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A Zinc Finger Transcription Factor ART1 Regulates Multiple Genes Implicated in Aluminum Tolerance in Rice [C][W]

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Aluminum (Al) toxicity is the major limiting factor of crop production on acid soils, but some plant species have evolved ways of detoxifying Al. Here, we report a C2H2-type zinc finger transcription factor ART1 (for Al resistance transcription factor 1), which specifically regulates the expression of genes related to Al tolerance in rice (*Oryza sativa*). *ART1* is constitutively expressed in the root, and the expression level is not affected by Al treatment. ART1 is localized in the nucleus of all root cells. A yeast one-hybrid assay showed that ART1 has a transcriptional activation potential and interacts with the promoter region of *STAR1*, an important factor in rice Al tolerance. Microarray analysis revealed 31 downstream transcripts regulated by ART1, including *STAR1* and *2* and a couple of homologs of Al tolerance genes in other plants. Some of these genes were implicated in both internal and external detoxification of Al at different cellular levels. Our findings shed light on comprehensively understanding how plants detoxify aluminum to survive in an acidic environment.

INTRODUCTION

Ionic aluminum (mainly Al^{3+}) inhibits root elongation rapidly at low concentrations (Kochian et al., 2004; Ma, 2007; Poschenrieder et al., 2008). Subsequent inhibition of water and nutrient uptake results in reduced crop production and increased susceptibility to environmental stresses on acid soils, where Al toxicity is the major limiting factor for crop production (von Uexkull and Mutert, 1995). Approximately 55, 39, and 37% of the soil in tropical America, tropical Africa, and tropical Asia, respectively, are acidic, the total area being 1.6 billion hectares (Sanchez and Salinas, 1981). Therefore, enhancing Al tolerance of crops has been considered a key to increasing crop productivity on acidic problem soils, which would subsequently help solve the problem of food shortage and biofuel production. Some plants have evolved strategies to detoxify Al. Elucidation of these strategies will help us generate crops with increased Al tolerance.

Some Al-tolerant plant species or cultivars are able to detoxify Al both internally and externally. Internal detoxification in Al-accumulating plants is achieved by sequestration of Al into the vacuoles and chelation with organic acids such as citrate and oxalate (Ma, 2007). The most well-documented mechanism for external detoxification is the secretion of organic acid anions, such as oxalate, citrate, and/or malate, from the roots in response to Al. These organic acid anions chelate toxic Al and thereby detoxify Al in the rhizosphere (Ryan et al., 2001; Kochian et al., 2004; Ma, 2007; Poschenrieder et al., 2008). Genes responsible for Al-induced secretion of malate (*ALMT1*) have been identified in wheat (*Triticum aestivum*), *Arabidopsis thaliana*, and rape (*Brassica napus*; Sasaki et al., 2004; Hoekenga et al., 2006; Ligaba et al., 2006). Recently, the genes, involved in Al-induced secretion of citrate have also been identified in barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and *Arabidopsis* (Furukawa et al., 2007; Magalhaes et al., 2007; Liu et al., 2009). All these genes encode a citrate efflux transporter that belongs to the multidrug and toxic compound extrusion (MATE) family.

Japonica cultivars of rice (*Oryza sativa*) show the most tolerance to Al among the small-grain cereal crops (Foy, 1988). Recently, two genes (*STAR1* and *2*) required for Al tolerance in rice have been cloned (Huang et al., 2009). *STAR1* and *2* encode ATP binding and transmembrane domains of a novel ABC transporter, respectively. The complex between STAR1 and 2 transports UDP-glucose, which is used for modification of the cell wall although the exact mechanism remains unknown. Here, we report a gene (*Al resistance transcription factor 1* [*ART1*]) that encodes a transcription factor that regulates 31 genes implicated in Al tolerance, including *STAR1* and *2* in rice.

RESULTS

Isolation and Phenotypic Analysis of the *art1* Mutant

A mutant sensitive to Al rhizotoxicity (*art1*) was isolated by screening M3 lines derived from an Al-tolerant cultivar of rice

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(Koshihikari) irradiated with γ-rays according to the procedures described previously (Ma et al., 2005). There is no difference in the root and shoot morphology between the *art1* and its wild type. In the absence of Al, the mutant showed root growth similar to that of the wild type (Figure 1A). However, in the presence of Al, the root elongation of *art1* was inhibited significantly more than in the wild type. At 10, 30, and 50 μM Al, root elongation was inhibited by 64, 83, and 88%, respectively, in *art1* and was inhibited by 27, 49, and 68%, respectively, in the wild type (Figure 1A). In neutral soil, both lines grew similarly (Figure 1B), while in acid soil, the root growth of *art1* was completely inhibited. The wild type and *art1* were equally sensitive to a low pH and to other metals, including Cd, La, Zn, and Cu (Figures 1C and 1D). In addition, when grown in a field at a pH of 6.5 (without Al toxicity stress, but with other natural biotic and abiotic stresses), the plant growth and grain yield did not differ significantly between the wild type and *art1* (see Supplemental Figure 1 online). All these results indicate that *art1* is a mutant specifically sensitive to Al.

Map-Based Cloning of *ART1*

Genetic analysis with 164 F2 seedlings derived from a cross between the mutant and the wild type showed that plants tolerant and sensitive to Al segregated at a ratio of 3:1, indicating that a single recessive gene is responsible for the sensitivity of Al in the *art1* mutant (see Supplemental Figure 2 online). To map the *ART1* gene, we constructed an F2 population by crossing *art1* with Kasalath, an indica cultivar. Bulked segregant analysis with 59 polymorphic InDel markers covering the whole rice genome showed that the C62896 marker on chromosome 12 was linked to the *ART1* gene (see Supplemental Figure 3A online). Cosegregation analyses using 46 Al-sensitive F2 plants indicated that *ART1* was located between MaOs1219 and MaOs1229 on the short arm of chromosome 12, with a distance of 1.1 and 4.5 centimorgans, respectively (see Supplemental Figure 3A online). By fine mapping with a large F2 population (986 plants), we located *ART1* between MaOs1250 and MaOs1246, which covered two overlapping BAC clones (see Supplemental Figure 3B online). The candidate region between these two markers covers

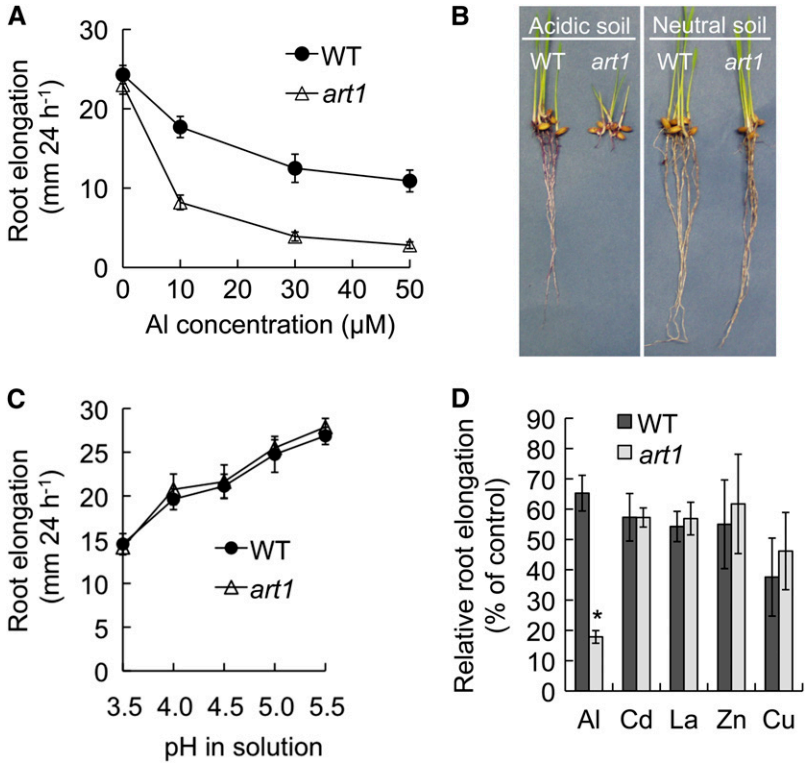

Figure 1. Phenotype of the *art1* Mutant.

