Running head: *STOP1*-like proteins in Al tolerance

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Characterization of AtSTOP1 orthologous genes in tobacco and other plant species

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One sentence summary: Diverse land plant species possess STOP1-like proteins that function in transcriptional regulation of aluminum tolerance.
Footnotes
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Abstract
Aluminum (Al) and proton (H\(^+\)) tolerances are essential traits for plants to adapt to acid soil environments. In *Arabidopsis*, these tolerances are mediated by a zinc-finger transcription factor, AtSTOP1 (*Arabidopsis thaliana* sensitive to proton rhizotoxicity\(^1\)), which regulates transcriptions of multiple genes critical for tolerance to both stressors. Here, the functions of orthologous proteins (STOP1-like proteins) in other plant species were characterized by reverse genetics analyses and *in planta* complementation assays. RNA interference of a gene for *NtSTOP1* (*Nicotiana tabacum* STOP1) repressed Al- and H\(^+\)-tolerances of tobacco roots. Tobacco roots released citrate in response to Al, concomitant with up-regulated transcription of an ortholog of an Al-tolerance gene encoding a citrate-transporting MATE (multi-drugs and toxic compound extrusion) protein. The RNAi repression of *NtSTOP1* blocked this process and also repressed transcription of another orthologous gene for Al tolerance, *ALS3* (*aluminum sensitive 3*), which encodes a prokaryotic-type transporter. These results demonstrated that *NtSTOP1* regulates Al tolerance in tobacco through transcriptional regulation of these genes. The *in planta* complementation assays revealed that other plant species, including woody plants, a legume, and a moss (*Physcomitrella patens*), possess functional STOP1-like proteins that can activate several H\(^+\)- and Al-tolerance genes in *Arabidopsis*. Knocking out the gene encoding the STOP1-like protein decreased the Al-tolerance of *P. patens*. Together, our results strongly suggest that transcriptional regulation by STOP1-like proteins is evolutionarily conserved among land plants, and that it confers the ability to survive in acid soils through transcriptional regulation of Al- and H\(^+\)-tolerance genes.
Introduction
Worldwide, more than 30% of land is affected by acid soils (pH <5.5). The area of acid soils is approximately 4 billion ha, accounting for approximately one-half of the world’s potential arable lands (Macdonald and Bruce Martin, 1988; Uexküll and Mutert, 1995). Growth of crop plants in acid soil is severely suppressed because of acid soil syndrome, which includes rhizotoxicities of solubilized ions such as aluminum (Al) and deficiencies of nutrients such as inorganic phosphorus (Pi) (Kochian et al., 2004). Because root growth inhibition is the main symptom of acid soil syndrome, many crops become susceptible to drought stress in acid soil regions, including those in African countries (Eswaran et al., 1997). Thus, it is believed that resistance to rhizotoxicities in acid soil is one of the most important traits to protect food production around the world against drought stress.

In acid soils, Al\(^{3+}\) is the main ion responsible for rhizotoxicity, but H\(^+\) is also harmful to sensitive plant species (Kinraide 1998). Although research on crop plants has been limited, the growth of H\(^+\)-sensitive genotypes of Arabidopsis accessions was impaired when they were grown in acid soil (Ikka et al., 2007). Al\(^{3+}\) and H\(^+\) also interact with each other as a result of their chemical and biological properties. Low pH (i.e., high H\(^+\) concentration and toxicity) increases the solubility of Al and the proportion of the most toxic Al-ion species, Al\(^{3+}\), in soil solutions, while it decreases the negativity of the plasma membrane (PM) surface, decreasing its ability to attract Al\(^{3+}\) (Kinraide, 1998; Kinraide, 2003). H\(^+\) rhizotoxicity disturbs ion homeostasis and destabilizes the cell wall, causing severe damage to the growing root tips (e.g., Koyama et al., 1995; Koyama et al., 2001). This might be alleviated by Al, because Al can solidify pectin in the cell wall (Blamey et al., 1993), although plant species must have functional Al-tolerance mechanisms to employ this strategy. In this situation, plants need to develop a series of strategies to tolerate Al- and H\(^+\)-rhizotoxicities to survive in acid soil environments.

In Arabidopsis, resistance mechanisms to acid soil conditions are transcriptionally regulated by the zinc-finger transcription factor STOP1 (Sensitive to Proton rhizotoxicity 1). The stop1 mutant was first selected as a mutant showing hyper-sensitivity to H\(^+\) rhizotoxicity, but it also showed hyper-sensitivity to Al\(^{3+}\) rhizotoxicity (Iuchi et al., 2007). The dual hypersensitivities could be explained by the loss of ability to induce expressions of a series of genes related to Al\(^{3+}\) and H\(^+\) tolerance (Sawaki et al., 2009). The STOP1 gene regulates Al-inducible expression of the AtALMT1 (Arabidopsis thaliana aluminum-activated malate transporter 1; Hoekenga et al., 2006) malate transporter and ALS3 (aluminum sensitive 3; Larsen et al., 2005), which encodes a prokaryotic-type ABC transporter with a possible role in UDP-glucose transport. The Arabidopsis stop1 mutant also showed repressed transcriptions of genes with roles in ion homeostasis, nitrogen and carbohydrate metabolism, and those encoding proteins that stabilize the cell wall, all of which contribute to H\(^+\) tolerance (Sawaki et al., 2009). As a result, stop1 mutant showed hyper-sensitivity in acid soil (Sawaki et al., 2009). This indicated that further research on the STOP1 system would provide further details about the complex system of Al- and H\(^+\)-tolerances in plants.

