PLANT SIGNALING & BEHAVIOR
2021, VOL. 16, NO. 5, e1899487 (7 pages)
https://doi.org/10.1080/15592324.2021.1899487

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SHORT COMMUNICATION

The SUMO E3 ligase SIZ1 partially regulates STOP1 SUMOylation and stability in *Arabidopsis thaliana*

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ABSTRACT

The zinc finger transcription factor STOP1 plays a crucial role in aluminum (Al) resistance and low phosphate (Pi) response. Al stress and low Pi availability do not affect *STOP1* mRNA expression but are able to induce STOP1 protein accumulation by post-transcriptional regulatory mechanisms. We recently reported that STOP1 can be mono-SUMOylated at K40, K212, or K395 sites, and deSUMOylated by the SUMO protease ESD4. SUMOylation of STOP1 is important for the regulation of STOP1 protein function and Al resistance. In the present study, we further characterized the role of the SUMO E3 ligase SIZ1 in STOP1 SUMOylation, Al resistance and low Pi response. We found that mutation of *SIZ1* reduced but not eliminated STOP1 SUMOylation, suggesting that SIZ1-dependent and -independent pathways are involved in the regulation of STOP1 SUMOylation. The STOP1 protein levels were decreased in *siz1* mutants. Nevertheless, the expression of STOP1-target gene *AtALMT1* was increased instead of reduced in *siz1* mutants. The mutants showed enhanced Al resistance and low Pi response. Our results suggest that SIZ1 regulates Al resistance and low Pi response likely through the modulation of *AtALMT1* expression.

ARTICLE HISTORY
Received 12 January 2021
Revised 1 March 2021
Accepted 2 March 2021

KEYWORDS
Aluminum resistance; low phosphate response; STOP1; *AtALMT1*; SIZ1; SUMOylation

Malate exudation from roots via ALUMINUM-ACTIVATED MALATE TRANSPORTER 1 (AtALMT1) transporter is critically required for aluminum (Al) resistance and low phosphate (Pi) response in *Arabidopsis thaliana*.[1–3] *AtALMT1* expression is triggered by different stresses, including Al toxicity and low phosphate availability.[2–5] One key upstream regulator of *AtALMT1* is the zinc-finger transcription factor SENSITIVE TO PROTON RHIZOTOXICITY 1 (STOP1) because mutation of *STOP1* nearly abolishes *AtALMT1* transcription.[6] STOP1 plays a crucial role in the regulation of Al resistance and low Pi response, which is achieved mainly through controlling *AtALMT1* expression.[2,3,6] STOP1 also regulates the expression of Al-inducible genes *AtMATE* and *ALS3*.[7] *AtMATE* encoding a citrate transporter was demonstrated to mediate root citrate exudation to be involved in the Al resistance.[8] ALS3 interacts with AtSTAR1 to form a functional complex of an ATP-binding cassette (ABC) transporter, which might modify the cell wall to modulate Al resistance and low Pi response although the exact underlying mechanism remains elusive.[9–11] Al stress does not affect transcript level of *STOP1* but is able to induce its protein accumulation,[6,12] indicating that *STOP1* is regulated by Al stress at post-transcriptional levels. An F-box protein RAE1 and a core component of THO/TREX complex, HPR1, were recently reported to modulate STOP1 protein levels through the regulation of STOP1 degradation and nucleocytoplasmic *STOP1* mRNA export, respectively.[12,13]

