Identification and characterization of suppressor mutants of stop1

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Abstract

Background: Proton stress and aluminum (Al) toxicity are major constraints limiting crop growth and yields on acid soils (pH < 5). In Arabidopsis, STOP1 is a master transcription factor that controls the expression of a set of well-characterized Al tolerance genes and unknown processes involved in low pH resistance. As a result, loss-of-function stop1 mutants are extremely sensitive to low pH and Al stresses.

Results: Here, we report on screens of an ethyl-methane sulphonate (EMS)-mutagenized stop1 population and isolation of nine strong stop1 suppressor mutants, i.e., the tolerant to proton stress (tps) mutants, with significantly enhanced root growth at low pH (4.3). Genetic analyses indicated these dominant and partial gain-of-function mutants are caused by mutations in single nuclear genes outside the STOP1 locus. Physiological characterization of the responses of these tps mutants to excess levels of Al and other metal ions further classified them into five groups. Three tps mutants also displayed enhanced resistance to Al stress, indicating that these tps mutations partially rescue the hypersensitive phenotypes of stop1 to both low pH stress and Al stress. The other six tps mutants showed enhanced resistance only to low pH stress but not to Al stress. We carried out further physiologic and mapping-by-sequencing analyses for two tps mutants with enhanced resistance to both low pH and Al stresses and identified the genomic regions and candidate loci in chromosomes 1 and 2 that harbor these two TPS genes.

Conclusion: We have identified and characterized nine strong stop1 suppressor mutants. Candidate loci for two tps mutations that partially rescue the hypersensitive phenotypes of stop1 to low pH and Al stresses were identified by mapping-by-sequencing approaches. Further studies could provide insights into the structure and function of TPSs and the regulatory networks underlying the STOP1-mediated processes that lead to resistance to low pH and Al stresses in Arabidopsis.

Keywords: ALMT1, Aluminum toxicity, MATE, Proton toxicity, STOP1, Suppressor mutants

Background

Acid soils are associated with excess levels of toxic ions such as aluminum (Al3+), manganese (Mn2+), and proton (H+), which cause stunted growth and significant yield reductions of crops grown on acid soils [1–3]. Although applications of calcium carbonate could mitigate the acid soil associated stresses [4], these practices are expensive in financial and energy costs and, thus, are unsuitable for large scale applications, especially in developing and under-developing countries [5]. Therefore, improving crop plants’ resistance to proton and Al stresses would provide an effective solution to enhance crop yields on acid soils.

Plants have adopted two major mechanisms to cope with Al stresses, namely the Al exclusion/avoidance and the internal Al tolerance mechanisms [1, 2]. The exclusion mechanism relies on Al-activated root exudation of organic acid (OA) anions, mainly malate, citrate and oxalate, into the rhizosphere, where the OAs chelate Al3+ ions, forming nontoxic compounds that are unable to enter the root apex, the primary site of Al toxicity [1, 2, 6–10]. Through the internal Al tolerance mechanisms, Al retained in the root cell wall is actively removed by Al transporters, such as NRAT1 in rice [11, 12] and NIP1;2 in Arabidopsis [13], into the root cytosol. Then, Al in the root cell cytosol is further sequestered into root cell vacuoles and/or translocated and stored in the
vacuoles of shoot cells [14–16]. In *Arabidopsis*, we have demonstrated that the NIP1.2-mediated removal of Al from the root cell wall into the root cytosol and the subsequent root-to-shoot Al translocation require a functional Al-activated and ALMT1-facilitated malate release into the root cell wall [13]. Thus, a coordinated functioning of the Al exclusion mechanism and the internal Al tolerance mechanism is required to attain overall Al tolerance in *Arabidopsis* [13].

Recently, increasing lines of evidence indicate that the root cell wall is a major target for Al toxicity [17–20], and modifications in root cell wall carbohydrate polymers (pectins and hemicelluloses), which limits binding of toxic Al$^{3+}$ ions to the cell wall, could play an important role in Al tolerance in plants [17, 18, 21–24].

In *Arabidopsis*, STOP1 encodes a zinc finger transcription factor that plays a critical role in plants’ resistance to proton (H$^+$) and Al stresses [25]. As a result, root growth of the loss-of-function stop1 mutants is extremely sensitive to low pH and the expression of a set of key Al tolerance genes, including *ALMT1*, *MATE*, *ALS3*, which encode an Al-activated malate transporter; an Al-activated citrate transporter and a putative transporter involved in Al redistribution, respectively, is strongly suppressed in the loss-of-function stop1 mutant [7, 25, 26]. The fact that mutants of the key Al resistance genes, *ALMT1*, *MATE* and *ALS3*, are not hypersensitive to low pH stress indicates that the STOP1-mediated Al tolerance and low pH tolerance are independent events and tolerance to Al stress is not a prerequisite for resistance to low pH stress in *Arabidopsis* [7, 27–29]. Currently, the molecular mechanisms underlying the STOP1-mediated low pH resistance remain unknown in plants, however.