(A) Response to Al. Five-day-old seedlings of both the wild-type rice and *art1* were exposed to a 0.5 mM $CaCl_2$ solution containing 0, 10, 30, or 50 μM $AlCl_3$, pH 4.5, for 24 h. Data are means ± SD (n = 10).

(B) Growth on acid soil. Germinated seeds were sowed on acidic soil, pH 4.5, or neutral soil, pH 6.5, and grown for 6 d.

(C) Response to different pHs. Seedlings were exposed to a buffered solution at different pHs for 24 h. Data are means ± SD (n = 10).

(D) Effect of toxic metals on root elongation. Five-day-old seedlings were exposed to a 0.5 mM $CaCl_2$ solution, pH 4.5, containing 0, 30 μM Al, 20 μM Cd, 5 μM La, 100 μM Zn, or 0.5 μM Cu in their chloride form for 24 h. Root elongation was measured before and after the treatment and relative root elongation, (root elongation with metals)/(root elongation without metals) × 100. Data are means ± SD (n = 8 to 10). The asterisk shows a significant difference between the wild type and *art1* ($P < 0.05$ by Student's t test).

38.5 kb and contains six candidate genes based on the prediction from the RAP-DB website. Sequencing the open reading frames (ORFs) of these genes in the mutant revealed that a putative gene Os12g0170400 has a 1-bp deletion at 953 bp from the ATG start codon in its coding sequence, suggesting that this gene is *ART1* (see Supplemental Figure 3C online; Figure 2A).

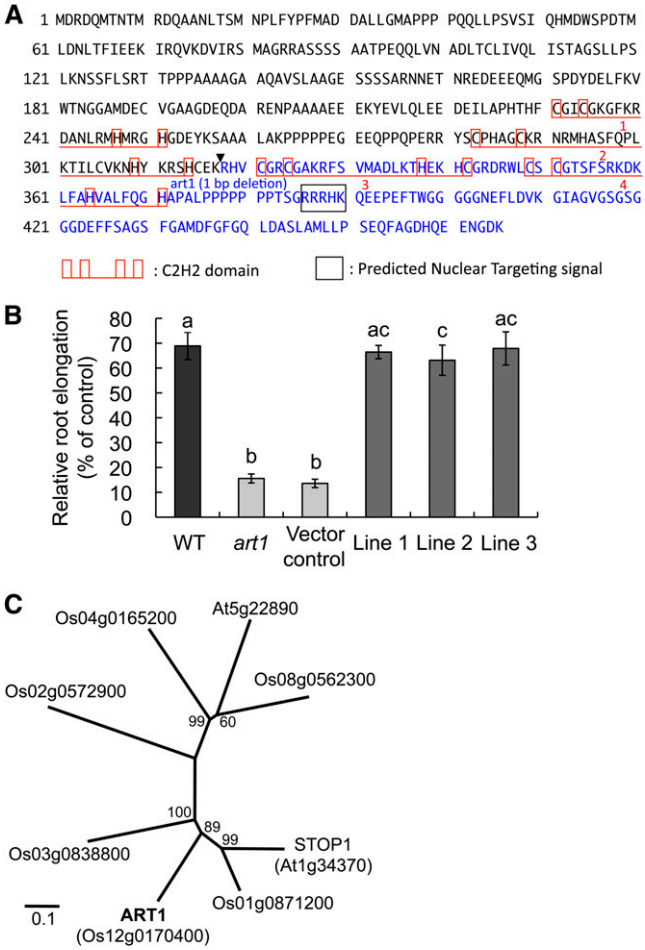

Figure 2. Sequence and Complementation Test of *ART1*.

(A) Amino acid sequence of ART1. Predicted C2H2 domain (red frame with underline), putative nuclear targeting signal (black frame), mutation site (reversed triangle), and defect region in *art1* due to frame shift by 1-bp deletion (blue letters) are shown.

(B) Complementation test. Relative root elongation of the wild type, *art1*, transgenic rice with empty vector (vector control), and three independent transgenic lines with *ART1* genomic region (lines 1 to 3) were measured. Data are means ± SD (n = 13 to 15 biological replicates). Different letters indicate significant differences at $P < 0.05$ by Tukey's test.

(C) Phylogenic tree of ART1-like C2H2 zinc finger proteins in rice (Os-) and *Arabidopsis* (At-). The sequence alignment used to generate the phylogeny is presented in Supplemental Data Set 1 online. Bootstrap values from 1000 trials are indicated. The 0.1 scale shows substitution distance.

[See online article for color version of this figure.]

Complementation Test

To confirm this mapping result, we performed a complementation test by introducing a 6.0-kb DNA fragment containing the candidate gene Os12g0170400 plus a 3.4-kb upstream region into *art1* mutant by *Agrobacterium tumefaciens*–mediated transformation. The tolerance to Al in the three independent transgenic lines harboring the candidate gene recovered to the similar level of the wild type (Figure 2B), whereas the tolerance in the vector control line remains the same as the *art1* mutant. These results indicate that the one-base deletion in Os12g0170400 is responsible for the Al hypersensitivity observed in the mutant *art1*. In addition, we checked transcript levels of these three independent complementation lines by quantitative RT-PCR. Although these levels include both endogenous *ART1*/*art1* and transgenic *ART1*, one of the complementation lines, line 2, showed significantly higher expression of *ART1*, probably due to positional effect or copy number of the transgene (see Supplemental Figure 4 online). However, the Al tolerance of this line was not significantly different from other two complementation lines (Figure 2B). These results suggest that ART1 activity is probably also controlled by posttranslational activation as discussed later.

Phylogenic Analysis of ART1

ART1 is predicted to encode a putative C2H2 zinc finger protein with 465 amino acids (Figure 2A), which has four potential zinc finger domains. The 1-bp deletion in the mutant resulted in a frame shift and loss of the last two C2H2 domains. BLAST searches revealed that *ART1* has five close homologs in rice (Figure 2C). There are two close homologs in *Arabidopsis* genome, and the closest one (At1g34370, STOP1) shared 41.2% identity with ART1. STOP1 has been identified as a putative transcriptional factor regulating both H^+ and Al tolerance (Iuchi et al., 2007). However, ART1 is not the likely rice ortholog of STOP1, as the closest homolog of STOP1 in rice is Os01g0871200 (Figure 2C), not ART1. Also, STOP1 regulates H^+ and Al tolerance in *Arabidopsis*, but ART1 specifically regulates Al-responsive genes in rice (Figure 1C). Indeed, the specific downstream genes regulated by ART1 and STOP1 are also different as discussed later.