More recently, we discovered that SUMOylation is involved in the regulation of STOP1 protein function.[14] We found that the SUMO protease ESD4, but not other SUMO proteases, can specifically interact with and deSUMOylate STOP1. In *esd4* mutants, the levels of STOP1 SUMOylation were increased while the STOP1 protein levels were not affected. Interestingly, the expression of *AtALMT1* was increased but that of another STOP1-target gene *AtMATE* was decreased in the *esd4* mutants, which could be attributed to the increased and decreased association of STOP1 to the promotes of *AtALMT1* and *AtMATE*, respectively. We further demonstrated that STOP1 is mono-SUMOylated at K40, K212, or K395 sites. Mutation of STOP1 SUMOylation site at K40 decreases *AtALMT1* expression and increases *AtMATE* expression, which leads to reduced Al resistance and low Pi response, while mutation at K212 site does not affect STOP1 function. In contrast, mutation of STOP1 SUMOylation at K395 or all the three (K40, K212 and K395) sites reduces STOP1 protein levels and the expression of *AtALMT1* and *AtMATE*, leading to the decreased Al resistance and low Pi response. Our results thus reveal the important role of SUMOylation in the regulation of STOP1 protein function, Al resistance and low Pi response.[14]

In this study, we further investigated whether the SUMO E3 ligase SIZ1 is involved in the regulation of STOP1 SUMOylation, and found that SIZ1 was partially required for the SUMOylation of STOP1. We also determined the role of SIZ1 in Al resistance and low Pi response and found that mutation of *SIZ1* enhanced Al resistance and low Pi response, which is likely caused by the increased expression of *AtALMT1* expression.

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Materials and methods

Plant materials

The *Arabidopsis thaliana* ecotype Col-0 was used as the wild-type (WT) for all the control experiments. T-DNA insertions mutants, *siz1-2* (SALK_065397), *siz1-3* (SALK_034008) and *hyp2-2* (SAIL_77_G06), were derived from Arabidopsis Biological Resource Center (ABRC). The *pSTOP1:STOP1-3 HA* transgenic line, which has been produced before,[12] was crossed with *siz1-2* and *siz1-3*, respectively, to generate *siz1* mutants carrying homozygous *pSTOP1:STOP1-3 HA* transgene.

Split-LUC complementation analysis and GST pull-down assay

For split-LUC complementation analysis, the coding sequence of *STOP1* was cloned into pCAMBIA-nLUC vector and the coding sequences of *SIZ1, SUMO1, SUMO2*, and *SUMO3* were, respectively, introduced into pCAMBIA-cLUC.[15] *STOP1-nLUC* was co-transformed with *cLUC* fused *SIZ1, SUMO1, SUMO2*, or *SUMO3* into *N. benthamiana* leaves by *Agrobacterium*-mediated transformation, respectively. After growth in the dark for 2 d, transformed leaves were subjected for LUC signal detection to determine the interaction between two proteins.

For pull-down assay, CDS of *SIZ1* and *STOP1* were cloned into pGEX4T and PET29a to generate *GST-SIZ1* and *STOP1-His* recombinant plasmids, respectively. *GST-SIZ1* transformed cells were lysed and incubated with GST Bind Resin (C60031; Sangon Biotech Co., Ltd., China). After gentle rotation on ice for 1 h, the beads were washed with 1× TBS buffer for four times and mixed with lysates of *STOP1-His* transformed cells, and then rotated on ice for 2 h. Subsequently, the beads were washed for five times, and the protein complex on the beads was then eluted with elution buffer (5 mM glutathione in TBS buffer). The dissolved proteins were subjected to immunoblot analysis by using anti-His or anti-GST antibodies.

RNA isolation and real-time RT-PCR analysis

Seedlings of WT, *siz1-2* and *siz1-3* were grown on a one-tenth strength Hoagland nutrient solution for four weeks. The plants were then pretreated with a 0.5 mM $CaCl_2$ solution at pH 4.8 for 6 h and subsequently exposed to the same solution containing 0 or 30 μM $AlCl_3$ at pH 4.8 for 12 h. Roots were excised for RNA isolation. Total RNA was extracted using TaKaRa M iniBEST plant RNA Extraction Kit. About 1 μg of total RNA was first digested with DNase I and then used for the synthesis of first-strand cDNAs with the HiScript® 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, China). The cDNA products were used for real-time RT-PCR analysis by the SYBR® Green Master Mix kit (Vazyme Biotech Co., Ltd., Nanjing, China) and gene-specific primers of *STOP1, AtALMT1, AtMATE*, and *ALS3*, which have been designed previously.[14] *UBQ10* was used as an internal control in the real-time RT-PCR analysis. Real-time RT-PCR data were recorded and analyzed using the CFX96 Touch real-time PCR detection system (Bio-Rad).