Homologs of the STOP1 gene in Arabidopsis (hereafter AtSTOP1) exist in a wide range of plant species (Iuchi et al., 2008). In rice, a functional homolog of AtSTOP1 was identified from studies on an Al-sensitive mutant (Huang et al., 2009; Yamaji et al., 2009). The mutant carried a dysfunctional ART1 transcription factor with the conserved zinc-finger domains of STOP1. ART1 regulates expression of the putative ALS3 homolog in rice, STAR2 (sensitive to Al rhizotoxicity 2; Huang et al., 2009). In addition, transcriptome analysis of near-isogenic wheat lines with contrasting Al tolerances suggested that the AtSTOP1 homolog is involved in determining variations of Al tolerance (Houde and Diallo, 2008), although its exact function remains unknown. AtSTOP1 regulates the expression of a type of citrate-transporting transporter AtMATE (A. thaliana multi-drug and toxic compounds extrusion), which makes a
minor contribution to Al tolerance in Arabidopsis (i.e., knocked-down transgenic lines showed small but significant increases in Al sensitivity; Liu et al., 2009). However, MATE-dependent citrate excretion is an important Al-tolerance mechanism in some plant species (Magalhaes et al., 2007). Together, all of these findings suggest that STOP1 transcription factors are ubiquitous in a wide range of plants and regulate Al-tolerance mechanisms, even though the Al-tolerance mechanisms may differ among plant species.

A reverse genetics approach is one way to analyze the function of STOP1 homologs from different plant species. In addition, in planta complementation assays using the Arabidopsis stop1-mutant (Atstop1) can be used to evaluate the transcription-factor activities of the homologs in a plant system. In the present study, we performed RNAi suppression of the STOP1-like protein in tobacco, and analyzed the phenotypes of complemented transgenic Atstop1 carrying homologous genes isolated from various plant species, including dicots, woody plants, and a bryophyte. These analyses strongly suggested ubiquitous and important roles of STOP1 in acid-soil resistance in a wide range of land plant species.

Results
Isolation of a gene encoding a STOP1-like protein from tobacco
A complementally DNA (cDNA) encoding an ortholog of AtSTOP1 (i.e., a gene encoding a STOP1-like protein) was isolated from tobacco (Nicotiana tabacum) by degenerate PCR (polymerase chain reaction) followed by RACE (rapid amplification of cDNA ends) PCR. The degenerate primers were designed from sequences of putative STOP1-like proteins from various plants available in the GenBank database. We conducted sequencing analysis using approximately 30 independent partial cDNA clones (all sharing the same sequence). Based on the results of that analysis, we isolated a unique cDNA encoding a protein with 506 amino acids and containing four Cys2-Hys2 zinc-finger domains that were homologous (Fig. 1) to those of AtSTOP1 and other STOP1-like proteins (i.e., ART1 and other putative STOP1-like proteins that were used for in planta complementation assays in our study; see Fig. 7). The tobacco ortholog, hereafter referred to as NtSTOP1 (Nicotiana tabacum STOP1), was characterized by producing an RNAi-suppressed line.

Growth response of RNAi transgenic tobacco suppressing NtSTOP1
We generated transgenic tobacco plants suppressing NtSTOP1 by Agrobacterium-mediated transformation using a vector carrying an RNAi that targeted sequences of NtSTOP1 (Fig. 2A). These transgenic plants showed repressed expression of NtSTOP1 (Fig. 2B), and plants in the T3 generation were sensitive to Al- and H+-rhizotoxicities (Fig. 2C). We conducted root growth assays for three independent RNAi lines (Knock-Down, KD1, 2, and 3) in low-pH and Al3+ conditions, and compared them with wild-type (WT). All KD lines showed significantly inhibited root growth, compared with that of WT, at pH 5.2, 5.0, and 4.7, when relative values (% of root growth at pH 5.5) were compared (Fig. 2C). At pH 5.5, there were no significant differences in root growth among the various lines, indicating that repression of NtSTOP1 enhanced H+-sensitivity. Al-tolerance was evaluated at pH 5.0 by comparing relative values for root growth (% of root growth in medium without Al at pH 5.0; Supplemental Fig. S1). Al sensitivity was significantly enhanced in KD1 and KD3 in the presence of 2 μM Al, and in all KD lines in the presence of 4 μM Al (Fig. 2C). The line showing greatest repression of NtSTOP1 (KD1) was the most sensitive to Al3+ rhizotoxicity (Fig. 2C); however, KD1 did not show increased sensitivity to other rhizotoxic ions (Fig. 3). This suggested that NtSTOP1 regulates both H+ and Al- tolerance in tobacco, but it does not regulate tolerance to other stressors.

Repression of Al-activated citrate excretion and Al-tolerance genes in tobacco KD1
After incubation in Al-rhizotoxic conditions, the root tips of KD1 showed brighter fluorescence
than that of WT when stained with morin (an Al-detecting fluorescent probe; control plants grown in Al-free medium showed no staining) (Fig. 4A). This result showed that KD1 accumulated higher levels of Al than did the WT, suggesting that RNAi inhibition of NtSTOP1 decreased the ability of the root tips to exclude Al. We also analyzed the concentrations of the major organic acids (OAs; malate, citrate, and oxalate) in Al-toxic culture solution (24-h incubation), because excretion of these OAs is one of the most common mechanisms of Al-exclusion from the root tip. In the WT, the OA with the highest concentration was citrate, while oxalate was not detected. Citrate showed an Al-inducible excretion pattern (Fig. 4B). All KD lines showed suppressed Al-inducible citrate excretion (Fig. 4B). These results suggested that the repression of Al-induced citrate excretion is one mechanism underlying the enhanced Al-sensitivity in KD lines, accompanied by increased accumulation of Al in the root tip (Fig. 4A).

