

STOP1 Regulates Multiple Genes That Protect Arabidopsis from Proton and Aluminum Toxicities^{1[C][W][OA]}

Yoshiharu Sawaki², Satoshi Iuchi², Yasufumi Kobayashi², Yuriko Kobayashi, Takashi Ikka, Nozomu Sakurai, Miki Fujita, Kazuo Shinozaki, Daisuke Shibata, Masatomo Kobayashi, and Hiroyuki Koyama*

Laboratory of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan (Y.S., Ya.K., T.I., H.K.); Experimental Plant Division, RIKEN BioResource Center, Tsukuba, Ibaraki 305-0074, Japan (S.I., Yu.K., M.K.); Laboratory of Genome Biotechnology, Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan (N.S., D.S.); and RIKEN Plant Science Center, Tsurumi-ku, Yokohama 230-0045, Japan (M.F., K.S.)

The Arabidopsis (*Arabidopsis thaliana*) mutant *stop1* (for sensitive to proton rhizotoxicity1) carries a missense mutation at an essential domain of the histidine-2-cysteine-2 zinc finger protein STOP1. Transcriptome analyses revealed that various genes were down-regulated in the mutant, indicating that STOP1 is involved in signal transduction pathways regulating aluminum (Al)- and H⁺-responsive gene expression. The Al hypersensitivity of the mutant could be caused by down-regulation of *AtALMT1* (for Arabidopsis ALUMINUM-ACTIVATED MALATE TRANSPORTER1) and *ALS3* (ALUMINUM-SENSITIVE3). This hypothesis was supported by comparison of Al tolerance among T-DNA insertion lines and a transgenic *stop* mutant carrying cauliflower mosaic virus 35S::*AtALMT1*. All T-DNA insertion lines of *STOP1*, *AtALMT1*, and *ALS3* were sensitive to Al, but introduction of cauliflower mosaic virus 35S::*AtALMT1* did not completely restore the Al tolerance of the *stop1* mutant. Down-regulation of various genes involved in ion homeostasis and pH-regulating metabolism in the mutant was also identified by microarray analyses. *CBL-INTERACTING PROTEIN KINASE23*, regulating a major K⁺ transporter, and a sulfate transporter, *SULT3;5*, were down-regulated in the mutant. In addition, integral profiling of the metabolites and transcripts revealed that pH-regulating metabolic pathways, such as the γ -aminobutyric acid shunt and biochemical pH stat pathways, are down-regulated in the mutant. These changes could explain the H⁺ hypersensitivity of the mutant and would make the mutant more susceptible in acid soil stress than other Al-hypersensitive T-DNA insertion lines. Finally, we showed that STOP1 is localized to the nucleus, suggesting that the protein regulates the expression of multiple genes that protect Arabidopsis from Al and H⁺ toxicities, possibly as a transcription factor.

Plants modulate tolerant mechanisms to adapt to various environmental stresses, such as drought, salinity, and soil acidity (Seki et al., 2003; Kochian et al., 2004). Molecular characterization of such systems is critical to develop efficient breeding programs to make crop plants with high productivity under stress environments. In this context, tolerance mechanisms to various stress factors involved in acid soils (e.g. rhizo-

toxicities of Al³⁺ and H⁺ [Kinraide, 2003], excess of manganese [Horst, 1988], and phosphorus deficiency [Neumann et al., 1999]) are important targets, because about 40% of world arable land is categorized as acid soil.

A number of genes that are responsible for Al tolerance have been isolated from various plant species. Genes encoding malate and citrate transporters were identified as key genes in the Al-exclusion mechanism, such as those encoding Al-activated malate transporters in wheat (*Triticum aestivum*; *TaALMT1* [Sasaki et al., 2004]) and in Arabidopsis (*Arabidopsis thaliana*; *AtALMT1* [Hoekenga et al., 2006]), and for the gene encoding a citrate transporter in sorghum (*Sorghum bicolor*; Magalhaes et al., 2007). *ALS3*, encoding a half-type ATP-binding cassette transporter that may be involved in the redistribution process of Al from sensitive tissues in Arabidopsis, was isolated by a mutant study (Larsen et al., 2005). On the other hand, molecular physiological studies identified several key genes regulating efficient phosphorus acquisition, such as the high-affinity phosphate transporter (Mitsukawa et al., 1997). Although H⁺ is highly rhizotoxic to various plant

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² These authors contributed equally to the article.

* Corresponding author; e-mail koyama@gifu-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: Hiroyuki Koyama (koyama@gifu-u.ac.jp).

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species (Koyama et al., 1995; Yokota and Ojima, 1995; Kinraide, 2003; Watanabe and Okada, 2005), our knowledge of the molecular basis for its toxicity is limited compared with that of other coexisting stress factors in acid soils, such as manganese toxicity (Delhaize et al., 2003).

Studies on rhizotoxicity indicate that patterns of damage caused by H^+ are distinct from those induced by Al (Koyama et al., 1995). In addition, these stress factors were distinguished by a modeling of root elongation in acidic forest soil (Kinraide, 2003). These reports suggest that H^+ and Al rhizotoxicities induce damage in the roots by different processes. However, several studies selecting Al-tolerant cultivars suggested that H^+ tolerance and Al tolerance are genetically linked in some plant species (Rangel et al., 2005; Yang et al., 2005). These suggest that, although the mechanisms of the toxicities of H^+ and Al would involve distinct biological processes, tolerance to these stresses might be concomitantly regulated in some plant species, possibly by the same genetic factors. Recently, we isolated an Arabidopsis mutant that shows hypersensitivity to H^+ rhizotoxicity and, interestingly, hypersensitivity to Al (Iuchi et al., 2007). The mutant might be a useful model to dissect the mechanism(s) of H^+ tolerance at the molecular level and the interaction of tolerance mechanisms to Al^{3+} and H^+ .

The *stop1* (for *sensitivity to proton rhizotoxicity1*) mutant of Arabidopsis was isolated from an ethyl methanesulfonate-mutagenized population using growth inhibition of the roots in the root-venting assay on low-pH gelled medium as a selection index (Iuchi et al., 2007). Interestingly, the *stop1* mutant was also hypersensitive to Al, although it was selected by H^+ sensitivity. Chromosome-walking analyses, followed by genomic DNA sequencing analysis, revealed that the *stop1* mutant carries a missense mutation that substitutes an essential His residue with Tyr in the Cys-2-His-2 motif of a putative zinc finger protein, STOP1. On the other hand, one of the critical genes for Al tolerance in Arabidopsis, namely *AtALMT1*, encoding an Al-activated malate transporter (Hoekenga et al., 2006), was down-regulated in the *stop1* mutant (Iuchi et al., 2007). This turns off the major Al tolerance mechanism of Arabidopsis in the *stop1* mutant, namely Al-responsive malate release (Kobayashi et al., 2007). However, reverse genetics studies revealed that *AtALMT1* is not involved in the H^+ -tolerant mechanisms, because a knockout mutant (i.e. T-DNA insertion line) of *AtALMT1* was not sensitive to H^+ (Kobayashi et al., 2007). These results suggest that STOP1 functions to regulate the expression of other genes that critically affect hypersensitivity to H^+ and also to Al. Recently, Liu et al. (2009) reported that one of the other genes for Al tolerance in Arabidopsis (i.e. a type of multidrug and toxic compound exclusion protein [*AtMATE*]; At1g51340) is regulated by STOP1. Although zinc finger proteins have various functions (Englbrecht et al., 2004), these results suggest that STOP1 is one of the key factors involved in the signal transduction pathways

regulating tolerance mechanisms to Al and H^+ rhizotoxicities, which would consist of various genes other than *AtALMT1* (Iuchi et al., 2007) and *AtMATE* for Al tolerance (Liu et al., 2009).

In this study, we performed molecular physiological characterization of the hypersensitivities of the *stop1* mutant to both Al and H^+ . DNA microarray analysis revealed that multiple genes critical for Al tolerance (i.e. *AtALMT1* and *ALS3*) and those potentially contributing to low pH resistance were down-regulated in the *stop1* mutant, such as genes belonging to biological processes of ion homeostasis (i.e. potassium and others). In addition, an integrated analysis of metabolites and transcripts revealed that metabolic pathways that have been assumed to regulate cytosolic pH of the plant (biochemical pH stat [Sakano, 1998] and γ -aminobutyric acid [GABA] shunt [Bouche and Fromm, 2004] pathways) were also down-regulated in the mutant. Interestingly, both ion homeostasis and pH-regulating metabolism are critical for low pH resistance in other organisms, such as *Escherichia coli* (Yohannes et al., 2004) and fish (Hirata et al., 2003) and possibly in higher plants (Yan et al., 1992). These results indicated that STOP1 regulates multiple tolerant mechanisms to major stress factors of acid soil and that some of the H^+ -resistant systems down-regulated in the *stop1* mutant are similar to those of other organisms.

RESULTS

Transcriptomic Identification of Repressed Genes in the *stop1* Mutant

As reported previously, one of the genes critical for Al tolerance, *AtALMT1*, was significantly down-regulated in the *stop1* mutant (Iuchi et al., 2007). However, the gene does not contribute to H^+ tolerance (Kobayashi et al., 2007). This suggested that STOP1 is involved in signal transduction pathways that regulate the expression of the Al- and H^+ -responsive genes other than *AtALMT1*. To examine this possibility and to identify these other genes, we performed a series of DNA microarray analyses for gene expression. First, we identified greatly repressed genes in the *stop1* mutant in Al and H^+ treatments by competitive microarray analysis between the *stop1* mutant and the wild type. Using fold change (FC) values (*stop1* mutant/wild type) of three biologically independent replications, 101 and 45 genes were identified as significantly down-regulated in the *stop1* mutant with Al and H^+ treatments, respectively. These genes belonged to the lowest 1% of detected genes in all three replications, indicating that down-regulation of the genes was reproducible (Fig. 1A). From the identified genes, 32 genes were down-regulated with both Al and H^+ treatments.