The hypersensitive phenotypes of stop1 to low pH provide us a unique opportunity to identify stop1 suppressor mutants with enhanced root growth under low pH conditions. Here, we report on the screens of an ethylmethane sulphonate (EMS)-mutagenized stop1 population and the identification of nine tolerant to proton stress (tps) mutants with significantly enhanced root growth at low pH. Three of the tps mutants also displayed increased tolerance to Al stress, two of which, i.e., tps1 and tps2, were selected for further physiological characterization and mapping-by-sequencing analyses. Candidate genes and map locations were identified for these two mutants. Thus, our work could potentially open new avenues aimed at identifying previously uncharacterized genetic, cellular and regulatory components functioning in regulation of the STOP1-mediated functional networks.

**Methods**

**Plant materials and growth conditions**

The loss-of-function T-DNA insertion line, SALK_114180 (*stop1*), was acquired from the Arabidopsis Biological Resource Center (ABRC). Homozygous stop1 seeds were mutagenized with EMS followed the procedures of previously reported [30]. About 500,000 M2 seeds were surface sterilized, cold-treated for 2 d, and sown onto plastic mesh floating on the Murashige and Skoog (MS) [31] solution (pH 4.3) in Magenta boxes as previously described [7, 29]. Plants were grown in a growth chamber with continuous light (130 μmol/m$^2$ sec) at 23 °C. As at pH 4.3, root growth of stop1 is severely inhibited [25], the tps mutants could be easily identified from the M2 population by their long-rooted phenotypes. Putative tps mutants were rescued from the Magenta boxes and transferred to soils. After 2 wk, young leaf tissues of individual plants were collected for genomic DNA extraction with DNeasy Plant Mini Kit (Qiagen). PCR analyses were conducted to examine the state of the original T-DNA insertions at the STOP1 locus. The STOP1/T-DNA-specific primers (5′-GCTGTTGCCTCCTA CACTGGTG-3′ and 5′-GT GGTGCCTCAGAGTTCGAT-3′) were used for testing T-DNA insertions at the STOP1 locus; the STOP1-specific primers (5′-GTGGTGACTCAGAGTTCGAT-3′ and 5′-CCACATTTGGCGAGAAA-3′) were used for PCR amplification of the flanking sequence encompassing the T-DNA insertion. Only those tps mutants that remained homozygosity of the T-DNA insertion at the STOP1 locus were kept for further studies.

The M3 tps mutants were further tested for their stable long-rooted phenotypes at pH 4.3. In brief, surface-sterilized M3 seeds of individual lines were germinated on 1.2% agar plates (pH 5.6) containing 1/2 (w/v) MS salts and 1% (w/v) sucrose. Then, 4-d-old seedlings were transferred to 0.8% (pH 4.3) containing 600 μM AlCl$_3$ nutrients as solution (pH 4.3) containing 600 μM AlCl$_3$ nutrients as described previously [13, 27] with a modified concentration of KH$_2$PO$_4$ of 0.1 mM and an addition of 1.1 mM K$_2$SO$_4$ was added onto the surface of gellan gum plates (pH 4.3) and dried in hood for 6 h, which resulted in

**Genetic analysis of the tps mutants**

For testing dominant/recessive nature of the tps mutants, individual homozygous tps mutants were crossed with stop1. Surface-sterilized seeds of stop1, tps’s and their corresponding F1 progenies were germinated on gellan gum plates (pH 4.3) as described above. Root growth was measured for 5-d-old seedlings.

**Responses of tps mutants to excess levels of aluminum and other metal ions**

To test the effects of Al toxicity, 10 ml of hydroponic solution (pH 4.3) containing 600 μM AlCl$_3$ nutrients as described previously [13, 27] with a modified concentration of KH$_2$PO$_4$ of 0.1 mM and an addition of 1.1 mM K$_2$SO$_4$ was added onto the surface of gellan gum plates (pH 4.3) and dried in hood for 6 h, which resulted in

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final concentration of 200 μM AlCl₃. For testing the effects of other metal ions, 1/2 (w/v) MS plates (pH 5.6) were made containing 1.2% (w/v) agar, 3% (w/v) sucrose and one of the following chemicals: 500 μM ZnSO₄, 10 mM LiCl, 150 mM NaCl or 50 μM CdCl₂. Then, 4-d-old seedlings were transferred from 1/2 (w/v) MS agar plates (pH 5.6) to the above mentioned treatment plates. And, 5 d root growth was measured for individual plants.

RNA isolation and quantitative real-time qRT-PCR
About 10 mg of surface-sterilized seeds were germinated individually in Magenta boxes containing sterile hydroponic growth solution [13, 27] (pH 5.6) inside a growth chamber with a continuous light and a temperature of 23 °C. After 6 d, seedlings were transferred to fresh hydroponic growth solutions (pH 4.3) supplemented with or without 1.5 μM Al³⁺ activity for 2 d.

Total RNAs were extracted from root tissues with the RNeasy Mini Kit (Qiagen) following the manufacturer’s instruction. First-strand cDNAs were synthesized from 5 μg DNasel-digested total RNAs using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time qRT-PCR was performed with a 7500 Fast Real-Time PCR System according to manufacturers’ protocols (Applied Biosystems, Inc.). The relative expression levels of the target genes were referred to an endogenous calibrator gene, 18S rRNA. The sequences of the qRT-PCR primers for ALMT1 are: CTCAGATTTTCAGATCCCTAG AGTGGAC and TTCCCGATTCGGAGCTCATATTG; MATE: GCATAGCCATCTCGGTGTTGGCA and CGAACACAAACGCTAAGGCA; 18S: CGCTATTGGAGCTGGAA TTACC and AATCCCCATACAGGATCCATTG.