**Repressed transcriptions of Al-responsive MATE and ALS3**

Previous studies identified that the citrate-transporting MATE protein regulates Al-inducible citrate excretion in various plant species (e.g., Magalhaes et al., 2007). Studies on mutants of AtSTOP1 and rice ART1 identified that both mutants showed repressed transcriptions of orthologous genes [ALS3 in *Arabidopsis*, STAR2 (*SENSITIVE TO ALUMINUM RHIZOTOXICITY 2*) in rice] that play roles in excluding Al from the root tip. It is possible that repression of these genes could enhance the Al-susceptibility of KD lines. To test this possibility, we isolated orthologous genes from tobacco (i.e., orthologous genes encoding citrate-transporting MATE and ALS3-like proteins) and analyzed their transcript levels.

We used degenerate PCR followed by RACE PCR to isolate genes homologous to citrate-transporting MATE (hereafter *NtMATE1*) and ALS/STAR2 (hereafter *NtALS3*). They were homologous to previously isolated genes (see phylogenetic trees in Supplemental Fig. S2 and S3). These two genes were Al-inducible, but their transcriptions were repressed in the KD lines (Fig. 5A and B). The Al-induced transcript level of *NtMATE* was much lower in KD1 than in other KD lines, and the transcript level was correlated with citrate excretion and Al-tolerance levels (Fig. 5C). In addition, *NtALS3* transcript levels were repressed in Al-treated KD lines (ranging from 0.13- to 0.35-fold that in WT). These findings suggested that *NtSTOP1* regulates expression of a set of genes that could explain the enhanced Al-susceptibility of KD lines.

**In planta complementation assays with genes encoding STOP1-like proteins from various plant species**

Database searches revealed putative STOP1 genes (i.e., genes encoding STOP1-like proteins) in other plant species (Fig. 6), although the transcription-factor functions have been analyzed only for AtSTOP1 and ART1. To analyze the transcription-factor activity of other putative STOP1-like proteins (shown in bold in Fig. 6), we performed *in planta* complementation assays using the Atstop1-mutant as the complementation host. A homologous gene from tea (hereafter *CsSTOP1*, from *Camellia sinensis*) was newly isolated by degenerate PCR, while a cDNA was isolated by RT-PCR from the model legume *Lotus japonicas* (hereafter *LjSTOP1*) based on the genomic DNA sequence. Two other homologous genes (hereafter *PnSTOP1* from black poplar (*Populus nigra*) and *PpSTOP1* from the moss *Physcomitrella patens*) were obtained from the full-length cDNA collection of the RIKEN BioResource Center. These cDNAs and *NtSTOP1* were used for the complementation assays.

The relatedness among the orthologous proteins (AtSTOP1, ART1, and other putative STOP1 proteins from various plant species) is shown in Fig. 6. AtSTOP1 was grouped with putative STOP1 proteins from *Arabidopsis lyrata* and *Brassica rapa*, while *LjSTOP1*, *NtSTOP1*, *CsSTOP1*, and *PnSTOP1* were grouped with those from closely related plant species. For example, *LjSTOP1* was grouped with putative STOP1 proteins from other legumes; *NtSTOP1* was grouped with an ortholog from tomato; tea and black poplar were grouped with putative
STOP1 proteins from other broadleaf trees. Rice ART1 and PpSTOP1 (moss) were grouped with a putative STOP1 ortholog from *Picea glauca* (white spruce; a coniferous tree), while the other rice homologous proteins were grouped with putative orthologs from monocots. This analysis suggested that genes for STOP1/ART1-like proteins are conserved among a wide range of plant species and that rice has multiple copies.

Each isolated cDNA was introduced into the *Atstop1* mutant, and then transcript levels of several genes that are known to be repressed in the *Atstop1* mutant were analyzed (see Table 1, Sawaki et al., 2009) (Fig. 7A). All of the putative orthologs introduced into *Atstop1* activated transcriptions of several genes, suggesting that each of these STOP1-like proteins functioned as a transcription factor. However, there were differences among the orthologous genes in terms of which genes they activated. For example, under low-pH treatment, the transcript levels of *PGIP1* (*POLYGALACTURONASE INHIBITING PROTEIN 1*), *CIPK23* (*CBL-INTERACTING PROTEIN KINASE 23*), and *AtSTOP2* (*A. thaliana SENSITIVE TO PROTON RHIZOTOXICITY 2*, a unique homolog of *AtSTOP1*) were generally recovered by all of the orthologous genes. For all of these genes, their transcript levels were recovered to at least 0.5 compared with their respective transcript levels in WT. In contrast, in the Al-treatment, the homologous genes showed different abilities to recover the transcript levels of genes. *AtALMT1* transcription was activated by many of the homologous genes, but only *PpSTOP1* recovered transcription of *AtALMT1* to a level comparable to that in WT. The ability to recover expressions of *ALS3* and *AtMATE* also varied among the orthologs (Fig. 6A). These results revealed that all of the orthologous genes have transcription factor activity *in planta*, but their encoded proteins did not have the full function of *AtSTOP1* to activate all of the regulated genes in *Arabidopsis*. All these orthologous genes conferred H⁺-tolerance as determined by root growth assays (Fig. 6B). In contrast, the recovery of Al³⁺-tolerance varied among the complemented lines (Fig. 6B) because of the lower ability of most of the transformed genes to activate transcriptions of Al-tolerance genes.