Expression Patterns and Localization of *ART1*

We investigated the expression pattern of *ART1* with quantitative real-time RT-PCR. *ART1* was mainly expressed in the roots (Figure 3A), and the expression level in the root tips (0 to 1 cm) was similar to that in the basal region roots (1 to 2 cm). Furthermore, the expression level of this gene was not significantly affected by Al (Figure 3A).

To examine the subcellular localization of ART1, we transiently introduced a gene (ART1-GFP) fused between *ART1* and a gene encoding a green fluorescent protein (GFP) into protoplasts derived from rice callus. The ART1-GFP green fluorescence was observed only in the nuclei (Figure 3B), whereas GFP alone as a control was found in both the nuclei and cytoplasm. These results indicate that ART1 is localized to the nucleus.

We also performed immunostaining with the anti-ART1 antibody to investigate the cell specificity of localization of ART1. ART1 was localized to the nuclei of all cells in the root tip (Figure

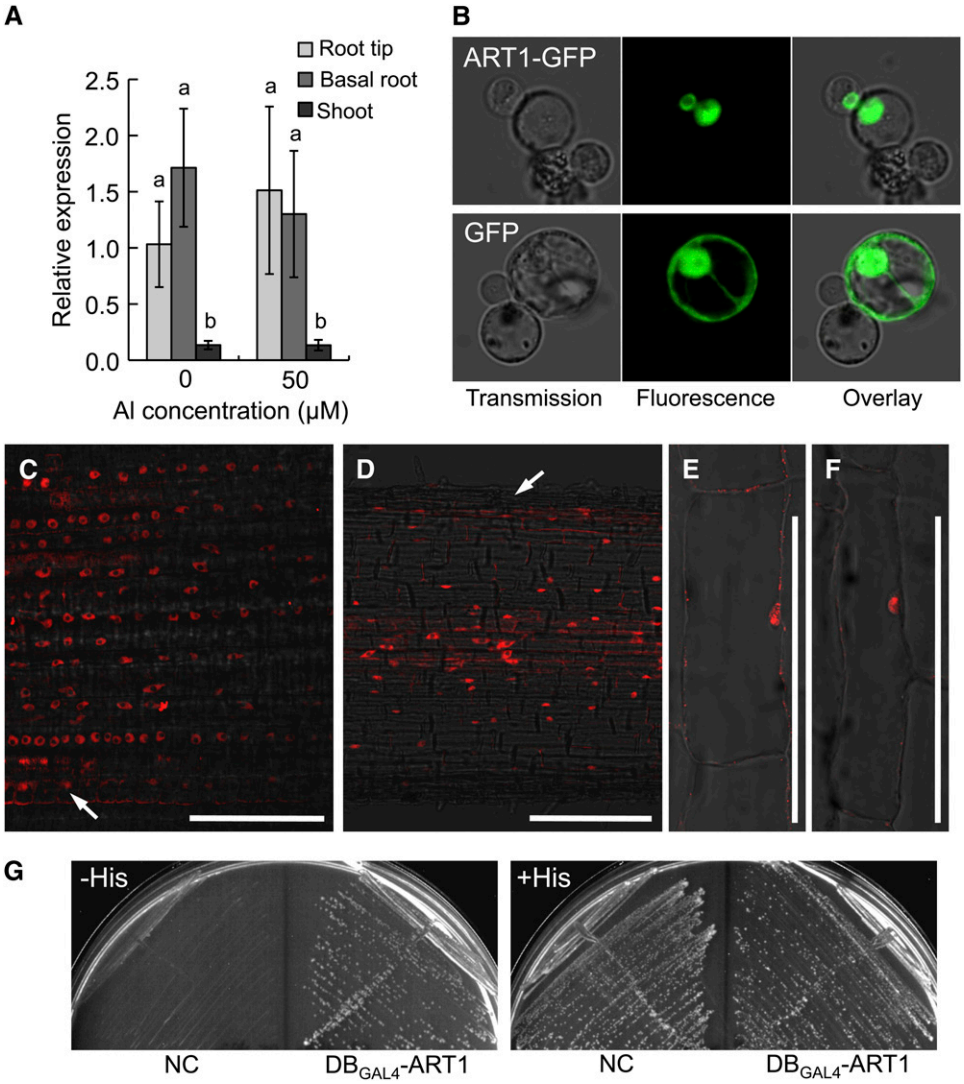

Figure 3. Expression, Localization, and Transcriptional Activation Analysis of ART1.

(A) Relative mRNA expression of *ART1* in different tissues. Five-day-old seedlings (cv Koshihikari) were exposed to a 0.5 mM $CaCl_2$ solution containing 0 or 50 μM Al for 6 h, and then root tips (0 to 10 mm), basal roots (10 to 20 mm), and shoots were used for quantitative RT-PCR to determine relative expression of *ART1*. Histone H3 was used as an internal control. Expression relative to the root tip expression without Al treatment is shown. Data are means ± SD (n = 6; three biological replicates and two technical replicates). Different letters indicate significant differences at $P < 0.05$ by Tukey's test.
(B) Transient expression of the ART1-GFP fusion and GFP alone as control in rice callus protoplasts.
(C) and **(D)** Immunostaining of root longitudinal section with an anti-ART1 antibody at 1 mm **(C)** and 20 mm **(D)** from the tip treated with Al (50 μM, 6 h). Arrows indicate root epidermal cells with **(C)** or without **(D)** ART1 expression.
(E) and **(F)** Magnified images for single root cells at 20 mm from the tip treated with **(F)** or without **(E)** Al (50 μM, 6 h). Bars = 100 μm in **(C)** to **(F)**.
(G) Transcriptional activation analysis in yeast. Yeast strain AH109 carrying fusion gene of GAL4 DNA binding domain and ART1 (DB$_{GAL4}$-ART1) or DB$_{GAL4}$ alone as control (NC) were cultured on SD medium with or without histidine (His) at 30°C for 3 d.

3C) and all cells except the epidermal cells in the basal region (Figure 3D). Exposure to Al did not affect the subcellular localization of ART1 (Figures 3E and 3F).

Determination of Transcriptional Activation Potential

To examine whether ART1 has transcriptional activation potential, we conducted a modified yeast one-hybrid analysis using a chimeric protein with the DNA binding domain of the yeast GAL4 transcription factor and full-length ART1 (DB$_{GAL4}$-ART1). If ART1 has transcriptional activation potential, DB$_{GAL4}$-ART1 bound to the GAL1 upstream activating sequence will induce *HIS3* reporter gene expression and accordingly complement the histidine requirement of the host yeast strain (AH109). The yeast carrying DB$_{GAL4}$-ART1 grew well on the medium without histidine, but the control yeast carrying DB$_{GAL4}$ alone (NC) did not

(Figure 3G). These results indicate that ART1 has a transcriptional activation potential, at least in yeast.