Detection of organic acid exudation from roots
Surface sterilized seeds (~2–3 mg) were germinated in Magenta boxes containing sterile hydroponic growth solution [13, 27] (pH 5.6) in a growth chamber with a continuous light and a temperature of 23 °C. After 6 d, seedlings were transferred to fresh hydroponic growth solutions (pH 4.3) supplemented with or without 1.5 μM Al³⁺ activity for another 2 d [27]. The exudation solutions were collected and the numbers of plants were counted at the end. Malate and citrate contents were determined by an enzymatic method described by Ryan et al., 2009 [32].

Mapping-by-sequencing approach for identification of candidate gene regions of TPS1 and TPS2
Surface-sterilized F2 seeds derived from a cross between tps1 and stop1 or between tps2 and stop1 were germinated and grown on vertical growth plates (pH 4.3) for 10 d. The tps mutant (long root) and non-mutant (short root) phenotypes were segregated in these F₂ populations. Roughly equal amounts of leaf tissues were collected from each of ~80 long-rooted or shoot-rooted plants from corresponding F₂ populations and pooled together correspondingly. Genomic DNAs were extracted from the pooled leaf samples via the E.A.N.A. Plant DNA Midi Kit (Omega Bio-tek, Inc.). Hi-Seq DNA libraries were constructed with ~2 μg DNAs via a PRC-free TruSeq prep method according to the manufacturer’s instructions (Illumina, http://illumina.com). The long-rooted and short-rooted DNA libraries were individually subjected to next generation sequencing with a High Output mode (single-end 100 bp) via a HiSeq2500 instrument (Illumina, https://illumina.com). At least 7 Gbp of sequences were generated with 50 x genome coverage for each of the libraries.

Sequencing assembly, alignments and data analyses were performed via the DNASTAR SeqMan NGen 14 software (https://www.dnastar.com). The reference genomic template, i.e., the Arabidopsis-TAIR10-dbSNP138.genome template, was downloaded from the DNASTAR SeqMan NGen 14 software for identification of non-reference SNPs/INDELS in individual DNA libraries.

Results
Isolation of stop1 suppressor mutants
At pH 5.6, root growth of the loss-of-function Arabidopsis T-DNA knock-out stop1 line (SALK_114108) was comparable to that of the wild type (WT, Col-0) (Fig. 1a). However, at low pH (4.3), root growth of stop1 was inhibited by >90%, whereas root growth of the WT was inhibited by ~35% (Fig.1a, b). These results confirmed that the stop1 mutant is extremely hypersensitive to low pH stress [25].

We screened ~500,000 ethyl-methane sulphonate (EMS)-generated M2 seedlings with a homozygous stop1 background and identified a total of 284 putative tolerant to proton stress (tps) mutants with enhanced root growth at pH 4.3. Subsequently, progenies of these putative tps mutants were rescreened and thirty stable tps mutants were confirmed. PCR analysis indicated that all of the thirty tps mutants retained a homozygous T-DNA insertion at the STOP1 locus, indicating that the partially enhanced root growth phenotypes of these tps lines were caused by second-site gain-of-function mutations. A large portion of false putative tps mutants from the initial screens could be due to multiple factors, including environmental effects and high density of seedlings at the initial screen, which could jeopardize the accuracy of the initial identification of the stop1 suppressor mutants.

Among the thirty stable tps mutants, nine displayed significantly enhanced root growth compared with stop1 (Fig. 1a), whereas the rest tps mutants showed moderately enhanced root growth at low pH. These nine strong tps mutants were selected for further characterization here.
Relative root growth (RRG%) (i.e., root growth at pH 4.3 vs. at pH 5.6) of these nine tps mutants ranged from ~35–55%, compared with the RRG% of 65% and <10% for the WT and stop1, respectively (Fig. 1b). These results indicated that although the tps mutations led to significantly enhanced root growth, they could not completely recover the WT phenotype at low pH. Thus, they are partial stop1 suppressor mutants in terms of resistance to low pH stress.

Among the nine tps mutants, tps’s 1, 2 and 5 displayed significantly higher RRG% than the rest of tps mutants: the RRG% of tps’s 1, 2 and 5 were closed to or higher than 50%, whereas the RRG% of the rest tps mutants ranged from 34 to 46% (Fig. 1). This result suggests that tps’s 1, 2 and 5 could be distinguished from the rest tps mutants.

Genetic analysis of suppressor mutants of stop1
To test the dominant/recessive nature of the tps mutations, each of the nine tps mutants was crossed with stop1. At low pH (4.3), all of the F1 progenies resembled their corresponding tps parents when judged by their patterns of root growth, indicating that all of these tps mutations are dominant (Fig. 2). The F2 progenies of tps1 x stop1 were selected for further segregation analysis. Among the F2 progenies, the long-root and the short-root phenotypes were segregated at a ~ 3:1 ratio (Table 1). A Chi-square analysis indicated that no statistically significant difference in the expected and the observed ratio of 3:1 for long-root versus short-root phenotypes (Table 1), confirming that tps1 is caused by a dominant mutation of a single nuclear gene.

