# F-box protein RAE1 regulates the stability of the aluminum-resistance transcription factor STOP1 in *Arabidopsis*

Yang Zhang[a,b,1], Jie Zhang[a,1], Jinliang Guo[a,b,1], Fanglin Zhou[a], Somesh Singh[a], Xuan Xu[b], Qi Xie[c], Zhongbao Yang[d], and Chao-Feng Huang[a,b,2]

[a]National Key Laboratory of Plant Molecular Genetics, Shanghai Center for Plant Stress Biology, Center of Excellence for Molecular Plant Sciences, Chinese Academy of Sciences, 200032 Shanghai, China; [b]College of Resources and Environmental Sciences, Nanjing Agricultural University, 210095 Nanjing, China; [c]State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 100101 Beijing, China; and [d]Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, College of Life Science, Shandong University, 250100 Jinan, China

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**Aluminum (Al) toxicity is a major factor limiting crop production on acid soils, which represent over 30% of the world's arable land. Some plants have evolved mechanisms to detoxify Al. *Arabidopsis*, for example, secretes malate via the AtALMT1 transporter to chelate and detoxify Al. The C2H2-type transcription factor STOP1 plays a crucial role in Al resistance by inducing the expression of a set of genes, including *AtALMT1*. Here, we identify and characterize an F-box protein-encoding gene *regulation of Atalmt1 expression 1* (*RAE1*) that regulates the level of STOP1. Mutation and overexpression of *RAE1* increases or decreases the expression of *AtALMT1* and other STOP1-regulated genes, respectively. RAE1 interacts with and promotes the degradation of STOP1 via the ubiquitin-26S proteasome pathway, while Al stress promotes the accumulation of STOP1. We find that STOP1 up-regulates *RAE1* expression by directly binding to the *RAE1* promoter, thus forming a negative feedback loop between STOP1 and RAE1. Our results demonstrate that RAE1 influences Al resistance through the ubiquitination and degradation of STOP1.**

aluminum toxicity | AtALMT1 | E3 ligase | low phosphate response | ubiquitination

Approximately 30–40% of the world arable land consists of soils with a pH of 5.5 or lower (1). In these acidic soils, aluminum (Al) is solubilized and becomes highly toxic to plants. Consequently, Al toxicity is a severe global problem for crops growing on acid soils (2).

To chelate and detoxify Al, some plants secrete organic acids, including malate, citrate, and oxalate (3–5). *Arabidopsis* plants, for example, release both malate and citrate in response to Al stress, with malate being essential for Al detoxification (6–8). The gene responsible for Al-activated secretion of malate in roots, *Al-activated malate transporter 1* (*ALMT1*), was first identified in wheat and encodes an anion transporter/channel (9). There are 12 ALMT members in *Arabidopsis*, and only AtALMT1 is indispensable for root malate secretion and Al detoxification (7). Unlike *TaALMT1*, whose expression is largely constitutive and unaffected by Al stress (9), *AtALMT1* expression is induced by Al in all *Arabidopsis* ecotypes (7). In addition to Al, plant hormones and other stresses such as indole-3-acetic acid (IAA), abscisic acid (ABA), low pH, and hydrogen peroxide trigger *AtALMT1* transcription (10), which suggests that the regulation of *AtALMT1* expression is complex. The mechanisms that underlie the regulation of *AtALMT1* expression, however, are not fully understood.

Through a forward genetic screen of mutants exhibiting increased sensitivity to proton toxicity, a zinc-finger transcription factor sensitive to proton rhizotoxicity 1 (STOP1) was identified and found to be critical for both proton and Al tolerance (11). Mutation of *STOP1* greatly suppresses *AtALMT1* transcription (11), demonstrating that STOP1 is a crucial regulator of *AtALMT1* expression. *STOP1* transcription is not affected by Al stress, but the expression of STOP1-downstream Al-resistance genes, including *AtALMT1*, *AtMATE*, and *ALS3*, is induced by Al (8, 12). These results suggest the possibility that *STOP1* might be regulated by Al at posttranscriptional or posttranslational levels.

Ubiquitin-mediated protein degradation is an important post-translational mechanism that regulates numerous biological processes (13). Ubiquitin conjugation is mediated through the sequential action of three enzyme complexes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. The E3 ligases are responsible for substrate recognition and ubiquitin transfer. Among various E3 families, the SKP1–CUL1–F-box (SCF) is the largest and best characterized and is composed of four major subunits: SKP1, Cullin1, RBX1, and an F-box protein (13, 14). Cullin functions as a scaffold and interacts with SKP1 and RBX1 to form a complex that generates the core ligase activity. F-box proteins selectively interact with target proteins and deliver the targets to the complex (15). The *Arabidopsis* genome contains nearly 700 genes that potentially encode F-box proteins (16). The F-box proteins can be further divided into subgroups based on C-terminal protein-interaction domains. Members of the leucine-rich repeat (LRR) subgroup are involved in the regulation of auxin, ethylene, jasmonate, and strigolactone hormone signaling (17–20), but no roles in metal stress tolerance or signaling have been reported.