Genes Regulated by ART1

To examine the genes regulated directly or indirectly by ART1, we compared genome-wide transcriptional profiles between treatments with Al and without Al in both the wild type and the mutant by microarray analysis with the rice 44 K oligo microarray. The roots were exposed for a short period (4 h) to a low Al concentration (10 μM), at which concentration the root elongation of the wild type was hardly inhibited so that genes affected by Al toxicity could be excluded. The microarray analysis identified differentially expressed genes between the wild type and mutant (false discovery rate [FDR] < 0.1). Then, genes upregulated by more than threefold in the wild type, but hardly changed in the mutant (less than twofold change) were picked up. As a result, a total of 31 genes were selected as candidates for genes regulated by ART1. Based on the annotation database, the candidate genes regulated by ART1 are classified into four groups: (1) cell wall maintenance and root elongation, (2) membrane protein, (3) metabolism, and (4) unknown (Table 1). These genes are probably involved in Al detoxification at different cellular levels in rice as discussed later.

To confirm the microarray result, we examined the expression pattern of two genes, *STAR1* and *STAR2*, belonging to group 2 (Table 1). Both genes have been demonstrated to be required for Al tolerance in rice (Huang et al., 2009). In the absence of Al, there was no difference between the wild type and the mutant in the expression level of *STAR1* and *2* (Figures 4A and 4B). However, in the presence of Al, the expression levels of both *STAR1* and *2* were upregulated in the wild type but not in the mutant (Figures 4A and 4B). Interestingly, we found that the expression of both *STAR1* and *2* is not responsive to a low pH treatment as reported previously (Figures 5A and 5B) (Huang et al., 2009). Furthermore, we examined the direct interaction between the ART1 protein and the promoter region of *STAR1* using a yeast one-hybrid assay. ART1 protein can interact with promoter regions of *STAR1* (−939, −629, and −436 to −172 from the start codon), but we found no interaction in the region (−297 to −172) in the yeast assay (Figure 4C). These results suggest that a *cis*-acting element(s) recognized by the ART1 transcription factor is present in the region between −436 and −298 of the *STAR1* promoter. The exact *cis*-element(s) in this region remains to be identified in the future.

To further demonstrate that ART1 also regulates other genes involved in Al tolerance, we made a double mutant between *star1* and *art1*. Al tolerance tests showed that the double mutant is more sensitive to Al than the single mutant *star1* at 5 μM Al (Figure 4D). These results support that ART1 also regulates other Al tolerance genes, although *STAR1* may play a major role in Al tolerance. The Al sensitivity of *star1* is higher than *art1* (Figure 4D). Interestingly, the distribution of Al in root cells observed by both morin and eriochrome cyanine R staining was different between *star1* and *art1* (see Supplemental Figure 5 online). Al reached all cortex cells in the *star1* mutant root, but not in *art1*. These differences are due to the basic level expression of functional *STAR1* and other downstream genes in the *art1* mutant (Figure 4D). In other words, knockout of the ART1 transcription factor does not result in silence of the downstream genes (Figures 4A and 4B). This result also suggests that other downstream genes and their upregulation by ART1 in response to Al are required for Al tolerance in rice.

DISCUSSION

Genes Regulated by ART1 Are Implicated in the Detoxification of Al at Different Cellular Levels

Our results clearly show that ART1 is a transcription factor localizing to the nucleus and having transcriptional activation potential (Figure 3). Furthermore, specific response of *art1* mutant to Al, but not to low pH and other metals, suggests that genes regulated by ART1 are specifically implicated in Al tolerance (Figure 1). Although the exact mechanisms for Al-induced root inhibition have not been elucidated, it is clear that Al targets multiple cellular sites, including the cell walls, plasma membranes, and cellular processes, such as signal transduction pathways and homeostasis mechanisms (Barceló and Poschenrieder, 2002; Kochian et al., 2004; Ma, 2007; Poschenrieder et al., 2008). Therefore, plants have to detoxify Al at different cellular levels for survival. Among candidate genes regulated by ART1, most of them are implicated in the internal and external detoxification of Al (Table 1). Candidate genes in group 1 are probably involved in the cell wall maintenance and/or root elongation. For example, *Os04g0583500* encodes an expansin, EXPA10. Expansins were identified as proteins that mediate extension of isolated cell walls (Lee and Kende, 2002), and the effect of Al on expansin has been reported (Gao et al., 2008). Os10g0524600 is the closest homolog of *Arabidopsis* subtilisin-like Ser protease, SDD1, which is exported to the apoplast and associates with the plasma membrane and then acts as a putative generator of extracellular signal (von Groll et al., 2002). *Os01g0652100* encodes a close homolog of *Arabidopsis* PMR5. The *pmr5* mutant exhibited pectin enrichment in the cell wall, although the function of PMR5 protein is still unknown (Vogel et al., 2004). *Os01g0178300* encodes a small Cys-rich peptide, CDT3. A similar peptide, CDT1, was proposed to be localized in the cell wall, and overexpression of this peptide gave cadmium tolerance in transgenic *Arabidopsis* (Kuramata et al., 2009). Os01g0860500 is a close homolog of *Arabidopsis* putative chitinase At5g24090, which is a cell wall–associated protein in *Arabidopsis* suspension cells and probably involved in the mechanical properties of the cell wall (Kwon et al., 2005). Since >90% of Al is bound to the cell wall and affects cell wall extensibility (Horst et al., 1983; Ma et al., 2004), the upregulation of these genes by ART1 will likely contribute to Al tolerance, although further confirmation is required.

Group 2 includes nine membrane protein genes (Table 1). Among them, a bacterial-type ABC transporter composed of STAR1 and STAR2 has been demonstrated to play an essential role in detoxifying Al (Huang et al., 2009). Os03g0755100/ALS1 is a homolog of *Arabidopsis* ALS1, which has been reported as a putative half-type ABC transporter localized to the tonoplast and probably responsible for sequestration of Al into the vacuoles in *Arabidopsis* (Larsen et al., 2007). A putative citrate efflux transporter, Os10g020131800, which showed high similarity with an