Down-regulation in the *stop1* mutant may occur if the expression of the genes is related to growth rate, because growth is inhibited in the mutant more

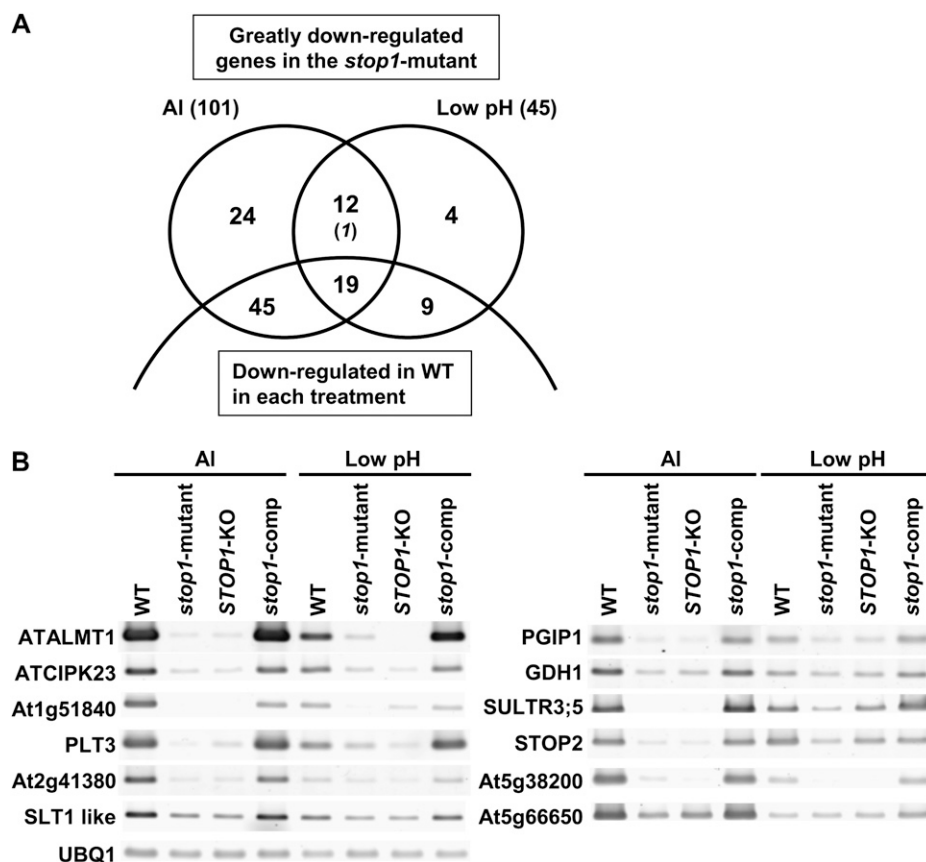


Figure 1. A, Genes repressed in the *stop1* mutant compared with the wild type (WT) that were identified by competitive microarray analyses. Greatly repressed genes in the *stop1* mutant, when they were compared with the wild type, were identified by three biologically independent replications of the comparative microarray of the *stop1* mutant/wild type under Al toxic (10 μ M) and low-pH (pH 4.5) conditions. The genes that were in the lowest percentile of the FC values (*stop1* mutant/wild type) in all three replications were defined as “significantly down-regulated genes.” Numbers of down-regulated genes in the wild type in each treatment (FC in treatments/control in the wild type were < 1 with $P < 0.05$ and $FC < 0.8$) are shown in the lower parts of the Venn diagram. The genes down-regulated in *AtALMT1*-KO compared with the wild type in the upper parts are shown by italics. B, Expression levels of 12 overlapped genes in the wild type, the *stop1* mutant, *STOP1*-KO, and a complemented *stop1* mutant carrying *CaMV35S::STOP1* (*stop1*-comp) were determined by semiquantitative RT-PCR.

severely than in the wild type. Such genes would be down-regulated in the wild type with Al and H^+ treatments, where the growth rate of the wild type was reduced compared with the control treatment. Such genes were filtered from the above selected genes using FC values of the competitive microarrays in the wild type (i.e. Al treatments/control and H^+ /control). To maximize the effect of this filtering, both the genes significantly down-regulated in the treatments ($FC < 1$ with $P < 0.05$ by *t* test), and those with $FC < 0.8$ were filtered out. By this procedure, 64 (Al) and 28 (H^+) genes, including 19 of the overlapping genes (detected by both Al and H^+), were filtered out from the list of significantly down-regulated genes in the *stop1* mutant (Fig. 1; Supplemental Table S1). More than half of such filtered genes were down-regulated in the T-DNA insertion mutant of *AtALMT1* (*AtALMT1*-KO) with Al treatment (FC [*AtALMT1*-KO/wild type] < 1 with $P < 0.05$ by *t* test and/or $FC < 0.8$; Table I; Supplemental Table S1), where the growth of *AtALMT1*-KO is repressed compared with the wild type. This suggests that the above filtering was effective at eliminating false positives linked to growth suppression in the sensitive lines (i.e. in the *stop1* mutant). There was one gene (At1g53480) in the shared gene group by both Al and H^+ treatments that was not down-regulated in the wild type with Al treatment and was significantly down-regulated in the *AtALMT1*-KO line. This gene

might be repressed in the *stop1* mutant due to the block of Al-activated malate release but not by the mutation of *STOP1*. Finally, we identified a total of 39 down-regulated genes that included 12 genes that were shared with both Al and H^+ treatments (Table I; Fig. 1A).

To further evaluate whether the repression of the genes in the mutant was caused by the mutation of *STOP1*, the expression levels of the 12 shared genes with both treatments were examined in *STOP1*-KO (T-DNA insertion in *STOP1*) and with the transgenic mutant carrying normal *STOP1* by introducing cauliflower mosaic virus (CaMV) 35S::*STOP1* (Iuchi et al., 2007). Very similar expression profiles were found in the *stop1* mutant and *STOP1*-KO (Fig. 1B). Furthermore, expression of these down-regulated genes recovered in the transgenic mutant carrying normal *STOP1* (Fig. 1B). These results indicate that mutation of *STOP1* affects the expression of these identified genes.

Function of the Genes Repressed in the *stop1* Mutant

The shared gene group, whose members were repressed by both Al and H^+ treatments in the *stop1* mutant, contained a previously identified gene critical for Al tolerance, *AtALMT1* (Hoekenga et al., 2006; Table I), and a homolog of *STOP1*, encoding a zinc finger protein (referred to here as *STOP2*). Several genes for transporters (i.e. *SULTR3;5* encoding a sulfate trans-

Table 1. List of repressed genes in the *stop1* mutant among up-regulated or stable genes in the wild type under AI and low-pH conditions (Fig. 1)

Three biologically independent replications of competitive microarray analyses were carried out as follows: *stop1* mutant versus the wild type in AI (10 μ M, 24 h) or low pH (pH 4.5, 24 h); *AtALMT1*-KO versus the wild type in AI (10 μ M, 24 h); control (pH 5.0, no AI, 24 h) versus AI (10 μ M, pH 5.0, 24 h) or low pH (pH 4.5, 24 h) in the wild type. FC \pm SD values are shown, as are *P* values from *t* tests for significant differences from FC = 1.00.