Complementation test

A 2.2-kb promoter and the coding sequence of SIZ1 were, respectively, amplified and fused by PCR and then the fused DNA fragment was cloned into pCAMBIA1305 vector. The construct was subsequently introduced into *siz1-2* by *Agrobacterium tumefaciens* (strain GV3101)-mediated transformation. To perform complementation test on *AtALMT1* expression, seedlings of WT, *siz1-2* and a T3 complementation line were grown on a half-strength MS medium with 1.2% agar and 1% sucrose for 8 d and then treated with a 0.5 mM $CaCl_2$ solution containing 0 or 30 μM $AlCl_3$ at pH 4.7 for 8 h. Roots of each line were collected for RNA extraction and expression analysis of *AtALMT1*.

Determination of STOP1 protein levels in roots

Ten-day-old seedlings of WT, *siz1-2* and *siz1-3* harboring *pSTOP1:STOP1-3 HA* transgene were exposed to a 0.5 mM $CaCl_2$ solution containing 0 or 30 μM $AlCl_3$ at pH 4.7 for 8 h and then roots (~200 mg) were excised and frozen for protein extraction using the extraction buffer composed of 50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10% Glycerol, 0.25% NP-40, 0.5% Triton X-100, 0.05% SDS, 50 μM MG132, 20 mM **N**-Ethylmaleimide, and 1× complete protease inhibitor mixture. Immunoblot analysis was carried out to determine STOP1 protein level using anti-HA antibody (12013819001; Roche). The Actin protein was used as the loading control, which was detected by anti-Actin antibody (CW0264M; CoWin Biosciences Co., Ltd., China).

STOP1 SUMOylation analysis

Two milliliter protoplasts (~$2x10^6$ cells/ml) of WT, *siz1-2* or *hyp2-2* were co-transformed with 50 μg *35S:6Myc-SUMO1* and 50 μg *35S:STOP1-2Flag*, which were constructed previously.[14] After the protoplasts were incubated overnight, total protein fraction was extracted and subjected to be immunoprecipitated by anti-FLAG M2 magnetic beads (M8823, Sigma-Aldrich). The SUMOylated form of STOP1 was detected by using anti Myc-HRP antibody (A00704-100; Genscript Biotech Co., Ltd., China).

Evaluation of Al resistance and low Pi response

Seedlings of WT *siz1-2* and *siz1-3* were grown on a modified soaked gel medium[9,14] containing 0 (-Al) or 1.25 mM $AlCl_3$ (+Al) for 7 d. Relative root length expressed as root length with Al treatment/root length without Al × 100 was used to evaluate Al resistance. For the evaluation of low Pi response, seedlings were grown on 0.35% Gellan gum medium (pH5.7) containing 1% sucrose and 1× Hoagland nutrient solution 0.5 mM $(NH_4)_2SO_4$ and 10 μM or 1 mM $NH_4H_2PO_4$ (Pi), and with or without 40 μM Fe(III)-EDTA. After growth for 7 d, the seedlings were subjected for picture taken and root length measurement.

Results and discussion

The SUMO E3 ligase SIZ1 is involved in STOP1 SUMOylation

To investigate whether SIZ1 is required for STOP1 SUMOylation, we first performed protein interaction assays between SIZ1 and STOP1. A pull-down assay using GST-fused SIZ1 and His-fused STOP1 showed that GST-SIZ1 specially pulled down STOP1-His (Figure 1a), indicating that SIZ1 can directly interact with STOP1 in vitro. To determine whether SIZ1 can interact with STOP1 in planta, we conducted split luciferase