Responses of tps mutants to toxic levels of different metal ions
Although the dominant nature makes it difficult to determine the allelic relationships between the tps mutants by complementation tests, their responses to treatment of different metal ions might provide clues for classification of these mutants. Therefore, we began to test the sensitivity of the tps mutants to Al stress. At low pH (4.3), stop1 is extremely sensitive to Al stress: the RRG% (i.e., root growth + Al vs. root growth –Al) of stop1 was ~6%, whereas the RRG% of the WT ~78% (Fig. 3). This result was consistent with the previously reported [25]. Although, compared with stop1, all of the nine strong tps mutants displayed significantly enhanced root growth at pH 4.3 (Fig. 1), only tps’s 1, 2 and 5 showed partially, but significantly, enhanced root growth under Al stress (Fig. 3). The RRG% of tps’s 1, 2 and 5 were 4.0, 4.9 and 4.8 fold higher than the RRG% of stop1, but 60, 52 and 53% lower than that of WT, respectively (Fig. 3), indicating that they are partial revertant mutants of stop1 in terms of Al resistance. As tps’s 1, 2, and 5 also displayed the highest root growth under low pH stress compared with the rest tps mutants (Fig. 1), they could be distinguished from the rest of the tps mutants.

All tps mutants were further subjected to treatment with other metal ions, including Zn\(^{2+}\), Li\(^+\), Na\(^+\) and Cd\(^{2+}\). Under Zn treatment, WT and stop1 displayed comparable

![Fig. 1](image-url)  
**Fig. 1** Isolation of the stop1 suppressor mutants. Here, 4-d-old seedlings of WT (Col-0), stop1 and the stop1 suppressor mutants, tps1–9, were transferred from pH 5.6 agar plates to pH 5.6 or pH 4.3 gellan gum plates and grown vertically for 5 d. **a** Five-day root growth of individual lines at pH 5.6 (upper panel) and pH 4.3 (lower panel). **b** Relative root growth (RRG%) of individual lines. RRG% = root growth at pH 4.3/root growth at pH 5.6. Data are means ± SD (n = 10). Scale bar = 1 cm. Red arrows point to the initial root growth positions. Letters represent groups with significant differences (P ≤ 0.05) as determined by Fisher’s LSD test.

![Fig. 2](image-url)  
**Fig. 2** Determination of the dominant/recessive nature of the tps mutants. Here, 4-d-old seedlings of st (stop1), tps’s and their corresponding F1 progenies were transferred onto pH 5.6 or 4.3 gellan gum plates. After 5 d, root growth was measured for each seedling and RRG% was calculated for each line. RRG% = root growth at pH 4.3/root growth at pH 5.6. Values are means ± SD, n = 15.
root growth patterns (Fig. 4a). In contrast, tps’s 2 and 5 were much sensitive to Zn toxicity than the other tps lines which showed similar or slightly increased sensitivity to Zn toxicity compared with WT and stop1 (Fig. 4a). As tps’s 1, 2 and 5 could be grouped together based on their similar responses to low pH and Al stresses (Figs. 1, 3), the differential responses to Zn could further separate tps1 from tps’s 2 and 5.

The stop1 mutant was more sensitive to Li stress than did WT as indicated by a 31% decrease in RRG% of stop1 compared with that of WT under Li treatment (Fig. 4b). All tps mutants displayed similar sensitivity to Li stress as stop1 did except that tps’s 3 and 6 were more sensitive to Li stress than stop1, whereas tps4 displayed a higher level of resistance compared with WT (Fig. 4b). The WT, stop1 and tps mutants manifested comparable levels of RRG% under Na stress except that tps4 displayed slightly increased tolerant to Na stress (Fig. 4c). Surprisingly, although WT and stop1 displayed similar sensitivity to Cd stress (Fig. 4d), all tps mutants, except for tps6, were more tolerant to Cd stress (Fig. 4d).

Expression of key aluminum resistance genes in tps1 and tps2
In Arabidopsis, the Al-activated and ALMT1-facilitated root malate exudation plays a major role, whereas the MATE-facilitated root citrate exudation plays a smaller but significant role, in Al resistance [7, 27]. In addition, expression of ALMT1, MATE as well as ALS3 is controlled by STOP1 [7, 26]. Therefore, it is interesting to understand the effects of tps mutations on the expression of these Al resistance genes.

To begin with, we investigated the expression of ALMT1, MATE and ALS3 in the root of tps1 and tps2, both of which showed enhanced resistance to proton and Al stresses (Figs. 1 and 3). Real-time qRT-PCR analyses indicated that Al stress induced a strong upregulation of ALMT1, MATE and ALS3 expression in the root of WT and the levels of the Al-activated ALMT1 expression were much higher than those of MATE and ALS3 in WT (Fig. 5a–d). These results confirmed the major role of ALMT1 in Al resistance in Arabidopsis [7, 28, 29]. In addition, we confirmed that the expression of ALMT1, MATE and ALS3 was greatly suppressed in the loss-of-function stop1 background (Fig. 5a–d).

We notice that in stop1, Al treatment caused small, but significant, increases in ALMT1 and MATE transcript levels, whereas ALS3 expression was not affected by Al treatment (Fig. 5b, c and d). These results suggest that although STOP1 plays a key role in controlling the Al-induced expression of ALMT1 and MATE, there exist non-STOP1 regulatory factors that control a smaller portion of Al-induced ALMT1 and MATE expression, whereas the expression of ALS3 is likely to be solely controlled by STOP1.