Arabidopsis Genome Initiative Code	FC (<i>stop1</i> Mutant or <i>AtALMT1</i> -KO/Wild Type)						FC in the Wild Type with AI or Low-pH Treatments (Treatment/Control)				Description
	AI <i>stop1</i> Mutant		Low-pH <i>stop1</i> Mutant		AI <i>AtALMT1</i> -KO		AI		Low pH		
	FC \pm SD	<i>P</i>	FC \pm SD	<i>P</i>	FC \pm SD	<i>P</i>	FC \pm SD	<i>P</i>	FC \pm SD	<i>P</i>	
Repressed in the <i>stop1</i> mutant and up-regulated or stable in the wild type with AI and low-pH treatments; 12 (+1 of the gene repressed in <i>AtALMT1</i> -KO) genes											
At1g08430	0.01 \pm 0.00	0.001	0.01 \pm 0.00	0.000	0.01 \pm 0.00	0.001	24.40 \pm 13.8	0.009	7.41 \pm 4.07	0.021	ATALMT1 (AI-activated malate transporter 1)
At1g30270	0.13 \pm 0.03	0.003	0.19 \pm 0.02	0.001	1.02 \pm 0.14	0.866	1.44 \pm 0.09	0.009	1.42 \pm 0.25	0.073	ATCIPK23 (CBL-interacting protein kinase 23)
At1g51840	0.25 \pm 0.06	0.010	0.40 \pm 0.06	0.008	1.03 \pm 0.15	0.765	1.31 \pm 0.42	0.272	1.21 \pm 0.30	0.305	Similar to Leu-rich repeat protein kinase, putative
At2g18480	0.06 \pm 0.01	0.002	0.12 \pm 0.09	0.031	1.08 \pm 0.15	0.433	6.97 \pm 1.12	0.002	1.89 \pm 0.08	0.001	PLT3 (probable polyol transporter 3)
At2g41380	0.04 \pm 0.01	0.003	0.14 \pm 0.08	0.025	1.11 \pm 0.16	0.327	6.98 \pm 3.70	0.021	2.28 \pm 0.57	0.028	Similar to embryo-abundant protein-related
At5g02480	0.19 \pm 0.03	0.003	0.31 \pm 0.08	0.015	1.07 \pm 0.15	0.491	2.02 \pm 0.31	0.015	1.18 \pm 0.07	0.040	Similar to SLT1 (sodium- and lithium-tolerant 1)
At5g06860	0.05 \pm 0.00	0.000	0.13 \pm 0.06	0.017	1.17 \pm 0.17	0.201	1.93 \pm 1.46	0.232	1.00 \pm 0.74	0.992	PGIP1 (polygalacturonase-inhibiting protein 1)
At5g18170	0.08 \pm 0.01	0.001	0.27 \pm 0.11	0.030	1.26 \pm 0.18	0.109	2.91 \pm 0.39	0.005	1.19 \pm 0.10	0.068	GDH1 (glutamate dehydrogenase 1)
At5g19600	0.01 \pm 0.00	0.002	0.29 \pm 0.06	0.008	1.33 \pm 0.19	0.074	1.50 \pm 0.45	0.138	2.08 \pm 0.37	0.019	SULTR3;5 (sulfate transporter 3;5)
At5g22890	0.06 \pm 0.01	0.001	0.16 \pm 0.08	0.023	1.07 \pm 0.15	0.520	1.03 \pm 0.20	0.809	1.15 \pm 0.15	0.200	STOP2 (a STOP1 homolog)
At5g38200	0.02 \pm 0.00	0.001	0.10 \pm 0.04	0.009	1.06 \pm 0.15	0.552	4.16 \pm 1.79	0.027	1.77 \pm 0.19	0.012	Similar to hydrolase
At5g66650	0.24 \pm 0.03	0.003	0.19 \pm 0.13	0.043	1.03 \pm 0.15	0.766	9.13 \pm 1.11	0.001	2.68 \pm 1.17	0.055	Similar to unknown protein
At1g53480	0.02 \pm 0.01	0.002	0.02 \pm 0.01	0.007	0.01 \pm 0.00	0.003	1.02 \pm 0.02	0.341	1.10 \pm 0.05	0.060	Similar to unknown protein
Repressed in the <i>stop1</i> mutant and up-regulated or stable in the wild type with AI treatment; 24 genes											
At1g35670	0.34 \pm 0.02	0.001	0.76 \pm 0.06	0.030	1.05 \pm 0.15	0.598	1.35 \pm 0.07	0.011	0.53 \pm 0.08	0.020	ATCDPK2 (calcium-dependent protein kinase 2)
At1g60610	0.26 \pm 0.05	0.006	0.54 \pm 0.05	0.008	0.95 \pm 0.14	0.618	1.68 \pm 0.23	0.023	1.16 \pm 0.10	0.095	Similar to protein-binding/zinc ion-binding
At1g61560	0.26 \pm 0.03	0.003	0.61 \pm 0.09	0.029	0.99 \pm 0.14	0.955	3.34 \pm 0.75	0.011	2.65 \pm 1.02	0.045	MLO6 (mildew resistance locus O6)
At1g72870	0.22 \pm 0.09	0.021	0.59 \pm 0.15	0.064	0.92 \pm 0.13	0.406	1.29 \pm 0.32	0.213	0.60 \pm 0.05	0.008	Putative disease resistance protein (TIR-NBS class)
At1g77760	0.18 \pm 0.05	0.008	0.88 \pm 0.30	0.574	1.29 \pm 0.19	0.088	1.66 \pm 0.30	0.038	1.68 \pm 0.13	0.007	NIA1 (nitrate reductase 1)
At2g01180	0.31 \pm 0.03	0.003	0.59 \pm 0.09	0.028	1.03 \pm 0.15	0.779	2.13 \pm 0.32	0.013	1.04 \pm 0.09	0.525	ATPAP1 (phosphatidic acid phosphatase 1)
At2g16660	0.34 \pm 0.01	0.001	0.53 \pm 0.06	0.009	1.23 \pm 0.18	0.126	2.62 \pm 0.66	0.021	1.29 \pm 0.43	0.310	Similar to nodulin family protein
At2g23150	0.31 \pm 0.03	0.002	0.61 \pm 0.13	0.054	1.04 \pm 0.16	0.661	2.80 \pm 0.55	0.012	1.27 \pm 0.08	0.020	NRAMP3 (manganese ion transporter)
At2g28270	0.08 \pm 0.03	0.006	0.41 \pm 0.26	0.114	0.98 \pm 0.23	0.873	2.52 \pm 0.92	0.046	0.74 \pm 0.34	0.357	Similar to DC1 domain-containing protein
At2g39380	0.28 \pm 0.05	0.005	0.83 \pm 0.07	0.064	1.03 \pm 0.15	0.753	1.41 \pm 0.21	0.054	2.63 \pm 0.22	0.003	ATEXO70H2 (exocyst subunit EXO70 family protein H2)
At2g39510	0.27 \pm 0.07	0.013	1.32 \pm 1.12	0.579	1.35 \pm 0.20	0.072	9.60 \pm 7.83	0.032	1.97 \pm 1.57	0.235	Similar to nodulin MtN21 family protein
At2g43590	0.25 \pm 0.10	0.025	1.09 \pm 0.50	0.754	0.84 \pm 0.15	0.157	3.20 \pm 0.63	0.009	2.22 \pm 2.00	0.214	Putative chitinase
At2g45220	0.26 \pm 0.04	0.005	0.69 \pm 0.02	0.003	1.13 \pm 0.29	0.458	1.98 \pm 0.26	0.012	0.43 \pm 0.11	0.026	Similar to pectin esterase family protein
At3g05400	0.19 \pm 0.04	0.004	0.63 \pm 0.15	0.076	1.20 \pm 0.17	0.158	2.98 \pm 1.04	0.031	1.07 \pm 0.09	0.293	SUGTL5 (sugar transporter ERD6-like 12)
At3g28290	0.13 \pm 0.03	0.004	0.46 \pm 0.16	0.055	0.87 \pm 0.12	0.237	1.51 \pm 0.13	0.014	0.93 \pm 0.28	0.713	Similar to AT14A
At4g13420	0.03 \pm 0.00	0.000	2.21 \pm 1.35	0.135	0.84 \pm 0.12	0.165	7.54 \pm 4.39	0.023	6.86 \pm 1.12	0.002	HAK5 (high-affinity K ⁺ transporter 5)
At4g16563	0.09 \pm 0.07	0.026	0.22 \pm 0.27	0.113	1.13 \pm 0.28	0.449	1.22 \pm 0.31	0.307	0.35 \pm 0.04	0.004	Similar to aspartyl protease family protein
At4g30270	0.28 \pm 0.06	0.010	0.84 \pm 0.63	0.693	0.91 \pm 0.15	0.407	12.56 \pm 5.39	0.009	2.05 \pm 0.76	0.074	MERI5B (endoxyloglucan transferase)
At4g38470	0.29 \pm 0.05	0.007	0.80 \pm 0.22	0.297	1.17 \pm 0.17	0.200	1.13 \pm 0.20	0.343	1.15 \pm 0.17	0.245	Similar to protein kinase family protein

(Table continues on following page.)

Table I. (Continued from previous page.)

Arabidopsis Genome Initiative Code	FC (<i>stop1</i> Mutant or <i>AtALMT1</i> -KO/Wild Type)						FC in the Wild Type with Al or Low-pH Treatments (Treatment/Control)				Description
	Al <i>stop1</i> Mutant		Low-pH <i>stop1</i> Mutant		Al <i>AtALMT1</i> -KO		Al		Low pH		
	FC ± SD	P	FC ± SD	P	FC ± SD	P	FC ± SD	P	FC ± SD	P	
At5g07440	0.29 ± 0.05	0.005	0.54 ± 0.15	0.060	1.16 ± 0.17	0.208	2.55 ± 0.16	0.001	1.23 ± 0.17	0.123	GDH2 (glutamate dehydrogenase 2)
At5g11670	0.26 ± 0.07	0.012	0.67 ± 0.06	0.017	1.02 ± 0.14	0.834	3.62 ± 0.78	0.009	1.53 ± 0.15	0.018	ATNADP-ME2 (malic enzyme 2)
At5g17860	0.31 ± 0.03	0.003	0.98 ± 0.02	0.208	0.98 ± 0.17	0.837	1.28 ± 0.06	0.012	1.02 ± 0.06	0.681	CAX7 (calcium exchanger 7)
At5g47560	0.12 ± 0.02	0.002	0.75 ± 0.15	0.129	1.09 ± 0.19	0.423	1.05 ± 0.12	0.557	1.06 ± 0.12	0.457	ATTDT (tonoplast malate/fumarate transporter)
At5g66800	0.33 ± 0.03	0.003	0.58 ± 0.06	0.011	0.71 ± 0.11	0.057	1.04 ± 0.05	0.248	0.91 ± 0.06	0.124	Similar to unknown protein
Repressed in the <i>stop1</i> mutant and up-regulated or stable in the wild type with low-pH treatment; four genes											
At1g62280	0.97 ± 0.62	0.936	0.47 ± 0.01	0.001	0.84 ± 0.15	0.161	0.33 ± 0.30	0.133	1.06 ± 0.51	0.858	SLAH1 (SLAC1 homolog 1)
At1g75840	0.58 ± 0.04	0.005	0.47 ± 0.03	0.002	0.99 ± 0.15	0.879	0.94 ± 0.14	0.570	1.03 ± 0.06	0.460	ATROP4 (rho-like GTP-binding protein 4)
At3g12750	0.60 ± 0.05	0.010	0.46 ± 0.09	0.020	1.42 ± 0.21	0.053	0.55 ± 0.04	0.004	1.62 ± 0.13	0.009	ZIP1 (zinc transporter 1)
At5g24780	0.65 ± 0.57	0.431	0.43 ± 0.01	0.000	2.21 ± 0.42	0.004	1.06 ± 1.02	0.918	4.00 ± 1.69	0.027	VSP1 (vegetative storage protein1)

porter 3;5 and *PLT3* encoding a probable polyol transporter 3) and regulatory proteins in ion transport (*CBL-INTERACTING PROTEIN KINASE23* [*CIPK23*] regulating *AKT1* activity and a homolog of *SLT1* [for sodium and lithium tolerant 1] regulating Na^+/K^+ transport) were identified in this down-regulated gene group. One of major genes for nitrogen metabolism, *GLUTAMATE DEHYDROGENASE1* (*GDH1*), and a gene that might belong to biotic stress defense systems, *POLYGALACTURONASE-INHIBITING PROTEIN1* (*PGIP1*), were also found in the same down-regulated gene group.