## Significance

**Aluminum (Al) toxicity is a major constraint of crop production on acid soils. *Arabidopsis* can secrete malate via the AtALMT1 transporter to chelate and detoxify Al. The transcription factor STOP1 is essentially required for Al resistance, mainly through the control of *AtALMT1* expression. Here, we report an F-box protein RAE1 that regulates STOP1 stability. RAE1 interacts with STOP1 to promote STOP1 degradation via the ubiquitin-26S proteasome pathway, whereas Al stress stabilizes STOP1. Together, our results reveal an important role for RAE1 in the regulation of Al resistance.**

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[1]Y.Z., J.Z., and J.G. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: cfhuang@sibs.ac.cn.

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To gain insights into how *AtALMT1* or *STOP1* are modulated at transcriptional or posttranscriptional levels, we conducted a forward genetic screen on an EMS-mutagenized population in an *AtALMT1* promoter-driven luciferase reporter (LUC) line. Through this screen, we identified an F-box protein-encoding gene *RAE1* that interacts with STOP1 to regulate its stability via the ubiquitin-26S proteasome pathway.

## Results

**Identification of Mutants with Altered *AtALMT1* Expression.** To identify components involved in the regulation of *AtALMT1* expression, we fused the *AtALMT1* promoter to the luciferase reporter gene (*pAtALMT1:LUC*) and then introduced the construct into the wild-type (WT) [Columbia-0 (Col-0)] seedlings. We screened for single-copy transgenic lines displaying high luminescence and then performed thermal asymmetric interlaced (TAIL)-PCR on these lines to pinpoint the location of the reporter gene in the genome. With this procedure, we identified a line in which the reporter gene was inserted in the intergenic region between At4g23000 and At4g23010. We generated an EMS-mutagenized library in the background of this transgenic line for mutant screening. After screening ~10,000 M2 lines, we finally identified 17 *regulation of AtALMT1 expression* (*rae*) mutants with altered luminescent signal. Two mutants with extremely low luminescence had different mutations in the key Al-resistance gene *STOP1* (*SI Appendix*, Fig. S1*A*), which indicated that our screening system was effective. In this study, we focused on *rae* mutants that showed increased LUC signal (Fig. 1*A*), and we identified eight alleles (*SI Appendix*, Fig. S1*B*) that had different mutations in the same gene (designated as *rae1*) as described below. We selected two *rae1* mutants (*rae1-1* and *rae1-2*) that were allelic to each other (Fig. 1*A*) for most analysis.

**Mutation of *RAE1* Increases the Expression of STOP1-Regulated Genes.** The expression levels of both the *LUC* transgene and the endogenous *AtALMT1* gene were significantly higher in *rae1* mutants than in the WT, especially under −Al conditions (Fig. 1 *B* and *C*). In the *stop1-3* mutant, the expression of *LUC* and *AtALMT1* was almost eliminated under both −Al and +Al conditions, which is consistent with previous reports that STOP1 is an essential regulator of *AtALMT1* expression (11). We also generated *pAtALMT1:GUS* transgenic lines by fusing the *AtALMT1* promoter to the *uidA* gene, which encodes β-glucuronidase (GUS) and selected a transgenic line that had a one-locus segregation pattern for GUS expression and introduced the transgene into the *rae1-1* background by crossing. GUS staining showed that GUS was expressed at higher levels in *rae1-1* than in the WT in both the absence and presence of Al (*SI Appendix*, Fig. S1 *C* and *D*).

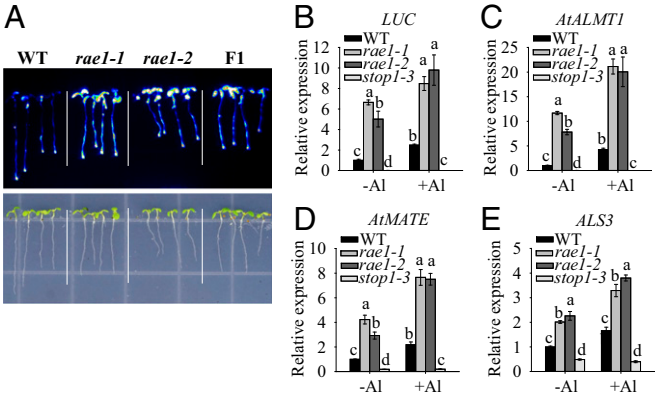


**Fig. 1.** Mutation of *RAE1* increases expression of *LUC* reporter gene and of STOP1-regulated genes. (*A*) Elevated LUC signal of *pAtALMT1:LUC* in *rae1* mutants and F1 plants of *rae1-1*/*rae1-2*. (*B*–*E*) Increased mRNA expression of *LUC* and endogenous STOP1-regulated genes in *rae1* mutants. Real-time RT-PCR analysis was carried out to determine the expression of *LUC* (*B*), *AtALMT1* (*C*), *AtMATE* (*D*), and *ALS3* (*E*) in roots of WT, *rae1-1*, *rae1-2*, and *stop1-3*. Values are means ± SD ($n$ = 3). Means with different letters are significantly different ($P < 0.05$, Duncan's multiple-range test).

