Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance

Satoshi Iuchi*†, Hiroyuki Koyama‡, Atsuko Iuchi*, Yasufumi Kobayashi‡, Sadako Kitabayashi*, Yuriko Kobayashi‡, Takashi Ikka‡, Takashi Hirayama§¶, Kazuo Shinozaki‖, and Masatomo Kobayashi*

*BioResources Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan; ‡Laboratory of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan; §Graduate School of Integrated Science, Yokohama City University, 1-7-29 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan; ¶RIKEN Wako Institute, Environmental Molecular Biology Laboratory, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; and ‖RIKEN Plant Science Center, 1-7-22 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

Edited by Jim Peacock, Commonwealth Scientific and Industrial Research Organization, Canberra, ACT, Australia, and approved April 22, 2007 (received for review January 5, 2007)

Acid soil syndrome causes severe yield losses in various crop plants because of the rhizotoxicities of ions, such as aluminum (Al^{3+}). Although protons (H^+) could be also major rhizotoxicants in some soil types, molecular mechanisms of their tolerance have not been identified yet. One mutant that was hypersensitive to H^+ rhizotoxicity was isolated from ethyl methanesulfonate mutagenized seeds, and a single recessive mutation was found on chromosome 1. Positional cloning followed by genomic sequence analysis revealed that a missense mutation in the zinc finger domain in a predicted Cys_2His_2-type zinc finger protein, namely sensitive to proton rhizotoxicity (STOP)1, is the cause of hypersensitivity to H^+ rhizotoxicity. The STOP1 protein belongs to a functionally unidentified subfamily of zinc finger proteins, which consists of two members in *Arabidopsis* based on a Blast search. The *stop1* mutation resulted in no effects on cadmium, copper, lanthanum, manganese and sodium chloride sensitivitities, whereas it caused hypersensitivity to Al^{3+} rhizotoxicity. This *stop1* mutant lacked the induction of the AtALMT1 gene encoding a malate transporter, which is concomitant with Al-induced malate exudation. There was no induction of *AtALMT1* by Al^{3+} treatment in the *stop1* mutant. These results indicate that *STOP1* plays a critical role in *Arabidopsis* tolerance to major stress factors in acid soils.

aluminum toxicity | *Arabidopsis thaliana* | Cys_2His_2-type zinc finger protein | proton-rhizotoxicity | sensitive to proton rhizotoxicity

Acid soil syndrome causes severe yield losses in various crop plants (see reviews in refs. 1 and 2). The syndrome consists of phytotoxicity to excess ions, such as aluminum (Al^{3+}), manganese (Mn^{2+}), and protons (H^+), and a deficiency of essential nutrients including phosphorus (P), calcium (Ca), and magnesium (Mg) (3). Heavy application of limestone and P fertilizer is commonly used to prevent such growth losses in commercial plantations, but these amendments are not applicable for low-input farming systems in developing countries because of their high cost (3). Also, this approach could cause environmental problems because of an overenrichment of P in fresh waters (4) and greater energy costs. Molecular breeding of plants for enhanced tolerance to acid stress factors is one approach to solve these problems.

In this context, much research has been conducted to isolate genes that are involved in Al tolerance, because Al^{3+} rhizotoxicity is believed to be the most significant constraint that leads to serious yield loss in acid soils under drought. Several Al-tolerant genes such as ROS scavenging enzymes, namely GST and catalase (5), have been identified from a model plant, *Arabidopsis*, by screening Al inducible genes. Biochemical approaches on wheat (6) and tobacco (7) also clarified the involvement of ROS scavenging enzymes in Al tolerance. In addition, several genes other than ROS have been identified as Al-tolerance genes. For example, a malate transporter gene *TaALMT1*, which was isolated from wheat by comparing gene expression between Al-tolerant and Al-sensitive near-isogenic lines, is considered a key factor that controls Al tolerance (8). A study on *Arabidopsis* succeeded in the isolation of a gene encoding a transporter that exudes cytosolic Al into xylem (9). A study on rice mutants that are sensitive to Al stress has also been reported (10). These approaches could provide gene constracts for the molecular breeding of Al tolerance in crop plants.

In contrast, little is known of the genes that control H^+ tolerance, although H^+ rhizotoxicity causes severe inhibition of root growth of wheat, *Arabidopsis*, and spinach in hydroponic culture (11–14). Proton rhizotoxicity is also observed under certain soil conditions such as organic acid soil (15) and acid sulfate soil (16). Therefore, identification of genes that regulate H^+ tolerance is also important for the molecular breeding of crops tolerant to acid soils.

To identify key genes that regulate tolerance to rhizotoxicities, isolation of a hypersensitive mutant from *Arabidopsis* could be a promising approach. For example, studies on *sos* mutants revealed a critical mechanism of salt tolerance (17), and studies on *cad* mutants clarified that metallothionein is important for Cd tolerance (18). An experimental system has been developed that can separate H^+ toxicity from Al^{3+} toxicity (13). Recent advances of public resource centers and databases could accelerate functional biological studies of Al^{3+} and H^+ tolerance in this model plant (19). Thus, we performed the isolation and characterization of a mutant that showed sensitivity to proton rhizotoxicity.