The other two gene groups, which were down-regulated by either Al or H^+ treatment, contained several genes that are functionally related to the above gene group detected by both treatments (Table I). One of major potassium transporters, *HAK5*, and major enzymes in nitrogen metabolism (*NIA1* [for nitrate reductase] and *GDH2*) belong to the gene group identified by Al^{3+} treatment. Different types of malate transporters, namely *AtTDT* (a vacuolar malate/ Na^+ cotransporter), a homolog of *SLAC1* (for slow anion channel 1), and a malate metabolism enzyme (*ME2* encoding a malic enzyme) belong to the gene group identified with Al (*AtTDT* and *ME2*) or low pH (*SLAC1* homolog) treatment, respectively. These data suggest that mutation of *STOP1* modifies ion homeostasis, malate transport, and metabolism in the *stop1* mutant.

Down-Regulation of *ALS3* in the *stop1* Mutant

Although genes critical for H^+ sensitivity have not been clarified yet, several genes responsible for Al tolerance have been identified in Arabidopsis. In the above data mining, we only identified genes signifi-

cantly down-regulated in the *stop1* mutant, or genes in the bottom 1% of FC values. However, genes that were relatively less down-regulated might contribute to the enhancement of Al hypersensitivity of the *stop1* mutant if the genes are critical for Al tolerance. This possibility was examined with the reported Al-resistant genes using FC values (*stop1* mutant/wild type) of the same microarray data. *ALS1* and *ALS3* were identified by mutant studies (Larsen et al., 2005), while *WAK1* (for cell wall-associated kinase 1 [Sivaguru et al., 2003]; *At1g21250*), *AtBCB* [for Arabidopsis blue copper-binding protein [Ezaki et al., 2004]; *At5g20230*), and *AtCS* (for Arabidopsis citrate synthase [Koyama et al., 1999]) were identified by transgenic approaches. Among these genes, only *ALS3* (*At2g37330*), which is involved in the redistribution process of Al from sensitive tissues, was down-regulated in the *stop1* mutant, with an FC of 0.33 (Supplemental Fig. S1). The expression level of *ALS3* was determined by quantitative reverse transcription (RT)-PCR, which showed that it was down-regulated in the *stop1* mutant and *STOP1*-KO, and it recovered its expression level in the complemented line of the mutant carrying *CaMV35S::STOP1* (Fig. 2A). This result indicated that *STOP1* regulates at least a set of genes critical for Al tolerance in Arabidopsis (i.e. *AtALMT1* and *ALS3*).

We compared the growth of KO mutants to evaluate the down-regulation of multiple Al-resistant genes on the Al-hypersensitive phenotype of the *stop1* mutant. As reported previously (Iuchi et al., 2007), *STOP1*-KO is sensitive to H^+ , while *AtALMT1*-KO is not (Fig. 2, B and C; Kobayashi et al., 2007). In the control solution (no Al), pH 5.5, all KO mutants grew comparably to the wild type. On the other hand, H^+ rhizotoxicity (i.e. inhibition of root growth with comparison to pH 5.5) in the wild

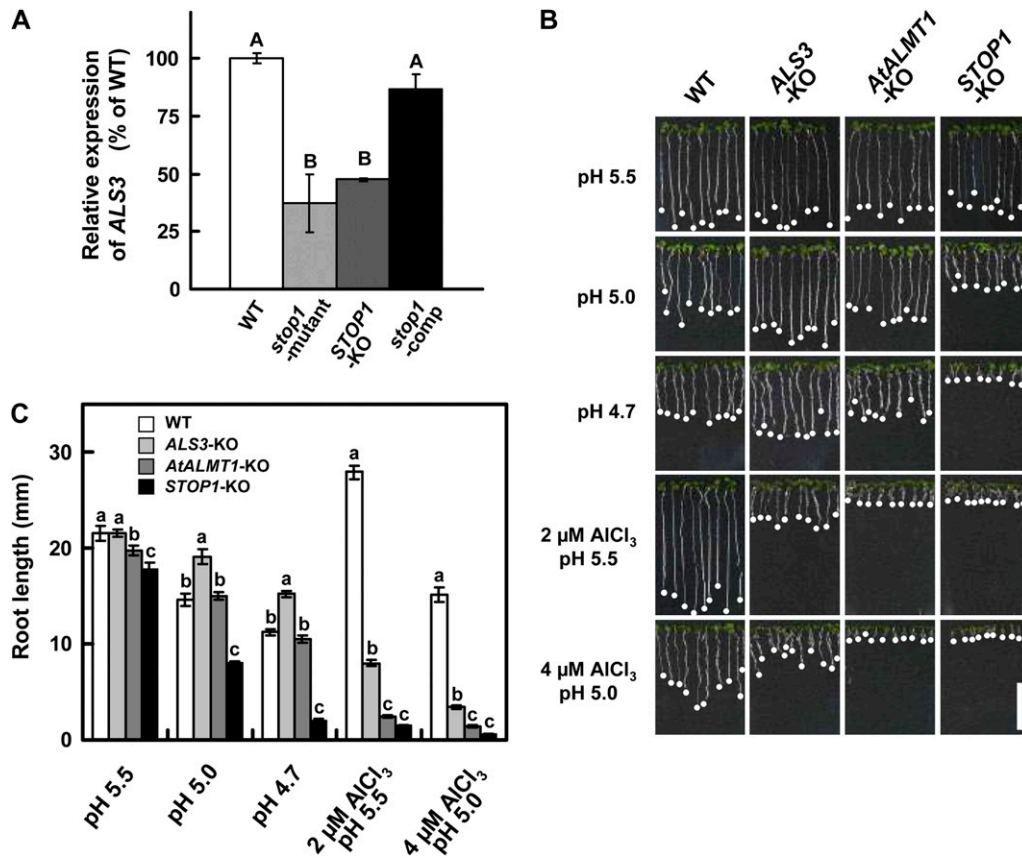


Figure 2. Repression of *ALS3* in the *stop1* mutant and comparison of growth among T-DNA insertion mutants. A, *ALS3* expression in the wild type (WT), the *stop1* mutant, *STOP1*-KO, and a complemented line of the *stop1* mutant carrying CaMV35S::*STOP1* (*stop1*-comp), which were grown for 10 d in normal conditions and exposed to Al (10 μ M, pH 5.0) for 24 h. *ALS3* expression was normalized by *UBQ1* expression and compared with that of the wild type. B and C, Growth response of *ALS3*-KO compared with that of *STOP1*-KO and *AtALMT1*-KO with Al and H⁺ rhizotoxicities. White bar in B = 1 cm. Different letters indicate significant differences by LSD test ($P = 0.05$). [See online article for color version of this figure.]

type appeared slight at pH 5.0 (about 30% inhibition) and became greater at pH 4.7 (about 50% inhibition). Under these conditions, *ALS3*-KO grew slightly better than the wild type at pH 5.0 and 4.7, indicating that down-regulation of *ALS3* was not related to H⁺ hypersensitivity of the *stop1* mutant. To evaluate the Al sensitivity of these KO mutants, we employed a relatively high pH to minimize the effect of H⁺ toxicity. pH 5.0 was previously used in quantitative trait locus analyses of Al tolerance (Kobayashi et al., 2005; Ikka et al., 2008), while pH 5.5 was also employed to minimize H⁺ rhizotoxicity in this study. Under both conditions, *ALS3*-KO showed a hypersensitive phenotype to Al in comparison with the wild type, while it was significantly more resistant than *STOP1*-KO and *AtALMT1*-KO (Fig. 2, B and C). These results indicate that down-regulation of *ALS3* contributes to the greater hypersensitivity to Al of the *stop1* mutant than that of *AtALMT1*-KO. This possibility was supported by an in planta complementation assay with the *stop1* mutant by introducing CaMV35S::*AtALMT1*. Overexpression of *AtALMT1* in the *stop1* mutant restored Al³⁺ tolerance as the expression level of *AtALMT1* was increased. How-

ever, it did not completely restore Al tolerance, even if the expression levels of *AtALMT1* in the transgenic lines were greater than in the wild type (Fig. 3). On the other hand, growth inhibition of *STOP1*-KO in the Al toxic solution at pH 5.5 was significantly more severe than that of *AtALMT1*-KO when these two lines were statistically compared (t test, $P = 7.71 \times 10^{-5}$). These results indicated that the hypersensitivity of *STOP1*-KO to Al rhizotoxicity is caused by down-regulation of multiple genes critical for Al tolerance in Arabidopsis.