To determine whether *rae1* mutations influence the expression of other Al-resistance genes, we analyzed the expression levels of *AtMATE*, *ALS3*, *AtSTAR1*, *ALS1*, and *STOP1* in the WT and the *rae1* mutants. The results showed that *AtMATE* and *ALS3*, two additional target genes of STOP1 (Fig. 1 *D* and *E*) (8, 12), were also expressed at higher levels in the *rae1* mutants than in the WT under both −Al and +Al conditions (Fig. 1 *D* and *E*). In contrast, the expression levels of *AtSTAR1* and *ALS1*, which are not regulated by STOP1 (*SI Appendix*, Fig. S2 *A* and *B*) (12, 21), did not differ between the WT and the *rae1* mutants (*SI Appendix*, Fig. S2 *A* and *B*). The expression of *STOP1* was also not affected in *rae1* (*SI Appendix*, Fig. S2*C*). In addition, introduction of the *stop1-3* mutation into the *rae1-1* mutant background fully suppressed the increased expression of LUC in the *rae1-1* mutant (*SI Appendix*, Fig. S1*E*). These results suggest that RAE1 acts upstream of STOP1 to regulate the expression of Al-resistance genes.

Because the expression of *AtALMT1* was increased in *rae1* mutants, we compared malate secretion between the WT and *rae1* mutants. Without Al treatment, roots from both the WT and from *rae1-1* and *rae1-2* released basal amounts of malic acid, but *rae1* mutants released more malic acid than the WT (Fig. 2*A*). In the presence of Al, the level of Al-activated malate secretion was also significantly higher in the mutants than in the WT (Fig. 2*A*). In the *stop1-3* control, the Al-activated exudation of malate was completely suppressed. In accordance with the increased malate secretion in *rae1*, the mutants accumulated less Al in their roots than WT seedlings in response to Al treatment (Fig. 2*B*). We then compared the Al resistance of *rae1-1*, *rae1-2*, and the WT at different Al concentrations, and found that Al resistance was higher in the *rae1* mutants than in the WT (Fig. 2 *C* and *D*). Recently, malate secretion mediated by the STOP1-AtALMT1 pathway was also reported to repress root growth under low phosphate (Pi) conditions (22, 23). Therefore, we also compared the root growth under low Pi conditions and found that *rae1* mutants were more sensitive to low Pi than the WT, whereas the *stop1-3* control showed less sensitivity to low Pi than the WT (Fig. 2 *E* and *F*). Together, these data indicate that RAE1 probably regulates Al resistance and low Pi response by modulating the expression of STOP1-regulated *AtALMT1*.

**Cloning of *RAE1*.** We performed a genetic analysis of *rae1-1* by using an F2 population from a cross between *rae1-1* and the WT. Observation of the LUC signal showed that among the 322 F2 seedlings examined, 77 exhibited high luminescence, while the rest had low luminescence like the WT control. The number of plants with high LUC expression vs. the number with low LUC expression fit 1:3 ($\chi^2 = 0.15$, $P = 0.7$), suggesting that the increased LUC expression in the *rae1-1* mutant was controlled by a single recessive gene.

To clone the *RAE1* gene, we used the same F2 population as above and sequenced the whole genome of pooled DNA from F2 plants with high LUC signal by second-generation high throughput DNA sequencing. We also sequenced WT plants as a control. Through Mutmap analysis (24), we found that the candidate gene was likely located on a small region of the short arm of chromosome 5 (*SI Appendix*, Fig. S3*A*). To confirm the mapping-by-sequencing results, we developed four derived cleaved amplified polymorphic sequence (dCAPS) markers surrounding the candidate region based on the mutations in the *rae1-1* mutant (*SI Appendix*, Table S1), and we genotyped each of the 34 F2 plants

