Knockout of a Bacterial-Type ATP-Binding Cassette Transporter Gene, *AtSTAR1*, Results in Increased Aluminum Sensitivity in Arabidopsis[1][C][OA]

Chao-Feng Huang, Naoki Yamaji, and Jian Feng Ma*

Institute of Plant Science and Resources, Okayama University, Kurashiki 710–0046, Japan

ATP-binding cassette (ABC) transporters represent a large family in plants, but the functions of most of these transporters are unknown. Here we report a gene, *AtSTAR1*, only encoding an ATP-binding domain of a bacterial-type ABC transporter in Arabidopsis (*Arabidopsis thaliana*). AtSTAR1 is an ortholog of rice (*Oryza sativa*) OsSTAR1, which has been implicated in aluminum (Al) tolerance. Knockout of *AtSTAR1* resulted in increased sensitivity to Al and earlier flowering. Unlike *OsSTAR1*, *AtSTAR1* was expressed in both the roots and shoots and its expression was not induced by Al or other stresses. Investigation of tissue-specific localization of *AtSTAR1* through β-glucuronidase fusion revealed that *AtSTAR1* was predominantly expressed at outer cell layers of root tips and developing leaves, whose localization is also different from those of OsSTAR1. However, introduction of *OsSTAR1* into *atstar1* mutant rescued the sensitivity of *atstar1* to Al, indicating that AtSTAR1 has a similar function as OsSTAR1. Furthermore, we found that AtSTAR1 may interact with ALS3, a transmembrane-binding domain in Arabidopsis to form a complex because introduction of *OsSTAR1*, a functional substitute of *AtSTAR1*, into *als3* mutant resulted in the loss of OsSTAR1 protein. All these findings indicate that *AtSTAR1* is involved in the basic detoxification of Al in Arabidopsis.

ATP-binding cassette (ABC) proteins constitute a large, diverse, and ubiquitous superfamily. In the genomes of rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*), there are 121 and 120 members estimated, respectively (Garcia et al., 2004). In a model legume, *Lotus japonicus*, there are at least 91 ABC proteins (Sugiyama et al., 2007). Although the function of most these proteins are unknown, recent studies have shown that plant ABC proteins not only are involved in the transport of hormones, lipids, metals, secondary metabolites, and xenobiotics, but also contribute to plant-pathogen interactions and the modulation of ion channels (Rea, 2007; Verrier et al., 2008).

Most plant ABC proteins are characterized by having both a nucleotide-binding domain (NBD) and a transmembrane domain (TMD), forming full-size (two NBDs and two TMDs) or half-size (one NBD and one TMD) proteins. However, a few ABC proteins only contain NBD or TMD. Recently, some of these types of proteins have been reported to be implicated in the tolerance to aluminum (Al) toxicity, which is a major limiting factor for plant growth and production on acid soils (von Uexkull and Mutert, 1995). Larsen et al. (2005) reported that an Arabidopsis gene, *ALS3*, which encodes only TMD, is involved in Al tolerance. Knockout of *ALS3* resulted in increased sensitivity to Al (Larsen et al., 2005). *ALS3* is primarily expressed in leaf hydathodes and the phloem throughout the plants, along with root cortex following Al treatment. However, ALS3 has not been functionally characterized. Also it is unknown whether ALS3 functions alone or requires NBDs to form a complex.

More recently, a rice gene, *STAR1* (for sensitive to Al rhizotoxicity), which encodes only NBD of an ABC transporter, was reported to be involved in Al tolerance (Huang et al., 2009). Knockout of this gene resulted in hypersensitivity to Al (Ma et al., 2005; Huang et al., 2009). STAR1 interacts with STAR2, a TMD protein, to form a complex, which functions as a bacterial-type ABC transporter. The complex of STAR1/STAR2 is localized at the membrane of vesicles in the root cells and transports UDP-Glc (Huang et al., 2009). UDP-Glc may be used for the modification of the cell wall, thereby detoxifying Al, although the exact mechanism remains to be elucidated (Huang et al., 2009). The

---

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 21248009 to J.F.M.), a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation grant no. IPG–0006 to J.F.M.), and by a Sunbor Grant and the Ohara Foundation for Agricultural Science.

* Corresponding author; e-mail maj@rib.okayama-u.ac.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jian Feng Ma (maj@rib.okayama-u.ac.jp).

[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.110.155028

expression of both *STAR1* and *STAR2* is up-regulated by Al and controlled by ART1, a C2H2 zinc-finger transcription factor (Yamaji et al., 2009).

STAR1 is single-copy gene in the rice genome and its putative orthologous genes were found in maize (*Zea mays*), Arabidopsis, grape (*Vitis vinifera*), and moss (*Physcomitrella patens*; Huang et al., 2009). However, it is unclear whether *STAR1* represents a universal Al tolerance gene in plant species or is only required for Al tolerance in rice. In this study, we characterized *AtSTAR1*, an ortholog of rice *STAR1*, in a dicot species Arabidopsis. We found that *AtSTAR1* is also required for Al tolerance in Arabidopsis, but the expression patterns and localization are different from those of *OsSTAR1*. Furthermore, we found the flowering time in a T-DNA insertion mutant of *AtSTAR1* is altered.

RESULTS

Structure of *AtSTAR1* Gene

A BLAST search using OsSTAR1 protein sequence resulted in identification of a unique homolog, *AtSTAR1* (At1g67940), in the Arabidopsis genome. *AtSTAR1* has two exons and one intron, encoding a 263-amino acid protein (Fig. 1A). AtSTAR1 shows 62% identity and 77% similarity with OsSTAR1 (Fig. 1B). Similar to OsSTAR1, AtSTAR1 is characterized by conserved domains of a NBD of an ABC transporter, such as Walker A, Q loop, ABC signature, Walker B, and H motif (Fig. 1B).

Phenotype of *AtSTAR1* Knockout Line

To examine the biological function of *AtSTAR1* in Arabidopsis, we obtained a knockout line (762A06, *atstar1*) that has a T-DNA insertion at the coding sequence of *AtSTAR1* (724 bp from ATG; Fig. 1A). Reverse transcription (RT)-PCR analysis indicated that the full-length transcript of *AtSTAR1* was absent in *atstar1* (Fig. 2A). When the wild-type Arabidopsis and *atstar1* knockout line were grown in the absence of Al, their root growth was similar (Fig. 2B). However, in the presence of 2 μM Al, the roots of the knockout line were severely inhibited, whereas those of the wild type were hardly affected (Fig. 2B).

To confirm that disruption of *AtSTAR1* was responsible for the Al sensitivity in the knockout line, a complementation test was performed. A 3.7-kb DNA fragment harboring 1.7-kb *AtSTAR1* promoter and the candidate gene was introduced into the knockout line by *Agrobacterium*-mediated transformation method. Examination of two independent T2 transgenic lines carrying *AtSTAR1* showed that their tolerance to Al toxicity was recovered to a similar level to that of the wild type, whereas the tolerance of the vector control plants to Al was not (Fig. 2C). The growth of all lines was similar in the absence of Al (Fig. 2C). These results indicate that Al-sensitive phenotype in the knockout line was caused by loss of function of *AtSTAR1* gene.