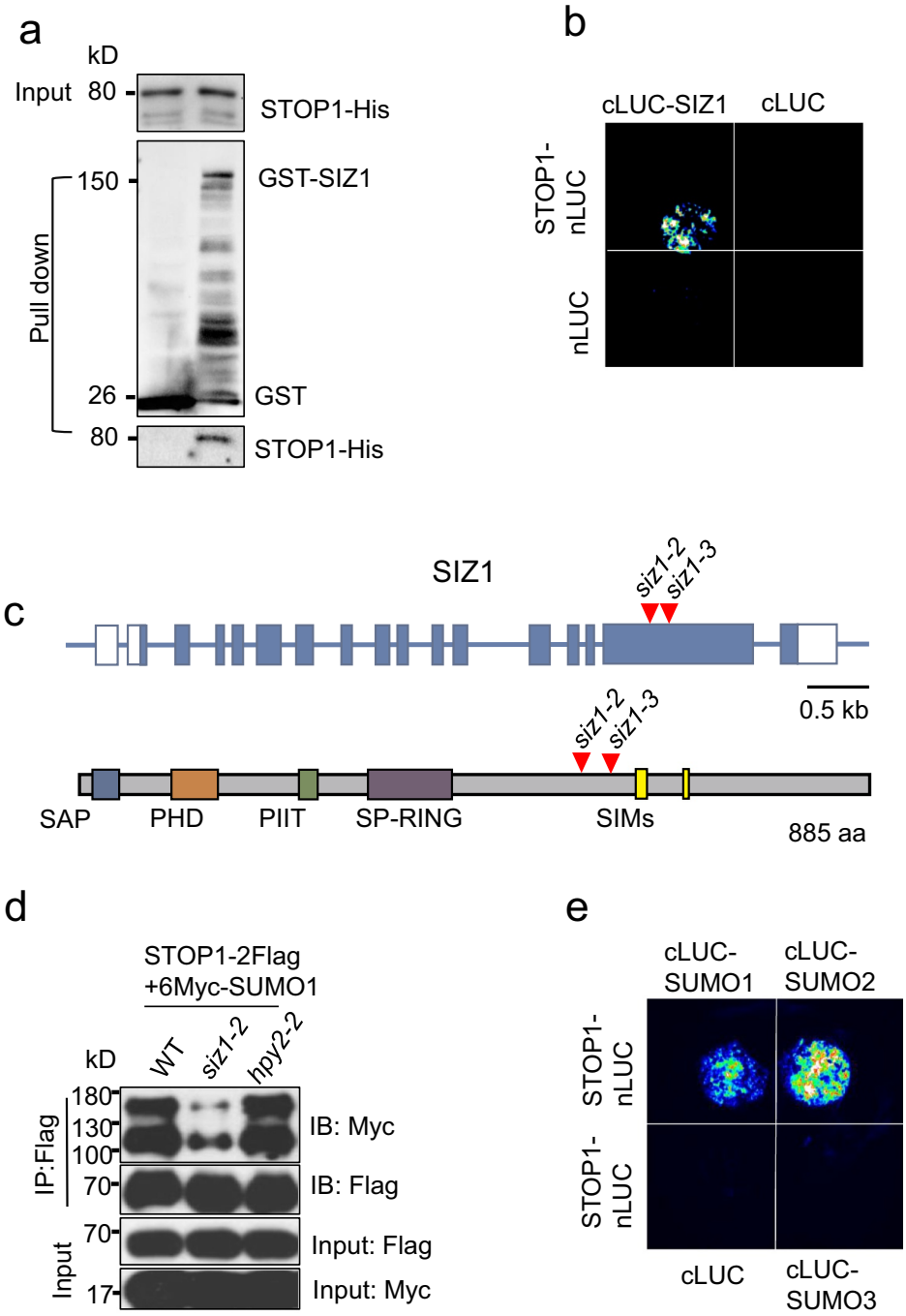

Figure 1. The SUMO E3 ligase SIZ1 can interact with STOP1 and regulate STOP1 SUMOylation. (a and b) Interaction of SIZ1 with STOP1 in a the GST pull-down assay (a) and a split-LUC complementation analysis (b). (c) Gene structure (upper figure) and protein domains (lower figure) of *SIZ1* with the indication of T-DNA insertion sites for *siz1-2* and *siz1-3*. Untranslated regions and introns are shown as white boxes and lines, respectively. The SAP, PHD, PIIT, SP-RING, and SIM domains are highlighted in rectangle boxes with different colors. (d) Comparison of the levels of STOP1 SUMOylation in WT, *siz1-2* and *hpy2-2*. Protoplasts were co-transfected with STOP1-2Flag and 6Myc-SUMO1. Crude total protein lysates (input) were used for STOP1 immunoprecipitation (IP) with anti-FLAG magnetic beads and 6Myc-SUMO1 modified STOP1 was detected using an anti-Myc antibody. (e) SUMO1 and SUMO2 can interact with STOP1 on the basis of split-LUC complementation analysis.

complementation assays (Split-LUC) using cLUC-fused SIZ1 and nLUC-fused STOP1. We detected the LUC signal with the combination of cLUC-SIZ1 and STOP1-nLUC, but not with other control combinations (Figure 1b), which indicates that SIZ1 is able to interact with STOP1 in planta.

We then examined whether mutation of *SIZ1* affected STOP1 SUMOylation by co-expressing STOP1-2Flag with 6Myc-SUMO1 precursor in WT and *siz1-2* protoplasts. *siz1-2* mutant has a T-DNA insertion in the 16th exon of *SIZ1* (Figure 1c), causing SIZ1 polypeptide nearly undetected.[16,17] The result showed that the level of STOP1 SUMOylation was reduced but not eliminated in *siz1-2* (Figure 1d), suggesting that STOP1 can also be SUMOylated through SIZ1-independent pathway. To investigate whether HPY2/MMS21, which can also act as a SUMO E3 ligase,[18,19] is involved in the regulation of STOP1 SUMOylation, we compared the levels of STOP1 SUMOylation in WT and *hpy2-2* protoplasts. The result showed that the SUMOylation levels of STOP1 were similar between WT and *hpy2-2* (Figure 1d), suggesting that HYP2 might not be involved in the regulation of STOP1 SUMOylation.