**Table 1** The tps1 mutant is caused by a dominant mutation of a single nuclear gene

| Cross       | Observed Number of Progenies | Expected Number of Progenies | χ² | P  |
|-------------|------------------------------|------------------------------|----|----|
|            | Suppressor Phenotypea        | stop1 Phenotypeb             |     |    |
| tps1 x stop1 | 166                          | 50                           | 183.75 | 61.25 | 3.01 | 0.24 |

*a*Long root

*b*Short root

**Fig. 3** Response of tps mutants to Al stress. **a** Here, 4-d-old seedlings of WT, stop1 and tps mutants were transferred from pH 5.6 agar plates to pH 4.3 gellan gum plates supplemented without or with 200 μM AlCl₃. Root growth was measured for each plant 5 d after transfer. **b** RRG% = root growth (+Al) /root growth (−Al). Vertical scale bar = 1 cm. Red arrows point to the initial root tip positions. Values are means ± SD (n = 10). Letters represent groups with significant differences (P ≤ 0.05) as determined by Fisher’s LSD test.
Compared with stop1, the levels of Al-induced ALMT1 transcripts increased 1.3 and 1.9 fold in tps1 and tps2, respectively (Fig. 5b), suggesting that the wild-type TPS1 and TPS2 might function as suppressors for the Al-induced and STOP1-independent ALMT1 expression. In contrast, no significant differences were found in the patterns of MATE expression between stop1, tps1 and tps2 (Fig. 5c), suggesting that TPS1 and TPS2 are not involved in regulation of the Al-induced and STOP1-independent MATE expression.

**Root organic acid exudation in tps1 and tps2**

Root OA exudation was measured for WT, stop1, tps1 and tps2. In WT, Al triggered a large increase in root malate exudation and a smaller increase in root citrate exudation (Fig. 6). Compared with the WT, the Al-activated malate and citrate exudation was strongly suppressed in stop1: the rates of Al-activated malate and citrate exudation in stop1 decreased by 96 and 73%, respectively (Fig. 6). These results were consistent with previously reported [7]. Interestingly, compared with stop1, Al treatment caused 3.6- and 3.1-fold increases in Al-activated root malate exudation in tps1 and tps2, respectively (Fig. 6a). In contrast, patterns of root citrate exudation remained comparable between stop1, tps1 and tps2 (Fig. 6b).

Regression analyses indicated that levels of Al-induced ATMT1 expression (Fig. 5) and Al-activated malate exudation were highly associated among WT, stop1, tps1 and tps2 ($R^2 = 0.98$). These results suggest that the increased Al resistance in tps1 and tps2 (Fig. 3) was due, at least partially, to enhanced Al-induced and STOP1-independent ALMT1 expression and the associated ALMT1-mediated root malate exudation. In contrast, no correlations could be found between Al resistance (Fig. 1), MATE expression and root citrate exudation, suggesting that Al-induced MATE expression (Fig. 5c) and Al-activated root citrate exudation (Fig. 6b) had few contributions to enhanced Al resistance in tps1 and tps2.

**Identification of candidate genomic regions that harbor tps1 and tps2 mutations by whole genome sequencing**

To understand the molecular bases underlying how TPSs function in the STOP-mediated signaling/regulatory networks, we started to map and clone the TPS1 and TPS2 loci via a mapping-by-sequencing technique. In contrast to traditional map-based cloning techniques, the mapping-by-sequencing approach is a combination of bulked segregant analysis [33, 34] and whole genome sequencing [35]. To identify candidate gene regions for TPS1 and TPS2, HiSeq DNA libraries from bulked long-rooted or short-rooted F2 progenies derived from a cross between stop1 (Col-0) and tps1 or tps2 were individually subjected to next generation whole genome sequencing. The sequencing data were then subjected to reference-guided assemblies and analyses with the SeqMan NGen 14 software (DNASTAR Lasergene). From each pool, non-reference SNPs/INDELs were identified and their allele frequencies calculated. As both tps1 and tps2 are dominant mutants, the causal non-reference SNPs/INDELs could be characterized by their allele frequencies >75% in the long-rooted mutant DNA pools, but <25% in the short-rooted non-mutant DNA pools. In addition, there would be a group of non-reference SNPs/INDELs tightly linked to the causal SNPs/INDELs with high allele frequencies in the mutant libraries due to linkage effects. On the basis of these criteria, TPS1 was mapped to the long arm of chromosome 2 between molecular markers CDS297A and SM80_193.1, whereas TPS2 to chromosome 1 between NGA692 and SM235_460.1 (Fig. 7). Eight and six strong candidate genes with
nonsynonymous mutations were identified in the TSP1 and TSP2 regions, respectively (Tables 2 and 3).

**Discussion**

STOP1 encodes a master transcription factor that controls both low pH and Al resistance in *Arabidopsis* [25, 26]. The fact that mutations in the STOP1-controlled Al tolerance genes, such as *ALMT1* and *MATE*, caused hypersensitivity to Al stress but not to low pH stress indicates that STOP1 mediates independent processes leading to resistance to low pH stress or Al stress [7]. However, the genetic and regulatory networks underlying STOP1-mediated resistance to low pH stress and Al stress remains unknown.