**Suppression of Al tolerance in a moss by gene knock-out**
To evaluate the function of *PpSTOP1* in moss, *PpSTOP1* was knocked-out (KO) by homologous recombination. In the recombinant lines, the *PpSTOP1* genomic region was replaced by a kanamycin resistance gene (neomycin phosphotransferase II) (Fig. 8A). The growth of KO lines was comparable to that of WT on control plates (without Al), but was severely suppressed on Al-containing medium (Fig. 8B). Growth inhibition was associated with the reduction of chlorophyll (green color), which is one of the most sensitive indicators of cellular damage in this plant. These results showed that the STOP1 ortholog functioned in regulating Al tolerance in this host. The H⁺-sensitivity was unclear in the KO lines evaluated under these experimental conditions. WT and KO lines grew comparably on control medium (pH 4.2).

**Discussion**
Acid soils are associated with both Al³⁺ and H⁺ rhizotoxicities, which cause severe damage to many crops. Plant species have developed various strategies to tolerate acidity, including internal tolerance and Al-exclusion mechanisms (Kochian et al., 2004). Recent studies identified that these tolerance mechanisms are regulated by the transcription factors *AtSTOP1* (*Arabidopsis*; Sawaki et al., 2009) and *ART1* (rice; Yamaji et al., 2009), which have homologous Cis2-Hys2 zinc-finger domains. In the present study, we identified functional orthologous proteins (STOP1-like proteins) in other plant species. We conducted *in planta* complementation of the *Atstop1* mutant of *Arabidopsis* using each of the genes encoding STOP1-proteins. The complemented lines showed recovered transcriptions of various genes (Fig. 7A) that are suppressed in the mutant due to the dysfunctional *AtSTOP1* gene (Sawaki et al., 2009). Repression of the STOP1 ortholog in tobacco enhanced its sensitivity to H⁺- and Al³⁺
toxicities (Fig. 2C). RNAi inhibition of NtSTOP1 resulted in repressed transcriptions of a set of putative ortholog of genes reported to play roles in Al-tolerance in other plant species. We evaluated the transcript levels of a gene encoding the citrate-transporting MATE protein (NtMATE), which is homologous to other previously identified citrate-transporting MATEs (see Supplemental Fig. S2). The transcript level of NtMATE was correlated with the capacity to excrete citrate, which determined the Al sensitivity of NtSTOP1 RNAi lines (Fig. 5C). This strongly suggested that Al-inducible citrate excretion, which is regulated by NtMATE and NtSTOP1, is an Al-tolerance mechanism of tobacco. This is consistent with the previous finding that Al-tolerant cultured tobacco cells showed enhanced citrate excretion and Al tolerance (Ojima et al., 1989).

Al tolerance was repressed in Arabidopsis AtSTOP1 (Iuchi et al., 2007) and rice (art1) (Yamaji et al., 2009) mutants, both of which carried dysfunctional STOP1-ART1-type transcription factors. However, these two plant species have different Al-tolerance mechanisms. Almost all of the Al-tolerance mechanisms of rice can be categorized as internal Al tolerance (e.g., Kochian et al., 2004), whereas Arabidopsis uses a typical Al-excretion mechanism; mainly Al-activated malate excretion through the AtALMT1 malate transporter (Hoekenga et al., 2006). In the present study, we identified that a different type of Al tolerance (i.e., MATE-dependent citrate excretion, another Al-exclusion mechanism) is also regulated by the same transcription factor family in different plant species such as tobacco. This finding suggests that the RNAi approach could be used to find genes that play critical roles in Al-tolerance mechanisms in different plant species. For example, an in planta complementation assay indicated that tea contains a functional CsSTOP1, while physiological studies indicated that it has Al-tolerance mechanisms different from those in other species. Tea plants and cultured cells released oxalic acid in response to Al (Morita et al., 2008; Morita et al., 2011). The release of oxalic acid is one of the major Al-tolerance mechanisms previously identified in other plants, including buckwheat (Zheng et al., 1998). A similar approach in tea would be useful to identify the oxalate excretion system at the molecular level. Although we have not yet characterized the Al-tolerance mechanisms of P. patens, disruption of the homologous gene PpSTOP1 enhanced its Al susceptibility (Fig. 7). Further research on P. patens will uncover as-yet unknown mechanisms of Al tolerance in basal land plants.

In other studies, the rice art1 mutant showed enhanced Al sensitivity, but no significant changes in H⁺ sensitivity (Yamaji et al., 2009), whereas the Arabidopsis Atstop1 mutant showed enhanced H⁺ sensitivity (Iuchi et al., 2007). In the present study, we found similar conflicting results in two different plant species using reverse genetics approaches. Tobacco showed a small but significant increase in H⁺ sensitivity after suppression of NtSTOP1 (Fig. 2C), while the P. patens KO-line (PpSTOP1-KO) did not show enhanced H⁺-sensitivity, at least on a gelled medium at pH 4.2 (Fig. 8). These results suggest that the contribution of STOP1 orthologs to H⁺ resistance varies among different plant species. A previous study revealed that multiple genes for H⁺-resistance are regulated by AtSTOP1 in Arabidopsis. However, other genes (i.e. those not strictly regulated by AtSTOP1) and proteins associated with H⁺-tolerance have been also reported. For example, ROF2 encoding a peptidyl-prolyl cis-trans isomerase (Bissoli et al., 2012) and RALF-proteins (rapid-alkalization factor proteins; Srivastava et al., 2009) were identified to regulate cellular pH homeostasis. Such systems and other unknown mechanisms of H⁺-tolerance that are not regulated by STOP1-like proteins may operate in some plant species such as rice and P. patens.