Growth of the *STOP1*-KO and Al-Sensitive KO Mutants in Soil Culture

In our previous study, we showed that H⁺-sensitive accessions of Arabidopsis grew poorly in acid soil compared with H⁺-resistant accessions (see Fig. 5 in Ikka et al., 2007). Under the same soil culture system, the growth of *STOP1*-KO and other Al-hypersensitive KO mutants was compared to evaluate negative impacts of H⁺ hypersensitivity due to dysfunction of *STOP1*. On the neutral-pH forest brown soil (pH 6.6), *STOP1*-KO and other KO mutants grew comparably to

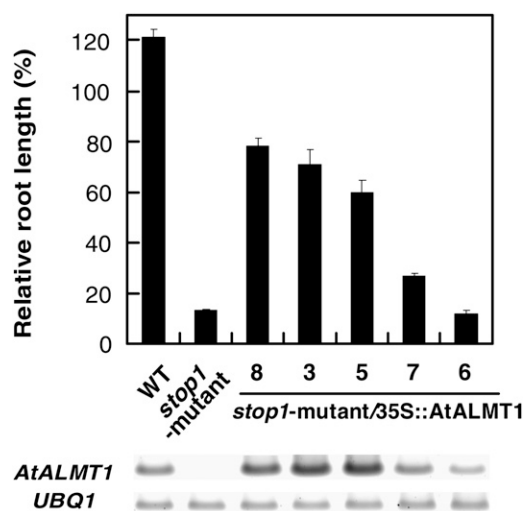


Figure 3. Root growth of the wild type (WT), the *stop1* mutant, and a transgenic *stop1* mutant carrying CaMV35S::AtALMT1. Seedlings were grown hydroponically in Al toxic solution containing 2 μ M Al at pH 5.5. Culture solution was renewed every 2 d, and relative root length (percentage of the root growth in the absence of Al) was determined at 7 d. Gel image of semiquantitative RT-PCR of *AtALMT1* and *UBQ1* transcripts of each line is shown at the bottom. Means \pm SE are shown ($n = 3$).

the wild type, indicating that disruption of STOP1 did not affect growth at neutral pH in soil culture (Fig. 4). On the other hand, growth of *STOP1*-KO and other Al-hypersensitive KO mutants was inhibited in acid soil containing rhizotoxic exchangeable Al and low pH. At the lowest liming plot, growth of all KO mutants was almost totally inhibited due to Al rhizotoxicity, but it recovered when Al rhizotoxicities in soil were alleviated by CaCO_3 . Growth of *ALS3*-KO and *AtALMT1*-KO became comparable to that of the wild type in the middle (*ALS3*-KO) and high (*AtALMT1*-KO) liming plots, respectively. This pattern could account for the difference of Al sensitivity of these KO mutants, as judged by hydroponic culture (Fig. 2, B and C), and also suggests that Al rhizotoxicity was totally alleviated in the high liming plot. However, in the high liming plot, growth of *STOP1*-KO was significantly inhibited compared with the wild type and other Al-hypersensitive KO mutants. This indicated that the hypersensitivity to H^+ of the *stop1* mutant has a negative impact on its growth capacity in acid soil, possibly due to hypersensitivity to H^+ . In fact, growth of *STOP1*-KO was significantly less than that of the wild type at the pH of high liming soil (pH 5.2) when judged in hydroponic conditions (see Fig. 6A in Iuchi et al., 2007).

Effect of the *stop1* Mutation on Metabolic Pathways Related to pH Regulation

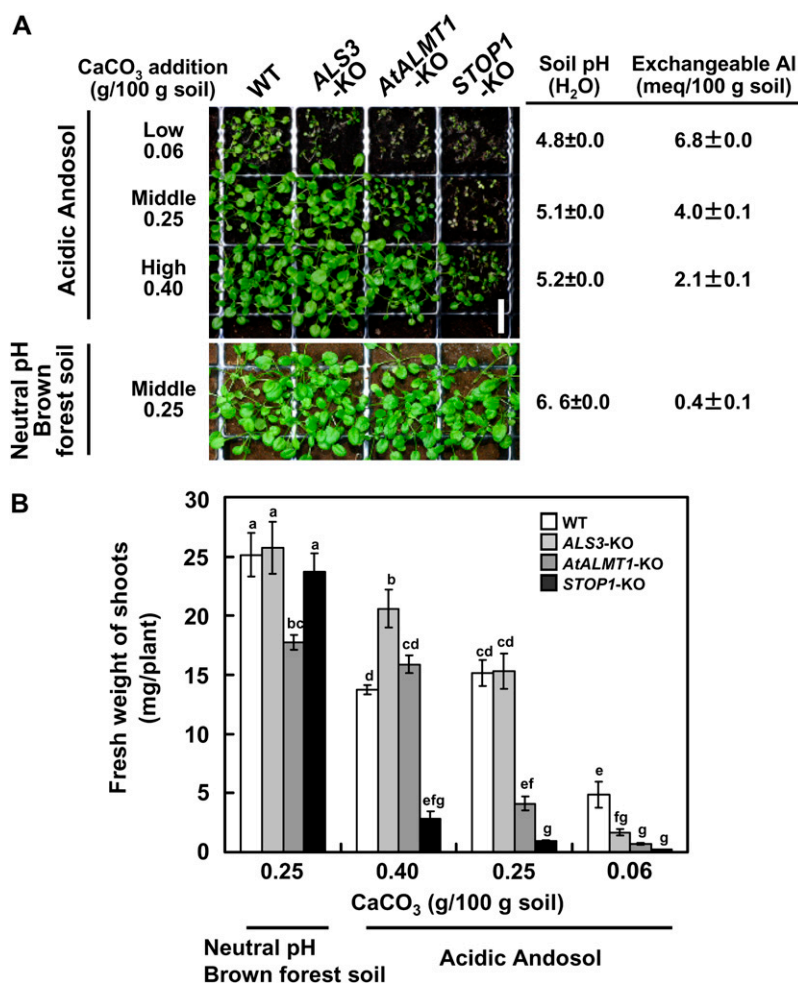
AtTDT, which encodes a vacuolar malate transporter that is involved in the pH regulation process in Arabidopsis, was identified as a significantly down-regulated gene after Al treatment in the *stop1* mutant. However,

the gene was not greatly down-regulated in the low-pH treatment; thus, the gene may not solely account for the H^+ hypersensitivity of the *stop1* mutant. This suggested that other genes cooperatively affect the H^+ hypersensitivity of the mutant. One possible mechanism that could explain H^+ hypersensitivity is the block of pH-regulating metabolic pathways, because some of the genes (e.g. GDH in the GABA shunt [Bouche and Fromm, 2004] and malic enzyme in the biochemical pH stat [Sakano, 1998]) were found in the significantly down-regulated genes group in the *stop1* mutant (Table I).

Both comparative DNA microarray data (*stop1* mutant/wild type) and that of metabolites (*STOP1*-KO/wild type) with H^+ treatment (pH 4.5, 24 h) were integrated on a pathway map including pH-regulating pathways (Fig. 5). In this experiment, we rejected the genes with low intensity (intensity < 1,000) for comparison because their impacts on metabolism would be relatively small compared with those with greater intensity. Among a total of 222 genes in the pathway map (i.e. glycolysis pathway, tricarboxylic acid cycle, and related organic acid and amino acid metabolic pathways; Supplemental Table S3), 11 genes were relatively more down-regulated in the *stop1* mutant than other genes (*stop1* mutant/wild type < 0.8 and $P < 0.05$ in t test between the FC and 1), including four genes encoding enzymes in the pH-regulating metabolic pathways (Fig. 5; Supplemental Table S3). Genes encoding enzymes in the GABA shunt pathway (*GDH1* and *GAD1*; Bouche and Fromm, 2004) and two others in the biochemical pH stat pathway (*ME1* and *ME2*; Sakano, 1998) were down-regulated in the *stop1* mutant. In addition, quantitative RT-PCR analysis revealed that *GABA-T* (GABA aminotransferase) and *GDH2* tended to be down-regulated in *STOP1*-KO, even if it was rejected by a slightly larger P value than the significance threshold of microarray data analysis (*GABA-T*, FC = 0.76, $P = 0.06$ in t test for *stop1* mutant/wild type < 1; *GDH2*, FC = 0.54, $P = 0.06$ in t test for *stop1* mutant/wild type < 1). These trends were confirmed by quantitative RT-PCR analysis in *STOP1*-KO (Fig. 5). Taken together, we could assume that down-regulation of these genes in the *stop1* mutant, which belong to pH-regulating pathways, was caused by the dysfunction of STOP1 (Fig. 5). Only four genes on the pathway map were significantly up-regulated in the *stop1* mutant (pH 4.5; *stop1* mutant/wild type > 1.25, $P < 0.05$ in t test between the FC and 1), including a gene for one of the isozymes of pyruvate decarboxylase (*PDC2*) and another encoding a putative homolog (At4g33070; Supplemental Table S3).

Under these conditions, some metabolites in the pathway were significantly changed in the *STOP1*-KO mutant. A total of 24 metabolites were detected by our analyses, and four metabolites were significantly altered in the *STOP1*-KO mutant (Fig. 5; Supplemental Table S2). The amounts of GABA and succinate, which belonged to the GABA shunt pathway, were significantly lower in the KO mutant. In addition, products

Figure 4. A, Growth of *ALS3*-KO, *AtALMT1*-KO, and *STOP1*-KO in neutral forest brown soil (bottom) and acidic andosol (top). Seedlings were grown for 4 weeks on both soil types. Acidic andosol was neutralized by adding various amounts of CaCO_3 . Soil pH and amount of exchangeable Al were determined. WT, wild type. White bar = 1 cm. B, Fresh weight of the shoots plotted as means \pm SE ($n > 4$). Different letters indicate significant differences at $P = 0.05$ by LSD test. [See online article for color version of this figure.]



of malic enzymes of the pH stat pathway were also significantly lower in the mutant. These results indicated that pH-regulating pathways are down-regulated by the *stop1* mutant. On the other hand, Suc concentration was significantly increased in the *stop1* mutant, suggesting that dysfunction of STOP1 also affects Suc availability in the roots. This was accompanied by the repression of several homologs of hexokinase (*HXK3* and *HXK4*).

