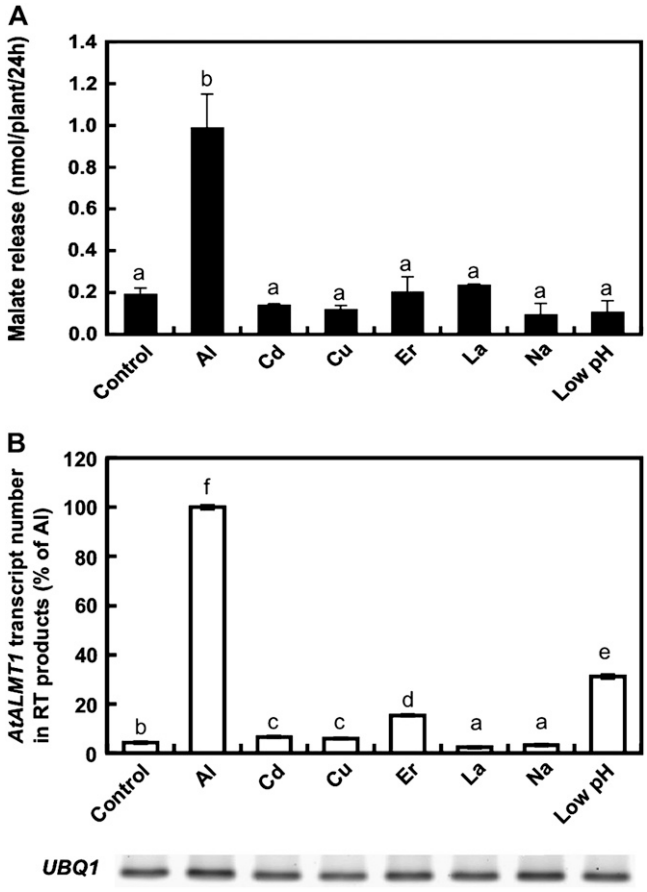

Figure 2. Effect of abiotic stressors on malate release and *AtALMT1* expression. All media contained abiotic stressors at similar levels of root growth inhibition (10 μM Al, 4.0 μM Cd, 2.0 μM Cu, 1.3 μM Er, 1.3 μM La, and 50 mM Na in solution at pH 5.0, low pH [pH 4.4]), which yield approximately 90% root growth inhibition. A, Root malate release in the Col-4. The means and SE values are shown ($n = 3$). B, Transcript levels of *AtALMT1* in the Col-4, means are for relative transcript levels (standardized to Al treatment at 100%), and SE values of three independent experiments are shown ($n = 3$). Gel image shows ubiquitin expression with RT-PCR analysis using primers specific for *UBQ1*. Different letters indicate significant difference by LSD test ($P < 0.05$).

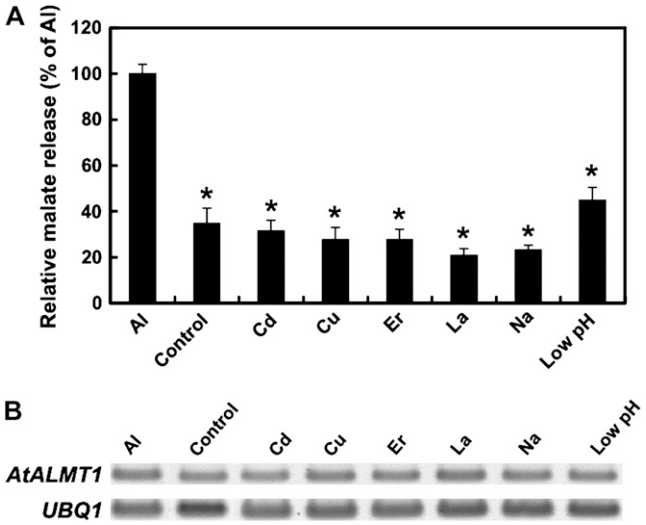

Figure 3. Effect of various ions on malate release in Al-treated Arabidopsis roots. Seedlings were incubated for 2 h in various rhizotoxic solutions after activating malate efflux by incubating in Al (10 μM) containing media for 24 h. All media contained abiotic stressors at similar levels of root growth inhibition (10 μM Al, 4.0 μM Cd, 2.0 μM Cu, 1.3 μM Er, 1.3 μM La, and 50 mM Na in solution at pH 5.0, low pH [pH 4.4]), which yield approximately 90% root growth inhibition. Malate efflux (means and SE; A) and *AtALMT1* expression level quantified by real-time PCR (B) were determined. *, Significant difference to Al (LSD test $P < 0.05$). *UBQ1* expression is shown as control for gene expression.

AtALMT1 mutant exhibited very low levels of root growth (Fig. 4A) and malate release in the presence of Al (0.2 $\pm$ 0.11 nmol plant^{-1} 24 h^{-1}). As might be expected, both wild-type and mutant varieties demonstrated equivalent responses to the suite of rhizotoxic abiotic stresses, verifying that *AtALMT1* is not essential for these stress responses (Fig. 4B).