Table 1. Fold Changes in Gene Expression

RAP-DB	*Arabidopsis* Homolog	Description	Fold Change by Al Treatment						Harboring QTL
			Wild Type	SD	Mutant	SD	FDR	P Value	
(1) Cell wall maintenance and root elongation									
Os01g0178300	None	OsCDT3	4.42	0.45	1.10	0.06	0.02	0.000	
Os01g0652100	At1g29050	PMR5-like DUF231 domain containing protein	3.08	0.10	1.37	0.05	0.01	0.000	
Os01g0860500	At5g24090	Chitinase	8.69	0.54	1.15	0.08	0.02	0.000	
Os03g0760800	At2g39540	Gibberellin-regulated Cys-rich protein family	5.11	0.62	1.27	0.10	0.02	0.000	
Os04g0583500	EXP14/At3g03220	Expansin-A10	3.38	0.95	1.11	0.13	0.06	0.004	
Os09g0479900	At5g59810	Subtilisin-like Ser protease	3.15	0.29	1.54	0.03	0.02	0.000	
Os10g0524600	SDD1/At1g04110	Subtilisin-like Ser protease	3.25	0.62	1.51	0.14	0.06	0.003	
(2) Membrane protein									
Os01g0869200	At3g19640	Putative Mg^{2+} transporter	4.19	0.22	1.26	0.05	0.01	0.000	Ma et al. (2002)
Os02g0131800	At1g80830	Nramp	5.43	0.25	1.96	0.10	0.02	0.000	Ma et al. (2002)
Os02g0755900	At1g22400	UDP-glucuronosyl/ UDP-glucosyltransferase	3.43	0.98	1.15	0.10	0.07	0.004	
Os03g0755100	ALS1/At5g39040	At ALS1 homolog	3.06	0.09	1.21	0.05	0.01	0.000	
Os05g0119000	At2g37330	STAR2	3.78	0.29	1.64	0.05	0.02	0.000	
Os06g0695800	At1g67940	STAR1	3.60	0.24	1.07	0.06	0.02	0.000	
Os09g0426800	WAX2/At5g57800	GLOSSY1-like	4.48	0.92	1.60	0.11	0.04	0.001	
Os10g0206800	At1g51340	MATE	4.14	0.33	1.45	0.02	0.02	0.000	
Os10g0578800	At1g32080	LrgB-like	4.10	0.38	1.84	0.11	0.02	0.000	
(3) Metabolism and detoxification									
Os01g0716500	At5g10830	SAM-dependent methyltransferase	4.39	0.62	1.78	0.38	0.06	0.003	
Os02g0186800	At2g30750	Cytochrome P450 family protein	4.69	0.59	1.38	0.16	0.03	0.000	
Os02g0770800	NIA1/At1g77760	Nitrate reductase	9.39	2.63	1.96	0.44	0.02	0.000	
Os12g0227400	At3g03080	Allyl alcohol dehydrogenase	4.19	0.85	1.18	0.15	0.04	0.001	
(4) Unknown									
Os01g0731600	At1g78780	Hypothetical protein	11.62	1.19	1.19	0.21	0.02	0.000	
Os01g0766300	none	Hypothetical protein	3.58	0.70	1.13	0.09	0.03	0.000	
Os01g0919200	none	Hypothetical protein	3.10	0.88	1.29	0.19	0.08	0.008	Wu et al. (2000)
Os03g0126900	none	Hypothetical protein	4.31	0.99	1.09	0.04	0.03	0.001	
Os03g0304100	At1g56320	Hypothetical protein	3.45	0.67	1.40	0.08	0.05	0.002	
Os04g0419100	none	Hypothetical protein	3.36	0.35	1.34	0.10	0.02	0.000	
Os04g0494900	At5g11420	Unknown function DUF642 family	10.05	2.15	1.92	0.24	0.02	0.000	
Os07g0493100	none	Non-protein coding transcript	10.12	1.70	1.16	0.18	0.02	0.000	
Os07g0587300	none	Hypothetical protein	3.42	0.24	1.08	0.08	0.02	0.000	Nguyen et al. (2003)
Os11g0488100	none	Hypothetical protein	3.14	0.63	1.06	0.04	0.04	0.001	Nguyen et al. (2001)
Os11g0490100	At1g67330	Uncharacterized plant-specific DUF579 family	5.42	0.87	1.35	0.25	0.03	0.001	Nguyen et al. (2001)

Microarray analysis was performed with wild-type rice (wild type) and *art1* mutant exposed to 10 μM Al for 4 h. Data are means ± SD from three independent biological replicates. Genes upregulated by Al more than threefold in the wild type but less than twofold change in the mutant (cutoff by FDR < 0.1 of Benjamini-Hochberg FDR method) were extracted.

Al-activated citrate transporter (AACT1) in barley (Furukawa et al., 2007), may also play a role in conferring Al tolerance by secretion of citrate from the roots. *Os01g0869200* and *Os02g0131800* encode a putative Mg^{2+} transporter and an Nramp family protein, respectively. It is reported that Mg^{2+} transporters are one of the cellular targets of Al toxicity, and overexpression of the *Arabidopsis* transporter gene *MGT1* in *Nicotiana benthamiana* confers Al tolerance on the plant (Deng et al., 2006). Nramp proteins exhibit functional divergence, and most Nramp proteins are able to transport multiple metal ions such as Fe, Mn, Zn, and Cd (Oomen et al., 2009). Therefore, *Os01g0869200* and *Os02g0131800* seem to be involved in maintenance of metal homeostasis under Al stress.

Genes in group 3 (Table 1) are predicted to be involved in metabolism and detoxification. However, the functions of most genes in this group and those in group 4 are unknown and remain to be examined.

Although the genes regulated by ART1 are upregulated by Al, they are not newly induced, but constitutively expressed at basal levels (Figures 4A and 4B). This is probably because, unlike temporary environmental stresses such as water and temperature stresses, Al toxicity stress is prevalent in acid soils.

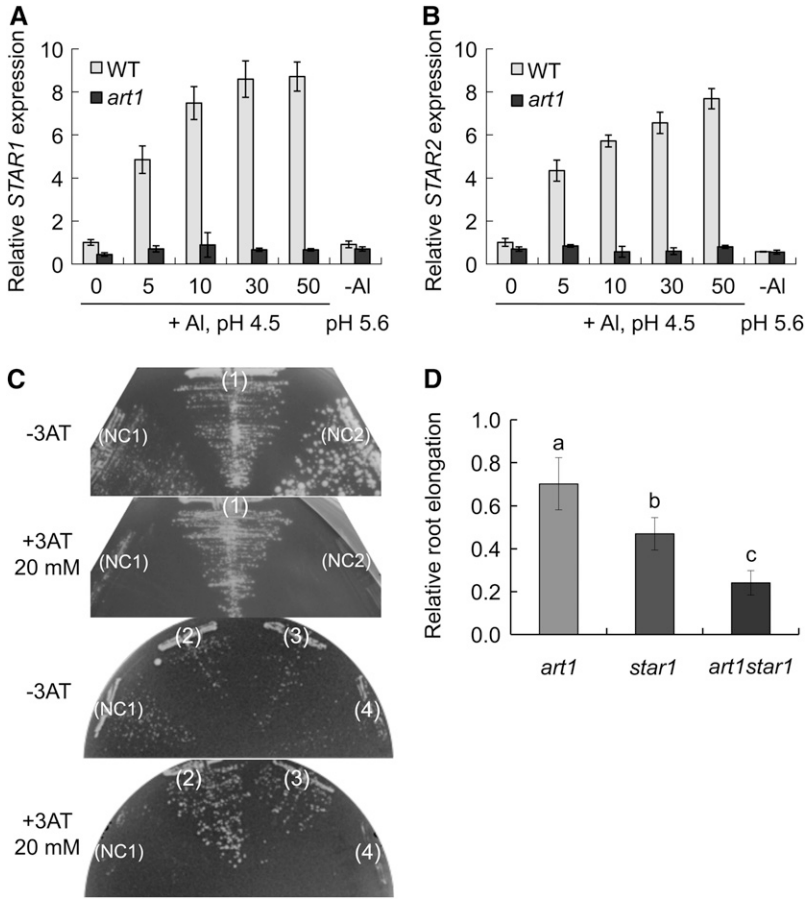

Figure 4. Regulation of *STAR1* and *STAR2* by ART1.