Intracellular Localization of the STOP1 Protein

Down-regulation of various genes in the *stop1* mutant suggested that STOP1 belongs to the gene expression pathways responsive to H^+ and Al stresses. To further examine this possibility, we determined the intracellular localization of the STOP1 protein by transient assay in onion (*Allium cepa*) epidermis cells using a STOP1 fusion protein with GFP. When the control construct, CaMV35S::GFP, was introduced to the epidermis cells by particle bombardment, the whole cell fluoresced in green and the nucleus fluoresced slightly (Fig. 6). This was a typical subcellular localization pattern for GFP in plant cells. By contrast, the cells

harboring CaMV35S::STOP1:GFP fluoresced only in the nucleus (Fig. 6). This clearly showed that STOP1 localizes in the nucleus and suggests that it might be involved in the regulation of transcription in the nucleus, either as a transcription factor or by affecting other members of the H^+ or Al signal transduction pathways.

DISCUSSION

Comparative microarray analyses followed by quantitative RT-PCR revealed that dysfunction of STOP1 caused down-regulation of two of genes critical for Al^{3+} tolerance, *AtALMT1* (Kobayashi et al., 2007) and *ALS3* (Larsen et al., 2005; Table I, Fig. 2; Supplemental Fig. S1). In addition, another gene for Al tolerance, *AtMATE*, was identified as one of the genes regulated by STOP1, because the expression of the gene was greatly repressed in *STOP1*-KO (Liu et al., 2009). We arbitrarily rejected the *AtMATE* gene during quality control of the comparative microarray data because it has abnormal scanning data in two of the three replications in the low-pH induction of the wild type, but the repression

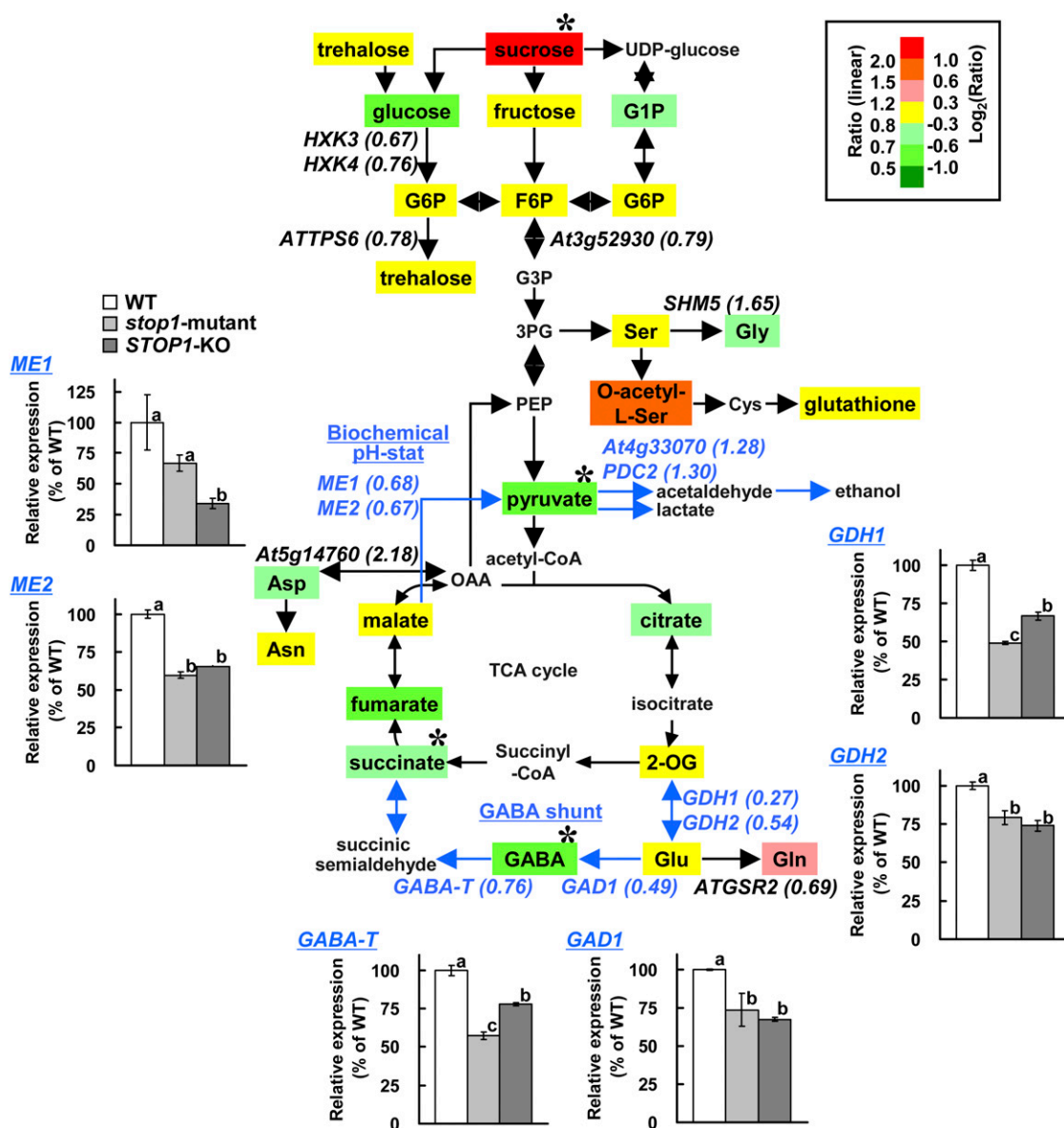
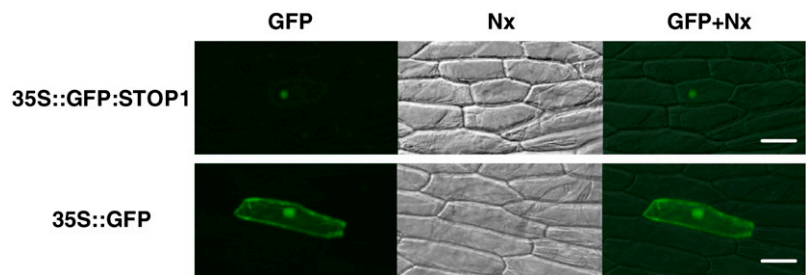


Figure 5. Comparison of metabolites and transcripts in glycolysis, tricarboxylic acid (TCA) cycle, and pH-regulating pathways between the wild type (WT) and the *stop1* mutant. Seedlings of the wild type and the *stop1* mutant were grown for 10 d in stress-free control conditions, then transferred to low-pH (pH 4.5) solution and incubated for 24 h. The transcriptomes of the *stop1* mutant and the wild type on the pathway (222 genes in Supplemental Table S3) were compared, while the metabolomes were compared between *STOP1*-KO and the wild type. Determined metabolites are shown in colored boxes, and the ratio of *STOP1*-KO to the wild type is indicated by color as shown at top right. Among the genes with intensities greater than 1,000, relatively more down-regulated (*stop1* mutant/wild type < 0.8) or up-regulated (*stop1* mutant/wild type > 1.25) genes than others are indicated in italics with the means of FC in parentheses (Supplemental Table S3). Asterisks show significant difference from FC = 1 (t test, $P < 0.05$). Transcript levels of selected genes in the wild type, the *stop1* mutant, and *STOP1*-KO at pH 4.5 for 24 h are shown in small panels. Different letters in the small panels indicate significant differences by LSD test ($P = 0.05$). GABA shunt and biochemical pH stat pathways are emphasized by blue color.

level of the gene was comparable to that of genes listed as significantly repressed by Al in Table I (i.e. FC of the *stop1* mutant/wild type in Al was 0.23 ± 0.07 ; Supplemental Table S4). Taken together, these results indicated that multiple genes for Al tolerance were coregulated in Arabidopsis in a gene expression pathway that requires the STOP1 protein (Fig. 7). In addition, dysfunction of

STOP1 caused down-regulation of several genes that could affect H^+ tolerance, belonging to ion homeostasis and pH regulation pathways (Table I; Fig. 5). This was consistent with the growth response of *STOP1*-KO and Al-hypersensitive KO mutants, *AtALMT1*-KO and *ALS3*-KO, in hydroponics and acid soil culture conditions (Figs. 2 and 4). *STOP1*-KO was sensitive to H^+

Figure 6. Localization of the GFP::STOP1 protein that was transiently expressed in onion epidermis cells. Vectors containing CaMV35S::GFP:STOP1 (top) or CaMV35S::GFP (bottom) were introduced by particle bombardment. Fluorescence images (left) and bright-field images (middle) are merged at right. Bars = 100 μ m.



rhizotoxicity, and its growth in acid soil was more repressed than that of other Al-sensitive KO mutants, which were not sensitive to H^+ rhizotoxicity. These results clearly indicated that both the tolerance systems for Al and H^+ rhizotoxicities are coregulated by a signal transduction pathway that involves STOP1 (Fig. 7). The intracellular localization of GFP:STOP1 indicated that STOP1 acts in the nucleus (Fig. 6), either as a transcription factor or as another critical factor regulating transcription.