Using a root bending assay, we succeeded in the isolation of a proton-sensitive mutant, which is designated as sensitive to proton rhizotoxicity (*stop*)*1* from an ethyl methanesulfonate-

Author contributions: S.I. and H.K. contributed equally to this work; S.I., H.K., T.H., K.S., and M.K. designed research; S.I., H.K., A.I., Yasufumi Kobayashi, S.K., Yuriko Kobayashi, and T.I. performed research; Yasufumi Kobayashi and Yuriko Kobayashi analyzed data; and S.I. and H.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: L*er*, Landsberg *erecta*; STOP, sensitive to proton rhizotoxicity; ZF, zinc finger domain.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AB300236 (L*er*-0, NW20), AB300237 (Est-1, JA297), AB300238 (Kl-5, JA124), AB300239 (Col-4, N933), AB300240 (Tu-0, JA350), AB300241 (Lö-1, JA143), AB300242 (Kb-0, JA120), AB300244 (Fr-3, JA299), AB300245 (Gö-0, JA93), AB300246 (Van-3, JA353), and AB300247 (Li-1, JA321)].

†To whom correspondence should be addressed. E-mail: iuchi@brc.riken.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0700117104/DC1.

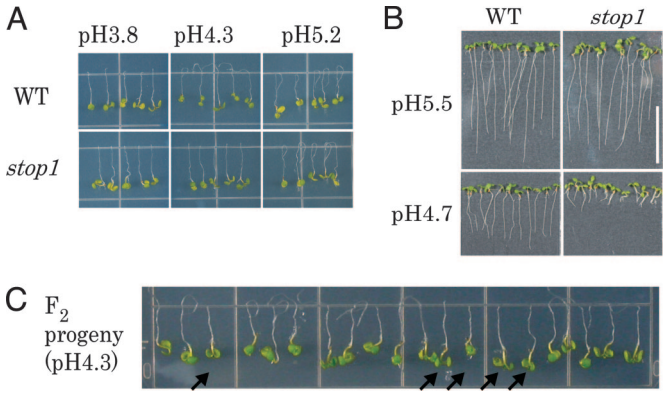

Fig. 1. Selection of an *Arabidopsis thaliana* mutant STOP. (*A*) Growth of Col-0 and *stop1* mutant seedlings on various pHs in a root bending assay. Pregrown seedlings were transferred to various pH gelled media and grown upside down. (*B*) Growth of mutant and WT (Col-0) in hydroponic culture at pH 4.7 and 5.5. Hydroponic culture can enhance rhizotoxicity to a greater degree than a gelled medium. (Scale bar: 1 cm.) (*C*) Segregation of mutant phenotype among F_2 population derived from a cross between the mutant (Col-0 background) and L*er*-0. Arrows indicate the mutant phenotype.

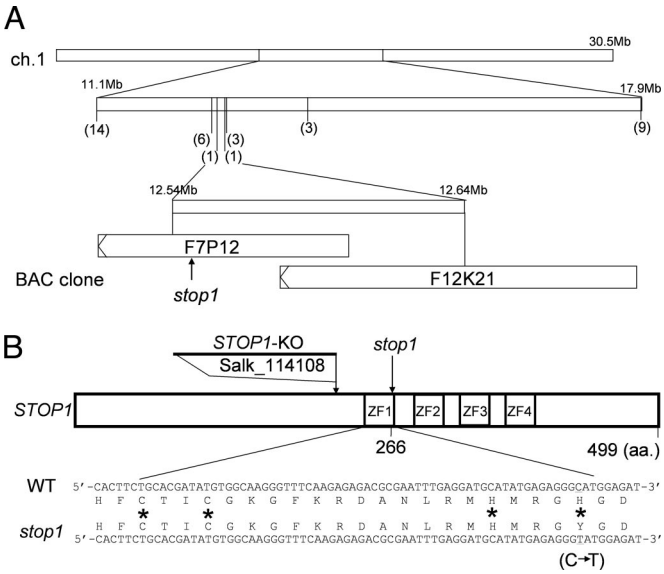

Fig. 2. Positional cloning of the STOP1 gene and overall domain structure of the STOP1 gene. (*A*) Schematic representation of the *STOP1* region on chromosome I. Numbers on the upper of the chromosome diagrams indicated the distance (in megabases) from the top of the chromosome. The number of recombination events detected in the F_2 progeny crossed with L*er* are shown in the lower part of the chromosome diagrams. *STOP1* was located on bacterial artificial chromosome clone F7P12. (*B*) Schematic representation of the overall domain structure and *stop1* mutation. The four ZFs (ZF1–ZF4) are indicated. The position of the *stop1* mutation and T-DNA insertion of SALK_114108 are also indicated. Asterisks indicate conserved motif of ZF.

mutagenized population. The mutant involved a single recessive mutation in the gene for a Cys_2His_2-type transcriptional factor that replaced a conserved His residue by Tyr. Because the *stop1* mutant also showed altered *AtALMT1* expression and greater sensitivity to Al stress, we conclude the STOP1 gene operates in the signal transduction pathway controlling acid-soil tolerance in *Arabidopsis*.