(A) and **(B)** Relative expression levels of *STAR1* **(A)** and *STAR2* **(B)** in root tip (0 to 10 mm) of the wild type and *art1* mutant treated with 0 to 50 μM Al, pH 4.5, or without Al, pH 5.6, for 6 h, were determined by quantitative RT-PCR. Expression relative to the wild type expression without Al treatment, pH 4.5, is shown. Data are means ± SD (n = 3).

(C) Yeast one-hybrid assay. A pair of plasmids, (1 to 4) pHIS-ProSTAR1 [HIS reporter gene with *STAR1* promoter, containing a region between −939 (1), −629 (2), −436 (3), or −297 (4) to −172 from *STAR1* start codon] and pGAD-ART1 (fusion of GAL4 activation domain and ART1), (NC1) pHIS2.1 (without *STAR1* promoter) and pGAD-ART1, (NC2) pHIS-ProSTAR1 and pGADT7 (GAL4 activation domain only) were introduced into yeast strain Y187 and cultured on SD medium containing 0 or 20 mM 3-amino-1,2,4-triazole, a competitor of HIS3 at 30°C for 3 d in the absence of His.

(D) Relative Al sensitivity of *art1*, *star1*, and the *art1 star1* double mutant. Five-day-old seedlings were exposed to a 0.5 mM $CaCl_2$ solution containing 0 or 5 μM Al, pH 4.5, for 24 h. Relative root elongation (root elongation with Al/root elongation without Al × 100) is shown. Data are means ± SD (n = 10). Different letters indicate significant differences at $P < 0.05$ by Student's t test.

Therefore, it seems that plants have acquired basic strategies to constitutively detoxify Al at different cellular levels.

Different Pathways of Al Tolerance between Rice and *Arabidopsis*

Rice and *Arabidopsis* differ greatly in the tolerance to Al toxicity (Ma et al., 2002; Iuchi et al., 2007). Recently, *STOP1*, a C2H2 zinc finger-type putative transcriptional factor, was reported as a responsible gene for both proton and Al hypersensitivity in *Arabidopsis* (Iuchi et al., 2007). ART1 is not the closest homolog of STOP1 in the rice genome (Figure 2C), and ART1 and STOP1 only share 41.2% identity. Comparison between ART1 and STOP1 shows that they differ in the response to stresses and downstream genes. The *stop1* mutant showed increased sensitivity to both Al and low pH (Iuchi et al., 2007), whereas *art1* only showed increased sensitivity to Al, but not to low pH (Figure 1C). The fact that *STAR1* and *STAR2* do not respond to low pH (Figures 4A and 4B) (Huang et al., 2009) also supports this conclusion. Most importantly, the downstream genes regulated by STOP1 and ART1 are different except two genes (*STAR2*/*ALS3* and *MATE*) (Sawaki et al., 2009; Table 1). STOP1 and ART1 each regulate an Nramp gene (Table 1; Sawaki et al., 2009), but the two Nramp genes belong to different branches of the Nramp family. These differences may contribute to different mechanisms for Al tolerance. The major Al tolerance mechanism in *Arabidopsis* is the secretion of malate from the roots, whereas rice does not secrete malate in response to Al (Ma et al., 2002). Furthermore, Al

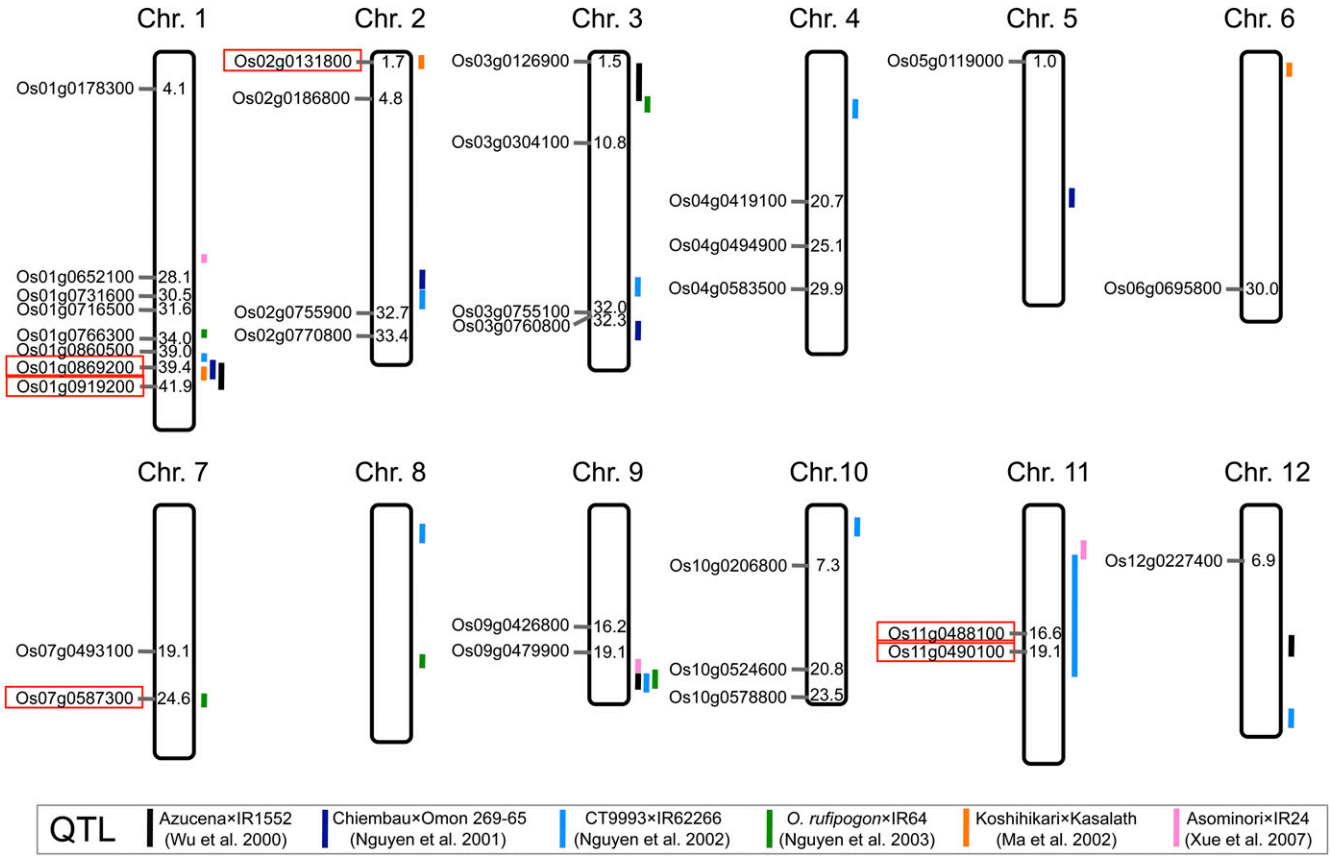

Figure 5. Position of Candidate Genes Regulated by ART1 and QTLs for Al Tolerance.