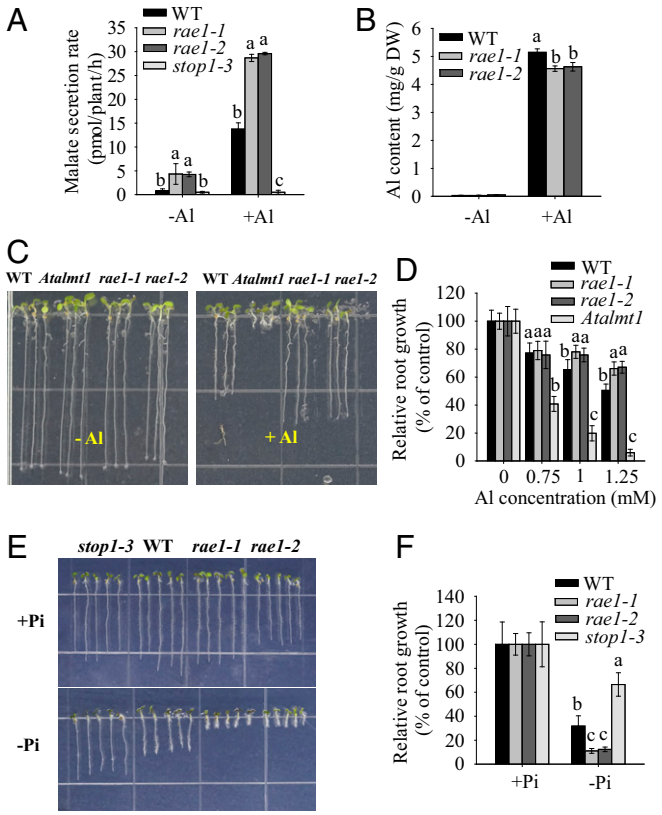


**Fig. 2.** Phenotype of *rae1* mutants. (*A* and *B*) Malate secretion (*A*) and Al accumulation (*B*) in the WT and *rae1* mutants. Plants were treated with 0 (−Al) or 20 μM Al (+Al) in *A*, or 0 (−Al) or 30 μM Al (+Al) in *B* for 12 h. Values are means ± SD ($n = 3$ in *A*; $n = 4$ in *B*). (*C* and *D*) Images (*C*) and quantitative data (*D*) of increased Al resistance in *rae1* mutants. Values are means ± SD ($n = 18$–23). (*E* and *F*) Images (*F*) and quantitative data (*G*) of low Pi-induced root growth inhibition. Values are means ± SD ($n = 15$–17). Means with different letters are significantly different ($P < 0.05$, Tukey test).

with high LUC expression to perform linkage analysis. All of the markers were linked to the LUC expression phenotype to different degrees (*SI Appendix*, Table S1), confirming that the *RAE1* gene was located on this region. We found that only marker A01 in the coding sequence of At5g01720 was completely linked to the mutant phenotype. This G-to-A substitution at position 499 bp from ATG is predicted to cause a G167R amino acid change in At5g01720 (*SI Appendix*, Fig. S4 *A* and *B*). The other *rae1* mutant alleles were found to have nonsynonymous mutations in this gene (*SI Appendix*, Fig. S4 *A* and *B*). Phenotypic analysis on four of these *rae1* mutant alleles showed that malate secretion, Al resistance, and low Pi-induced root growth inhibition were all enhanced in the mutants (*SI Appendix*, Fig. S5). To further confirm that At5g01720 is *RAE1*, we conducted a complementation test on *rae1-1* by transgenically introducing the WT *RAE1* gene into the mutant. Observation of luminescent signal showed that the increased LUC expression in *rae1-1* was fully rescued in two independent complementation lines (*SI Appendix*, Fig. S3*B*). The elevated expression of STOP1-regulated genes was also rescued in the two lines (*SI Appendix*, Fig. S3*C*). These results demonstrate that the mutation in At5g01720 is responsible for the increased LUC expression in the *rae1-1* mutant.

***RAE1* Encodes a Nuclear-Localized F-Box Protein.** *RAE1* has eight exons and seven introns and encodes a peptide of 665 amino acids. BLAST searches revealed that *RAE1* encodes an uncharacterized member of the F-box protein superfamily involved in protein degradation through the 26S proteasome pathway (15). Domain analysis predicted that RAE1 has an F-box domain at the N-terminal side (*SI Appendix*, Fig. S4*B*) and 18 LRRs at the C-terminal region that presumably participate in protein interaction and substrate recognition. RAE1 is conserved in land plants and has putative orthologs in both dicots and monocots and also in the moss *Physcomitrella patens* (*SI Appendix*, Fig. S4*C*).

To determine the subcellular localization of RAE1, we made a translational fusion between *RAE1* and *GFP* and introduced the construct into *Arabidopsis* protoplasts. Observation of GFP fluorescence revealed that, whether GFP was fused to the C- or to the N-terminal of RAE1, RAE1-dependent fluorescence overlapped with the signal of Hoechst 33342 nuclear staining dye (*SI Appendix*, Fig. S4*D*), indicating that RAE1 is localized to the nucleus.

**Expression Pattern of *RAE1*.** *RAE1* was expressed in roots as well as in leaves, stems, flowers, and young siliques (Fig. 3*A*). The expression of *RAE1* in roots was induced by Al stress (Fig. 3*B*). *RAE1* expression was significantly higher in the *rae1-1* mutant than in the WT (Fig. 3*B*).