Results

Isolation of a Hypersensitive Mutant to Proton Rhizotoxicity. When pregrown *Arabidopsis* seedlings were placed on the agar plates that were kept in an upside-down orientation, root elongation could be easily recognized by the bending of root tips. We call this assay system the "root bending assay" and applied it to screen mutants related with proton rhizotoxicity. As shown in Fig. 1, roots of WT seedlings [Columbia (Col)-0] can grow at pH 4.3, but their growth is totally inhibited at pH 3.8. We screened 25,000 M2 seedlings of ethyl methanesulfonate mutants with this assay and isolated one seedling that showed no root growth at pH 4.3. Homozygous seedlings of the mutant showed no root growth under the screening conditions (Fig. 1*A*) and also showed severe inhibition of root growth even at pH 4.7 in a hydroponic culture system, which can enhance proton rhizotoxicity more than a gelled medium (13) (Fig. 1*B*). Thus, we designated this mutant line as *stop1* and further characterized the mutation.

Positional Cloning of the *stop1* Mutation. We used Landsberg *erecta* (L*er*)-0 as a mating partner of mapping analysis because its roots can grow better than those of Col-0 under low pH conditions [see supporting information (SI) Fig. 9]. The *stop* phenotype segregated into approximately a 1:3 ratio, indicating a single recessive mutation (Fig. 1*C*). A total of 610 F_2 plants (*stop1* × L*er*-0) were used for mapping analysis, and the mutation was mapped between SNPs located 12.54 Mb and 12.64 Mb from the top of chromosome 1 that correspond to the BAC clones F7P12 and F12K21 and include 26 genes (Fig. 2*A*). When the genomic DNA sequence of *stop1* was compared with that of Col-0, one missense mutation was identified within the ORF of At1g34370 which encodes a predicted Cys_2His_2-type zinc finger transcriptional regulator. The mutation (Cyt to Thy) caused a replacement of a conserved His residue of the zinc finger protein by Tyr (Fig. 2*B*). To confirm the involvement of At1g34370 in the *stop* phenotype, we tested the growth response of a T-DNA insertion mutant of At1g34370 (designated as *STOP1*-KO) as well as a transgenic *stop1* mutant carrying a CaMV35s driven At1g34370 gene (designated as the complemented line, *stop1*-comp) at pH values 5.5 and 4.7 in hydroponic culture. Both the *stop1* mutant and the *STOP1*-KO lines showed reduced root growth at pH 4.7. By contrast, root growth of the *stop1*-comp lines recovered to a comparable level with Col-0 (Fig. 3). From these results, we concluded that the acid-sensitive phenotype of *stop1* is caused by a missense mutation at At1g34370.

Sequence Analysis and Homologue of *STOP1*. Deduced amino acid sequence of *STOP1* consists of 499 aa containing four potential

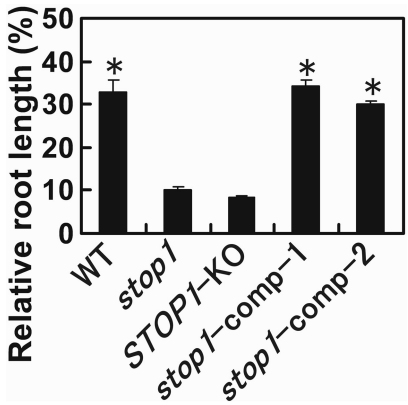

Fig. 3. Complementation test for pH hypersensitivity of the *stop1* mutation. WT (Col-0), *stop1* (*stop1* mutant), *STOP1*-KO (SALK_114108), and *stop1*-comp-1 and -2 (transgenic *stop1* mutant carrying a CaMV35s-driven WT STOP1 gene) were grown hydroponically at pH 5.5 and 4.7 for 7 days. Mean ± SE of relative root length (%) (pH 4.7/pH 5.5) are shown ($n = 5$). Asterisks indicate significant difference from stop1 and *STOP1*-KO (Student's *t* test, $P < 0.05$).

Fig. 4. Multiple alignment of potential ZFs of the STOP1 gene and homologues. Homologues were identified from *A. thaliana* (At5g22890), *Oryza sativa* (CT832156) and *Z. mays* (AY106636) by TBlastN search. Horizontal bars indicate ZFs and asterisks indicate conserved motif of Cys_2His_2 or Cys_2His_2-Cys. The arrow head shows the mutation point of *stop1*. Conserved amino acids are shaded dark, and the residues that have a positive Blosum62 score (43) are shaded light.

zinc finger domains (ZFs) (ZF1–ZF4) (Fig. 4). Three ZFs (ZF1, ZF2, and ZF4) are predicted as the Cys_2His_2 type, whereas ZF3 is predicted as the Cys_2His-Cys or the Cys_2His_2 type. Nuclear localization of the STOP1 protein was predicted by the WOLF–PSORT program, indicating possible localization in nuclei as a Cys_2His_2-type zinc finger transcriptional regulator. The *stop1* mutation (replacement of His by Tyr) was identified in the essential motif of Cys_2His_2 of the ZF1 domain, which had the lowest E-value ($4.9\ e^{-06}$) among four potential ZFs by the Pfam prediction. Based on the similarity of whole protein and ZFs, we identified one closely related homologue (At5g22890) from the *Arabidopsis* genome (Fig. 4). A TBlastN search revealed several homologues in rice and *Zea mays*, in which all four ZFs are conserved (Fig. 4).