Response of Al-Induced Malate Release to Protein Kinase and Phosphatase Inhibitors

Reversible protein phosphorylation has been repeatedly implicated in abiotic stress responses (for review, see Zhu, 2001; Sreenivasulu et al., 2007). Protein kinase inhibitors have been demonstrated to block Al-activated malate release in wheat roots (Osawa and Matsumoto, 2001). Al treatment alters protein kinase activity in Arabidopsis, although the targets are still unknown (Sivaguru et al., 2003). We examined whether reversible protein phosphorylation affects *AtALMT1* expression or function by perturbing protein kinase and phosphatase function using the protein kinase inhibitors, K-252a and staurosporine, and the protein phosphatase inhibitors, calyculin A and cyclosporin A. The specificities of these inhibitors have not fully elucidated in plant systems yet. However, given the differences in efficacy of these agents on mammalian signaling pathways, it is not unreasonable to observe differential responses in malate release and *AtALMT1* expression from these chemical agents (see http://www.sigmaaldrich.com/Area_of_Interest/Biochemicals.html).

Kinase and phosphatase inhibitors were applied in two series of experiments. First, inhibitors were used to pretreat plants before Al stress treatment. After the 24-h Al treatments, Al-inducible malate release was significantly reduced in plants pretreated with K-252a, staurosporine, and calyculin A, while cyclosporin A had no effect (Fig. 5A). There are several levels at which reversible phosphorylation could regulate Al-inducible malate release: directly on the *AtALMT1* transporter to modulate transport activity, by influencing *AtALMT1* gene expression level, by either reducing or eliminating increases in AtALMT1 expression, or by some combination of both. K-252a treatment had no effect on *AtALMT1* gene expression at the 24-h time point but significantly reduced gene expression at the earlier time point tested. In the K-252a treatment, 24-h malate release rates were reduced by 60%. On the other hand, staurosporine and calyculin A both blocked all changes in *AtALMT1* gene expression and thus malate release was totally inhibited (Fig. 5).

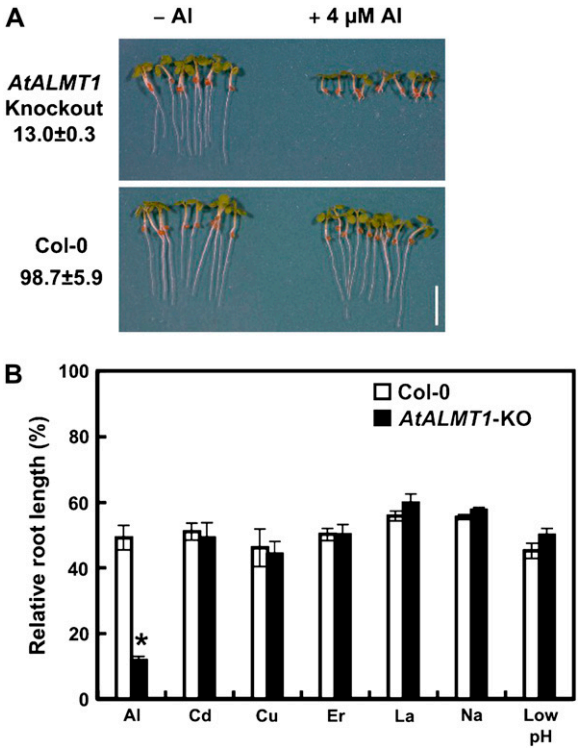

Figure 4. Root growth of a T-DNA insertion knockout mutant of *AtALMT1* in response to different abiotic stress treatments. A, A knockout mutant (SALK_009629) and wild-type Col-0 were grown for 1 week in the presence of 4 μM $AlCl_3$ or control solution (pH 5.0). Bar = 1.0 cm. B, The mutant (black bars) and wild-type Col-0 (white bars) were grown for 7 d in the abiotic stress (6.0 μM $AlCl_3$, 3.0 μM $CdCl_2$, 1.3 μM $CuCl_2$, 1.3 μM $ErCl_3$, 1.0 μM $LaCl_3$, and 8.0 mM NaCl in solution at pH 5.0, low pH [pH 4.7]) or control treatment (pH 5.0), which yield approximately 90% root growth inhibition for Col-0. Means and SE of percent relative root length are shown ($n = 3$). *, Significant difference from Col root growth (*t* test, $P < 0.05$).