To determine the tissue-specific expression pattern of *RAE1*, we generated *pRAE1:GUS* transgenic lines. GUS expression was found throughout roots, including both root tips and basal roots, and increased in roots of Col-0 WT background in response to Al treatment (Fig. 3 *C* and *D*). In root tips, GUS activity was detected in both epidermal cells and phloem tissues, and Al treatment increased GUS expression in the epidermal cells and the central vascular tissue (Fig. 3 *D* and *E*). In the mature root zone, GUS expression was mainly confined to the central vascular tissue, and its expression was also induced by Al (Fig. 3 *D* and *E*). These results indicate that regulation of *RAE1* expression by Al occurs at the transcriptional level. GUS activity was also evident in vascular tissues of rosette leaves, cauline leaves, flower tissues, and siliques (Fig. 3 *F*–*H*).

**STOP1 Directly Modulates *RAE1* Expression.** According to a recent study, STOP1 is able to bind to the *RAE1* promoter region (−373 bp to −342 bp from ATG) (25). We therefore determined whether mutation of *STOP1* affected *RAE1* expression. The expression level of *RAE1* was significantly lower in *stop1-2* than in the WT, and the Al-triggered expression of *RAE1* in the WT was fully depressed in *stop1-2* (Fig. 3*B*). We also introduced the *pRAE1:GUS* reporter gene into the *stop1-2* mutant background through crossing to examine the effect of the *stop1-2* mutation on GUS expression. GUS expression in the absence of Al treatment was clearly lower in the mutant than in the Col-0 WT (Fig. 3 *C* and *D*), and the Al-induced expression of GUS in the Col-0 WT was completely suppressed in the *stop1-2* mutant.

To determine whether STOP1 directly controls *RAE1* transcription, we fused a WT or mutated *RAE1* promoter to *LUC* and then transiently coexpressed each construct with or without *35S:STOP1* in *stop1-2* protoplasts (Fig. 3*J*). The mutated *RAE1* promoter harbored mutated nucleotides on the potential binding sites of STOP1 (Fig. 3*J*). The LUC signal was stronger with coexpression of *pRAE1:LUC* and *35S:STOP1* than with expression of *pRAE1:LUC* alone (Fig. 3*J*). Although *35S:STOP1* also promoted the LUC expression of *mpRAE1:LUC*, the LUC signal was lower in *mpRAE1:LUC* than in *pRAE1:LUC* (Fig. 3*J*). We also performed electrophoretic mobility shift assay (EMSA) experiments to determine whether STOP1 can directly bind to the *RAE1* promoter. The results showed that STOP1 was able to bind to biotin-labeled wild-type DNA probes, whereas the binding capacity of STOP1 on mutated DNA probes was greatly diminished (Fig. 3*K*). Application of excess unlabeled DNA was able to exclude the binding. Together, these data demonstrate