In this report, through classic forward genetic approaches, we have identified nine strong stop1 suppressor mutants, which displayed partially, but significantly, enhanced root growth at low pH (Fig. 1). Genetic analyses indicated that all of the nine tps mutants are caused by gain-of-function dominant mutations (Table 1).

Further physiological characterizations indicated that tps’s 1, 2 and 5 also showed partially enhanced Al resistance (Fig. 3). Thus, TPSs 1, 2 and 5 appear to act in a STOP1-mediated networks before the divergence of
Table 2 Candidate genes in the TPS1 region at chromosome 2

| Candidate Genes | Description | Predicted Subcellular Localization | GO Biological Process |
|-----------------|-------------|-----------------------------------|-----------------------|
| At2g17790       | Similar to yeast VPS35. | Intracellular membranes | Intracellular protein transport |
| At2g27880       | AGOS; required for antiviral RNA silencing | Cytosol | Defense response, Gene silencing |
| At2g29210       | Splicing factor PWW domain-containing protein | Nucleus | RNA splicing, mRNA processing |
| At2g31862       | B3 domain protein | Nucleus | Regulation of transcription |
| At2g31890       | RAP, containing putative RNA binding domain | Chloroplast, nucleus | Chloroplast RNA processing |
| At2g34810       | BBE16, FAD-binding Berberine family protein | Cytosol | Oxidation-reduction process, response to jasmonic acid, response to wounding |
| At2g43180       | Phosphoenolpyruvate carboxylase family protein | Chloroplast | Catalytic activity |
| At2g44440       | EML4, ENT domain-containing protein | Nucleus | Defense response to fungus |

Due to the dominant nature of all tps mutants identified here, it is hard to classify them through complementation tests. However, based on their responses to low pH and excess levels of different metal ions, these tps mutants could be classified into at least five different groups: Group 1 includes tps1 which displayed significantly enhanced resistance to both low pH (Fig. 1) and Al stress (Fig. 3); Group 2 contains tps's 2 and 5, which showed similar levels of enhanced resistance to low pH and Al stresses as tps1, but with enhanced sensitivity to excess Zn (Fig. 4a); Group 3 includes tps's 3 and 6, which are hypersensitive to Li⁺ (a more toxic analog for Na⁺) stress (Fig. 4b). Interestingly, tps's 3 and 6 did not show significant hypersensitivity to Na stress compared with stop1 (Fig. 4c). In addition, all tps mutants, except for tps 6, showed enhanced resistance to Cd stress, which might further distinguish tps 6 from tps 3; Group 4 contains tps4 which showed enhanced tolerance to Li stress (Fig. 4b); Group 5 includes tps's 7, 8 and 9, which lack the specific characteristics of the above groups besides their increased resistance to low pH stress (Fig. 1).

It has been well characterized that one of the deleterious effects of salt (Na) stress on plant growth is the disruption of cellular K⁺ homeostasis through inhibition of K⁺ uptake that is facilitated by K⁺ channels such as AKT1 [36, 37]. Biochemical studies indicate that CIPK23 (CBL-interacting protein kinase 23) is required for activation of the AKT1 channel through the phosphorylation of the ankyrin repeat domain of the AKT1 protein [38]. Interestingly, the expression of CIPK23 is induced by Al and low pH stresses in WT [26]. However, such an induction was strongly suppressed in the stop1 mutant, implicating a disruption of K⁺ homeostasis in stop1 under low pH and Al stresses [26]. This could explain the reason for the hypersensitivity of the stop1 mutant to Na and Li stresses (Fig. 4b and c). It will be interesting to investigate if the enhanced resistance of tps4 to Li and Na stresses is caused by up-regulation of CIPK23 expression, whereas the enhanced sensitivity of tps3 and tps 5 to Li and Na is due to further down-regulation of CIPK23 expression.

As the Al-induced ALMT1 and MATE expression and the Al-activated root malate and citrate exudation are strongly suppressed in the stop1 mutant background (Figs. 5 and 6), we decided to investigate how the tps1 and tps2 mutations affects ALMT and MATE expression and corresponding root OA exudation in the stop1 mutant background.
qRT-PCR analyses indicated that there exist AI-induced and STOP1-independent ALMT1 and MATE expression in Arabidopsis (Fig. 5b and c). Interestingly, this AI-induced and STOP1-independent ALMT1 expression was further enhanced by the tps1 and tps2 mutations (Fig. 5b), suggesting that TPS1 and TPS2 might function as negative regulators for the STOP1-independent ALMT1 expression. Therefore, the increased AI resistance in tps1 and tps2 mutants could be due, at least partially, to the increased AI-activated, STOP1-independent and ALMT1-mediated root malate exudation. Interestingly, the AI-induced and STOP1-independent MATE expression was not affected by these tps mutations (Fig. 5c).

To identify chromosome and genomic regions for TPS1 and TPS2, we carried out a mapping-by-sequencing approach to identify the candidate causal nonsynonymous SNPs in coding sequences. Compared with the traditional map-based cloning technique, which is time-consuming and labor intensive, the mapping-by-sequencing approach is a relatively simple and quick way to map and to identify the candidate causal genes. Through analyses of the distributions and the allele frequencies of the non-reference SNPs in the long-rooted and short-rooted DNA libraries, TPS1 was mapped to chromosome 2 between the molecular markers CDS297A and SM80_193.1, whereas TPS2 to chromosome 1 between NGA692 and SM235_460.1 (Fig. 7). Eight and six candidate genes with nonsynonymous mutations in the coding sequences were identified in the TPS1 and TPS2 regions, respectively (Tables 2 and 3). These candidate genes encode proteins involved in regulation of transcription, responses to hormone stimulus, gene silencing, defense responses, response to wounding and intracellular protein transport. Further functional characterization for these candidate genes will allow us to confirm the molecular identities and functions of TPS1 and TPS2.