Response of *stop1* Mutation to Various Rhizotoxicities. To evaluate the effect of *stop1* mutation on other abiotic rhizotoxicities, we compared the growth response of *stop1* and Col-0 (WT) roots in hydroponic culture with various treatments. Because *stop1* was sensitive to low pH, we used pH 5.5 for the test solution to minimize the effect of proton toxicity other than testing Al toxicity. There was no significant difference between *stop1* and Col-0 in test solutions containing a moderate level of toxic ions, namely cadmium (Cd^{2+}), copper (Cu^{2+}), sodium (Na^{+}), lanthanum (La^{3+}), and manganese (Mn^{2+}), which caused ≈30–60% inhibition in the root growth of Col-0 (Fig. 5). When Al toxicity was tested, medium pH was adjusted to pH 5.0 to prevent precipitation of Al in the medium. In this condition, the root growth of *stop1* was almost inhibited, but the WT root showed moderate growth inhibition by Al. Because there was growth inhibition at pH 5.0 for *stop1*, Al sensitivity of stop1 was further compared with a known Al hypersensitive accession, namely, a T-DNA insertion mutant of *AtALMT1* which encodes a malate transporter [designated as *AtALMT1*-KO (20)]. The *AtALMT1*-KO showed hypersensitivity to Al, and its root growth was totally inhibited with 2 μM Al at pH 4.7–5.5. However, a dose-response pattern to pH was similar with that of the parental accession (Fig. 6*A*). Growth of *stop1* and *STOP1*-KO was totally inhibited by Al, as was *AtALMT1*-KO (Fig. 6). In contrast, constitutive expression of *STOP1* in the mutant (*stop1-comp*) resulted in the recovery of Al tolerance (Fig. 6*B*). From these results, we inferred that the *stop1* mutation caused hypersensitivities to both H^{+} and Al^{3+} rhizotoxicities.

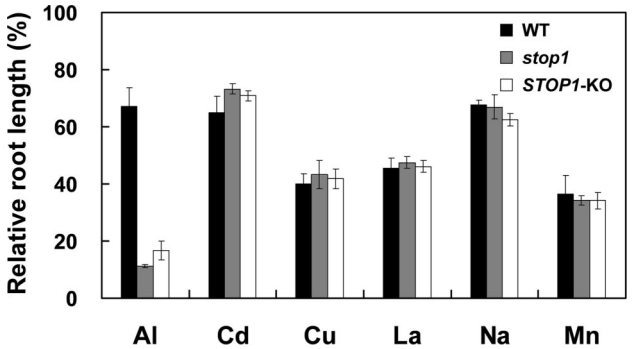

Fig. 5. Root growth of Col-0 (black bar, WT), *stop1* mutant (gray bar), and T-DNA insertion line of the STOP1 gene (white bar; SALK_114108, *STOP1-KO*) with various rhizotoxic ions in hydroponic culture. Seedlings were grown for 7 days in low-ionic strength nutrient solution that can enhance rhizotoxicity of ions. Seedlings were grown in test solutions containing 3.5 μM $CdCl_2$, 1.0 μM $CuCl_2$, 1.0 μM $LaCl_3$, 8.0 mM NaCl, 100 μM $MnSO_4$ at pH 5.5, or 4.0 μM $AlCl_3$ (pH 5.0). Means of relative root length (%) (toxic solution/nontoxic solution) ± SE are shown ($n = 5$). Asterisks indicate the significant difference from WT (Student's *t* test, $P < 0.05$).

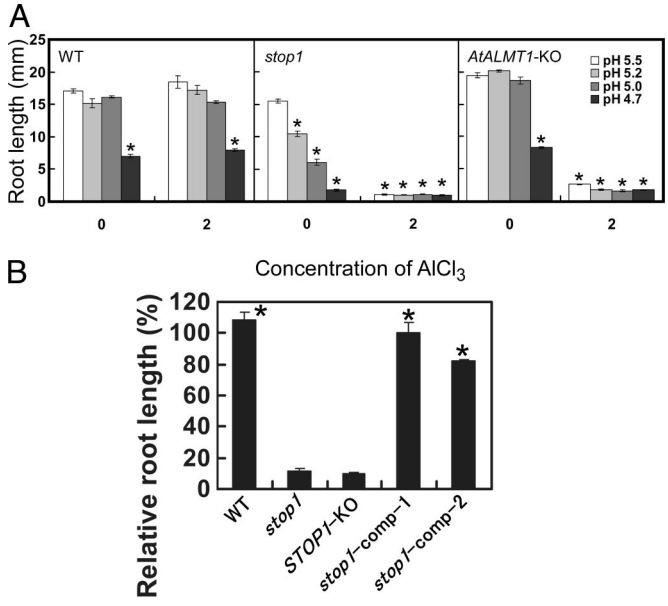

Fig. 6. Response of the *stop1* mutation to pH and Al rhizotoxicities. (*A*) Root growth of the *stop1* mutant, homozygous transgenic lines carrying a T-DNA insertion in *AtALMT1* (*AtALMT1*-KO; SALK_009629) and a parental accession Col-0 (WT) with various pH and Al treatments. Seedlings were grown hydroponically for 7 days in a test solution in the presence or absence of 2 μM $AlCl_3$ at various pH. Mean ± SE values are shown ($n = 5$). Asterisks indicate the significant difference from root growth at pH 5.5 (Student's *t* test, $P < 0.05$). (*B*) Complementation test for Al hypersensitivity of the *stop1* mutation. WT (Col-0), *stop1* (*stop1* mutant), *STOP1*-KO (SALK_114108), and *stop1*-comp-1 and -2 (transgenic *stop1* mutant carrying CaMV35s-driven WT STOP1 gene) were grown in hydroponic culture in the presence or absence of 2 μM $AlCl_3$ at pH 5.5 for 7 days. Mean ± SE values of relative root length (%) (+Al/−Al) are shown ($n = 5$). Asterisks indicate significant difference between *stop1* and *STOP1*-KO (Student's *t* test, $P < 0.05$).