In planta complementation assays showed that all of the tested STOP1-like proteins were able to activate transcriptions of several genes in Arabidopsis. This finding can be explained by the highly conserved zinc-finger domains that activate transcriptions of genes with the same target cis-motif in their promoter regions. The sequence of the cis-motif would have been conserved among plant species during evolution. However, the differences in activation of AtALMT1 expression among the different complemented lines (Fig. 7A) suggested that the
protein structure of AtSTOP1, which is critical for activating expression of AtALMT1, may not be completely conserved in some of the STOP1-like proteins. In fact, the zinc-finger domain was highly conserved among the STOP1-like proteins, but both the N- and C- termini showed much lower homologies (Supplemental Figure S4). Protein phosphorylation/dephosphorylation processes are involved in AtSTOP1-dependent AtALMT1 expression (Kobayashi et al., 2007), but it remains unclear whether AtSTOP1 is directly phosphorylated or whether it interacts with other proteins via protein phosphorylation. Such processes would be affected by differences in protein sequence and/or structure, possibly in the N- or C- terminus, among the various homologous proteins from different plant species. The recovery of transcriptions of other Al-tolerance genes (ALS3 and AtMATE) also varied among the complemented lines (Fig. 5A). As a result, the Al-tolerant phenotype was not conferred by many of the orthologous proteins. On the other hand, the H⁺-sensitive phenotype was conferred by almost all of the tested orthologous proteins. This finding suggested that the transcriptional activation of Al-tolerance genes is more sensitive to the protein structure of STOP1 than is the transcriptional activation of H⁺-tolerance genes. To explore this topic, further research is required to examine the structural and functional differences among STOP1-like proteins, and the conservation of cis-elements in the promoters of target genes.

Database searches of GenBank (www.ncbi.nlm.nih.gov/genbank) and individual plant databases [e.g. PHYSCObase for P. patens, http://moss.nibb.ac.jp/; miyakogusa.jp for Lotus japonicus, http://www.kazusa.or.jp/lotus/; black poplar, see Nanjiyo et al. (2004)] identified that most of the dicots and P. patens contain two or three copies of genes encoding putative STOP1-like proteins. In Arabidopsis, AtSTOP2 is a unique homolog with homologous zinc-finger domains (Englbrecht et al., 2004) that is expressed at much lower levels than AtSTOP1 (at least 10-times less; unpublished data) and whose expression is regulated by AtSTOP1 (Fig. 6A). Suppression of Al tolerance by knocking down orthologs suggested that AtSTOP1 orthologous proteins, but not AtSTOP2 orthologs, may play a critical role in Al tolerance in a wide range of plant species. The searches identified that monocots, especially rice, may have a greater number of homologous genes, while the function of each homolog has not yet been analyzed. The increased copy number of STOP1-like proteins in rice may explain its higher Al resistance compared with that of other crops. On the other hand, all tested plants carrying a dysfunctional STOP1-like protein (Arabidopsis and rice, Sawaki et al., 2009; Yamaji et al., 2009) or those with highly reduced STOP1 levels as a result of RNAi (tobacco, our ongoing research) showed repressed expressions of ALS3 orthologs. The origin of this protein in plants remains unclear, but it has the structural characteristics of a prokaryotic transporter (Larsen et al., 2005). In cancer cells in animals, many proteins of prokaryotic origin play critical roles in adaptation to cellular acidification (hypoxia) (Semenza, 2001; Fernandes et al., 2012). Further research on the STOP1 regulation system may reveal how plants acquired and evolved H⁺- and Al-tolerance systems that include STOP1-like and ALS3-like proteins.
Materials and Methods

Plant materials
Tobacco (Nicotiana tabacum, cv. Xianti) was used as the host for Agrobacterium tumefaciens-mediated transformation for RNAi suppression of the AtSTOP1 ortholog. The suppressed lines were designated as NtSTOP1-KD (Knock-Down). Arabidopsis (Arabidopsis thaliana) wild-type Col-0 (WT) and the stop1 mutant were identical to those used in a previous study (Iuchi et al., 2007). The stop1 mutant was used as the host for in planta complementation assays with STOP1 orthologs. P. patens subspecies patens (Gransden) was the WT strain for the gene knock-out experiment.

Isolation of cDNAs encoding STOP1 orthologs, NtMATE, and NtALS3
Full-length cDNAs of AtSTOP1 orthologs were obtained for a model tree (black poplar, Populus nigra; designated as PnSTOP1) and a model moss (Physcomitrella patens, designated as PpSTOP1) from the plant full-length cDNA library collection of the RIKEN BioResource Center [clone number pdp41649 for PnSTOP1 and pds27803 for PpSTOP1, developed by the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan]. Putative orthologs of AtSTOP1 in tobacco (Nicotiana tabacum, cv. Xianti), designated as NtSTOP1 and tea (Camellia sinensis L. Yabukita), designated as CsSTOP1, and homologs of the Al-inducible citrate transporting MATE (designated as NtMATE) and ALS3 (designated as NtALS3) in tobacco were isolated by degenerate PCR (primers listed in Supplemental Table S1) followed by 3’ and 5’ RACE (rapid amplification of cDNA ends) procedures as described previously (Kihara et al., 2003). Total RNA isolated from root samples (see below; RNA extraction) was used as the template for reverse transcription (RT)-PCR and degenerate PCR. A cDNA of the STOP1 homolog from the model legume Lotus japonicus (designated as LjSTOP1) was obtained by RT-PCR based on sequence information from the L. japonicas database (http://www.legumebase.brc.miyazaki-u.ac.jp/lotus/top/top.jsp). All newly isolated cDNAs were deposited into GenBank under the following accession numbers: PnSTOP1 (AB811779), PpSTOP1 (AB811779), NiSTOP1 (AB811781), CsSTOP1 (AB811780), LjSTOP1 (AK645689), NtMATE (AB811784), NtALS3 (AB811783).