Values indicate position of genes on each chromosome (Mb), and QTLs found by different studies are indicated by colored lines. Genes located at a similar position to the QTL are indicated with red boxes.
[See online article for color version of this figure.]

tolerance in rice is much higher than that in *Arabidopsis*; one possible mechanism for higher tolerance is that more tolerance genes are required in rice. In addition, rice is very tolerant to low pH independent of *ART1* (Figure 1C), whereas *Arabidopsis* is very sensitive to low pH (Iuchi et al., 2007). Another possibility for the difference is that the experimental conditions are different between different studies. To avoid genes induced by Al toxicity, we exposed the rice roots to a low Al concentration (10 μM) for a short period (4 h). However, Sawaki et al. (2009) exposed *Arabidopsis* to 10 μM Al at pH 5.0 for 24 h. Under such conditions, the root growth of *Arabidopsis* is severely inhibited even in the wild type (Iuchi et al., 2007). Therefore, most of genes extracted might not be directly regulated by STOP1 but might be induced indirectly by Al toxicity. It would be interesting to extract genes regulated by STOP1 under an experimental condition with low Al concentration and short exposure and compare these genes with those regulated by ART1.

ART1-Regulated Genes May Be Involved in Genotypic Variation of Al Tolerance

There is a genotypic variation in Al tolerance in rice. Usually, japonica cultivars show higher Al tolerance than indica cultivars, which are cultivated on acidic soil areas. More than 10 quantitative trait loci (QTL) for Al tolerance have been detected (Wu et al., 2000; Nguyen et al., 2001, 2002, 2003; Ma et al., 2002; Xue et al., 2007), but the genes for these QTLs have not been cloned so far. Among the genes regulated by ART1 (Table 1), several genes, Os01g0869200, Os01g0919200, Os02g0131800, Os07g0587300, Os11g0488100, and Os11g0490100, are located at the similar positions of Al tolerance QTLs (Figure 5). Therefore, these genes may be involved in genotypic differences in Al tolerance in rice, although further confirmation is required in the future.

ART1 Specifically Regulates Al-Responsive Genes in Higher Plants

Since these genes are implicated in the detoxification of Al at different cellular levels, further elucidation of these gene functions will help to understand comprehensively plant Al tolerance mechanisms, whose molecular bases are still poorly understood. Moreover, ART1 also provides an invaluable clue to identify plant sensing and signaling pathway for Al toxicity in the future. *ART1* is constitutively expressed in the roots (Figure 3A), and nuclear

localization of ART1 protein is not affected by Al treatment (Figures 3E and 3F), while genes regulated by ART1 are upregulated by Al. These observations suggest that posttranslational regulation of ART1, such as protein phosphorylation or interaction with some other factors, is required to activate ART1. This is supported by the finding that there was no correlation between the expression of *ART1* and Al tolerance in transgenic complementation lines (Figure 2B; see Supplemental Figure 4 online).

METHODS

Isolation of the *art1* Mutant

M3 seeds of rice (*Oryza sativa* cv Koshihikari) irradiated with γ-rays were used for isolation of Al-sensitive mutants in 2002, following the procedures described previously (Ma et al., 2005). After three rounds of screening based on root elongation inhibition, we obtained a mutant sensitive to Al, which we named *art1* based on the features of the responsible gene described in the text.

Physiological Analysis of the Mutant

Seeds of the mutant (*art1*) and its wild-type rice were soaked in deionized water overnight at 30°C in the dark and then transferred to nets that were floated on a 0.5 mM $CaCl_2$ solution in a 1.5-liter plastic container. After growth at 25°C for 4 or 5 d, the seedlings were used for subsequent experiments. To compare Al sensitivity, seedlings were exposed to a 0.5 mM $CaCl_2$, pH 4.5, containing various $AlCl_3$ concentrations for 24 h, and then relative root elongation (RRE) was used to evaluate Al sensitivity of each line. RRE was calculated as follows: (root elongation with Al treatment)/(root elongation without Al) ×100.

To further evaluate the sensitivity to Al, we grew both wild-type and mutant plants in acid soil (Andosol) at pH 4.5 and an alluvial soil at pH 6.5. After 6 d, the root length was measured.

The effect of pH on root elongation was investigated in a 0.5 mM $CaCl_2$ solution buffered with 10 mM Homo-PIPES at pH ranging from 3.5 to 5.5. Root length of 10 seedlings each from the wild type and *art1* was measured after 24 h. The root elongation was also compared by exposing seedlings to a 0.5 mM $CaCl_2$ solution, pH 4.5, containing 0, 30 μM $AlCl_3$, 20 μM $CdCl_2$, 100 μM $ZnCl_2$, 0.5 μM $CuCl_2$, or 5 μM $LaCl_3$ for 24 h.

Both wild-type rice (cv Koshihikari) and *art1* mutants were cultivated in a field (soil pH 6.5) at an experimental farm of Okayama University in 2006 as described previously (Tamai and Ma, 2008). Three replicates of a plot (1 × 0.4 m) were made for each line. Plant growth and yield were investigated at harvest.

Genetic Analysis

Seeds of F1 plants were derived from a cross between the mutant and wild-type rice plants. F2 seedlings (164) were exposed to a 0.5 mM $CaCl_2$ solution for 24 h and then transferred to a 0.5 mM $CaCl_2$ solution containing 20 μM $AlCl_3$ for another 24 h. RRE (see above) was used to evaluate Al sensitivity of each seedling.

Map-Based Cloning of *ART1*

For mapping the responsible gene, we constructed an F2 population derived from a cross between *art1* mutant and Kasalath. First, we performed a bulked segregant analysis to determine the molecular markers linked to *ART1* (Michelmore et al., 1991). Rough mapping of this gene was then done using 46 F2 Al-sensitive plants. For fine mapping of this gene, we used 986 F2 plants from the *art1*/Kasalath population. Polymorphic InDel markers were developed based on the rice InDel database at http://shenghuan.shnu.edu.cn/ricemarker (Shen et al., 2004) and the comparison of the genomic sequence of Nipponbare with that of 93-11 by BLASTn searches in Genbank (see Supplemental Table 1 online). Twenty-five crossover plants between InDel markers MaOs1219 and MaOs1237 from the *art1*/Kasalath population were further evaluated for their Al sensitivity in the F3 generation. *ART1* was finally defined to 38.5-kb region, and the six candidate genes were sequenced by BigDye Terminators V3.1 cycle sequencing kit and ABI PRISM 310 genetic analyzer. The putative C2H2 zinc finger was independently sequenced twice in the mutant.

Phylogenetic Analysis

Alignment was performed with ClustalW using default setting (http://clustalw.ddbj.nig.ac.jp/), and the phylogenetic tree was constructed using the neighbor-joining algorithm with MEGA version 4 (Tamura et al., 2007) with 1000 bootstrap trials.

RNA Isolation and Quantitative Real-Time RT-PCR

Seedlings of the wild type and *art1* were exposed to a 0.5 mM $CaCl_2$ solution, pH 4.5, containing 0 to 50 μM Al for 6 h, and the roots tips (0 to 10 mm), basal roots (10 to 20 mm), and the shoots were harvested. Total RNA was extracted using the RNeasy mini kit (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using a SuperScript kit (Invitrogen) following the manufacturer's instructions with an oligo $(dT)_{12-18}$ primer. One microliter of 10-fold dilution cDNA from each sample was used for the quantitative analysis of gene expression with SYBR Premix Ex Taq (Takara). Data were collected in accordance with using the 7500 Real Time PCR System (Applied Biosystems). *HistoneH3* was used as an internal control. Primer sequences used are as follows: *STAR1*, 5′-TCGCATTGGCTCGCACCCT-3′ (forward) and 5′-TCGTCTTCTTCAGCCGCACGAT-3′ (reverse); *STAR2*, 5′-ACCTCTTCATGGTCACCGTCG-3′ (forward) and 5′-CCTCAGCTTCTTCATCGTCACC-3′ (reverse); *ART1*, 5′- CAGTGCTTCTCGTGGGTCTT-3′ (forward) and 5′- CCTGTGCGTGAAGAACCACT-3′ (reverse); *HistoneH3*, 5′- AGTTTGGTCGCTCTCGATTTCG-3′ (forward) and 5′- TCAACAAGTTGACCACGTCAC-3′ (reverse).