When the wild type and the knockout line were cultivated to the mature stage, we further found that the flowering time of the knockout line was almost 4 d earlier than that of the wild type (28.6 ± 1.1 d for knockout line versus 32.3 ± 1.2 d for the wild type; Fig. 2D). In addition, we found that a knockout line of *ALS3* also showed early flowering like *atstar1* (Fig. 2D).

Physiological Characterization of *atstar1* Knockout Line

We further physiologically characterized *atstar1* knockout line in terms of dose response, metal specificity, and uptake of other cations. A dose-response experiment showed that the root growth of *atstar1* was inhibited by as high as 75% even at 1 μM Al, at which concentration the roots of the wild type were not

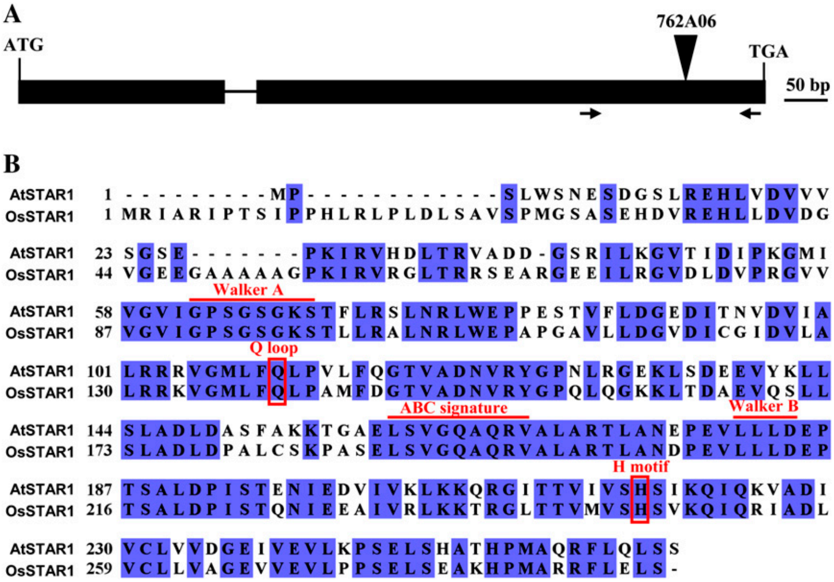

Figure 1. Gene structure of *AtSTAR1* in Arabidopsis (A) and sequence similarity with rice OsSTAR1 (B). The T-DNA insertion position of *AtSTAR1* in a line (762A06) was shown on the top (A). Arrows show a primer pair for detection of *AtSTAR1* expression by RT-PCR. B, Alignment of AtSTAR1 and OsSTAR1. Lines above the sequence indicate the conserved domains, and boxes show the conserved amino acids of two motifs. [See online article for color version of this figure.]

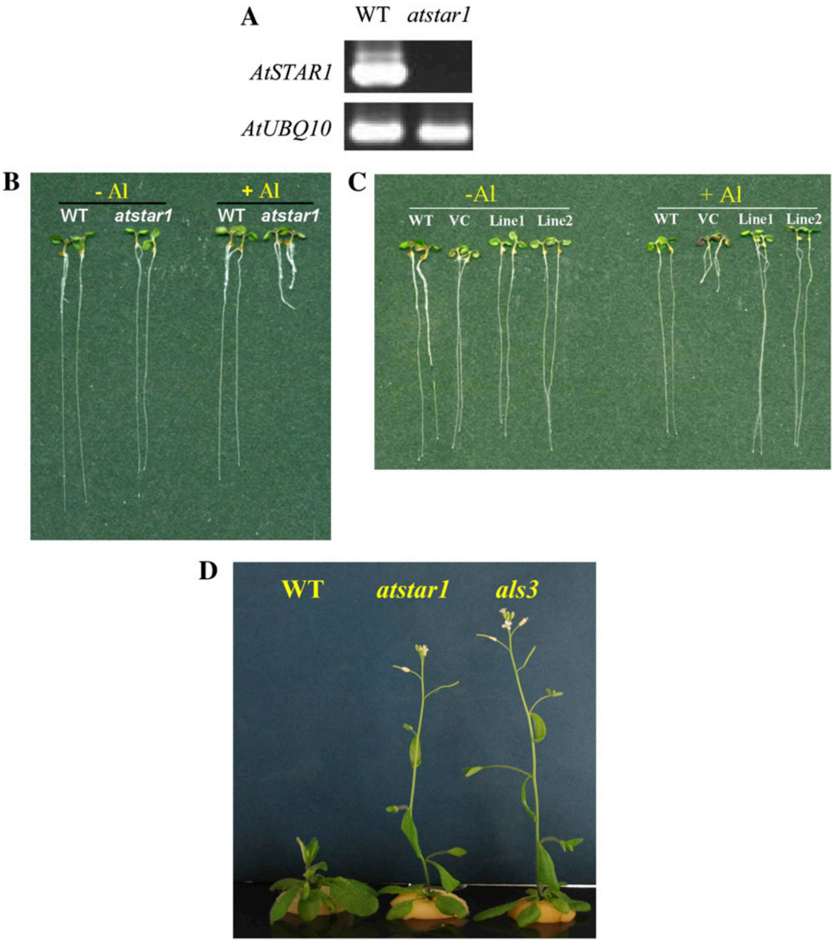

Figure 2. Phenotype of knockout line *atstar1*. A, mRNA expression of *AtSTAR1* in the wild type (WT) and *atstar1* knockout line. RT-PCR analysis was performed to detect the mRNA expression of *AtSTAR1* (35 cycles) and the internal control *AtUBQ10* (25 cycles). B, Al sensitivity in the wild type and the knockout line. Seedlings were grown in a nutrient solution containing 0 or 2 μM Al at pH 5.0 for 7 d. C, Complementation test of the *atstar1* knockout line. The wild type and *atstar1* transformed with empty vector (VC) and *AtSTAR1* (two lines), respectively, were grown in a nutrient solution containing 0 or 2 μM Al at pH 5.0 for 7 d. D, Flowering time of *atstar1*, *als3*, and the wild type. Plants were grown in a nutrient solution for 35 d.

inhibited (Fig. 3A). With increasing Al concentrations in the external solution, the Al-induced root inhibition was also observed in the wild type, but the inhibition was much lower at either Al concentration (Fig. 3A).

To investigate whether *atstar1* is specifically sensitive to Al stress or not, the sensitivity to other toxic metals, including cadmium (Cd) and lanthanum (La), was compared between the wild type and the knockout line. In the presence of 2 μM Cd or 0.1 μM La, the root growth inhibition caused by these metals was similar between the wild type and *atstar1* (Fig. 3B), although the difference in Al tolerance was significantly evident. These results indicate that the sensitivity of *atstar1* to Al was highly specific.