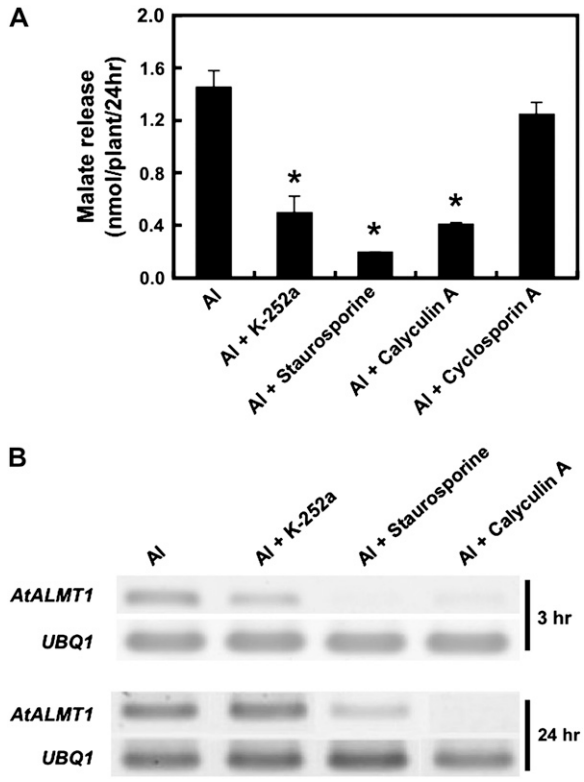

Figure 5. Effect of protein kinase and protein phosphatases inhibitors on the activation of malate efflux in Arabidopsis roots (pretreatment). K-252a and staurosporin A are protein kinase inhibitors, whereas calyculin A and cyclosporin A are protein phosphatase inhibitors. All inhibitors are added to give final concentration of 5 μM. Malate release at 24 h (A; means and SE, $n = 3$) and *AtALMT1* expression at 3 and 24 h (B) in 10 μM Al media were determined in the presence or absence of the inhibitors. *UBQ1* expression is shown as control. * in A, Significant difference from Al treatment (LSD test $P < 0.05$).

Second, kinase and phosphatase inhibitors were used to treat plants already stressed by Al. In this way, we could examine the effect of these agents on activated AtALMT1 transporters. Plants were treated with Al and then moved to nutrient solutions with Al and pharmacological agents or agents alone as a posttreatment (Fig. 6). The combination of K-252a with Al resulted in a small but significant reduction in malate release after 2-h treatment, while Al with phosphatase inhibitors had no effect on malate release rates (Fig. 6). When Al stress was removed, K-252a and staurosporine-treated plants exhibited reduced malate release (Fig. 6), similar to that seen in the control-treated plants (see Fig. 3A, control plants; Al activated then transferred to control solution). However, calyculin A treatment prevented this inactivation of malate release such that malate release rates were equivalent to those seen in Al-treated plants. This suggests that protein phosphatases are required to inactivate AtALMT1 transporters once they are activated by Al. These results expand upon the observations first made in wheat that reversible phosphorylation is required to activate malate release in response to Al and that these signaling pathways apparently act both at the transcriptional and posttranslational levels (Osawa and Matsumoto, 2001).

Tissue-Level Localization of *AtALMT1* Expression via Promoter-GUS Fusions

In previous transcriptomic studies, *AtALMT1* has been reported to have a developmentally sensitive and root-specific pattern of gene expression (Birnbaum et al., 2003; Meyers et al., 2004). To verify these observations and investigate Al-inducible changes in gene expression and location, an *AtALMT1* promoter::GUS reporter construct was generated and used to transform wild-type Arabidopsis. In the absence of Al stress, weak GUS staining was observed in the root apex (Fig. 7A). With Al stress (Fig. 7, B–F), GUS staining increased in intensity and locality. Root apices remained the most highly stained portion of the root, with the elongation zone in primary and lateral roots now also exhibiting *AtALMT1* expression (Fig. 7, B and C). In the mature root, the stele and endodermis were the sites of reporter activity, while the cortical and epidermal cells did not show expression (Fig. 7, C–E). Consistent with previous reports, *AtALMT1* expression was restricted to the root and absent from shoot tissues (Fig. 7, F–H).

AtALMT1 Allele of Al-Hypersensitive Accession, Warschau-1

Natural variation exists for Al tolerance among accessions of Arabidopsis (Hoekenga et al., 2006). We

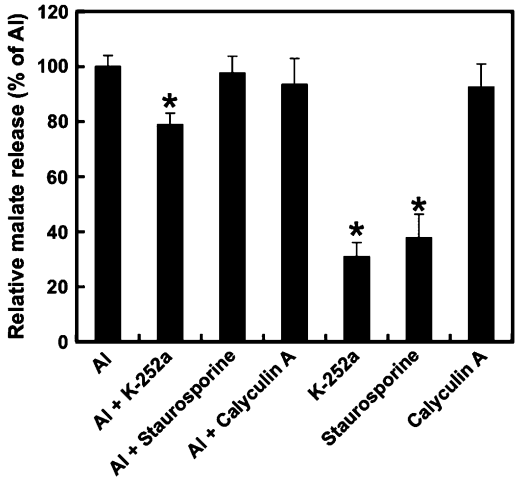

Figure 6. Effect of protein kinase (K-252a, staurosporin A; 5 μM) and protein phosphatase inhibitor (calyculin A; 5 μM) on the preactivated malate efflux (posttreatment). Activated seedlings preincubated in Al (see Fig. 3 caption) were transferred to solutions containing the different inhibitors in the presence or absence of Al. Means and SE of the malate efflux during 2 h are shown ($n = 3$). *, Significant difference from Al treatment (LSD test $P < 0.05$).