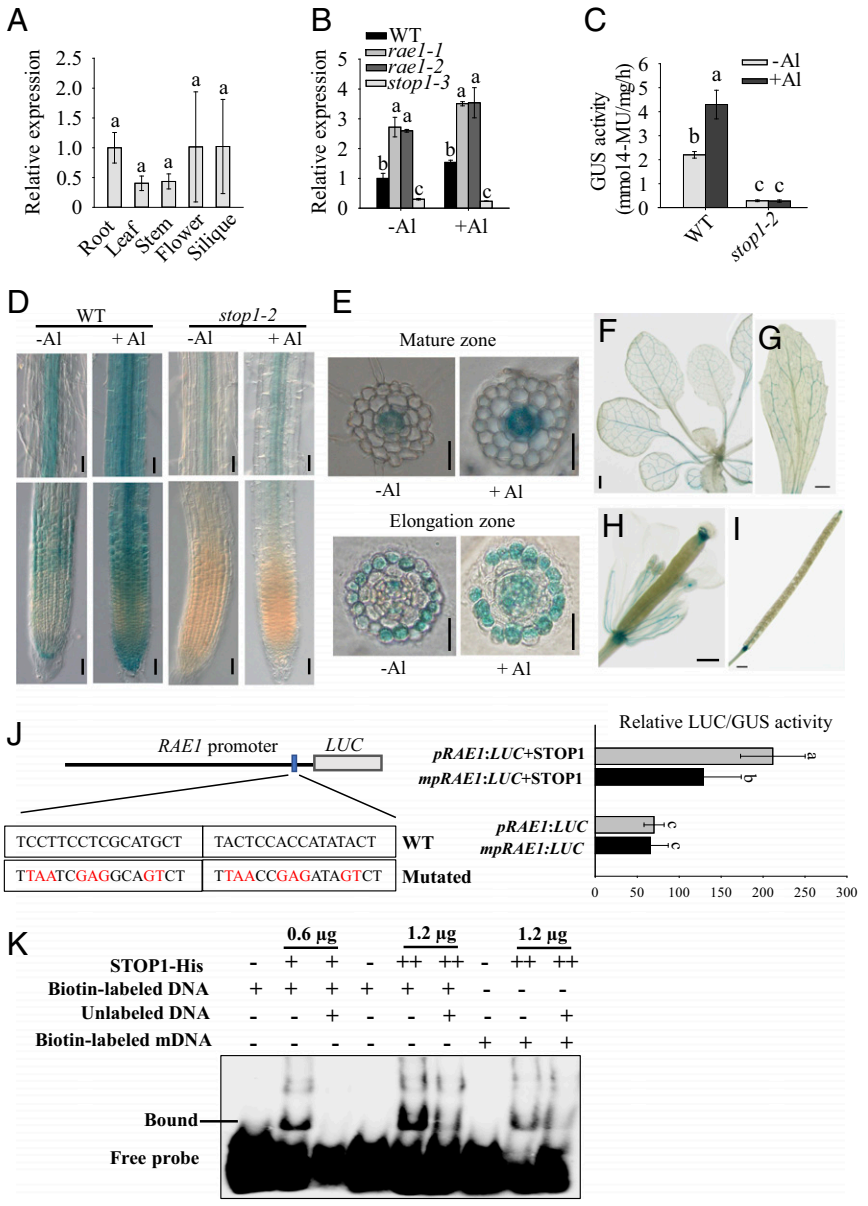


**Fig. 3.** Regulation of *RAE1* expression by STOP1. (*A*) Expression analysis of *RAE1* in roots, leaves, stem, flowers, and siliques. (*B*) Effect of Al stress and *rae1* mutations on *RAE1* expression. Expression analysis showed that *RAE1* expression is induced by Al and by the *rae1-1* and *rae1-2* mutations but is suppressed by the *stop1-2* mutation. (*C–I*) GUS expression of *pRAE1:GUS* in Col-0 WT and *stop1-2* with and/or without Al treatment. (*C*) Quantitative data of GUS expression in WT and *stop1-2*. (*D* and *E*) Longitudinal views (*D*) or cross-section observations (*E*) of GUS expression in root tips and basal root regions. GUS activity was also detected in rosette leaves (*F*), cauline leaves (*G*), flowers (*H*), and young siliques (*I*). (Scale bars: 50 μm in *D* and *E* and 1 mm in *F–I*.) (*J* and *K*) STOP1 binds to the *RAE1* promoter in *Arabdiopsis* protoplast expression systems (*J*) and EMSA assays (*K*). Two sites of the *RAE1* promoter (−373 to −342 bp from ATG) potentially targeted by STOP1 were indicated. For protoplast experiments, the WT (*pRAE1*) or mutated *RAE1* (*mpRAE1*) promoter was fused to *LUC* reporter gene and cotransformed with or without *35S:STOP1-2×FLAG* into *stop1-2* protoplasts. *pZmUBQ:GUS* was used as a control and was also cotransformed. Relative LUC/GUS activity was measured. For EMSA assays, the binding sites with WT or mutated versions were labeled with biotin and coincubated with 0.6 or 1.2 μg purified His-tagged STOP1 proteins for the assays. Values are means ± SD (*A–C*, $n = 3$; *J*, $n = 6$). Means with different letters are significantly different ($P < 0.05$, Duncan's multiple-range test).

that STOP1 positively modulates the expression of *RAE1* by directly binding to the *RAE1* promoter region.

**RAE1 Interacts with STOP1.** Because the expression of STOP1-regulated genes was induced in *rae1* mutants, and because *RAE1* expression was controlled by STOP1, we hypothesized that RAE1 might interact with STOP1 and regulate its stability. To test this hypothesis, we first conducted pull-down assays using GST or His fusions of RAE1 and STOP1. The results showed that GST-STOP1 specifically pulled down RAE1-His, and that GST-RAE1 could also specifically pull down STOP1-His (Fig. 4*A*), indicating that RAE1 is able to directly interact with STOP1 in vitro.

To determine whether RAE1 can interact with STOP1 in planta, we conducted split luciferase complementation assays (26). We detected the LUC signal with the combination of cLUC–rae1-1 and STOP1-nLUC but not with the combination of cLUC-RAE1 and STOP1-nLUC (Fig. 4*B*). The lack of the LUC signal in the combination of cLUC-RAE1 and STOP1-nLUC might be attributed to the functional RAE1-mediated