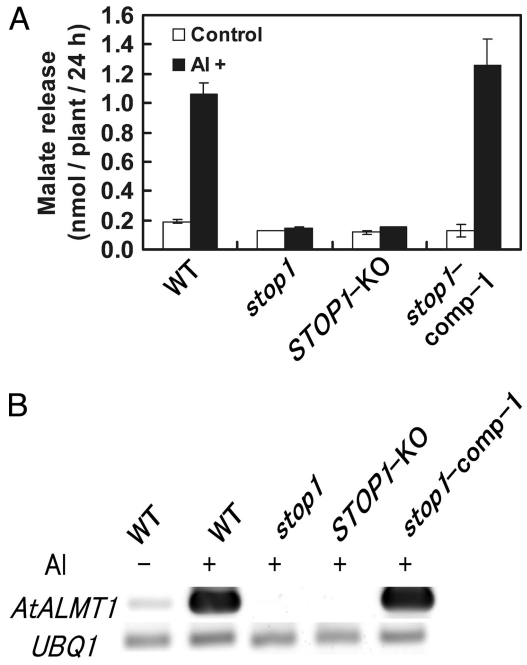

Fig. 7. Al responsive malate excretion and *AtALMT1* expression in the *stop1* mutation. (*A*) The 5-day-old roots of aseptically grown seedlings were incubated in malate collection medium with 10 μM $AlCl_3$ (solid bar) or without Al (open bar) at pH 5.0 for 24 h. Malate exudation was determined independently from three samples. Mean ± SE values are shown. (*B*) Analysis of *AtALMT1* expression by RT-PCR, using specific primers for *AtALMT1*. *UBQ1* expression is shown as a control.

Malate Excretion and *AtALMT1* Expression in *stop1* Mutation. Although molecular mechanisms of tolerance to H^+ rhizotoxicity have not been clarified yet, a critical Al-tolerance mechanism of *Arabidopsis* (i.e., malate exudation regulated by AtALMT1 expression) was reported (20). Thus, we characterized Al hypersensitivity of the *stop1* mutant in terms of the ability to excrete malate (Fig. 7*A*). Col-0 (WT) released a large amount of malate in Al medium, but the excretion was negligible in the control medium (no Al). This Al-dependent malate release was concomitant with the expression of the AtALMT1 gene (Fig. 7*B*). The *stop1* mutant and *STOP1*-KO showed no expression of the AtALMT1 gene and malate excretion after Al treatment, whereas *stop1-comp* recovered its ability to excrete malate and to express *AtALMT1* (Fig. 7 *A* and *B*).

Expression Level of *STOP1* by Low pH and Al Treatments. Expression level of *STOP1* at various pH and Al treatments was examined in Col-0 (WT) by quantitative RT-PCR. Under the physiologically relevant toxic conditions that were used for evaluating the *stop1* mutation (pH 4.7 and 4 μM Al; Figs. 3 and 5) or those that caused total inhibition (pH 4.4 and 10 μM Al) of root growth, no significant changes of *STOP1* expression were observed (Fig. 8). However, shock treatments (i.e., pH <3.0 and 200 μM Al at pH 4.6) induced *STOP1* expression (SI Fig. 9).

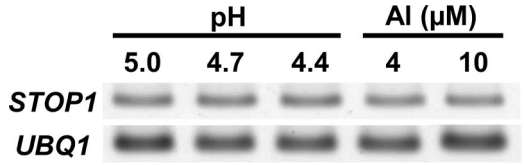

Fig. 8. Analysis of STOP1 gene expression by RT-PCR, using specific primers for the STOP1 gene. Col-0 seedlings were grown for 7 days in control medium (pH 5.0) then incubated for 24 h in various conditions (pH 5.0, 4.7, or 4.4 and 4 or 10 μM $AlCl_3$ at pH 5.0). *STOP1* and *UBQ1* expression level in the roots was determined by semiquantitative RT-PCR.

Discussion

Field studies of plant growth in acid soils have indicated that inhibition of root growth is caused by either Al^{3+} or H^+ rhizotoxicities (21, 22). The mechanisms of Al tolerance have been characterized at the molecular level (2, 23); however, there is no clear evidence for the molecular mechanism of H^+ rhizotoxicity. In the present study, we clearly demonstrate that a single, recessive mutation caused hypersensitivity to H^+ rhizotoxicity (Fig. 1).