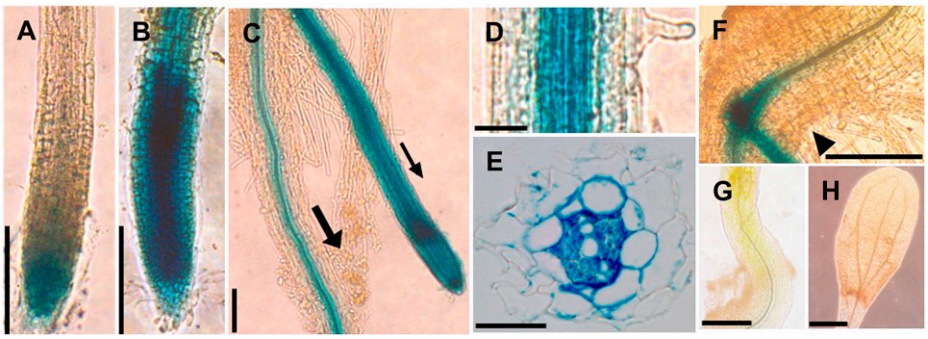

Figure 7. Profiling of *AtALMT1* expression in transgenic seedlings via GUS staining. Transgenic plants carrying a *AtALMT1* promoter::GUS construct were used for GUS staining after exposure to Al. After 10-d cultures in control solution, roots of transgenic seedlings (*AtALMT1* promoter::GUS) were exposed to nutrient solution containing 4 μM Al or control solution for 24 h. A, Apex of the primary root without Al treatment. B, Apex of the primary root with Al treatment. C, Al-treated primary (thick arrow) and secondary (thin arrow) root. Arrows indicate direction of root apex. D, Close-up views of mature part of the primary root. E, A cross section of the mature part of the primary root with Al treatment. F, The border between shoot and root indicated with triangle. G, Shoot. H, Leaf with Al treatment. Bar = 40 μm (A–F) and 100 μm (G and H).

have undertaken a broader and more deliberate survey of Al tolerance among 260 accessions of Arabidopsis (Ikka et al., 2007). Of these, we identified one accession, Warschau-1 (Wa-1), which was hypersensitive to Al stress. While Wa-1 is a natural autotetraploid (Henry et al., 2005), it has been reported to produce viable seeds at the F_2 generation in a cross with an accession of the ecotype Col (Adam and Somerville, 1996). These observations led us to construct a mapping cross between Wa-1 and Col-4 to permit the genetic characterization of the Al hypersensitivity. In the F_2 generation, Al tolerance similar to that seen in the Col-4 parent was in the minority (Fig. 8A). Using a bulk segregant analysis with highly Al-sensitive plants and a low-density genome-wide scan, we located markers linked to Al tolerance at the top of chromosome 1 (Supplemental Fig. S1; Fig. 8B). These markers define a chromosomal interval consistent with the location of *AtALMT1* (Fig. 8B). To test the possible involvement of *AtALMT1* directly, we performed a genetic complementation test using the *AtALMT1* knockout mutant (Fig. 8C). The F_1 hybrid was equally sensitive as either parent, which indicates that the accession Wa-1 carries a nonfunctional allele of *AtALMT1*. DNA sequence analysis of *AtALMT1-Wa-1* identified a single nucleotide polymorphism (SNP; T for G) in Wa-1 relative to Col, which generated a premature stop codon (Supplemental Fig. S2). This SNP is shared by all Wa-1 accessions from each of the different stock centers (Nottingham Arabidopsis Stock Centre [NASC], Arabidopsis Biological Resource Center [ABRC], and RIKEN Bio-Resource Center [BRC]) and indicates natural occurrence of this nonsense mutation. Computational methods predict that the AtALMT1 protein contains five membrane-spanning domains and a long, possibly cytoplasmic tail (Schwacke et al., 2003; Fig. 8D). The C-terminal tail region contains three Ser residues that the DISPHOS phosphorylation prediction software predicts as the most likely phosphorylation sites among all of the Ser, Thr, and Tyr residues present in the protein (Iakoucheva et al., 2006). The AtALMT1 protein encoded by Wa-1 is predicted to be truncated after the second membrane-spanning domain, thus lacking the majority of the protein and the three Ser residues identified by DISPHOS as targets of reversible phosphorylation (Fig. 8D).