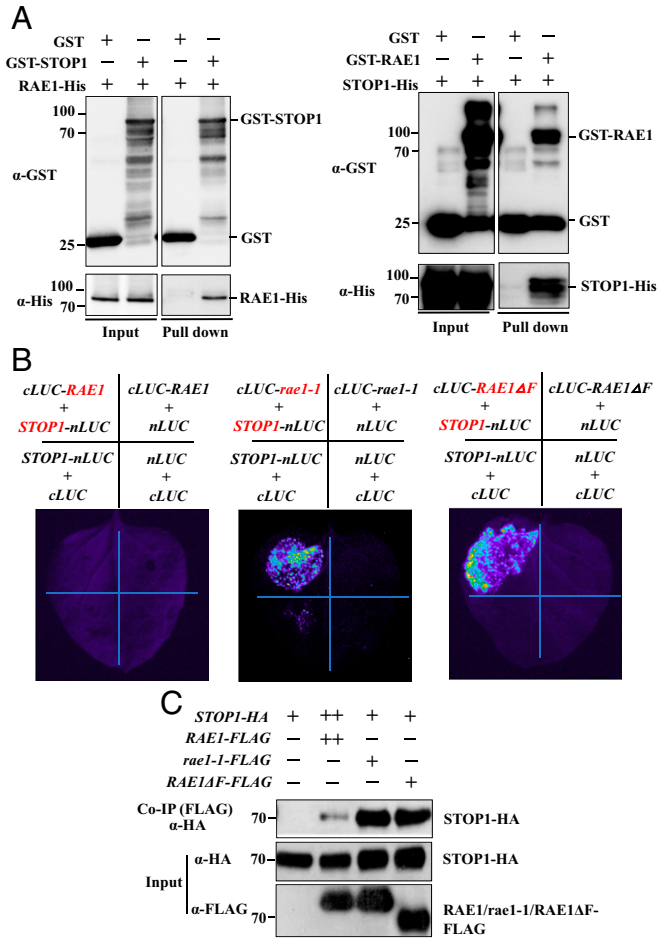


**Fig. 4.** Interaction of RAE1 with STOP1. (*A*) In vitro interaction of RAE1 with STOP1. His-tagged RAE1 was incubated with immobilized GST-STOP1 or GST proteins, and His-tagged STOP1 was incubated with immobilized GST-RAE1 or GST proteins. Bound proteins were detected by immunoblot using anti-His and anti-GST antibodies. (*B*) Interaction of RAE1 with STOP1 as indicated by split luciferase complementation assays. CaMV 35S promoter-driven construct pairs indicated in the figure were coexpressed in *N. benthamiana* leaves, and then the interaction-dependent luciferase activity was determined. (*C*) Coimmunoprecipitation of RAE1 with STOP1. FLAG-tagged WT or mutated RAE1 was coexpressed with HA-tagged STOP1 in *Arabidopsis* protoplasts. Crude proteins were immunoprecipitated using anti-FLAG antibody and then detected with HA antibody.

degradation of STOP1-nLUC, whereas dysfunctional cLUC–rae1-1 was unable to promote the degradation of STOP1-nLUC and hence the interaction-dependent LUC signal could be observed. To exclude the possibility that the interaction of STOP1 is specific to the rae1-1 variant, we coexpressed F-box domain-deleted RAE1 (cLUC-RAE1ΔF) with STOP1-nLUC. The result showed that cLUC-RAE1ΔF could also interact with STOP1-nLUC (Fig. 4*B*). Similarly, bimolecular fluorescence complementation (BiFC) assays revealed an interaction-dependent fluorescence signal between cYFP-RAE1ΔF and STOP1-nYFP, but not between cYFP-RAE1 and STOP1-nYFP (*SI Appendix*, Fig. S6). As a final test, we conducted coimmunoprecipitation (Co-IP) assays for RAE1 and STOP1 in *Arabidopsis* protoplasts. The results showed that FLAG-RAE1, FLAG–rae1-1, or FLAG-RAE1ΔF could all coimmunoprecipitate with STOP1-HA (Fig. 4*C*), although the coimmunoprecipitation was much weaker for WT RAE1 with STOP1 than for mutated RAE1 with STOP1. Together, these data confirm that RAE1 and STOP1 interact in planta. The finding that interactions between STOP1 and RAE1 are only stable with ligase-inactive forms of RAE1 supports the idea that the E3 ligase RAE1 may promote degradation of STOP1.

**Both RAE1 and Al Stress Regulate STOP1 Accumulation.** To investigate whether RAE1 regulates STOP1 expression, we generated native promoter-driven *STOP1-HA* transgenic lines. The *pSTOP1:STOP1-HA* construct complemented the Al-hypersensitive phenotype of *stop1-2* (*SI Appendix*, Fig. S7), indicating that the tagged protein is functional in vivo. We selected a single-copy homozygous transgenic line and introduced the tagged gene into the *rae1-1* mutant background through crossing. Western blot analysis showed that Al stress triggered STOP1 accumulation in both the WT and *rae1-1* (Fig. 5*A*), and that STOP1 levels were higher in *rae1-1* than in the WT under both conditions.