We also compared the uptake of some essential cations between the wild type and *atstar1* grown hydroponically in the absence of Al. Among cations tested, including potassium, calcium, magnesium, iron, zinc, and manganese, there was no difference in the concentration of these cations in the shoots between the wild type and *atstar1* (Fig. 3, C and D).

Expression Pattern of *AtSTAR1*

The tissue-specific expression pattern of *AtSTAR1* mRNA was examined with quantitative real-time PCR. *AtSTAR1* was expressed in the roots as well as in the leaves, stems, and flowers of Arabidopsis (Fig. 4A), but the expression level in the roots was relatively higher (Fig. 4A).

The expression of *AtSTAR1* mRNA in response to Al or other stresses was also examined. Exposure to Al, Cd, or La did not affect the expression of *AtSTAR1* in the roots (Fig. 4B). Low pH treatment also did not change the expression (Fig. 4C).

Localization of *AtSTAR1*

To investigate the tissue-specific localization of *AtSTAR1* in Arabidopsis, a DNA fragment consisting of 2.3-kb promoter and the full genomic sequence of *AtSTAR1* was fused in frame to the GUS reporter gene and the construct containing the fused gene was transformed into Arabidopsis wild-type plants. GUS analysis in the transgenic plants revealed that *AtSTAR1* was expressed in both the roots and shoots (Fig. 5). This result is consistent with the expression pattern of *AtSTAR1* (Fig. 4A). In the roots, *AtSTAR1* was predominantly expressed in the root tip region, including root caps, meristem, and elongation zone (Fig. 5A). In the shoots, GUS activity was strongly detected in newly generated leaves but not observed at

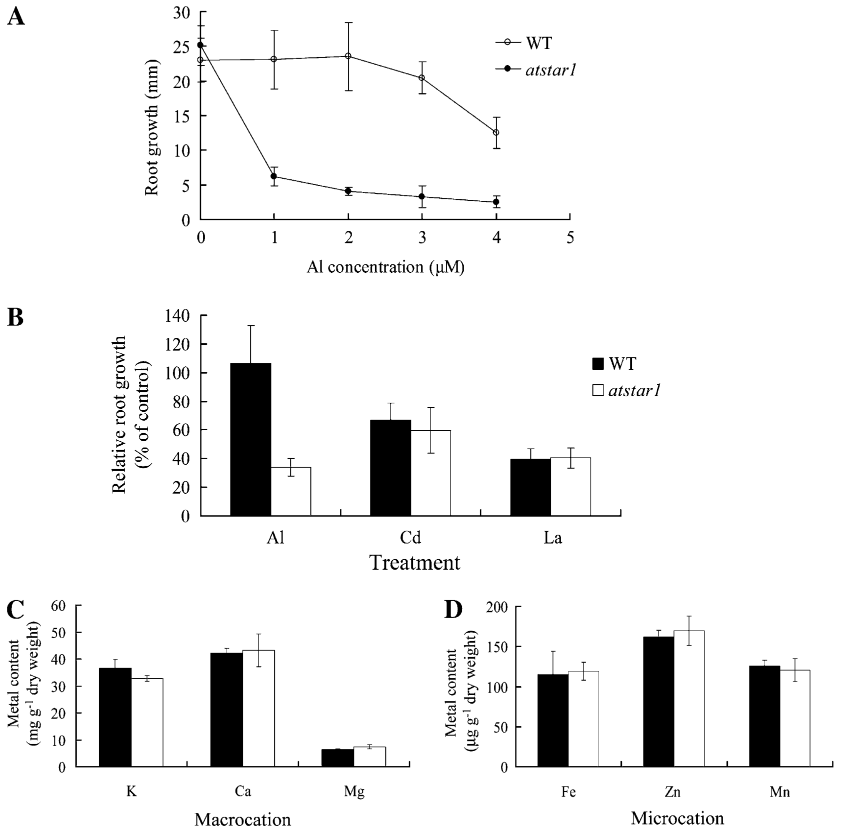

Figure 3. Physiological characterization of the knockout line *atstar1*. A, Dose response to Al in the wild type (WT) and *atstar1*. Seedlings were grown in a nutrient solution containing 0, 1, 2, 3, or 4 μM Al at pH 5.0 for 7 d. Root growth after the treatment was measured with a ruler. Data are means ± SD (n = 8–11). B, Specificity of *atstar1* to Al. Seedlings of the wild type and *atstar1* were grown in a nutrient solution containing 0, 2 μM Al, 2 μM Cd, or 0.1 μM La at pH 5.0 for 7 d. Relative root growth expressed as (root length with metal treatment/root length without metal) × 100 is shown. Data are means ± SD (n = 21–30). C and D, Shoot concentration of macrocations (C) and microcations (D). Both the wild type and *atstar1* were grown in one-thirtieth-strength Hoagland nutrient solution for 38 d. Data are means ± SD (n = 3).

cotyledon leaves (Fig. 5B). High GUS activity was also observed at developing young leaves, whereas in older leaves GUS expression was weak and became confined to epithem tissue of hydathodes (Fig. 5, C and D).

To further examine the cellular localization of *AtSTAR1* in the root tip region, we made cross sections through the GUS-stained roots. Results showed the cell specificity of localization of *AtSTAR1* differed with root regions. In the root cap and meristem region, *AtSTAR1* was mainly expressed at the outer cell layers; the outer cell layers of root caps (Fig. 5E), lateral root caps of root meristem (Fig. 5F). However, at the elongation zone, *AtSTAR1* was expressed in the epidermal cells, cortex, and endodermis, although the expression was relatively higher in the epidermal cells (Fig. 5G). The expression of *AtSTAR1* in the mature root region was much weaker compared with that of root tips (Fig. 5H).

Rescue of the Al Sensitivity in *atstar1* by *OsSTAR1*

To investigate whether rice OsSTAR1 has similar function to AtSTAR1 in Arabidopsis, we performed a complementation test by introducing *OsSTAR1* into *atstar1* mutant under the control of cauliflower mosaic virus *35S* promoter. RT-PCR analysis showed that *OsSTAR1* was expressed, whereas *AtSTAR1* expression was absent in the two independent *OsSTAR1; atstar1* transgenic lines (Fig. 6A). Al tolerance test showed that introduction of *OsSTAR1* into *atstar1* rescued the Al sensitivity of the knockout line, being a similar tolerance level to the wild type (Fig. 6B). These results indicate that OsSTAR1 and AtSTAR1 have similar function in conferring Al tolerance.