The SUMO E2 conjugating enzyme can also directly mediate target protein SUMOylation, without the help of E3 SUMO ligases.[20] We therefore tested whether SUMO proteins can interact with STOP1 directly. Split-LUC assays showed that SUMO1 and SUMO2, but not SUMO3, could directly interact with STOP1 in planta (Figure 1e). Together, these results suggest that both the SUMO E3 ligase SIZ1 and SUMO E2 conjugating enzyme can facilitate the SUMO-STOP1 linkage.

Effect of *siz1* mutations on STOP1 protein accumulation and the expression of STOP1-downstream genes

To investigate whether mutation of *SIZ1* influences STOP1 protein accumulation, we introduced *pSTOP1:STOP1-3 HA* transgene into *siz1-2* background by crossing and then performed immunoblot analysis using anti-HA antibody in WT and *siz1-2* under – Al and +Al conditions. The result showed that the level of STOP1-3 HA was clearly reduced in *siz1-2* compared to that in WT under both conditions (Figure 2a). We also introduced the *pSTOP1:STOP1-3 HA* transgene into the background of *siz1-3* allele, which also has a T-DNA insertion in the 16th exon of *SIZ1* (Figure 1c).[16] Whether *siz1-3* allele can produce truncated SIZ1 polypeptide is still unknown. Immunoblot analysis showed that STOP1-3 HA level was also decreased in *siz1-3* under +Al conditions, but the reduction in STOP1-3 HA level was less in *siz1-3* than in *siz1-2* (Figure 2a), suggesting that *siz1-2* allele is a strong allele compared to *siz1-3* in the regulation of STOP1 SUMOylation and protein accumulation. mRNA expression levels of *STOP1* in the *siz1* mutants were similar to that in WT (Figure 2b). Our previous work showed that blocking STOP1 SUMOylation reduced STOP1 protein accumulation.[14] Together, our results thus suggest that reduced STOP1 protein level in *siz1* might be partially caused by the impaired STOP1 SUMOylation.