STOP1-suppressing tobacco transgenic lines
The NtSTOP1-RNAi (RNA interference) vector for Agrobacterium-mediated transformation was prepared by replacing the β-glucuronidase gene of the binary vector pBI121 (Toyobo, Ohtsu, Japan) with the NtSTOP1-RNAi sequence. The NtSTOP1-RNAi sequence consisted of the partial cDNA of NtSTOP1 (312 bp; -87 to +225, 0 is first ATG) joined to 3’- and 5’-ends of the first intron of the A. thaliana isocitrate dehydrogenase (At1g65930) sequence in sense (3’ end) and antisense (5’ end) orientations. This intron is efficiently spliced in transformants and can produce double-stranded RNA to suppress the target gene (Nakagawa et al., 2007). The vector was introduced into A. tumefaciens LBA4404, and then introduced into tobacco by leaf-disc transformation (Horsch et al., 1985). Regenerated plants showing kanamycin resistance were grown in soil in a growth room (20 ± 3°C, 12 h illumination per day, 200 μmol m⁻¹s⁻¹) until seeding. T₂ and T3 generations were obtained by the single seed descent method. T₃ seeds were used for organic acid excretion assays, growth assays, and transcript analyses. Integration of T-DNA was confirmed by genomic PCR as described previously (Kihara et al., 2006).

In planta complementation assays of STOP1 orthologs
All putative STOP1 orthologs (genes encoding STOP1-like proteins) were connected to the AtSTOP1 promoter (-2848 from first ATG) and downstream region (+626 from stop codon) by
overlap extension PCR (Horton et al. 1989). All PCRs were carried out using Prime STAR MAX (Takara), a very high fidelity Taq polymerase. The STOP1 ortholog constructs were introduced into the T-DNA of the pBE2113 binary vector and transformed into *A. tumefaciens* strain GV3101 (a super-virulent strain). *Arabidopsis* transformation was carried out by the floral dip method (Clough and Bent, 1998) and T2 progeny were used for assays.

**Hydroponic culture**

*Arabidopsis* and tobacco seedlings were grown hydroponically according to methods for evaluating Al³⁺- and H⁺-tolerance of *Arabidopsis* seedlings (Kobayashi et al., 2007). Briefly, 10 seeds of each line were placed on a plastic mesh supported by a polystyrene float on culture solution. The basal solution lacked Pi, and contained 2% (w/v) MGRL nutrients and 200 μM CaCl₂. The H⁺- and Al³⁺- toxic solutions were prepared by adjusting the pH with HCl, and adding AlCl₃ to obtain concentrations known to be toxic to *Arabidopsis* (pH 4.7 for H⁺-toxicity and Al³⁺-toxicity, 2 μM at pH 5.0) and tobacco (pH 5.2, 5.0, and 4.7 for H⁺-toxicity and Al³⁺-toxicity, 2 and 4 μM at pH 5.0). Culture solution at pH 5.0 without Al served as the control to evaluate Al³⁺-toxicity, while solutions at pH 5.0 (*Arabidopsis*) and 5.5 (tobacco) were used as the controls to evaluate H⁺-toxicity. All solutions were renewed every 2 days, and seedlings were grown for 7 days at 25°C with 12 h illumination per day (70 μmole E m⁻¹ s⁻¹). Root length was measured using a video microscope and relative root length (%; stressed/control) values were calculated.

**Morin staining**

Staining with the Al-specific fluorescent probe morin (Sigma, St Louis, MO, USA) was performed following the method described by Tice et al. (1992). Briefly, roots of tobacco plants hydroponically grown in control solution for 7 days were incubated in a solution containing Al (20 μM, pH 5.0) and 2% (w/v) MGRL nutrients for 24 h. The roots were stained with 100 μM morin for 15 min, rinsed with distilled water, and then observed under a fluorescence microscope (AXIO Imager System, Carl Zeiss, Tokyo, Japan).

**Collection of root exudates and quantification of citrate and malate**

Tobacco seedlings were grown for 2 weeks in 150 ml 1/50 MGRL nutrients (CaCl₂ increased to 200 μM) solution containing 1% sucrose under aseptic conditions at pH 5.5 in plastic pots. Each plant was incubated for 24 h in 2 ml 1/50 MGRL solution containing 1% sucrose in a 6-well plate in the presence or absence of Al (20 μM; pH 5.0). Citrate and malate were quantified by the enzyme reaction (citrate lyase EC 4.1.3.8. for citrate; malate dehydrogenase, EC 1.1.1.82 for malate) -coupled NADH/NAD⁺ cycling method developed by Hampp et al. (1984) with minor modifications as described by Kihara et al. (2003). Oxalate in the medium was quantified calorimetrically using the Enzytec kit for oxalate (R-Biopharm AG, Darmstadt, Germany) according to manufacturer’s protocol.