Possible Interaction between AtSTAR1 and ALS3

To investigate the possible interaction between AtSTAR1 and ALS3 in Arabidopsis, we first obtained a T-DNA insertion line of *ALS3*. The T-DNA was inserted in the splicing site between the second intron and the third exon of *ALS3* (Fig. 7A), resulting in absence of the full-length transcript of *ALS3* (Fig. 7B). Mutation of *ALS3* resulted in hypersensitivity to Al (Fig. 7C), whose phenotype was consistent with that of previously reported allelic mutant (Larsen et al., 2005). To investigate the possible interaction between AtSTAR1 (OsSTAR1) and ALS3, we obtained lines carrying *OsSTAR1* in *als3* mutant background by crossing. Results showed that, in *OsSTAR1;atstar1* line, both *OsSTAR1* and *ALS3* mRNA were expressed (Fig. 7D), while there was no expression of *AtSTAR1*. By contrast, in the *OsSTAR1;als3* line, there was expression of both *OsSTAR1* and *AtSTAR1* but no expression of *ALS3* (Fig. 7D). When western-blot analysis was performed in these lines as well as the wild type and *atstar1* by using an antibody against OsSTAR1 (Huang et al., 2009), OsSTAR1 was detected in the *OsSTAR1;*

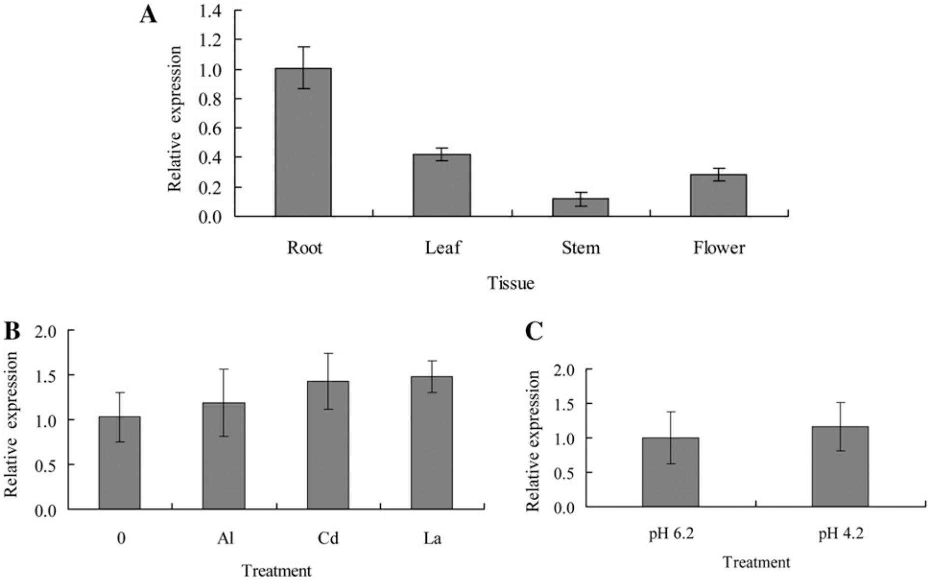

Figure 4. mRNA expression pattern of *AtSTAR1* in Arabidopsis. A, Quantitative real-time RT-PCR analysis of *AtSTAR1* expression in various tissues. *AtUBQ10* was used as an internal control. The data were normalized to *AtSTAR1* expression in roots. Data are means ± SD ($n = 3$). B, Quantitative real-time RT-PCR analysis of *AtSTAR1* expression in response to different metals. Seedlings were grown on a nutrient solution consisting of one-thirtieth-strength Hoagland nutrient solution ($NH_4H_2PO_4$ omitted) plus 1 mM $CaCl_2$ at pH 5.0 for 7 d and then exposed to the same nutrient solution containing 0, 3 μM Al, 3 μM Cd, or 3 μM La at pH 5.0 for 6 h and then the roots were sampled for RNA isolation. *AtUBQ1* was used as an internal control. The data were normalized to *AtSTAR1* expression under no metal treatment. Data are means ± SD ($n = 3$). C, Quantitative real-time RT-PCR analysis of *AtSTAR1* expression in response to low pH. Forty-day-old plants were exposed to one-tenth-strength Hoagland nutrient solution at pH 6.2 or pH 4.2 for 8 h and then the roots were sampled for RNA isolation. *AtUBQ1* was used as an internal control. The data were normalized to *AtSTAR1* expression at pH 6.2. Data are means ± SD ($n = 3$).

atstar1 line but not in the wild type and *atstar1*, indicating the specificity of the antibody for OsSTAR1 in Arabidopsis (Fig. 7E). Interestingly, although *OsSTAR1* mRNA was detected in the *OsSTAR1;als3* line (Fig. 7D), the protein of OsSTAR1 was not detected. This result indicates that ALS3 might be required for the expression of OsSTAR1 protein in Arabidopsis, indirectly implicating the interaction between AtSTAR1 (OsSTAR1) and ALS3.

DISCUSSION

AtSTAR1 Is Required for Al Tolerance in Arabidopsis

Al is the most abundant metal in the earth's crust, therefore plants have evolved different mechanisms to detoxify this metal (Kochian, 1995; Ma et al., 2001; Kochian et al., 2004; Ma, 2008). One of the well-documented mechanisms is the secretion of organic acid anions, including citrate, malate, and oxalate, from the roots. This mechanism has been observed in a wide variety of plant species, including both dicots and monocots such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), Arabidopsis, and *Cassia tora* (Delhaize et al., 1993; Ma et al., 1997; Hoekenga et al., 2003; Zhao et al., 2003). Genes responsible for the Al-induced secretion of malate in wheat (ALMT1; Sasaki et al., 2004), and citrate in barley (HvAACT1; Furukawa et al., 2007) and sorghum (SbMATE; Magalhaes et al., 2007) have been identified. Recently, progress in identification of other Al-tolerance genes has also been made. For example, a gene, *ALS1*, encoding a tonoplast-localized half-size of an ABC transporter, has been reported to be involved in the Al tolerance in Arabidopsis (Larsen et al., 2007). A bacterial-type ABC transporter OsSTAR1/OsSTAR2 is required for high Al tolerance in rice (Huang et al. 2009). In this study, we characterized the unique homolog of OsSTAR1 in Arabidopsis, AtSTAR1. All results show that like OsSTAR1, AtSTAR1 is also required for Al tolerance in Arabidopsis; knockout of *AtSTAR1* resulted in enhanced sensitivity specific to Al (Figs. 2 and 3). These results suggest that dicots like Arabidopsis and monocots like rice share similar mechanism of Al tolerance. This is supported by the finding that *OsSTAR1* was able to rescue the hypersensitivity of *atstar1* to Al in Arabidopsis (Fig. 6). However, the expression patterns and tissue cell specificity of localization are different between AtSTAR1 and OsSTAR1.