The STOP1 protein contains typical Cys_2His_2 ZFs and possible localization in the nucleus (Fig. 4). This finding indicates that the STOP1 protein belongs to a super family of Cys_2His_2-type zinc finger transcriptional factor proteins. The missense mutation that occurred at the 266 His residue disrupted the Zn binding activity and resulted in a loss of ability as a transcriptional regulator. In fact, we found that the expression of a critical gene in Al tolerance, namely *AtALMT1* that encodes a malate transporter (20), was affected by the *stop1* mutation. The gene *AtALMT1* is not expressed in the *stop1* mutant, but it can be recovered by constitutive expression of this gene (Fig. 7). This result indicates transcriptional regulation activity of *STOP1*. Furthermore, STOP1 may regulate another set of genes because disruption of the AtALMT1 gene did not affect H^+ sensitivity (Fig. 6), whereas constitutive expression of *STOP1* complemented H^+ hypersensitivity of the *stop1* mutant. This hypothesis is supported by previous physiological studies that revealed a distinct pattern in roots of damage by Al^{3+} and H^+ rhizotoxicities (11–13, 24–26). Further research on *stop1* may clarify other critical genes controlling H^+ tolerance.

Many transcription factors related to environmental stress can respond to multiple stress stimuli (27, 28) and regulate a distinct set of defense genes effective for each stress factor [e.g., dehydration responsive element binding protein (DREB)1A to cold stress (29)]. In this case, stress factors that can activate each transcriptional factor often coexist in the natural environment. For example, salinity, drought, and osmotic stress often coexist in a dried environment and activate a gene expression pathway regulated by the DREB2 transcriptional factor [high salinity, drought, and osmotic stress (30)] and AtDi19, a kind of Cys_2His_2 transcriptional factor [dehydration and high salinity (31)], respectively. Such multiple responses of a transcriptional factor to various stimuli would be important for the plant's survival under a dry environment. STOP1 could play a similar role in plant survival under an acid soil environment, where Al^{3+} and H^+ rhizotoxicities disturb root growth.

STOP1 has several common features of plant transcription factors that regulate tolerance to abiotic stress factors. *STOP1* belongs to a small gene family that have the same characteristics of stress response such as C-repeat DRE binding factor and the subfamily of NAC domain binding transcriptional factors (32, 33). In addition, *STOP1* expression can be induced by H^+ and Al^{3+} shock treatments similar to some transcriptional factors that belong to the DREB family [Fig. 8 and SI Fig. 9 (34)]. Activation of STOP1 under physiological conditions could be regulated by other mechanisms such as protein phosphorylation that is involved in the activation process of other stress responsive transcriptional factors [e.g., abscisic acid-responsive element binding protein 1 (35)]. In the future, it will be interesting to clarify whether the other homologue of STOP1 is involved in gene expression pathways of H^+ and Al^{3+} tolerances. Research is needed to determine how STOP1 activates downstream expression of genes, such as *AtALMT1*.

A genetic study (20) of the Al tolerance of L*er*-0/Col-4 recombinant inbred population indicates that both *STOP1* and

AtALMT1 loci cannot account for the major cause of the quantitative trait loci of Al tolerance, which is regulated by Al-responsive malate release (20, 36). This finding suggests that regulatory proteins activating the AtALMT1 protein or other transcriptional factors regulating *AtALMT1* expression could be the cause of phenotypic Al-tolerance variation between Col-4 and L*er*-0. In addition, *STOP1* expression is quite stable among other tolerant and sensitive accessions. Almost all accessions share the same protein sequence (SI Fig. 10 and SI Table 1). At this stage, we cannot conclude that *STOP1* is involved in the mechanism of the phenotypic variations of the proton and Al tolerance of *Arabidopsis*. Further genetic analyses such as a larger survey of *STOP1* polymorphisms and/or quantitative trait loci analyses with various allelic combinations could answer this question.

Materials and Methods

Isolation of the *stop* Mutant. M_2 seed progeny derived from ethyl methanesulfonate mutagenized seeds of Col-0 were used for screening a *stop* mutant. Firstly, seeds were sterilized for ≈5 min with 5% (vol/vol) commercially bleach. The seeds were rinsed 5 times with distilled water and kept in a refrigerator for 4 days. The seeds were then put on a neutral plates [MS agar plates (37) containing 0.5% sucrose (wt/vol) at pH 5.2] and kept at a vertical angle at 22–24°C under 16-h light/8-h dark cycles. At day 4, seedlings were transferred to selection medium [MS agar plates containing 0.5% sucrose (wt/vol) at pH 4.3] and kept upside down. After 2–4 days culture from transplants, the plants that did not grow on selection plates were rescued on the neutral plates and forwarded to the mapping analysis described below.

Positional Cloning of the *stop* Mutation. The F_2 mapping population was derived from a cross between *stop1* and L*er*-0 followed by a controlled self-pollination. The F_2 mapping population showed a 1:3 (sensitive and tolerant) segregation ratio when judged by the root bending assay. At first, linkage mapping of the *stop* mutation on the whole chromosome was performed by using simple sequence length polymorphism markers. These markers were generated by using the INDEL database derived from the Monsanto *Arabidopsis* polymorphism and L*er* sequence collections (www.arabidopsis.org/browse/cereon/index.jsp). To narrow the mapping range of the *stop1* mutation, genotypings of individual *stop1* plants were performed by using simple sequence length polymorphism and SNPs markers. Analysis of the F_2 population of 610 plants revealed one recombination event by using SNP markers which were located on the BAK clones F7P12 and F12K21. To identify the mutation, DNA sequence of this genomic region was analyzed by using an Applied Biosystems (Foster City, CA) BigDye Terminater system, Version 3.1 and an Applied Biosystems PRISM3100 DNA sequencer following the manufacturer's instructions, and the obtained sequence was compared with that of Col-0 on the public database.