Generation of Transgenic Rice

For the complementation test of *ART1*, we obtained a BAC clone (BAC AL731761) containing *ART1* by screening of BAC library of Koshihikari. After partial enzymatic cutting with *Sau*3AI, we selected a 6.1-kb clone containing *ART1* and its promoter (from 3.4 kb upstream of start codon). This clone was inserted into pPZP2H-lac vector (Fuse et al., 2001) and then transformed into *Agrobacterium tumefaciens* (strain EHA101). Calluses derived from the rice mutant *art1* were transformed by *Agrobacterium*-mediated transformation (Hiei et al., 1994). We obtained five independent transgenic lines. T2 seeds from three independent lines were further used to test the Al tolerance with a vector control line. The root elongation was measured for 24 h in a 0.5 mM $CaCl_2$ solution without Al and then to the same solution containing 20 μM Al, pH 4.5, for a further 24 h. Relative root elongation was calculated as (root elongation with Al/root elongation without Al) × 100.

Construction of Fluorescent Gene Fusion and Transient Expression

For construction of a translational *ART1-GFP* fusion, the ORF of *ART1* except the stop codon was amplified by PCR from rice (cv Koshihikari) root cDNA. Primer pairs used for amplification and introduction of restriction sites were 5′-AGATCACTCGAGATTATTCAGAAGCTTGCA-3′ and 5′-TAATCATGACTGATCCCTTGTCACCATTCTCCTCCTG-3′. The ORF was inserted between cauliflower mosaic virus 35S promoter and GFP-NOS terminator in pBluescript vector. Plasmid DNA was transiently introduced into rice callus protoplasts using the polyethylene glycol

method according to Kamiya et al. (2006). After overnight incubation, GFP fluorescence was observed with a fluorescence microscope (Axio Imager with Apotome; Carl Zeiss).

Immunohistological Staining

Antibodies against ART1 were obtained by immunizing rabbits with the synthetic peptide C-EQFAGDHQEENGDK (positions 452 to 465 of ART1). The roots of 1-week-old seedlings (cv Koshihikari) treated with or without Al (50 μM, 6 h) were used for immunostaining as described previously (Yamaji and Ma, 2007).

Yeast One-Hybrid Assay

The yeast one-hybrid assay was performed using MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) and MATCHMAKER One-Hybrid Library Construction and Screening Kit (Clontech) to examine the transcriptional activation potential of ART1. The ORF of *ART1* was amplified by PCR from rice (cv Koshihikari) root cDNA. Primer pairs used for amplification and introduction of restriction sites were 5′-AGATCACtCGAGATTATTCA-GAAGCTTGCA-3′ and 5′-CCGCTCGAGTCACTTGTCACCATTCTCC-3′. The ORF was cloned in frame after the DNA binding domain of yeast GAL4 transcription factor (without activation domain) in pGBKT7 vector (pGBK-ART1). These plasmids, pGBK-ART1 and control pGBKT7, were introduced into yeast strain AH109 that carried the GAL4-responsive GAL1 promoter and HIS3 reporter gene and cultured on SD medium with or without histidine (His) at 30°C for 3 d according to the manufacturer's manual.

To investigate the interaction between ART1 protein and *STAR1* promoter, we amplified the promoter sequence of *STAR1* (−939, −629, −436, or −297 to −172 bp from the start codon) by PCR from rice (cv Koshihikari) genomic DNA. Primer pairs used for amplification and introduction of restriction sites were 5′-TTCTAGATAGCATCTGGATAAT-GATAATC-3′ (from -939), 5′-AGAATTCAATTGGAGTTCTCTTTGCGG-3′ (from −629), 5′-AGAATTCCGTCGTACCGGTGATAAC-3′ (from −436), 5′-AGAATTCCCACACAGATCCACGGCA-3′ (from −297) and 5′-TTCTA-GATCGCCGTGGTCGGTTTGGA-3′. Two tandem copies of the amplified promoter region was cloned upstream of the *HIS3* reporter gene in pHIS2.1 vector (pHIS-ProSTAR1). The ORF of *ART1* described above was cloned in frame after transcriptional activation domain of yeast GAL4 transcription factor (without DNA binding domain) in pGADT7 (pGAD-ART1). A pair of these plasmids, pHIS-ProSTAR1 and pGAD-ART1, or control pHIS2.1 and pGADT7 were introduced into yeast strain Y187 and cultured on SD medium without His containing 0 to 20 mM 3-amino-1,2,4-triazole (a competitive inhibitor of HIS3) at 30°C for 3 d according to the manufacturer's manual.

Microarray Analysis

One-week-old seedlings of the wild type and *art1* were treated with 10 μM Al, pH 4.5, or without Al, pH 4.5, in 0.5 mM $CaCl_2$ solution for 4 h. RNA samples were prepared from root segments between 0 and 15 mm from the apex using the RNeasy Plant Mini Kit (Qiagen). Microarray analysis was performed according to Agilent Oligo DNA Microarray Hybridization protocols using the Rice Oligo DNA Microarray 44K RAP-DB (G2519F#15241; Agilent Technologies) with three biological replicates and color swap for each replicate. The hybridized slides were scanned using a DNA microarray scanner (Agilent Technologies). Signal intensities were extracted by Feature Extraction software (Agilent Technologies). For statistic analysis, we excluded genes with signal intensities below 100 in all the experiments after correction of the dye effect by averaging of the two color swaps. Significance test was performed by unpaired *t* test using GeneSpringGX10 (Agilent Technologies). The Benjamini-Hochberg FDR method was used to obtain P values corrected for multiple testing. Fold change and SD of each probe by Al treatment were calculated using the average of three biological replicates. Genes upregulated by Al more than threefold in the wild type but less than twofold change in the mutant (cutoff by FDR < 0.1) were extracted. The function of these genes was categorized based on the annotation database.

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number AB379846 (ART1).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Air-Dried Weight of Shoot and Grain Yield of Both Wild Type Rice and *art1* Grown in a Field.

Supplemental Figure 2. Frequency Distributions of Al Sensitivities in an F2 Population from a Backcross between the Mutant (*art1*) and Wild Type.

Supplemental Figure 3. Map-Based Cloning of ART1.

Supplemental Figure 4. Expression Levels of *ART1*/*art1* in Complementation Lines and the Control Plants.

Supplemental Figure 5. Root Al Staining in Wild Type, *star1*, and *art1.*

Supplemental Table 1. Primers for InDel Markers Used in Mapping ART1.

Supplemental Data Set 1. Alignment Used to Generate the Phylogeny Presented in Figure 2C.

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