AtSTAR1 was expressed in both the roots and shoots of Arabidopsis (Fig. 4), whereas *OsSTAR1* was only expressed in the roots of rice (Huang et al., 2009). Furthermore, the expression of *AtSTAR1* was mainly confined to root tip region (Fig. 5), while *OsSTAR1* was expressed at the whole root. Within the root tip region,

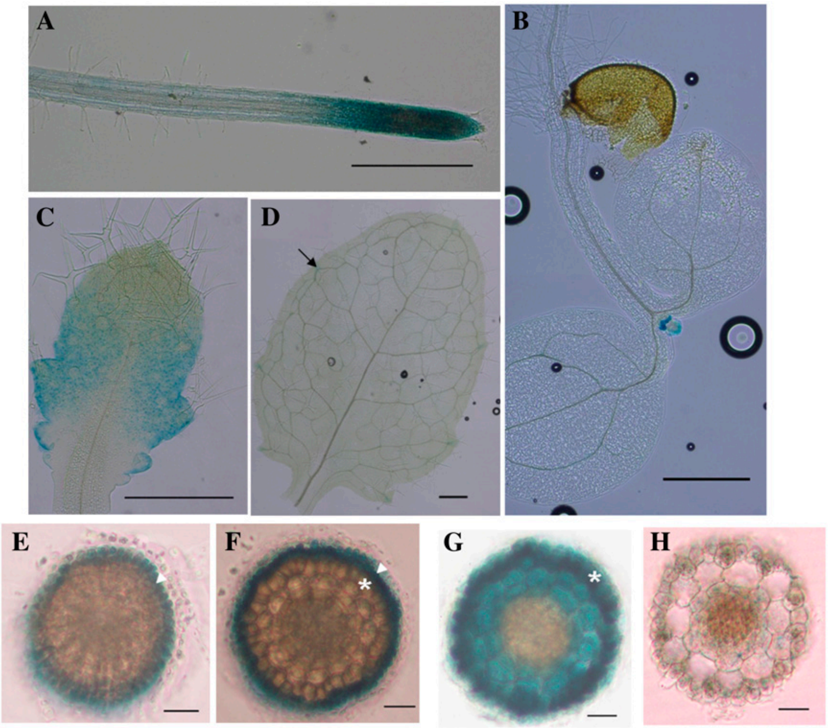

Figure 5. GUS analysis of *AtSTAR1* expression. T2 transgenic plants expressing *pAtSTAR1*:*AtSTAR1-GUS* were used for *AtSTAR1* expression analysis. A to D, GUS activity in the roots (A), cotyledon and newly generated leaves (B), developing leaves (C), and old leaves (D). Arrow indicates GUS expression in hydathode of an elder leaf. Scale bars for A to D: 500 μm. E to H, Cellular localization of GUS expression in roots. E, Root cap. Triangle indicates the outer cell layers. F, Meristem region. Triangle indicates the lateral root caps and asterisk indicates the epidermal cells. G, Elongation zone. Asterisk indicates the epidermal cells. H, Mature zone. Scale bars for E to H: 30 μm.

AtSTAR1 was mainly expressed at outer cell layers, including lateral root caps in root meristem region and epidermis in the elongation zone (Fig. 5, E–G). In contrast, rice *OsSTAR1* was expressed at all cells of root tips (Huang et al., 2009). Furthermore, the expression of *AtSTAR1* was not responsive to Al stress in Arabidopsis, whereas *OsSTAR1* is highly induced by Al treatment (Huang et al., 2009). These differences in the expression pattern and localization suggest that in addition to involvement of Al tolerance, AtSTAR1 has other roles different from that of OsSTAR1 in Arabidopsis. Early flowering in the knockout line of *AtSTAR1* supports this as described below.

Possible Interaction between AtSTAR1 and ALS3

Work in rice has shown that OsSTAR1 interacts with OsSTAR2 to form a complex and functions as an ABC transporter (Huang et al., 2009). This leads us to hypothesize that AtSTAR1 also requires a protein containing TMDs to be functional. ALS3 was previously reported to be involved in Al tolerance in Arabidopsis (Larsen et al., 2005). It is the unique homolog of OsSTAR2, which encodes only TMDs. Knockout of *ALS3* showed similar phenotype of *atstar1*; enhanced Al sensitivity and early flowering (Figs. 2D and 7C). Furthermore, OsSTAR1 protein, the functional substitute of AtSTAR1, was not detected in the *OsSTAR1;als3* line (Fig. 7E). These results suggest that ALS3 interacts with and stabilizes OsSTAR1 to prevent the degradation of OsSTAR1.

However, the tissue specificity of localization is not completely consistent between AtSTAR1 and ALS3. Although in the root tips, both *AtSTAR1* and *ALS3* are expressed at the outer cell layers, they have different localization in other tissues. *ALS3* was primarily expressed at the phloem of all organs, whereas no expression of *AtSTAR1* was found in the phloem (Fig. 5; Larsen et al., 2005). Furthermore, the expression of *ALS3* was induced by Al (Larsen et al., 2005), but that of *AtSTAR1* was not (Fig. 4B). These findings suggest that AtSTAR1 might form a complex with ALS3 in the root tip region to mediate Al tolerance, whereas in other tissues AtSTAR1 interacts with other proteins to fulfill its function. Deeken et al. (2008) isolated a number of potential mobile transcripts from leaf phloem exudates, which also include *ALS3*. Therefore, there is a possibility that *ALS3* is transcribed at the phloem and then moved to *AtSTAR1*-expressed tissues to perform protein synthesis and interact with AtSTAR1 although further investigation is required in future.

A complex of OsSTAR1/OsSTAR2 transports UDP-Glc, which may be used for modification of cell wall (Huang et al., 2009). Although the transport substrate of AtSTAR1/ALS3 complex was not examined in this study, the complementation of OsSTAR1 in *atstar1* suggests a similar function for AtSTAR1/ALS3.

A recent study showed that the expression of *OsSTAR1* and *OsSTAR2* is regulated by ART1 in rice, a zinc-finger-type transcription factor (Yamaji et al., 2009). Similarly, the expression of *ALS3* is regulated by a zinc-finger transcription factor STOP1 in Arabidopsis, but *AtSTAR1* was not in the list of downstream genes regulated by STOP1 (Sawaki et al., 2009). Consistently, we found that *AtSTAR1* was not induced by

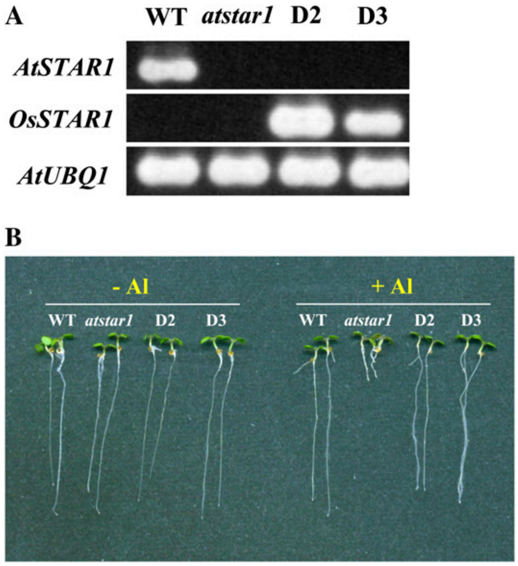

Figure 6. Complementation of *atstar1* by *OsSTAR1*. A, RT-PCR analysis of *AtSTAR1* and *OsSTAR1* expression in the wild type (WT), *atstar1* knockout line, and *OsSTAR1;atstar1* transgenic lines (D2 and D3). B, Al sensitivity in *OsSTAR1;atstar1* lines. Seedlings of the wild type, *atstar1*, and *OsSTAR1;atstar1* lines were grown in a nutrient solution containing 0 or 2 μM Al at pH 5.0 for 7 d. [See online article for color version of this figure.]