DISCUSSION

Al-activated malate release is a major Al tolerance mechanism in both dicots and monocots, mediated by members of a single family of plasma membrane-associated transporters called ALMT (summarized by Magalhães, 2006). Genetic variation in Al tolerance among accessions has been reported due to variation at the transporter locus (Raman et al., 2005). In wheat, variation in promoter structure and function seems to drive the variation observed at *TaALMT1* (Sasaki et al., 2006). Variation in tolerance also occurs at factors that work genetically upstream of the malate transporter (Hoekenga et al., 2003, 2006). Some of these upstream factors may include protein kinases and/or phosphatases, as pharmacological agents that interfere with reversible phosphorylation can block Al-activated malate release (Osawa and Matsumoto, 2001; Figs. 5 and 6). Signal transduction proteins have also been implicated in Al tolerance responses as the overexpression of *WAK1*, a wall-associated protein kinase, results in a modest increase in Al tolerance in Arabidopsis (Sivaguru et al., 2003). However, neither mutant analyses nor QTL mapping experiments have directly implicated protein kinases or phosphatases in regulating Al tolerance responses. Recently, a C2H2-type zinc finger transcription factor (*STOP1*) was identified as an upstream regulatory factor for AtALMT1 (Iuchi et al., 2007). Whether polymorphism at *STOP1* contributes to natural variation in Al tolerance or if *STOP1*

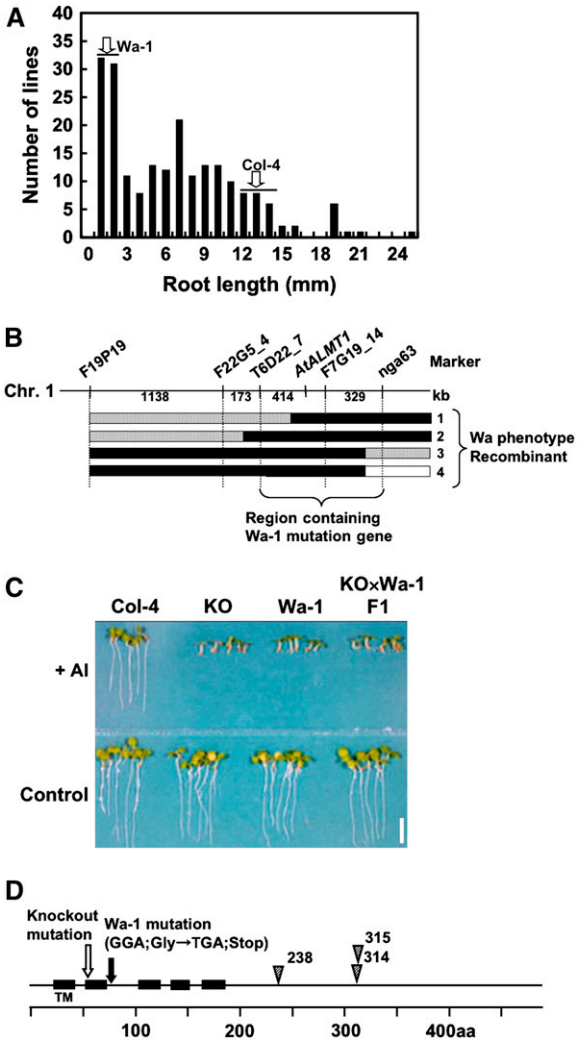

Figure 8. Characterization of Al sensitivity in Wa-1 ecotype. A, Distribution of root length of F_2 seedlings derived from a cross of Col-4 × Wa-1 in Al test solution (4 μM, pH 5.0) after 7 d culture. Arrows and horizontal bars indicate means and SE of parental line's root length (n = 3). B, Black bars are for the Wa-1 genotype, while the gray bars depict heterozygous plants and white bars the Col genotype in chromosome of the recombinant line. Marker names are showed above the chromosome. C, Root growth of Col-4, knockout mutant of *AtALMT1*, Wa-1, and F_1 for the cross of knockout mutant of *AtALMT1* × Wa-1 seedlings in control and Al test solution (4 μM, pH 5.0) for 7 d. Bar = 5 mm. D, Positions of mutations in the knockout mutant and Wa-1 are depicted with regards to an transmembrane helices model predicted by ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/), as well as in relation to amino acid number for the ATALMT1 protein, respectively. Predicted transmembrane domains are indicated by black rectangles. The mutation site is indicated by arrows. Arrowheads indicate predicted phosphorylation sites by DISPHOS (http://core.ist.temple.edu/pred/).

acts downstream from staurosporine or calyculin A-sensitive phosphatases has yet to be determined.

AtALMT1 gene expression is responsive to a few rhizotoxic stressors, most notably Al and low pH (Figs. 1 and 2). Basal expression of *AtALMT1* occurs within the root apex at a low level; with Al treatment, *AtALMT1* is expressed more strongly and widely within root apices and in the central core in mature portions of the root (Fig. 7). As *AtALMT1* responds to low pH stress, this likely explains the difference between basal expression levels reported here in and our previous work, which utilized a pH 4.2 nutrient solution (compare Fig. 2B in this article with figure 2 in Hoekenga et al., 2006). This transcriptional enhancement requires reversible phosphorylation, as the protein phosphatase inhibitors staurosporine and calyculin A can block increases in *AtALMT1* mRNA abundance (Fig. 5B). This indicates that whatever transcription factor regulates *AtALMT1* expression in response to Al treatment is at the end of a signal transduction pathway that utilizes protein phosphatases.