Next, we compared the expression levels of STOP1-regulated Al-resistance genes, including *AtALMT1, AtMATE* and *ALS3*, in WT and *siz1* mutants. Intriguingly, although the STOP1 protein levels were reduced in *siz1* mutants (Figure 2a), the expression of *AtALMT1* was increased in the mutants compared to WT under both – Al and +Al conditions (Figure 2c). This increased expression of *AtALMT1* in *siz1* could be recovered to the WT level in a complementation line (Figure 2d), confirming that mutation of *SIZ1* causes the elevated *AtALMT1* expression. In contrast, the expression of *AtMATE* and *ALS3* was decreased in the mutants under +Al conditions (Figure 2e and f), which is in accordance with the reduced STOP1 accumulation. These results imply that other unknown factors regulated by *siz1* mutations could override the negative impact of reduced STOP1 levels on the *AtALMT1* expression. In addition STOP1, the expression of *AtALMT1* has also been reported to be regulated by various plant hormones and stresses,[4] and by WRKY46 and CAMTA2,[21,22] suggesting that *AtALMT1* expression can be modulated by many factors. It remains to determine what factors regulated by the *siz1* mutation trigger the *AtALMT1* expression in the future.

Mutation of *SIZ1* enhances Al resistance and low Pi response

To assess Al resistance phenotype of *siz1* mutants, we grew WT, *siz1-2* and *siz1-3* on agar plates with or without Al. Under Al conditions, root growth of *siz1* mutants was similar to that of WT (Figure 3a). In the presence of Al, root growth of the mutants was faster than that of WT, indicating that *siz1* mutants are more resistant to Al stress than WT (Figure 3a and b). Compared to previously reported *rae1* mutant, the increased resistance to Al in *siz1* was less strong (Data not shown).

We then compared root growth of WT and the mutants under high and low Pi conditions. The result showed that in the presence of iron (Fe), the mutants was more sensitive to low Pi than WT (Figure 3c and d), which is consistent with the previous report.[16] Miura et al. (2005) suggested that SIZ1 might regulate Pi deficiency response through the modulation of SUMOylation of the MYB transcription factor PHR1 and other unknown factors.[16] Nevertheless, the exact mechanism responsible for enhanced low Pi response in *siz1* mutants is still elusive. In this study, we found that *AtALMT1* expression was increased in *siz1* mutants (Figure 2c). Since AtALMT1-mediated malate secretion has been reported to promote apoplastic Fe toxicity and consequently inhibit root growth under low Pi conditions,[2,3] it