Conclusions
We have identified nine strong stop1 suppressor mutants, which could be classified into five groups. Two of the tps mutants with enhanced resistance to both low pH and AI stresses were chosen for further physiological analyses and mapping-by-sequencing gene identification procedures [39–41]. Candidate causal genes have been identified for these two mutants. Our studies represent the first steps towards the identification of the molecular identities of all TPS genes, which will provide insights into the structure and function of the gene products and their roles in the STOP1-mediated genetic, cellular and regulatory networks that are involved in resistance to low pH and AI stresses in Arabidopsis.

Abbreviations
ALMT: Aluminum activated malate transporter; ALS: Aluminum sensitive; EMS: Ethyl-methane sulphonate; MATE: multidrug and toxic compound extrusion; MS: Murashige and Skoog; NGS: Next-generation sequencing; OA: Organic acid; RRG: Relative root growth; st: STOP1; STOP1: Sensitive to proton rhizotoxicity 1; TPS: Tolerant to proton stress

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Availability of data and materials
The datasets during or analyzed during the current study have been deposited to the NCBI Sequence Reading Archive (SRA) with the submission name of SUB2796538.

Authors’ contributions
JL designed the experiments. FJ and JL performed the experiments. FJ, TW, YW, LVK, FC and JL analyzed the data. FJ, TW, FC and JL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. Kochian L, Piñeros MA, Liu J, Magalhaes J. Plant adaptation to acid soils: the molecular basis for crop aluminum resistance. Annu Rev Plant Biol. 2015;66:571–98.
2. Liu J, Piñeros MA, Kochian LV. The role of aluminum sensing and signaling in plant aluminum resistance. J Integr Plant Biol. 2014;56:221–30.
3. Von Uexküll H, Muten E. Global extent, development and economic impact of acid soils. Plant Soil. 1995;171:1–15.
4. Alva A, Asher C, Edwards D. The role of calcium in alleviating aluminum toxicity. Crop Pasture Sci. 1986;37:95–82.
5. Ishihata M, Rao L, Wend P, Beebe S, Tohme J. Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: drought and aluminum toxicity as case studies. Field Crops Res. 2004;90:305–45.
6. Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF. An aluminum-activated citrate transporter in barley. Plant Cell Physiol. 2007;48:1081–91.
7. Liu J, Magalhaes JV, Shafl J, Kochian LV. Aluminum-activated citrate and maltate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. Plant J. 2009;57:389–99.
8. Magalhaes JV, Liu J, Guimaraes CT, Lara UG, Alves VM, Wang Y-H, Schaffert RE, Hoekenga OA, Piñeros MA, Shafl JF. A gene in the multidrug and toxic compound export (MATE) family confers aluminum tolerance in sorghum. Nat Genet. 2007;39:1156–61.
9. Sasaki T, Yamamoto Y, Etsuki B, Katsurahara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H. A wheat gene encoding an aluminum-activated malate transporter. Plant J. 2003;34:645–53.
10. Sivaguru M, Horst WJ. The distal part of the transition zone is the most aluminum-sensitive apical root zone of maize. Plant Physiol. 1998;116:155–63.
11. Xi J, Yamami N, Kasa T, Ma JF. Plasma membrane-localized transporter for aluminum in rice. Proc Natl Acad Sci U S A. 2010;107:18381–5.
12. Li JY, Liu J, Dong D, Jia X, McCouch SR, Kochian LV. Natural variation underlies alterations in Nrapm aluminum transporter (NRT1.1) expression and function that play a key role in rice aluminum tolerance. Proc Natl Acad Sci U S A. 2014;111:5603–8.
13. Wang Y, Li R, Li D, Ji X, Zhou D, Li J, Lyi SM, Hou S, Huang Y, Kochian L, et al. NIP1;2 is a plasma membrane-localized transporter mediating aluminum uptake, translocation and tolerance in Arabidopsis. Proc Natl Acad Sci U S A. 2017;114:5047–52.
14. Ma JF, Hiradate S. Form of aluminum for uptake and translocation in buckwheat (Fagopyrum Esculentum Moench). Planta. 2000;211:355–60.
15. Ma JF, Hiradate S, Nomoto K, Izawata T, Matsumoto H. Internal detoxification mechanism of aluminum in hakea (identification of a new form in the leaves). Plant Physiol. 1997;113:1033–9.
16. Zheng SJ, Ma JF, Matsumoto H. High aluminum resistance in buckwheat: I. Al-induced specific secretion of oxalic acid from root tips. Plant Physiol. 1998;117:745–51.
17. Horst WJ, Wang Y, Etsuki D. The role of the root apoplast in aluminum-induced inhibition of root elongation and in aluminum resistance of plants: a review. Ann Bot. 2010;106:185–97.
18. Ma JF, Shen R, Nagao S, Tanimoto E. Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. Plant Cell Physiol. 2004;45:8–9.