T-DNA-Tagged Mutants. The T-DNA insertion mutants for *STOP1* and *AtALMT1*, which had been registered as SALK_114108 and SALK_009629 on the Salk Institute (San Diego, CA) genomic analysis laboratory T-DNA express database (http://signal.salk.edu), were derived from the the *Arabidopsis* Biological Resource Center (Columbus, OH). The homozygous T-DNA-tagged lines were designated as *STOP1*-KO and *AtALMT1*-KO, respectively.

Complementation Test for *stop1* Mutation by 35sCaMV-Driven Authentic STOP1 Gene. STOP1 gene (At1g34370) cDNA (RAFL09-20-I22) is preserved and distributed from RIKEN BioResources Center through the National BioResource Project (www.brc.riken.go.jp/lab/epd). The *STOP1* cDNA was inserted into the binary vector pBE2113 at downstream of the cauliflower mosaic virus 35S promoter (34). The binary construct was then introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *stop1* plants by a floral dip method (38). The homozygous transformants at the T_3 generation were obtained by controlled self-pollination of T_2 plants.

Hydroponic Culture. Growth experiments in hydroponics were carried out with the hydroponic culture system used for an Al-tolerant quantitative trait loci study (39). Briefly, seedlings were grown in test solutions containing various toxicants for the root growth within the control solution, namely modified MGRL medium (40) (1/50 strength but with Pi eliminated and Ca concentration adjusted to 200 μM). The concentrations of toxicants were as follows: $AlCl_3$ 2.0, 4.0, and 10.0 μM; $LaCl_3$ 1.0 μM, $CdCl_2$ 3.5 μM, $CuCl_2$ 1.0 μM, $MnSO_4$ 100 μM, and NaCl 8 mM. The initial pH was adjusted to 5.5 for La, Cd, Cu, and Na, whereas the Al test solution was adjusted to various pHs (4.7–5.5). The effect of pH was examined by the control solution adjusted to various pHs (4.7–5.5). Solutions were renewed every 2 days, and root length was measured by using a video microscope as described in ref. 13. Plants were kept under a 12-h day (photosynthetic photon flex density, 250 μmol m^{-2} s^{-1})/12-h night cycle for 7 days at 25°C.

Malate Excretion and Measurement. Fifteen seedlings were grown aseptically on a plastic mesh (1 cm^2) floating on the control growth solution (see above) in the presence of 1% sucrose at pH 5.5. Seedlings grown on the plastic mesh were transferred at day 5 to separate wells of a 6-well plate containing 2 ml of the control malate collection medium for preincubation. After 1 h, media were switched to the control or Al-containing (10 μM Al) malate collection media. Both malate collection media were prepared by adding 1% (wt/vol) sucrose to the control growth solution, and the initial pH was adjusted to 5.0. Seedlings were gently shaken on a rotary shaker (40 rpm; Tokyo Rikakikai, Tokyo, Japan; Shaker MMS-5010) at 25°C in the dark. Media were collected at 24 h after transfer and malate concentration in each medium was quantified by a NAD/NADH cycling coupled enzymatic method as described in ref. 41. All experiments were carried out at least 3 times and means and SE values were obtained.

RNA Isolation, Semiquantitative RT-PCR, and Real-Time RT-PCR. RNA was extracted from the roots with rhizotoxic treatments and then reverse transcribed by the method of Suzuki *et al.* (42). Semiquantitative RT-PCR to determine the transcript level of *STOP1* and *AtALMT1* was carried out by using specific PCR primers STOP1F 5′-CATCAGCCAGTACATCTACTCAGA-3′, STOP1R 5′-ATGGCAATGCCTTAGAGACTAGTA-3′, AtALMT1F 5′-GGCCGACCGTGCTATACGAG-3′, and AtALMT1R 5′-GAGTTGAATTACTTACTGAAG-3′ with appropriate PCR conditions [denaturing at 94°C for 30 s, anealing at 51°C (AtALMT1 and UBQ1) or at 53°C (STOP1), extension at 72°C for 30 s; STOP1, 21 cycles, AtALMT1, 22 cycles, and UBQ1, 20 cycles] The amplified fragments were quantified with Typhoon9410 (Amersham Biosciences, Piscataway, NJ) and ImageQuant (Amersham Biosciences) after staining with SYBR Green I (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Real-time PCR was carried out by using SYBR Green PCR master mix and a GeneAmp 7500 sequence detection system (Applied Biosystems). The gene-specific primers for real-time PCR analyses were designated by using the Primer Express, Version 2.0 (Applied Biosystems). The following primer set was used in this analysis; STOP1Fq 5′-TTTCCGCGACTGATGTTTGAT-3′, STOP1q 5′-ACAGGCATTCGCAATAAGCAT-3′.

DNA Sequence Analysis. A homologue search of STOP1 was carried out by TBlastN database search (www.ncbi.nlm.nih.gov/

BLAST). The Pfam (www.sanger.ac.uk/Software/Pfam/search.shtml) program was used for potential protein domains, whereas the WOLF-PSORT program (http://wolfpsort.seq.cbrc.jp/) was used to predict the intracellular localization of the deduced polypeptides. Multiple amino acid alignment was performed by using a CLUSTALW and Jalview 2.2 softwares.