Al (Fig. 4), while STOP1 regulates Al-inducible genes (Iuchi et al., 2007); therefore, *AtSTAR1* is supposed to be not regulated by STOP1. It would be interesting to compare the different regulation mechanisms between rice and Arabidopsis in the future.

AtSTAR1 and ALS3 Might Also Be Involved in the Regulation of Flowering Time

Knockout of either *AtSTAR1* or *ALS3* resulted in earlier flowering (Fig. 2D), suggesting that both AtSTAR1 and ALS3 might be also involved in the regulation of flowering time. This phenotype is different from that of *osstar1*, which showed similar flowering time as wild-type rice (Huang et al., 2009). This difference may be attributed to the expression of *AtSTAR1* and *ALS3* in the shoots. Flowering time is a complicated trait and many genes have been suggested to be involved (Simpson and Dean, 2002; Komeda, 2004; Roux et al., 2006). Altered mineral nutrient availability is also suggested to affect the flowering time in Arabidopsis (Kolar and Senkova, 2008). However, among nutrients tested, there was no difference in the concentration between wild type and the knockout line of *AtSTAR1* (Fig. 3, C and D), suggesting that earlier flowering in the mutant was not related to these mineral nutrient availability. It remains to be elucidated how AtSTAR1/ALS3 is involved in the control of flowering time in the future.

In summary, our results indicate that AtSTAR1 is required for Al tolerance in Arabidopsis, probably functioning as a bacterial-type ABC transporter by forming a complex with ALS3. Furthermore, we found that both AtSTAR1 and ALS3 may be also involved in the regulation of flowering time in Arabidopsis.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia-0) was used for all the control experiments. The T-DNA insertion lines *atstar1* (762A06) and *als3* (SALK_004094) were obtained from GABI-Kat (http://www.gabi-kat.de) and the Arabidopsis Biological Resource Center, respectively. The homozygous lines were identified through PCR analysis of the genotype of each line.

Evaluation of Sensitivity to Al, Cd, and La

Seeds of each line were soaked in deionized water and kept at 4°C for 2 to 4 d in the dark to synchronize germination. The seeds were then placed on a plastic mesh floating on a low-strength nutrient solution consisting of one-thirtieth-strength Hoagland nutrient solution without $NH_4H_2PO_4$ and 1 mM $CaCl_2$ in a growth chamber with a 14-h-light/10-h-dark cycle at 22°C. For evaluation of sensitivity to Al, seeds were grown on the low-strength nutrient solution containing 0, 1, 2, 3, or 4 μM $AlCl_3$ at pH 5.0 for 7 d. The solution was renewed every 2 d. After the treatment, root length was measured by a ruler and relative root growth expressed as (root length with Al treatment/root length without Al) × 100 was used to evaluate the Al sensitivity. For evaluation of sensitivity to other metals, seeds were exposed to the same nutrient solution containing 0, 2 μM $CdCl_2$ or 0.1 μM $LaCl_3$ for 7 d as described above.

Determination of Cation Concentrations

To compare the macro- or microcation concentration in the shoots of the wild type and *atstar1*, plants were cultured in a one-thirtieth-strength Hoagland nutrient solution. After growth for 38 d, the shoots were harvested and dried in an oven at 70°C for a week. The dried tissue was fully digested with concentrated HNO_3 (60%) at a temperature up to 140°C. The concentrations of potassium, calcium, magnesium, zinc, iron, and manganese in the digested solution were determined with flame atomic absorption spectrometry (model Z-2000, Hitachi), after appropriate dilution with 0.1 N HNO_3.

RNA Isolation and RT-PCR Analysis

To examine the expression pattern of *AtSTAR1* in Arabidopsis, different tissues of 40-d-old plants, including roots, leaves, stem, and flowers, were excised and frozen in liquid nitrogen within 5 min of harvest for RNA extraction. For the expression analysis of *AtSTAR1* in response to different metals, seedlings were grown on a nutrient solution consisting of one-thirtieth-strength Hoagland nutrient solution ($NH_4H_2PO_4$ omitted) plus 1 mM $CaCl_2$ at pH 5.0 for 7 d and then exposed to the same nutrient solution containing 0, 3 μM $AlCl_3$, 3 μM $CdCl_2$, or 3 μM $LaCl_3$ at pH 5.0 for 6 h and then the roots were sampled. For the expression analysis of *AtSTAR1* expression in response to low pH, 40-d-old plants were exposed to one-tenth-strength Hoagland nutrient solution at pH 6.2 or pH 4.2 for 8 h and then the roots were sampled for RNA isolation. Total RNA was extracted using the RNeasy mini kit (Qiagen). One microgram of DNase I-treated total RNA was used for first-strand cDNA synthesis using a SuperScript II kit (Invitrogen) and an oligo$(dT)_{12\text{-}18}$ primer (Invitrogen) following the manufacturer's instructions. One-tenth of the reaction volume was used as template for PCR of *AtSTAR1*, and *AtUBQ10* was used as an internal control (26 cycles). The primer sequences for RT-PCR of *AtSTAR1* were 5′-GTTGAAGAAACCTCTGTGCCATT-3′ and 5′-GTCGTAGAGTTGGAATGCTTTTTC-3′ and the forward and reverse sequences of *AtUBQ10* were 5′-GGAGGTGGAGAGTTCTGACA-3′ and 5′-AGACCAAGTGAAGTGTGGAC-3′, respectively. For real-time RT-PCR analysis, 1 μL of 10-fold-dilution cDNA from each sample was used for the quantitative analysis of gene expression performed with SYBR Premix Ex Taq (Takara). *AtUBQ1* (forward primer, 5′-TCTACACTTCATCTTGTGTTGAGGC-3′; reverse primer, 5′-CACTGAAACAAGAAAAACAAACCCT-3′) was used as an internal control in this experiment. Data were collected in accordance with the 7500 real-time PCR system (Applied Biosystems).