19. Yang Z-B, Geng X, He C, Zhang F, Wang R, Horst WJ, Ding Z. TAA1-regulated local auxin biosynthesis in the root-apex transition zone mediates the aluminum-induced inhibition of root growth in Arabidopsis. Plant Cell. 2014;26:2889–904.
20. Zhou D, Yang Y, Zhang J, Jiang F, Craft E, Thannhauser TW, Kochian LV, Liu J. Quantitative iTRAQ proteomics revealed possible roles for antioxidant proteins in sorghum aluminum tolerance. Front Plant Sci. 2017;7:2043.
21. Yang J, Li YZ, Zhang YJ, Sizai S, Wu YL, Wu P, Zheng SJ. Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. Plant Physiol. 2008;146:602–11.
22. Yang J, Zhu YF, Peng YX, Zheng C, Li GX, Liu Y, Shi YZ, Zheng SJ. Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in Arabidopsis. Plant Physiol. 2011;155:1885–92.
23. Zhu XF, Shi YZ, Lei GJ, Fu SC, Zhang BC, Zhou YH, Braam J, Jiang T, Xu XY, Mao C. XTH31, encoding an in vitro XEH/XET-active enzyme, regulates aluminum sensitivity by modulating in vivo XET action, cell wall xyloglucan content, and aluminum binding capacity in Arabidopsis. Plant Cell. 2012;24:4731–47.
24. Zhu XF, Wan JX, Sun Y, Shi YZ, Braam J, Li GX, Zheng SJ. Xyloglucan ENDOTRANSGLUCOSYLASE-Hydrolase1 interacts with xyloglucan Endotransglucosylase/Hydrolase31 to confer xyloglucan endotransglucosylase action and affect aluminum sensitivity in Arabidopsis. Plant Physiol. 2014;165:1566–74.
25. Iuchi S, Koyama H, Uchi A, Kobayashi Y, Kitabayashi S, Kobayoshi Y, Ikka T, Hirayama T, Shinozaki K, Kobayashi M. Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. Proc Natl Acad Sci U S A. 2007;104:9900–5.
26. Sawaki Y, Iuchi S, Kobayashi Y, Kobayashi Y, Ikka T, Sakurai N, Fujita M, Shinozaki K, Shibata D, Kobayashi M. STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminum toxicities. Plant Physiol. 2009;150:281–94.
27. Hoekenga OA, Moran LG, Piñeros MA, Cançado GM, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T. ATALNT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proc Natl Acad Sci U S A. 2006;103:9738–43.
28. Lanser P, Geisler MJ, Jones CA, Williams KM, Cancel JD. ALMA encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in Arabidopsis. Plant J. 2005;41:353–63.
29. Liu J, Luo X, Shaff J, Liang C, Xia J, Li Z, Ma JF, Kochian LV. A promoter-swapp strategy between the ATALNT1 and AMATE genes increased Arabidopsis aluminum resistance and improved carbon-use efficiency for aluminum resistance. Plant J. 2012;71:327–37.
30. Kim Y, Schumaker KS, Zhu J-K. EMS mutagenesis of Arabidopsis. Arabidopsis Protocols. 2006:101–3.
31. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum. 1962;15:473–97.
32. Ryan PR, Raman H, Gupta S, Horst WJ, Delhaize E. A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. Plant Physiol. 2009;149:340–51.
33. Giovannoni JJ, Wing RA, Galan MW, Tanksley SD. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res. 1991;19:6553–68.
34. Michelmore RW, Paran J, Kesseli R. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci U S A. 1991;88:9842–32.
35. Schneeberger K, Osowski S, Lanz C, Juel T, Petersen AH, Jørgensen JE, Weigel D, Andersen SU. SHOREmap: simultaneous mapping and mutation identification by deep sequencing. Nat Methods. 2009;6:530–1.
36. Schneeberger K, Weigel D, Andersen SU. SHOREmap: simultaneous mapping and mutation identification by deep sequencing. Nat Methods. 2009;6:530–1.
37. Rains D, Epstein E. Sodium absorption by barley roots: its mediation by mechanism 2 of alkaline cation transport. Plant Physiol. 1967;42:319–23.
38. Hirsch RE, Lewis BD, Spalding EP, Susman MR. A role for the AKT1 potassium channel in plant nutrition. Science. 1998;280:918–21.
39. Lee SC, Lan W-Z, Kim B-G, Li, Cheong YH, Pandey GK, Lu G, Buchanan BB, Luan S. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci U S A. 2007;104:15959–64.
40. Latinen RA, Schneeberger K, Jolly NS, Osowski S, Weigel D. Identification of a spontaneous frame shift mutation in a non-reference Arabidopsis accession using whole genome sequencing. Plant Physiol. 2010;153:652–4.
41. Tabata R, Kamiya T, Shigenobu S, Yamaguchi K, Yamada M, Hasebe M, Fujiwara T, Sawas S. Identification of an EMS-induced causal mutation in a gene required for boron-mediated root development by low-coverage genome re-sequencing in Arabidopsis. Plant Signal Behav. 2013;8:e22534.
42. Uchida N, Sakamoto T, Kurata T, Tsakata M. Identification of EMS-induced causal mutations in a non-reference Arabidopsis Thaliana accession by whole genome sequencing. Plant Cell Physiol. 2011;52:716–22.