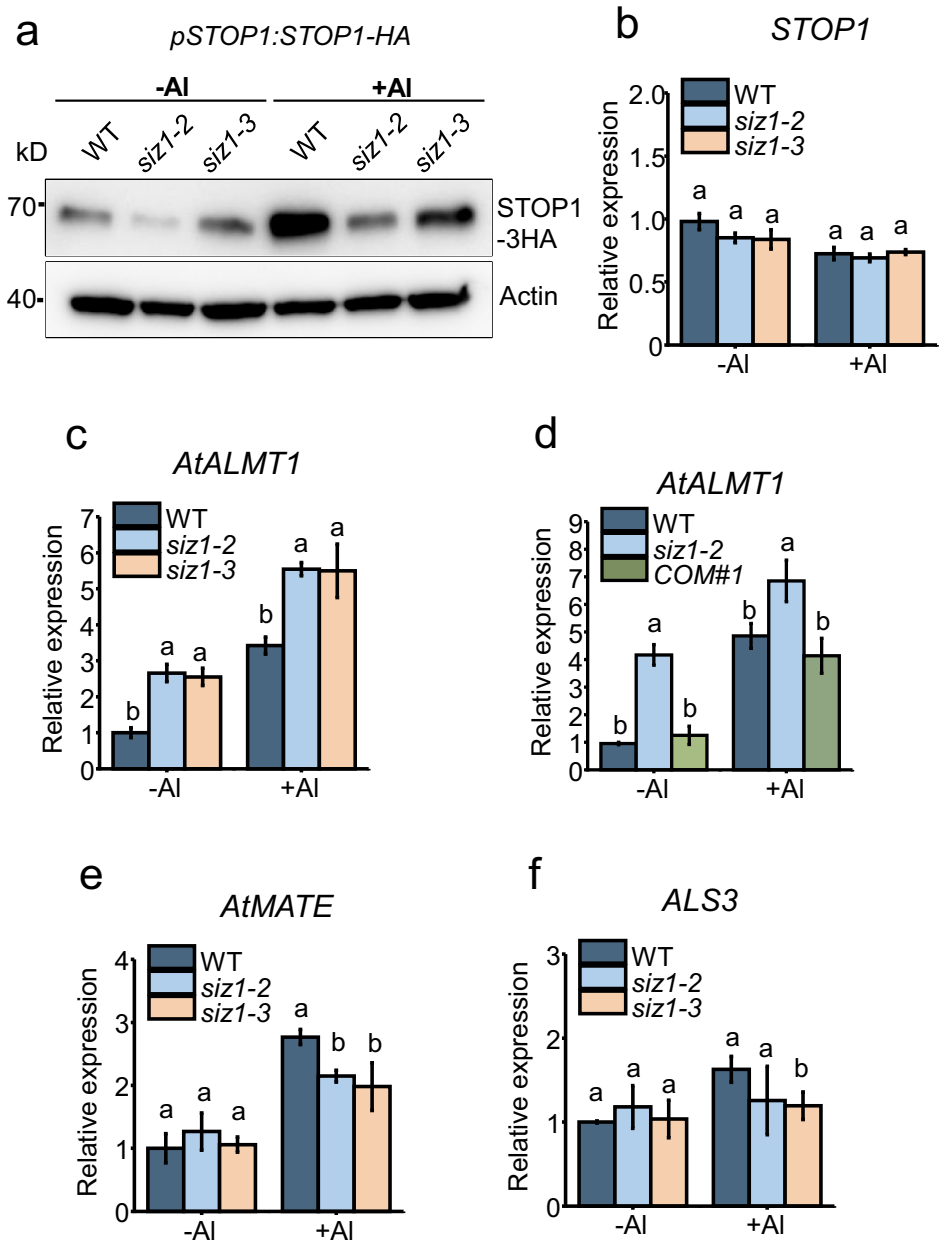

Figure 2. Effect of *siz1* mutations on STOP1 protein accumulation and the expression of STOP1-regulated genes. (a) Immunoblot analysis of STOP1-3 HA in WT, *siz1-2* and *siz1-3* under – Al and +Al conditions. Actin protein was used as an internal control. (b and c) Expression analysis of *STOP1* (b) and *AtALMT1* (c) in WT, *siz1-2* and *siz1-3* under – Al and +Al conditions. (d) Expression analysis of *AtALMT1* in WT, *siz1-2* and one complementation line of *siz1-2* (*COM#1*). (e and f) Expression analysis of *AtMATE* (e) and *ALS3* (f) in WT, *siz1-2* and *siz1-3*. Values are means ± SD of three replicates. Means with different letters are significantly different ($P < .05$, ANOVA test followed by Tukey test). Three independent experiments were performed with similar results.

is likely that the enhanced low Pi response in *siz1* mutants is caused by the elevated expression of *AtALMT1*. To further prove our hypothesis, we removed Fe from the medium to compare their root growth in high and low Pi concentrations. As expected, in the absence of Fe, the reduced root growth in the mutants under low Pi conditions was recovered and there was no difference in root growth between WT and the mutants under different Pi concentrations (Figure 3c and d). Our results thus suggest that increased *AtALMT1* expression might be responsible for the enhanced Al resistance and low Pi response in *siz1* mutants.