We thank Dr. Susan Miyasaka at the University of Hawaii and Dr. Thomas Kinraide at United States Department of Agricultural Research Service for critical reading of the manuscript, Ms. M. Nakashima and Mr. H. Itoh at Gifu University for their technical assistance, Ms. S. Kawamura and Ms. F. Mori of RIKEN BioResources Center for their assistance in the establishment and maintenance of *stop1*-comp and *STOP1*-KO lines, the Arabidopsis Biological Resource Center for providing T-DNA insertion mutants, and RIKEN BioResources Center for providing *STOP1* cDNA. This work is partially supported by the RIKEN Strategic Resource and Technologies Development Program (S.I. and M.K.) and Japan Society for the Promotion of Science grants (H.K.).

1. Delhaize E, Ryan PR (1995) *Plant Physiol* 107:315–321.
2. Matsumoto H (2000) *Int Rev Cytol* 200:1–46.
3. Ishitani M, Rao I, Wenzlb P, Beebea S, Tohme J (2004) *Field Crops Res* 90:35–45.
4. Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH (1998) *Ecol Appl* 8:559–568.
5. Ezaki B, Gardner RC, Ezaki Y, Matsumoto H (2000) *Plant Physiol* 122:657–665.
6. Basu U, Good AG, Taylor GJ (2001) *Plant Cell Environ* 24:1269–1278.
7. Yamamoto Y, Kobayashi Y, Devi S, Rikiishi S, Matsumoto H (2003) *Plant Soil* 255:239–243.
8. Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) *Plant J* 37:645–653.
9. Larsen PB, Geisler MJ, Jones CA, Williams KM, Cancel JD (2005) *Plant J* 41:353–363.
10. Ma JF, Nagao S, Huang CF, Nishimura M (2005) *Plant Cell Physiol* 46:1054–1061.
11. Kinraide TB (1998) *Plant Physiol* 118:513–520.
12. Kinraide TB (2003) *Eur J Soil Sci* 54:323–333.
13. Koyama H, Toda T, Hara T (2001) *J Exp Bot* 52:361–368.
14. Yang JL, Zheng SJ, He YF, Matsumoto H (2005) *J Exp Bot* 56:1197–1203.
15. Lumsdon DG, Stutter MI, Cooper RJ, Manson JR (2005) *Environ Sci Technol* 39:8057–8063.
16. Rosicky M, Sullivan L, Slavich P, Hughes M (2004) *Aust J Soil Res* 42:587–594.
17. Zhu J-K (2000) *Plant Physiol* 124:941–948.
18. Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995) *Plant Physiol* 107:1059–1066.
19. Rhee SY, Beavis W, Berardini TZ, Chen G, Dixon D, Doyle A, Garcia-Hernandez M, Huala E, Lander G, Montoya M, *et al.* (2003) *Nucleic Acids Res* 31:224–228.
20. Hoekenga OA, Maron LG, Piñeros MA, Cancado GM, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, *et al.* (2006) *Proc Natl Acad Sci USA* 103:9738–9743.
21. Rao I, Zeigler R, Vera R, Sarkarung S (1993) *BioScience* 43:454–465.
22. Kidd PS, Proctor J (2001) *J Exp Bot* 52:791–799.
23. Kochian LV, Hoekenga OA, Piñeros MA (2004) *Annu Rev Plant Biol* 55:459–493.
24. Koyama H, Toda T, Yokota S, Dawair Z, Hara T (1995) *Plant Cell Physiol* 36:201–205.
25. Watanabe T, Okada K (2005) *Anal Bot* 95:379–385.
26. Yokota S, Ojima K (1995) *Plant Soil* 171:163–165.
27. Blackwell HE, Zhao Y (2003) *Plant Physiol* 133:448–455.
28. Wang W, Vinocur B, Altman A (2003) *Planta* 218:1–14.
29. Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) *Plant J* 38:982–993.
30. Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Plant Mol Biol* 42:657–665.
31. Gosti F, Bertauche N, Vartanian N, Giraudat J (1995) *Mol Gen Genet* 246:10–18.
32. Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Proc Natl Acad Sci USA* 94:1035–1040.
33. Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) *Plant Cell* 16:2481–2498.
34. Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) *Plant Cell* 10:1391–1406.
35. Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Proc Natl Acad Sci USA* 97:11632–11637.
36. Lister C, Dean C (1993) *Plant J* 4:745–750.
37. Murashige T, Skoog F (1962) *Physiol Plant* 15:473–479.
38. Clough SJ, Bent AF (1998) *Plant J* 16:735–743.
39. Kobayashi Y, Furuta Y, Ohno T, Hara T, Koyama H (2005) *Plant Cell Environ* 28:1516–1524.
40. Fujiwara T, Hirai MY, Chino M, Komeda Y, Naito S (1992) *Plant Physiol* 99:263–268.
41. Takita E, Koyama H, Hara T (1999) *Plant Cell Physiol* 40:489–495.
42. Suzuki Y, Hibino T, Kawazu T, Wada T, Kihara T, Koyama H (2003) *Biotechniques* 34:988–990.
43. Henikoff S, Henikoff JG (1992) *Proc Natl Acad Sci USA* 89:10915–10919.