AtALMT1 protein activity is more tightly regulated than the pattern of gene expression. While low pH and Er treatments enhance *AtALMT1* mRNA levels, neither increases the rate of malate release from roots above control treatment levels (Fig. 2). Malate release rates take time to reach their maximum after 6 h of Al exposure, which closely follow the pattern of gene expression for the given time period, using both RT-PCR and promoter-reporter methodologies (Figs. 1 and 7). These experiments also clarify that Arabidopsis falls into the pattern II class for Al-activated OA release. *AtALMT1* expression level remained increased during 12 h after the Al exposure, but malate excretion reached its maximum rate at 6 h. This inconsistency suggests that AtALMT1 protein level is not completely correlated with the gene expression level and/or regulation of the transport capacity, which may depend upon some other activating factor. Protein phosphatase inhibitors applied as a pretreatment significantly reduce malate release rates, likely due to the aforementioned reduction in *AtALMT1* gene expression (Fig. 5A). Pretreatment with the protein kinase inhibitor K-252a blocks malate release without blocking changes in *AtALMT1* gene expression. This indicates that a protein kinase may also be directly involved in the activation of the AtALMT1 transporter. This is consistent with similar experiments in wheat, where K-252a was reported to be capable of blocking malate release (Osawa and Matsumoto, 2001). Reversible phosphorylation is also important for inactivating malate release; when Al is withdrawn from the nutrient solution, malate release rates fall within 2 h (Fig. 3). K-252a and staurosporine applications do not interfere with this recovery to basal rates; however, calyculin A treatment blocks the decline to basal malate release rates (Fig. 6). Thus, a protein phosphatase is quite likely involved in deactivating the AtALMT1 transporter.

Genetic experiments in this study build upon our understanding of AtALMT1 structure/function relationships. Electrophysiological examination of AtALMT1 in *Xenopus* oocytes demonstrated that the transporter alone has a substantial basal malate efflux activity that is stimulated by Al treatment (Hoekenga et al., 2006). *AtALMT1* is expressed in the absence of Al treatment under low pH (see figure 2 in Hoekenga et al., 2006; Fig. 2B in this article). These results suggest

that AtALMT1 in planta has accessory factors that regulate transporter activity, as malate release does not occur at substantial rates without Al exposure (Figs. 2B and 3A). The pharmacological agent experiments indicate that some of these accessory factors may be kinases or phosphatases, or the accessory factors themselves may be subject to reversible phosphorylation (Figs. 5 and 6). We have gained an additional insight into the AtALMT1 transporter, as experiments with the Wa-1 accession suggest a truncated allele may act as a dominant negative (Fig. 8, A and C). This possibility will be evaluated by growth response of F_1 plants and other experiments in future research. Many transporters form multimeric complexes that can consist of heteromers or homomers (e.g. Yellen, 2002). The *Shaker*-type K^+ channel forms a tetrameric transporter complex where each protein subunit has five transmembrane domains similar to that seen with AtALMT1 (Yellen, 2002). In mouse, the *mceph* mutation leads to megencephaly and occurs at the *Kv1.1* locus, which is an isoform of a *Shaker*-type K^+ channel (Petersson et al., 2003). Like *AtALMT1-Wa-1*, the *mceph* variant of *Kv1.1* has a premature stop codon after the second transmembrane-spanning domain, which leads to heteromeric channel complexes with significantly altered K^+ transport functionality (Petersson et al., 2003). Genetic experiments with a T-DNA insertion allele of *AtALMT1* (a null) and the L*er* ecotype indicate a single copy of *AtALMT1* can drive wild-type malate release rates (Hoekenga et al., 2006). In contrast, the majority of progeny of a cross between Wa-1 and Col-4 are Al sensitive (Fig. 8A). This result suggests that a mix of full-length and truncated AtALMT1 transporter subunits is more detrimental to Al stress responses than simply decreasing the number of transporters. This natural variant of *AtALMT1* offers us an excellent new tool to help clarify AtALMT1 transporter structure/function relationships.

AtALMT1 mutants, whether they are T-DNA-induced or natural nonsense variants, grow indistinguishably from wild-type plants under standard laboratory or greenhouse conditions (Hoekenga et al., 2006; Fig. 4A). Experiments with a range of abiotic stressors indicate that *AtALMT1* is required only for Al stress tolerance, as the mutants grow equally well as wild-type plants when treated with Cd, Cu, Er, La, Na, and low pH (Fig. 4). These emphasize the specificity of the stress response mediated by *AtALMT1*. Malate release is quickly reduced when environmental conditions change, as stress substitution experiments demonstrate (Fig. 3A). This inactivation of AtALMT1 comes without rapidly decreasing *AtALMT1* gene expression (Fig. 3B), which further emphasizes that posttranslational regulation of AtALMT1 occurs. The specificity and plasticity of AtALMT1 activity may make it an ideal candidate for use in transgenic crop improvement. TaALMT1 has already been demonstrated to be effective at increasing Al tolerance in barley (*Hordeum vulgare*), a species with very limited Al tolerance (Delhaize et al., 2004). It may be possible to increase the Al tolerance conferred by transgenic expression of an ALMT1 family transporter by cotransformation with a regulatory protein kinase or phosphatase, once they are identified. Given the specificity of malate release, it is certainly possible that unexpected consequences of ALMT1 expression, such as excessive carbon loss or disturbance to primary metabolism, could be minimized.