AtALMT1-KO and *ALS3*-KO were not sensitive to H^+ rhizotoxicity; therefore, other genes down-regulated in the *stop1* mutant should function as the tolerant system for H^+ rhizotoxicity. DNA microarray analyses (e.g. a comparative array with the *stop1* mutant and the wild type) followed by semiquantitative RT-PCR analyses with *STOP1*-KO and the transgenic complemented line (i.e. the *stop1* mutant carrying CaMV 35S-driven STOP1) revealed that various genes for ion homeostasis and pH regulation are coregulated by STOP1 (Fig. 7). For example, *AtTDT*, encoding a tonoplast dicarboxylate transporter (a kind of malate transporter), was identified as one of the down-regulated genes in the *stop1* mutant. A T-DNA insertion in this gene impaired pH regulation in the cytosol; thus, the gene was annotated as the gene for pH homeostasis in Arabidopsis (Hurth et al., 2005). Although the gene was not greatly repressed in the *stop1* mutant with low-pH treatment ($FC = 0.75$), it may partly explain the H^+ hypersensitivity of the *stop1* mutant. Several genes that are critical for pH tolerance in other organisms were also down-regulated in the *stop1* mutant. For example, K^+ transport and homeostasis are identified as major pH-regulating mechanisms in various organisms (Zhang and Kone, 2002). Repression of *CIPK23*, encoding the regulatory kinase of the major K^+ transporter AKT1 (Lee et al., 2007), might have an effect on pH regulation in the *stop1* mutant via reduced AKT1 activity. Other transporters relating to SO_4 and NO_3 could also contribute to pH regulation in plants. The addition of K_2SO_4 improved root growth of maize (*Zea mays*) in a low-pH medium (Yan et al., 1992), while high K^+ supply also blocked cytosolic acidification in Al-treated wheat root (Lindberg and Strid, 1997). On the other hand, addition of NO_3 alleviated acidosis of maize under anoxia (Libourel et al., 2006). Taken together, these data suggest that down-regulation of genes involved in ion homeostasis and transport may cause the proton-sensitive pheno-

type of the *stop1* mutant. This possibility is supported by a recent study of one of the K^+ transporters in Arabidopsis. Overexpression of a cation:proton antiporter, *CHX13*, slightly improved growth at low pH (pH 4.3 and 5.6; Zhao et al., 2008) in K^+ -limited conditions, suggesting that K^+ homeostasis in Arabidopsis is linked to H^+ sensitivity.

Dysfunction of STOP1 affected other mechanisms, which were predicted to be members of the pH homeostasis process in plant cells, namely pH-regulating metabolic pathways. One part of the pH stat pathway consists of malic enzyme, pyruvate decarboxylase, and alcohol dehydrogenase (or lactate dehydrogenase) and can consume H^+ (Roberts et al., 1992; Sakano, 1998). The other pH stat pathway, called the GABA shunt, consists of GDH, GAD, and GABA-T; it also contributes to regulating H^+ in the cytosol (Bown and Shelp, 1997). The GABA shunt is enhanced by hypoxia and by other stresses that cause acidosis of the cytosol (Crawford et al., 1994). Our results suggested that these pH-regulating pathways were down-regulated in the *stop1* mutant (Fig. 5). Major isoforms of these pathways, *GDH1* and *GDH2*, *GAD1*, *GABA-T*, and *ME1* and *ME2*, were repressed in the *stop1* mutant (Table I; Fig. 5). Although one *GAD* isoform, *GAD4*, was up-regulated in the *stop1* mutant (Supplemental Table S3), a smaller contribution of *GAD4* expression

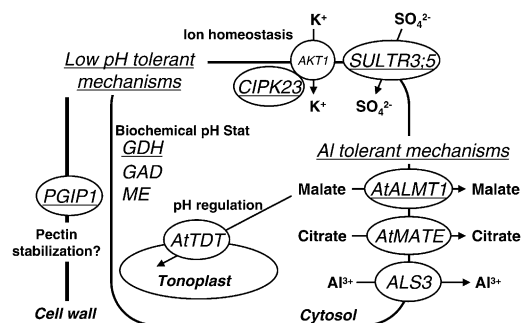


Figure 7. Schematic representation of regulating genes by STOP1 in relation to low pH and Al tolerance. Major genes identified as down-regulated in the *stop1* mutant are shown with possible functions in low pH and Al stress tolerance. Except for *AKT1*, other genes were down-regulated in the *stop1* mutant. *AtMATE* was reported by Liu et al. (2009). Underlined genes were greatly down-regulated in the *stop1* mutant with both low-pH and Al treatments (Table I).

than that of *GAD1* on total GAD activity was estimated by a recent study (Miyashita and Good, 2008). In addition, expression levels of the isoforms of the other down-regulated genes were quite similar between the wild type and the *stop1* mutant, indicating that these pathways were down-regulated in the *stop1* mutant. This might affect the H⁺ sensitivity of the *stop1* mutant. Interestingly, orthologs of these genes have been identified as critical for adaptation to acidic environments in other organisms (Castanie-Cornet et al., 1999). For *E. coli*, GAD is essential for surviving in low-pH medium (Yohannes et al., 2004). These genes, which are down-regulated in the *stop1* mutant, could be involved in low-pH-tolerant mechanisms in Arabidopsis.

It is possible that other genes regulated by STOP1 might have a function in low-pH tolerance, even if the genes have been previously characterized in different biological events but not identified as H⁺-resistant genes. For example, one of the down-regulated genes in the *stop1* mutant, *PGIP1*, has been well characterized as a protein involved in defense systems for pathogen infection. Rudrappa et al. (2008) recently reported that foliar infection of a pathogenic bacterium induces *AtALMT1* expression and recruits a beneficial bacterium in the rhizosphere. This suggests that *STOP1* is a pleiotropic gene that belongs to defense systems for both biotic and abiotic stressors; thus, it regulates *PGIP1* expression. However, *PGIP1* also has a potential role in H⁺ tolerance, according to its function in the root cell wall. The protein binds to the polygalacturonic acid region of pectin, forming a structure termed the "egg box" (Spadoni et al., 2006). This structure is essential for connecting pectin chains by covalent linkage (O'Neill et al., 2004), and its weakening by low-pH conditions is involved in a part of the H⁺ rhizotoxicity of Arabidopsis growing roots (Koyama et al., 2001). Although the role of *PGIP1* for H⁺ tolerance has been not clarified yet, a functionally similar protein stabilizing the cell wall has been identified as part of the low-pH-tolerant mechanisms in yeast. A protein carrying Man moieties, *CWP2* (for cell wall 2), is essential in H⁺ tolerance of yeast because it can stabilize the cell wall at low pH (Skrzypek et al., 1997). Interestingly, *PGIP1* inhibits polygalacturonase of the pathogen at neutral pH due to stabilizing substrate (i.e. pectin), while its synthesis is enhanced by low-pH treatment (Spadoni et al., 2006). In addition, a homolog of *PGIP1*, namely *PGIP2*, was also identified in the gene group significantly repressed in the *stop1* mutant, but it was rejected during quality control of the scanned data (Supplemental Table S4). Further research will be required for evaluating the contribution of these *PGIP* proteins in proton tolerance in Arabidopsis roots. Overall, the genes significantly repressed in the *stop1* mutant consisted of various genes that were functionally identified as the genes for pH tolerance or pH homeostasis in plants and other organisms. Although experimental evidence in plant research is limited, the combination of altered ion transport and carbon and nitrogen (CN) metabolism seems to be common in other organisms adapting to a

low-pH environment. For example, fish adapted to a low-pH environment show altered transport of potassium/sodium/hydrogen and CN metabolism (Hirata et al., 2003), while a bacterium surviving around pH 0 has more copies of genes encoding ion transporters and enzymes in CN metabolism than other bacteria adapting to neutral pH (Fütterer et al., 2004). Further research on the genes regulated by STOP1 might clarify the complex pH-tolerant systems in higher plants.

A series of regulatory proteins (e.g. protein kinases and transcriptional regulators) were down-regulated in the *stop1* mutant, including one of the two homologs of STOP1 existing in the Arabidopsis genome, designated STOP2. STOP2 carries a very similar Cys-2-Hys-2 zinc finger domain to STOP1 (Iuchi et al., 2007). Although STOP2's function has not yet been clarified, it may coregulate the expression of several genes with STOP1, similar to other zinc finger transcription factors. For example, a zinc finger transcription factor protein, *DOF1* (At1g51700), with its homolog *DOF2*, regulates the expression in root tissue (Yanagisawa, 2000) of genes encoding enzymes for amino acid and carbohydrate metabolism (e.g. PEP and PPK; Yanagisawa et al., 2004). Characterization of STOP2's role will raise important issues in understanding gene expression pathways regulated by STOP1. On the other hand, down-regulation of genes in the *stop1* mutant by H⁺ also occurred by Al treatment. This suggests that both stressors induce the same "signal" to trigger gene expression. One possible explanation is the induction of acidification of the cytosol by H⁺ and Al, which was identified in Arabidopsis using pH-sensitive GFP. Al treatment additively acidified cytosolic pH of the Arabidopsis roots under low-pH treatment (Moseyko and Feldman, 2001). The inhibition of plasma membrane H⁺-ATPase(s) by Al was reported in some plant species (Ahn et al., 2001), which can also acidify the cytosolic pH. Further research on these issues will clarify the molecular mechanism of the activation process of genes regulated by STOP1 under Al treatment.

The *stop1* mutant grew poorly on acid soil compared with *AtALMT1-KO*, while Al tolerance levels, judged by hydroponic culture, were very similar (Fig. 2, B and C; see Figure 6A in Iuchi et al., 2007). This indicates that H⁺ sensitivity affects the growth of the *stop1* mutant on acid soil. This could be supported by a previous growth experiment that used natural accessions of Arabidopsis. When natural accessions were grown on the same acid soil, growth was correlated with their H⁺ tolerance if their Al tolerance was similar (Ikka et al., 2007). This indicates that H⁺ tolerance is one of the factors critical for determining the growth of Arabidopsis in acid soil. Further research on the genes regulated by STOP1 for H⁺ tolerance (Fig. 7) might contribute to improving H⁺ tolerance of other crop plants by transgenic breeding or by establishing marker-assisted selection with comparative genomics approaches for the ortholog of STOP1, which have been identified in other plant species, such as rice and maize (Iuchi et al., 2007).