For RT-PCR analysis of *AtSTAR1* and *ALS3* expression, 1 mL of 10-fold-dilution cDNA from the wild type and the T-DNA lines was used as templates

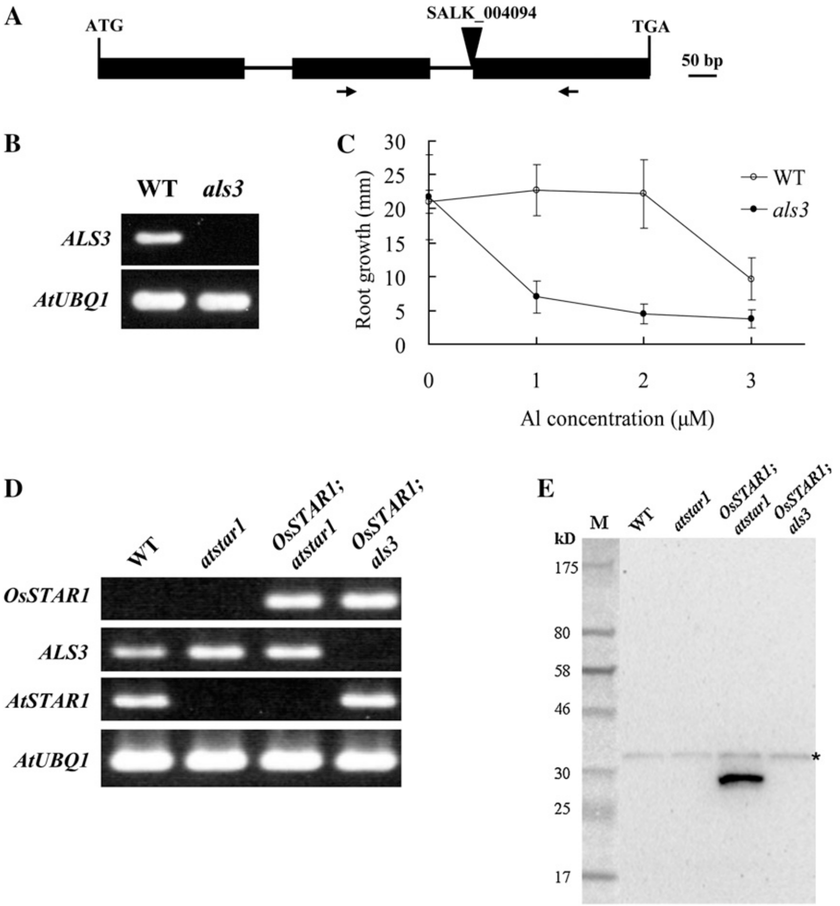

Figure 7. Possible interaction with ALS3. A, A T-DNA insertion line (*als3*, SALK_004094) of *ALS3*. Triangle on the top shows insertion position. Arrows show a primer pair for detection of *ALS3* expression by RT-PCR. B, mRNA expression of *ALS3* in the wild type (WT) and *als3*. RT-PCR analysis was performed to detect the mRNA expression of *ALS3* (30 cycles) and the internal control *AtUBQ1* (25 cycles). C, Al sensitivity in the wild type and *als3*. Seedlings of the wild type and the knockout line were grown in a nutrient solution containing 0, 1, 2, or 3 μM Al at pH 5.0 for 7 d. Data are means ± SD (n = 10–15). D, Expression of *OsSTAR1*, *ALS3*, and *AtSTAR1* in different lines. RT-PCR analysis was performed to detect mRNA expression of *OsSTAR1*, *ALS3*, *AtSTAR1*, and the internal control *AtUBQ1* (28 cycles) in the roots. E, Western-blot analysis of OsSTAR1 protein. Root total protein (40 μg) in each lane was used and detected with an antibody against OsSTAR1. The asterisk indicates a nonspecific band.

in a 20 μL solution containing 1× Ex Taq buffer, 0.5 μM of each primer, 0.2 mM deoxynucleotide triphosphates, and 0.1 unit of Ex Taq DNA polymerase. PCR was carried out as follows: 95°C for 3 min, 35 (*AtSTAR1*) or 30 (*ALS3*) cycles of 95°C denaturing for 30 s, 58°C annealing for 1 min and 72°C extension for 30 s, and a 5 min extension at 72°C. The primer sequences for *AtSTAR1* were 5′-GTTGAAGAAACCTCTGTGCCATT-3′ and 5′-GTCGTAGAGTTGGAA-TGCTTTTTC-3′ and those of *ALS3* were 5′-CAATGTTCTTGCTCGTCCT-CCT-3′ and 5′-CCGCCCATTATCATACCAGTCA-3′, respectively. *AtUBQ10* or *AtUBQ1* was used as internal control. Their primer sequences were 5′-GGAGGTGGAGAGTTCTGACA-3′ and 5′-AGACCAAGTGAAGTGTG-GAC-3′ for *AtUBQ10*, and 5′-TCTACACTTCATCTTGTGTTGAGGC-3′ and 5′-CACTGAAACAAGAAAAACAAACCCT-3′ for *AtUBQ1*.

Complementation Analysis

For complementation test of *atstar1* in Arabidopsis, a 3.8-kb DNA fragment harboring 1.7-kb promoter and *AtSTAR1* gene was directly amplified with a pair of primers: 5′-CTCGAGATGCGAAGGTATGAAAAATAAC-3′ and 5′-CACCACAGAAACCCATCCCAAAA-3′. The DNA fragment was inserted into the pPZP2H-lac binary vector (Fuse et al., 2001) and transformed into *atstar1* mutant by *Agrobacterium tumefaciens*-mediated floral-dip method (Clough and Bent, 1998). For the introduction of *OsSTAR1* into *atstar1* mutant, the open reading frame of *OsSTAR1* was amplified from rice (*Oryza sativa*) root cDNA by PCR using 5′-agaaTTCTCAAGATTACAGGTACACATC-3′ and 5′-aatgtacaaaggGATGCGCATTGCCCGGATTCCC-3′ primer pair. The DNA fragment was cloned into pPZP2Ha3 binary vector that contains a cauliflower mosaic virus *35S* promoter (Fuse et al., 2001) and then the construct was transformed into *atstar1* mutant.

Cross between *OsSTAR1;atstar1* and *als3* Mutant

We made a cross between *OsSTAR1;atstar1* line and the T-DNA line (*als3*) to generate *OsSTAR1;als3* line. The resultant F1 plants were genotyped to select *OsSTAR1*-containing plants through direct genomic PCR. After self pollination, a large F2 population from one F1 progeny was subjected for genotyping by genomic PCR and F2 plants with the genotype of *OsSTAR1;AtSTAR1;als3* were selected for the collection of F3 seeds. mRNA expression analysis and western-blot analysis was performed in *OsSTAR1;atstar1* and *OsSTAR1; als3* lines to investigate the possible relationship between *OsSTAR1* and *ALS3*. Since the *OsSTAR1* locus was heterozygous in the two lines, we first genotyped each plant by genomic PCR and then mixed the roots of *OsSTAR1*-positive plants for both mRNA expression analysis and western-blot analysis.