In this study, we have shown that root malate exudation in Arabidopsis mediated by AtALMT1 is regulated specifically by Al stress. We have also demonstrated that this regulation of malate release operates on at least two levels of control, both transcriptional and posttranslational, and establishes that Arabidopsis follows the pattern II of OA release. In the absence of Al stress, *AtALMT1* expression is apparently confined to the root apex and occurs at a low level. With Al stress, *AtALMT1* is greatly up-regulated within the root apex and is restricted to the central cylinder of the mature root (Fig. 7). This restricted pattern of expression helps to explain how a relatively limited amount of malate release, based on bulk solution estimates, can achieve such a large degree of protection for the root apex. These observations fit the model proposed by Kinraide et al. (2005), which reconciled the degree of protection achieved by wheat root apex for the amount of malate released per plant. We also have identified a natural *AtALMT1* mutant, which is due to the production of a truncated AtALMT1 protein, and genetic analysis of this mutant suggests that the AtALMT1 transporter may function as a multimeric protein complex. This mutant will be an important resource for future structure/function research on AtALMT1.

MATERIALS AND METHODS

Arabidopsis Accessions

Col-4 (N933) and Wa-1 (JA245, CS1586, CS1587, CS6885, and CS22644) were obtained from NASC (N933), RIKEN BRC (JA245), or the ABRC (CS series), respectively, as per stock number. A SALK T-DNA insertion mutant carrying T-DNA in exon of *AtALMT1* (At1g08430; SALK_009629) was derived from ABRC and homozygous lines were identified from the accession.

Growth Condition and Root Measurement

Seedlings were grown hydroponically in 2% strength modified MGRL medium (lacking P_i and substituting calcium salts with $CaCl_2$ at a concentration of 200 μM) at pH 5.0 with 12-h photoperiod (120 μmol quanta $m^{-2}\,s^{-1}$) at 23°C. Abiotic stresses were imposed with the addition of the following species: 6.0 μM $AlCl_3$, 3.0 μM $CdCl_2$, 1.3 μM $CuCl_2$, 1.1 μM $ErCl_3$, 1.0 μM $LaCl_3$, or 8.0 mM NaCl. Low pH treatment was carried out using the standard 2% modified MGRL medium acidified to a lower pH (4.7). In each replicate experimental treatment, 10 seedlings per line were grown for 7 d, replacing the medium every 2 d. Plants were grown on culture apparatus made from plastic mesh and photo slide mount as described previously (Toda et al., 1999), and the root length was measured by capturing with a micro video camera (Pico Scopemen, Kenis) as described previously. Growth experiments were repeated at least three times and means and SES were obtained to calculate relative root length (toxic condition/control [%]).

Malate Release and Measurement

Seedlings were grown for 4 d aseptically in the manner of Hoekenga et al. (2003), substituting 100% MGRL solution with 1% (w/v) Suc at pH 5.0. Other

conditions were as described above. Seeds were sown on 10-mm squares of plastic mesh (15 plants) and then transferred to wells of a 6-well microtiter plate containing 2 mL of root exudation collection medium (2% MGRL nutrients with 1% [w/v] Suc). Seedlings were pretreated in 2 mL of control medium for 1 h then transferred to fresh medium containing the appropriate stress agent (i.e. 10 μM $AlCl_3$, 4.0 μM $CdCl_2$, 2.0 μM $CuCl_2$, 1.3 μM $ErCl_3$, 1.3 μM $LaCl_3$, 50 mM NaCl, or low pH [4.4]). Protein kinase and protein phosphatase inhibitors, K-252a (Calbiochem), calyculin A (Wako Pure Chemical), cyclosporin A (Wako Pure Chemical), and staurosporine (Sigma-Aldrich) were applied at a final concentration of 5 μM. All inhibitors were first dissolved in a small amount of dimethyl sulfoxide then added to 2% MGRL modified medium containing 1% (w/v) Suc. Plants were gently shaken on a rotary shaker (40 rpm) at 25°C in darkness. For the time course study, plants were transferred to fresh medium every 2 h and old solutions were collected at each time point. Effect of various ions and inhibitors was determined in two ways. The first set of experiments (Figs. 2 and 5A) were carried out by incubating the seedlings for 24 h in exudation media containing various ions or inhibitors with Al. For stress reversal experiments (Figs. 3 and 6), the seedlings were first incubated in the growth solutions containing Al for 24 h and roots were rinsed in fresh control solution and then transferred to various media. In this case, the exudation media were collected after 2 h. Malate concentrations in exudation media were determined by a NAD/NADH cycling-coupled enzymatic method as described previously (Takita et al., 1999) or capillary electrophoresis as described (Hoekenga et al., 2003). All experiments were carried out at least three times and means and SE values are reported.