MATERIALS AND METHODS

Arabidopsis Accessions

The *stop1* mutant of Arabidopsis (*Arabidopsis thaliana*) was derived from an ethyl methanesulfonate-mutagenized F2 population of ecotype Col-0 in a previous study (Iuchi et al., 2007). SALK T-DNA insertion mutants, *AtALMT1*-KO (SALK_009629), *STOP1*-KO (SALK_114108), and *ALS3*-KO (SALK_061074), were derived from the Arabidopsis Biological Resource Center. Col-0 (JA58) and Col-4 (N933) were obtained from RIKEN Bio-Resource Center and Nottingham Arabidopsis Stock Centre, respectively. Complementation lines of *STOP1* carrying CaMV35S::*STOP1* in the *stop1* mutant were identical to those used in a previous study (Iuchi et al., 2007).

Transient Expression of the Synthetic GFP Protein in Onion Epidermal Cells

The synthetic GFP (sGFP) carrying optimized codon usage for plant systems (Chiu et al., 1996) was fused with the N terminus of *STOP1* by connecting PCR-amplified fragments derived from pBE2113 (containing sGFP) and RAFL (the RIKEN full-length cDNA clone [RAFL09-20I22; Seki et al., 2002] containing *STOP1*) using the following primers: *SfiI*-GFP (5'-CATTGGCCAAATCGGCCATGTGAGCAAGGCGGAGGAGCTG-3'), GFP-*NotI* (5'-GGATTAGCGGCCGCCTTGTACAGCTCGTCCATGCCGTGAG-3'), *NotI*-*STOP1* (5'-ACAAGGCGGCCGCATGGAAACTGAAGACGATTGTGCAAC-3'), and *STOP1*-*SfiI* (5'-ACAAGGCGGCCGCATGGAAACTGAAGACGATTGTGCAAC-3'). The PCR fragments of sGFP and *STOP1* were digested with *SfiI* and *NotI* and cloned into the *SfiI* site of the pBI2113SF vector. The accuracy of the sequence was confirmed by DNA sequencing analysis. The DNA constructs were introduced into onion (*Allium cepa*) epidermal cells, as described previously, by particle bombardment (Fujita et al., 2004). After incubation at 22°C for 12 h, GFP fluorescence was observed as described previously (Fujita et al., 2004).

Transgenic Complementation of *AtALMT1* in the *stop1* Mutant

The DNA fragment of *AtALMT1* was amplified by PCR using Arabidopsis genomic DNA and the primers Forward (5'-CATTGGCCAAATCGGCCA-TGGAGAAAGTGAGAGAGATAGTGAGAGAAG-3') and Reverse (5'-AGA-ACGGCCTTATGGCCTTACTGAAGATGCCCATTAATG-3') and was inserted downstream of the CaMV 35S promoter of the binary vector pBE2113SF (Liu et al., 1998). The binary construct was then introduced into *stop1* mutant plants by the floral dip method (Clough and Bent, 1998). The transformants in the T2 generation were obtained by controlled self-pollination of T1 plants.

RNA and Metabolite Extraction from Root Samples

Seedlings were precultured for 10 d in 2% MGR1 nutrient with 200 μM CaCl_2 at pH 5.6 hydroponically as described by Toda et al. (1999). Solutions were renewed every 2 d, and seedlings were kept in a controlled environment (30 μmol quanta m^{-2} s^{-1} , 12-h day/night cycle, 25°C). After the preculture, seedlings were transferred to Al^{3+} toxic solution (pH 5.0, 10 μM Al) and H^+ toxic solution (pH 4.5), which were prepared as described previously (Iuchi et al., 2007). The seedlings were kept under continuous light, and the roots were harvested at 24 h after the transfer. The roots were excised with scissors, immediately frozen in liquid N_2 , and kept at -80°C until use. RNA was isolated from the root samples and quantified according to Suzuki et al. (2004). For microarray analysis, the quality of the RNA was evaluated using the Agilent 2100 Bioanalyzer. Metabolites for gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis (CE)-MS analyses were extracted from the root samples in 50% ethanol using the Qiagen Mixer Mill MM300 kit. Samples were then pretreated by *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide for GC-time of flight (TOF)-MS or separated into the cationic and anionic fractions for CE-MS as described by Urano et al. (2009).

Microarray Analyses and Data Mining

Microarray analyses were carried out using the Agilent microarray system (Arabidopsis version 3) according to the manufacturer's protocol. Competitive hybridization analyses (Cy3-labeled versus Cy5-labeled copy RNA samples) were performed between control (24 h, no Al at pH 5.0) and

rhizotoxin-treated (24 h in pH 4.5 solution or 10 μM Al solution at pH 5.0) samples of Col-4, and comparisons were made between Col-0 and the *stop1* mutant in both treatments. Al and H^+ tolerance of Col-0 and Col-4 were very similar (Ikka et al., 2007). Copy RNA synthesis and dye labeling were performed with a kit (Low RNA Input Linear Amplification Kit PLUS; Agilent). All comparisons were carried out three times using biologically independent samples, including one dye-flip treatment. Microarray scanner G2565BA (Agilent) equipped with Feature Extraction version 9.5.1 software (Agilent) was used for scanning of the slides and extracting the data. Data mining and statistical analyses for microarray data were performed using GeneSpring GX version 7.3.1 (Agilent). As a quality control of microarray data, the spot feature data were examined with GeneSpring using default settings for the quality control that indicate "flags" to data with lower quality (e.g. low intensity, not uniform, abnormal in the background; http://www.chem.agilent.com/cag/bsp/products/gsgx/downloads/pdf/FE_Plugin.pdf). If the genes had flagged two or more data in any treatment (i.e. FC of the wild type in Al and low-pH treatments, or comparisons between the *stop1* mutant and the wild type), they were not used for the data mining. Significantly down-regulated genes in the *stop1* mutant with Al and H^+ treatments were defined as genes that carried FC (*stop1* mutant/wild type) values that were in the lowest 1% among all detected data.

Profiling of Metabolites

GC-TOF-MS analysis was performed with the Agilent 6890 GC system connected to a LECO Pegasus III mass spectrometer (Agilent), while CE-MS analyses were performed using the Agilent CE system in conjunction with an Agilent 1100 series MSD mass spectrometer. Anion detection by CE-MS followed the methods described by Urano et al. (2009), while GC-TOF-MS detection was carried out according to Urano et al. (2009). Detected peaks were assigned to known compounds (e.g. organic acids, amino acids, etc.) by theoretical M_r or by direct comparison with standard compounds. The intensity of each peak was recorded, and the values were normalized to the fresh weight base. Three independent analyses were carried out for all samples, and statistical analyses were carried out using Excel. Relative values (i.e. ratio of normalized peaks of *STOP1*-KO/wild type, pH 4.5) of metabolites were integrated on the metabolic pathway map. Raw data are available in Supplemental Table S3.

RT-PCR and Quantitative RT-PCR

RT-PCR was carried out as described previously (Kobayashi et al., 2007), and quantitative RT-PCR analyses were carried out using a real-time PCR system (Applied Biosystems 7300) following the manufacturer's protocol. Transcripts were relatively quantified using *UBQ1* transcripts as a control and then compared to that of the wild type. Primer sequences and PCR conditions are shown in Supplemental Table S5.

Hydroponic Culture and Growth Test

Growth experiments in hydroponic culture were carried out as described previously (Iuchi et al., 2007). The longest three root length values from 10 seedlings were used for evaluating root growth of the wild type and KO mutants. On the other hand, those from 20 seedlings were used for complemented lines carrying CaMV35S::*AtALMT1* in the *stop1* mutant.

Soil Culture and Soil Analyses

Seedlings were grown on acidic andosol containing large amounts of organic matter and phytotoxic Al (Kawatani andosol was obtained from the Kawatabi Experimental Farm at Tohoku University). Forest brown soil (neutral pH and no toxic level of Al) was obtained from Zao in the northern part of Japan. The properties of these soils are well characterized and used in physiological experiments for Al and proton toxicities (Ikka et al., 2007). The soil was fertilized with both macronutrients and micronutrients as described previously (Kobayashi et al., 2005). Kawatabi soil was neutralized by adding CaCO_3 (60, 250, and 400 mg per 100 g of dry soil). The soil pH (water) was measured according to the method of Shoji et al. (1964). Exchangeable Al was quantified by the method described by Koyama et al. (2000).

Microarray data are available through the ArrayExpress database (<http://www.ebi.ac.uk/microarray-as/ae/>) with accession code E-MEXP-1908.

Supplemental Data

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

Supplemental Figure S1. Comparison of expression levels of known Al-tolerant genes in the *stop1* mutant compared with the wild type by competitive microarray.

Supplemental Table S1. List of down-regulated genes in the *stop1* mutant among down-regulated genes in the wild type under Al and low-pH conditions.

Supplemental Table S2. Metabolite profile of major carbohydrate metabolic pathways (Fig. 5) of *STOP1*-KO relative to those determined for the wild type under low-pH conditions.

Supplemental Table S3. Relative expression levels of significantly down-regulated genes in the *stop1* mutant among genes involved in major carbohydrate metabolic pathways (Fig. 5) and those of isoforms involved in pH-regulating pathways.

Supplemental Table S4. List of genes rejected during quality control of microarray data analysis, which might contain genes greatly down-regulated in the *stop1* mutant under Al and low-pH conditions.

Supplemental Table S5. Primer sequences used for quantitative RT-PCR of the markedly down-regulated genes of the *stop1* mutant.

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