**RNA extraction, reverse transcription, and real-time RT-PCR**

Total RNA was extracted from *Arabidopsis* and other plant species according to the methods described previously (Suzuki et al., 2001; Suzuki et al., 2004). For transcript analyses in tobacco and *Arabidopsis*, total RNA was isolated from the roots of plants subjected to Al-toxic (pH 5.0, 10 μM *Arabidopsis*, 20 μM tobacco) or control (pH 5.0, no Al) conditions for 24 h. Seedlings that were grown in pre-culture solution (pH 5.6, no Al) for 10 days (*Arabidopsis*) or 14 days (tobacco) were used for these analyses. The pre-culture solution contained 2% (w/v) MGRL nutrients and 200 μM CaCl₂, and the same solution without Pi was used to incubate roots for Al-treatments and the control. Total RNA was reverse-transcribed to cDNA using ReverTra Ace (Toyobo, Osaka, Japan) with oligo dT primers. Real-time RT-PCR for quantitative analysis was carried out using Power SYBR Green PCR master mix and an ABI PRISM 7000 instrument.
(Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol for the standard curve method. For all experiments, a diluted cDNA series was used to obtain standard curves of target genes and the internal control. The internal controls were the UBQ1 gene (Ubiquitin extension protein, At3g52590; for Arabidopsis) and the Actin gene (for tobacco). Primer information is shown in Supplemental Table S1. The transcript levels of PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN L, At5g06860), CIPK23 (CBL-INTERACTING PROTEIN KINASE 23, At1g30270), STOP2 (SENSITIVE TO PROTON TOXICITY 2, At5g22890), AtALMT1 (A. thaliana AL-_ACTIVATED MALATE TRANSPORTER 1, At1g08430), ALS3 (ALUMINUM SENSITIVE 3, At2g37330), AtMATE (A. thaliana MULTI DRUGS AND TOXIC COMPOUND EXTRUSION, At1g51340), GDH1 (GLUTAMATE DEHYDROGENASE L, At5g18170) and PLT3 (PROBABLE POLYOL TRANSPORTER 3, At2g18480) were quantified in Arabidopsis. The transcript levels of NtMATE1 (N. tabacum MULTI DRUGS AND TOXIC COMPOUND EXTRUSION; GenBank AB811784), NtALS3 (N. tabacum ALUMINIUM SENSITIVE 3; GenBank AB811783) were quantified in tobacco.

DNA sequencing and sequence analyses, and database analyses
DNA sequencing analysis was performed with an ABI PRISM 3130xl DNA Sequencer and an ABI BigDye Terminator System (ver. 3.1) according to the manufacturer’s recommended protocols. Orthologous genes were identified by searches of GenBank (www.ncbi.nlm.nih.gov/genbank/), Miyakogusa.jp (http://www.kazusa.or.jp/lotus/), and NIBB PHYSCObase (http://moss.nibb.ac.jp/). Sequence analyses, amino acid alignments, and phylogenetic tree analysis (neighbor-joining method, and construction of a non-rooted phylogram) were carried out using GENETYX software version 11.01 (Genetyx, Tokyo, Japan). The domains described by Englbrecht et al. (2004) were used to identify the zinc-finger domains of AtSTOP1 and other STOP1-like proteins.

Knock-out of PpSTOP1 and growth test of Physcomitrella patens
PpSTOP1 was disrupted using the homologous recombination method as described by Schaefer and Didier (1997). The targeting sequence was synthesized by overlap extension PCR by sandwiching the neomycin phosphotransferase II gene cassette with flanking genomic DNA of PpSTOP1. PpSTOP1 disruptants were obtained as described previously (Komatsu et al., 2009) and confirmed by genomic PCR (primers are listed in Supplemental Table S1). Disruptants were grown on MPS medium [2.0 mmol/L Ca(NO3)2, 3.0 mmol/L KNO3, 1.5 mmol/L MgSO4·7H2O, 67 μmol/L Na2-EDTA, 8.6 μmol/L FeSO4·7H2O, 11.1 μmol/L MnSO4·H2O, 30 μmol/L H3BO3, 1.0 μmol/L ZnSO4·7H2O, 1.0 μmol/L CuSO4·5H2O, 24 mmol/L (NH4)6Mo7O24·4H2O, 0.13 μmol/L CoCl2·6H2O and adjusted to pH 4.2] containing 0.7% (w/v) agar and 3% (w/v) sucrose in the presence or absence of 400 μM AlCl3. Plants were maintained at 25°C under continuous light (50–80 μmol E m⁻² s⁻¹).

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Supplemental Data
Supplemental Figure S1. Root growth of tobacco seedlings in hydroponic culture at pH 5.5 and 5.0.
Supplemental Figure S2. Phylogram of citrate-transporting MATE proteins in various plants.
Supplemental Figure S3. Phylogram of ALS3-like proteins in various plants.
Supplemental Figure S4. Deduced amino acid alignment of STOP1-like proteins.
Supplemental Table S1 Sequence information for PCR primers used for degenerate PCR, RT-PCR and genomic PCR.
Literature Cited


Figure legends

Figure 1 Deduced amino acid sequences of zinc-finger domains of tobacco NtSTOP1 protein. Zinc-finger domains (predicted in Arabidopsis by Englbrecht et al., 2004) were compared among AtSTOP1 (Arabidopsis), ART1 (rice STOP1 ortholog), PgSTOP1 (black poplar), LjSTOP1 (Lotus japonicas), CsSTOP1 (tea), and PpSTOP1 (Physcomitrella patens). Strictly conserved amino acids are highlighted in black, residues belonging to conserved amino acid groups are highlighted in grey. Asterisks indicate Cys and His of C2H2 motifs.

Figure 2 Al- and H⁺-tolerance of transgenic tobacco plants with NtSTOP1 suppressed by RNAi inhibition. Transgenic tobacco was obtained by Agrobacterium-mediated transformation using a vector carrying an NtSTOP1 RNAi construct. Partial cDNA of NtSTOP1 sandwiched the first intron of NADP-ICDH of Arabidopsis, and the gene construct was driven by the Cauliflower mosaic virus 35S promoter (A). Suppression of NtSTOP1 was analyzed in T3 seed progeny of three independent transgenic lines (NtSTOP1-KD1, 2, and 3) by RT-PCR using Actin as the internal control (B). Seedlings of T3 seed progenies of NtSTOP1-KD and wild-type (WT) were grown hydroponically for 1 week in Al-toxic solutions (pH 5.0, 2 and 4 μM) and different levels of H⁺ toxicity (pH 5.2, 5.0, and 4.7). Means and SD of relative values (% of control; pH 5.0, 0 Al for Al, pH 5.5 for pH; absolute root lengths are shown in Supplemental file Fig. S2) from five seedlings are shown (C).