In conclusion, we demonstrate that STOP1 SUMOylation is mediated by SIZ1-dependent and -independent pathways.

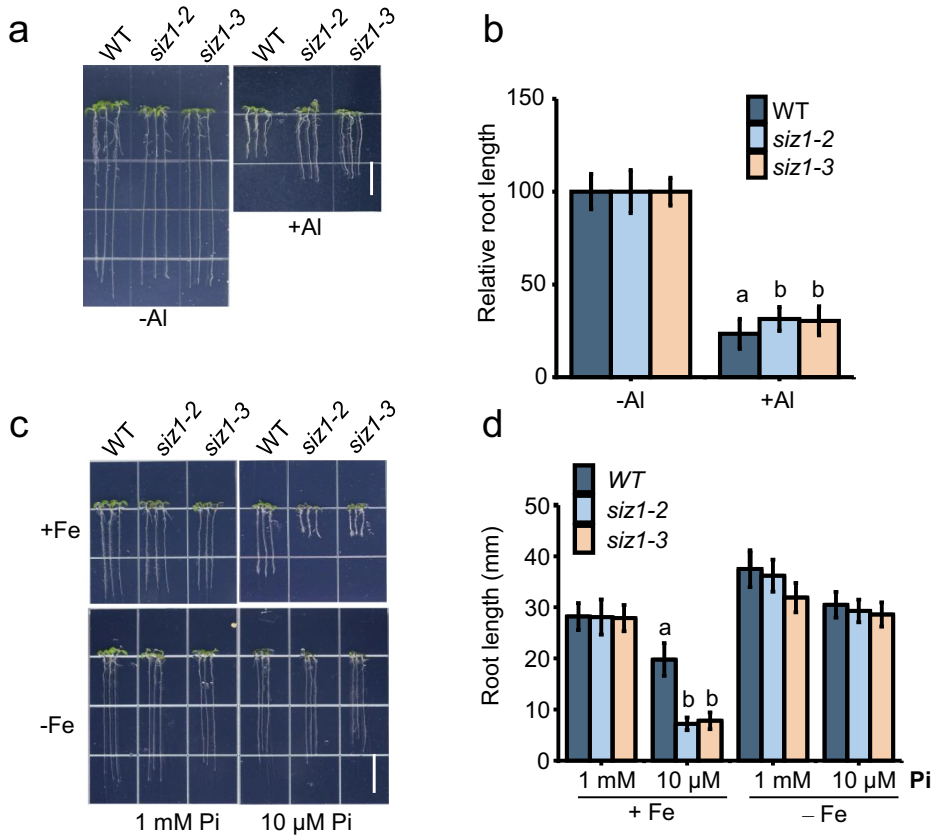

Figure 3. Effect of *siz1* mutations on Al resistance and low Pi response. (a and b) Increased Al resistance in *siz1-2* and *siz1-3* compared to WT. Values are means ± SD of relative root length of 25 to 32 seedlings. (c and d) Enhanced low Pi response in *siz1-2* and *siz1-3* under +Fe conditions. Values are means ± SD of root length of 14 to 19 seedlings. Means with different letters are significantly different ($P < .05$, ANOVA test followed by Tukey test). Scale bar = 1 cm.

Mutation of *SIZ1* increases *AtALMT1* expression likely through STOP1-independent pathways, which in turn leads to enhanced Al resistance and low Pi response.

Acknowledgments

This work was supported by Natural Science Foundation of Shanghai (Grant No. 20ZR1466500), the funding from Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, and National Key Laboratory of Plant Molecular Genetics.

Funding

This work was supported by the Natural Science Foundation of Shanghai [20ZR1466500]; Shanghai Center for Plant Stress Biology; National Key Laboratory of Plant Molecular Genetics.

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