Western-Blot Analysis

Total proteins were extracted from the roots of the wild type, *atstar1*, *OsSTAR1;atstar1*, and *OsSTAR1;als3* lines. The fresh roots (approximately 0.2 g) of each line were frozen in liquid nitrogen and ground with a homogenizer. The samples were then homogenized in 500 μL lysis buffer consisting of 100 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.5% (w/v) polyvinylpolypyrrolidone, 5 mM EDTA, 3.3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol. After that, the homogenates were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were then transferred to new tubes as total protein. Protein concentrations were measured by the Bradford method (Bradford, 1976). Equal amounts of samples (40 μg protein) were mixed with same volume of sample buffer containing 250 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 200 mM β-mercaptoethanol. The mixture was incubated at 65°C for 20 min and then loaded into a SDS-PAGE gel consisting of 11% polyacrylamide and 0.1% SDS for electrophoresis. The gel was then transferred to polyvinylidene difluoride membrane with a semidry blotting system, and the membrane was treated with the primary antibody anti-OsSTAR1 diluted at 1:100. Anti-rabbit IgG HRP conjugate (1:10,000 dilution; GE Healthcare) was used as a secondary antibody, and ECL plus (GE Healthcare) was used for detection via chemiluminescence.

GUS Analysis

For construct of *pAtSTAR*:*AtSTAR1-GUS*, a 3.3-kb DNA fragment harboring 2.4-kb promoter region and the *AtSTAR1* gene except for the stop codon was amplified by PCR of the Arabidopsis genomic DNA. The primer pair for the amplification and introduction of restriction sites was 5′-ttggtaccGCTATCGCACATAACAAGAGGACA-3′ and 5′-aatctAGAACTGAGTTGAAGAAACCTCTGTGC-3′. The obtained DNA fragment was then fused in frame to the GUS gene and introduced into the pPZP2H-lac binary vector, and finally transformed into Arabidopsis wild-type plants.

For histochemical staining of GUS activity, T2 transgenic plants were used. Roots and leaves were collected from 5-week-old plants grown in one-tenth-strength Hoagland nutrient solution at 22°C. The samples were pretreated with or without cold 90% acetone and immersed into staining buffer consisting of 50 mM sodium phosphate (pH 7.2), 1% Triton X-100, 2 mM potassium ferri- and ferrocyanide, and 1 mM X-Gluc under vacuum for 20 min and then incubated at 37°C overnight. Stained samples were subjected to an ethanol series (20%, 35%, 50%, and 70%) for the removal of chlorophyll or imbedded into 5% agarose for sections. Cross sections (50–100 μm) were made by using a micro-slicer (ZERO1, Dosaka EM). Stained tissues were observed by a microscopy (CKX41, Olympus) and photographed by a camera (DP20, Olympus).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB567722.

ACKNOWLEDGMENTS

We would like to thank GABI-Kat (Max Planck Institute for Plant Breeding Research, Cologne, Germany) and the Arabidopsis Biological Resource Center for providing T-DNA insertion lines.

Received February 17, 2010; accepted May 21, 2010; published May 24, 2010.

LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J **16:** 735–743

Deeken R, Ache P, Kajahn I, Klinkenberg J, Bringmann G, Hedrich R (2008) Identification of *Arabidopsis thaliana* phloem RNAs provides a search criterion for phloem-based transcripts hidden in complex datasets of microarray experiments. Plant J **55:** 746–759

Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.): II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol **103:** 695–702

Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF (2007) An aluminum-activated citrate transporter in barley. Plant Cell Physiol **48:** 1081–1091

Fuse T, Sasaki T, Yano M (2001) Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotechnol **18:** 219–222

Garcia O, Bouige P, Forestier C, Dassa E (2004) Inventory and comparative analysis of rice and Arabidopsis ATP-binding cassette (ABC) systems. J Mol Biol **343:** 249–265

Hoekenga OA, Vision TJ, Shaff JE, Monforte AJ, Lee GP, Howell SH, Kochian LV (2003) Identification and characterization of aluminum tolerance loci in Arabidopsis (Landsberg *erecta* x Columbia) by quantitative trait locus mapping: a physiologically simple but genetically complex trait. Plant Physiol **132:** 936–948

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. Plant Cell **21:** 655–667

Iuchi S, Koyama H, Iuchi A, Kobayashi Y, Kitabayashi S, Kobayashi Y, Ikka T, Hirayama T, Shinozaki K, Kobayashi M (2007) Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. Proc Natl Acad Sci USA **104:** 9900–9905

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol **46:** 237–260

Kochian LV, Hoekenga OA, Pineros MA (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol **55:** 459–493

Kolar J, Senkova J (2008) Reduction of mineral nutrient availability accelerates flowering of *Arabidopsis thaliana.* J Plant Physiol **165:** 1601–1609

Komeda Y (2004) Genetic regulation of time to flower in *Arabidopsis thaliana.* Annu Rev Plant Biol **55:** 521–535

Larsen PB, Cancel J, Rounds M, Ochoa V (2007) *Arabidopsis ALS1* encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. Planta **225:** 1447–1458

Larsen PB, Geisler MJB, Jones CA, Williams KM, Cancel JD (2005) *ALS3* encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in *Arabidopsis.* Plant J **41:** 353–363

Ma JF (2008) Syndrome of aluminum toxicity and diversity of aluminum resistance in higher plants. Int Rev Cytol **264:** 225–253

Ma JF, Nagao S, Huang CF, Nishimura M (2005) Isolation and characterization of a rice mutant hypersensitive to Al. Plant Cell Physiol **46:** 1054–1061

Ma JF, Ryan PR, Delhaize E (2001) Aluminum tolerance in plants and the complexing role of organic acids. Trends Plant Sci **6:** 273–278

Ma JF, Zheng SJ, Matsumoto H (1997) Specific secretion of citric acid induced by Al stress in *Cassia tora* L. Plant Cell Physiol **38:** 1019–1025

Magalhaes JV, Liu J, Guimarães CT, Lana UG, Alves VM, Wang YH, Schaffert RE, Hoekenga OA, Piñeros MA, Shaff JE, et al (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat Genet **39:** 1156–1161

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol **58:** 347–375

Roux F, Touzet P, Cuguen J, Corre VL (2006) How to be early flowering: an evolutionary perspective. Trends Plant Sci **11:** 375–381

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J **37:** 645–653

Sawaki Y, Iuchi S, Kobayashi Y, Kobayashi Y, Ikka T, Sakurai N, Fujita M, Shinozaki K, Shibata D, Kobayashi M, et al (2009) STOP1 regulates multiple genes which protect Arabidopsis from proton and aluminum toxicities. Plant Physiol **150:** 281–294

Simpson GG, Dean C (2002) Arabidopsis, the rosetta stone of flowering time? Science **296:** 285–289

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-rhizobium symbiosis. Plant Physiol **144:** 2000–2008

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, et al (2008) Plant ABC proteins—a unified nomenclature and updated inventory. Trends Plant Sci **13:** 151–159

von Uexkull HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant Soil **171:** 1–15

Yamaji N, Huang CF, Nagao S, Yano M, Sato Y, Nagamura Y, Ma JF (2009) A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. Plant Cell **21:** 3339–3349

Zhao ZQ, Ma JF, Sato K, Takeda K (2003) Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). Planta **217:** 794–800