Figure 3 Tolerance of NtSTOP1-KD1 to rhizotoxic metals was compared with that of WT. Means and SD of relative root length (+toxicants/control) are shown (n = 5). Tolerance to toxic metals was tested by adding the following compounds to control (pH 5.0, no toxicant) solution: 4 μM CdCl2, 0.4 μM LaCl3, 200 μM MnSO4, or 4.0 μM AlCl3. Asterisks indicate significant difference as compared with WT (t-test, p<0.05).

Figure 4 Aluminum accumulation in root tip and organic acid excretion in response to Al. Root tips of NtSTOP1-KD1 and wild-type (WT) were stained with morin after incubation in Al-containing solution (pH 5.0, 20 μM) for 24 h. Fluorescent image of root tip, scale bar = 50 μm (A). Citrate (left) and malate (right) excretion from roots of WT and NtSTOP1-KD lines in control (pH 5.0, Al 0) and Al-containing (20 μM, pH 5.0) solutions for 24 h. White bars, mean values in control solution (± SD). Black bars, mean values in Al solutions (n = 3).

Figure 5 Transcript levels of genes orthologous to those associated with Al tolerance in Arabidopsis in NtSTOP1-KD lines. Seedlings were incubated in Al-toxic (pH 5.0, 20 μM Al) or control (pH 5.0, no Al) solution for 24 h, and root samples were used for transcript analyses. Transcript levels of a citrate-transporting MATE, NtMATE (homologous to AtMATE) (A), and NtALS3 (homologous to AtALS3) (B), were compared between NtSTOP1 KD lines and wild-type (WT). Transcript levels were relatively quantified by real-time PCR using Actin as the internal control, and then fold-change (relative to WT control) values were calculated. Means and SD of three replications are shown. Relationship between NtMATE expression (identical to those in panel B) and citrate release (identical to Fig 4B) in different genotypes (open circle, KD; filled circle, WT) are shown in panel C. Means ± SD of three replications are shown.

Figure 6 Phylogram of STOP1-1 like proteins in various plant species. Distance indicator shows relatedness of proteins. Bold font indicates that function of protein has been supported by experiments using mutants [Arabidopsis thaliana, AtSTOP1 (GenBank ID, NM_103160); Oryza sativa, ART1 (NM_001072803)], RNAi suppression [Nicotiana tabacum, NtSTOP1]
gene knock-out [Physcomitrella patens, PpSTOP1 (AB811779)], and/or in planta complementation assays [Camellia sinensis (tea), CsSTOP1 (AB811780); Populus nigra (black poplar), PnSTOP1 (AB811779); Lotus japonicas, LjSTOP1 (AB811782)]. Putative orthologs are shown in regular font: Brassica rapa (AC232513.1); Arabidopsis lyrata (XM_002891054.1); Glycine max (XM_003556158), Vitis vinifera (XM_002270160), Picea glauca (BT117929), Vigna unguiculata (TC13125), Solanum lycopersicum (AK320912), Jatropha curcas (Jcr4S27000.20), Oryza sativa (NM_001051470), Malus × domestica (HM122494), Brachypodium distachyon (XM_003564671), Hordeum vulgare (AK252406).

Figure 7 Recovery of gene transcriptions (A) and H\(^+\)- and Al- sensitive-phenotypes (B) of Atstop1 after complementation with homologous genes encoding STOP1-like proteins. A; Recovery of transcriptions of suppressed genes in Atstop1-mutant (Sawaki et al., 2009) was analyzed in complemented lines after exposure to H\(^+\)-toxic (pH 4.5 for 24 h) and Al-toxic (10 \(\mu\)M Al, pH 5.0 for 24 h) conditions. Transcript levels of PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1), CIPK23 (CBL-INTERACTING PROTEIN KINASE 23), STOP2 (SENSITIVE TO PROTON TOXICITY 2), AtALMT1 (Arabidopsis thaliana AL-ACTIVATED MALATE TRANSPORTER 1), ALS3 (ALUMINUM SENSITIVE 3), AtMATE (Arabidopsis thaliana MULTI DRUGS AND TOXIC COMPOUND EXTRUSION 1), GDH1 (GLUTAMATE DEHYDROGENASE 1) and PLT3 (PROBABLE POLYOL TRANSPORTER 3) were quantified by real-time PCR. Mean values ± SE (n = 3) are shown. Asterisks indicate significant difference between complemented line and Atstop1 (MT) (t-test, p<0.05). B; Growth of complemented lines and wild-type (WT) in Al-toxic (pH 5.0, 4 \(\mu\)M) and H\(^+\)-toxic (pH 4.7) conditions. Relative root length (% of that in WT) of complemented lines is shown. Values are means ± SE (n = 3). Complemented lines carried AtSTOP1 or an ortholog of AtSTOP1 [from tobacco (NtSTOP1), black poplar (PnSTOP1), tea (CsSTOP1), Lotus japonicas (LjSTOP1), or Physcomitrella patens (PpSTOP1)].

Figure 8 Aluminum tolerance of gene knock-out (KO) line of Physcomitrella patens (PpSTOP1). Genomic PCR of wild-type and PpSTOP1-KO lines was conducted using primer pairs to amplify target sites (i.e., PpSTOP1 gene, NPTII gene) (A). Wild-type and KO-lines (1–3) were grown for 2 weeks on Al-toxic medium containing 400 \(\mu\)M Al at pH 4.2. Bar = 1 cm.
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