RNA Isolation and Quantitative RT-PCR

About 200 seedlings were grown for 10 d in the control solution for growth experiment plus Pi (2% MGRL nutrient solution) and transferred to the test solutions used for malate release experiments without Suc. After the experimental treatments, roots were rinsed in distilled water, wiped gently with tissues, harvested using scissors, and immediately frozen in liquid nitrogen. Total RNA was isolated and quantified by the method of Suzuki et al. (2003). Quantitative RT-PCR was carried out using real-time PCR system (Applied Biosystems 7300) with TaqMan probe and amplifying probes designed for *AtALMT1* by Applied Biosystems. The quantification process followed the manufacturer's protocol. Also, semiquantification of AtALMT1 transcripts by gel image was carried out according to Kihara et al. (2003) with modifications. Briefly, 1-μg aliquot of total RNA was reverse transcribed using oligo(dT) primer in the presence of pAW109 RNA (Applied Biosystems) as an internal control for the RT and PCR efficiency. Specific PCR primers obtained from the 3′-untranslated region were designed as follows: *AtALMT1* forward, 5′-GGCCGACCGTGCTATACGAG-3′, reverse, 5′-GAGTTGAATTACTTACTGAAG-3′. The amplification program consisted of 94°C for 3 min, followed by appropriate cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s, and a final extension period of 72°C for 3 min. *UBQ1* forward, 5′-TCGTAAGTACAATCAGGATAAGATG-3′, reverse, 5′-CACTGAAACAAGAAAAACAAACCCT-3′. The amplification program consisted of 94°C for 3 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension period of 72°C for 3 min. PCR products were separated by 3% agarose gel and stained with SYBR Green I, detected with Typhoon 9410, and then quantified by ImageQuant. As a standard for transcript number of *AtALMT1*, PCR was performed using a plasmid with the cloned cDNA at known concentration as template.

Preparation of *AtALMT1* Promoter GUS Fusion Transgenic Plants and GUS Assay

Genomic DNA sequence representing +6 to −2235 (first ATG as 0) of *AtALMT1* was cloned into a mini Ti plasmid vector pBI101, which contains a GUS reporter (Jefferson et al., 1987). *Agrobacterium tumefaciens*-mediated transformation was performed using strain C58C1pMP90 and the floral dip method (Clough and Bent, 1998). Homozygous transgenic plants were isolated in the T_3 generation by segregation test of Kan resistance. GUS staining was performed according to the method of Kosugi et al. (1990) after 24-h exposure to Al (4 μM) at pH 5.0.

Characterization of Wa-1 Mutation

A population consisting of 210 F_2 seedlings was derived from a cross between Col-4 and Wa-1, which were grown in Al toxic solution (4 μM, pH 5.0) for 7 d as described above, and was then measured for the root length and rescued to rockwool containing 100% MGRL nutrient solution. After 40 d, leaves were collected separately from individual plants for extracting genomic DNA with a kit (NucleoSpin Plant MACHEREY-NAGEL). Five DNA pools consisting of equal DNA from five Al-hypersensitive F_2 plants each were used to conduct bulk segregant linkage analysis of Al sensitivity on the genome. Genotyping was carried out by simple sequence length polymorphisms (Bell and Ecker, 1994) or cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) analyses using simple sequence length polymorphisms or CAPS markers derived from http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker, of which show polymorphisms between Col-4 and Wa-1. In addition, derived CAPS (Neff et al., 2002) and SNP markers were derived from sequence analysis of Col-4 and Wa-1. PCR conditions and primer sequences for above mapping analyses are shown in Supplemental Table S1.

For sequencing analysis of *AtALMT1* of Wa-1 and Col-4, cDNA encoding *AtALMT1* is amplified using specific primers 5′-CTGAAAGTAATCAGAGAATCAG-3′ and 5′-GATGGTCTCGTCTCTATAATCTT-3′ (30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min) and purified. DNA sequencing analysis was carried out using the above primer pairs, ABI BigDye Terminator system (ver 3.1), and ABI PRISM3100 DNA sequencer according to the manufacturer's recommended protocols.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU181365.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Segregation of genome-wide genetic makers in the pooled F_2 plants showed Wa-1 phenotype.

Supplemental Figure S2. DNA sequence around Wa-1 nonsense mutation.

Supplemental Table S1. The primer sequences used in mapping analysis of Al-sensitive gene in Wa-1 accession.

ACKNOWLEDGMENTS

We thank Dr. Takashi Ohno and Mr. Yoshiharu Sawaki in Gifu University for technical assistance. Arabidopsis seed stocks were kindly provided by NASC, ABRC, and RIKEN-BRC.

Received May 14, 2007; accepted September 9, 2007; published September 20, 2007.

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