To further validate the effect of Al on the accumulation of STOP1, we generated *pSTOP1:GUS* and *pSTOP1:STOP1-GUS* transgenic lines. We found that GUS activity in the *pSTOP1:GUS* transgenic line was detected in whole roots and was not affected by Al stress (Fig. 5 *B* and *C*), which is consistent with previous reports (11, 27). Consequently, GUS activity in response to Al stress in the *pSTOP1:STOP1-GUS* transgenic lines can be expected to reflect protein levels. We found that GUS activity in the *pSTOP1:STOP1-GUS* transgenic line was enhanced by Al treatment and was greatest at the root tip (Fig. 5 *D* and *E*). We then introduced *pSTOP1:STOP1-GUS* into the *rae1-1* background by crossing. GUS activity was greater in *rae1-1* than in the WT under both −Al and +Al conditions (Fig. 5 *D* and *E*). These results indicate that STOP1 accumulation is positively regulated by Al stress and negatively regulated by RAE1.

**RAE1 Promotes STOP1 Degradation via the Ubiquitination-26S Proteasome Pathway.** To determine whether STOP1 is degraded by the 26S proteasome pathway, we treated roots of the *pSTOP1:STOP1-HA* transgenic line with or without MG132, a specific inhibitor of the 26S proteasome. STOP1 accumulation was clearly increased after exposure to MG132 for 1 h (Fig. 6*A*). We also treated the roots with MG132 in combination with Al stress. The results showed that MG132 and Al stress synergistically promoted STOP1 accumulation (Fig. 6*B*). This phenomenon was further confirmed in the *pSTOP1:STOP1-GUS* transgenic line in which MG132 and Al stress had additive effects in the promotion of GUS activity

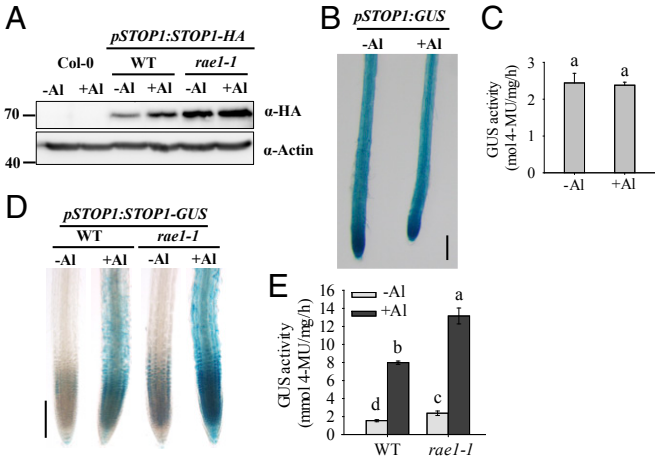


**Fig. 5.** Effect of Al stress and *rae1-1* mutation on STOP1 accumulation. (*A*) Western blot analysis of STOP1-HA in WT and *rae1-1* under −Al or +Al conditions. Actin protein was used as an internal control. (*B–E*) Images (*B* and *D*) and quantitative data (*C* and *E*) of GUS expression in *pSTOP1:GUS* (*B* and *C*) and *pSTOP1:STOP1-GUS* (*D* and *E*) transgenic lines. Values are means ± SD ($n = 3$). Means with different letters are significantly different ($P < 0.05$, Duncan's multiple-range test). (Scale bar: 200 μm.)

(Fig. 6 *C* and *D*). These results suggest that STOP1 is regulated by the 26S proteasome-degradation pathway.

Because RAE1 is an F-box protein that can interact with SKP1 to form a functional SCF-type E3 ligase (15), we tested the interaction of RAE1 with the *Arabidopsis* SKP1 homologs ASK1, ASK4, and ASK10. Split-LUC complementation assays in tobacco leaves revealed that RAE1 is able to interact with all three SKP1 homologs in planta (*SI Appendix*, Fig. S8), suggesting that RAE1 forms SCF complexes in *Arabidopsis*. To prove that RAE1 mediates the degradation of STOP1, we coexpressed *STOP1* with WT or mutated *RAE1* at different levels in *Arabidopsis* protoplasts. Increasing WT RAE1 expression promoted the degradation of STOP1 (Fig. 6*E*). In contrast, increasing the expression of rae1-1 or RAE1ΔF did not reduce the accumulation of STOP1 (Fig. 6*E*). These data indicate that functional RAE1 promotes STOP1 degradation.

To determine whether STOP1 can be ubiquitinated in vivo, we coexpressed *STOP1* with *UBQ10* in *Arabidopsis* protoplasts. Ubiquitinated forms of STOP1 were detected in the protoplasts (Fig. 6*F*), suggesting that STOP1 can be ubiquitinated in vivo. To determine whether RAE1 mediates the ubiquitination of STOP1, WT or mutated *RAE1* was coexpressed with *STOP1* and *UBQ10* in protoplasts, which were then treated with MG132 to inhibit the degradation of STOP1. The results showed that expression of WT RAE1 promoted the accumulation of the ubiquitinated forms of STOP1 (Fig. 6*G*), whereas expression of mutated RAE1 did not (Fig. 6*G* and *SI Appendix*, Fig. S9). These results indicate that STOP1 can be ubiquitinated by RAE1 in vivo.

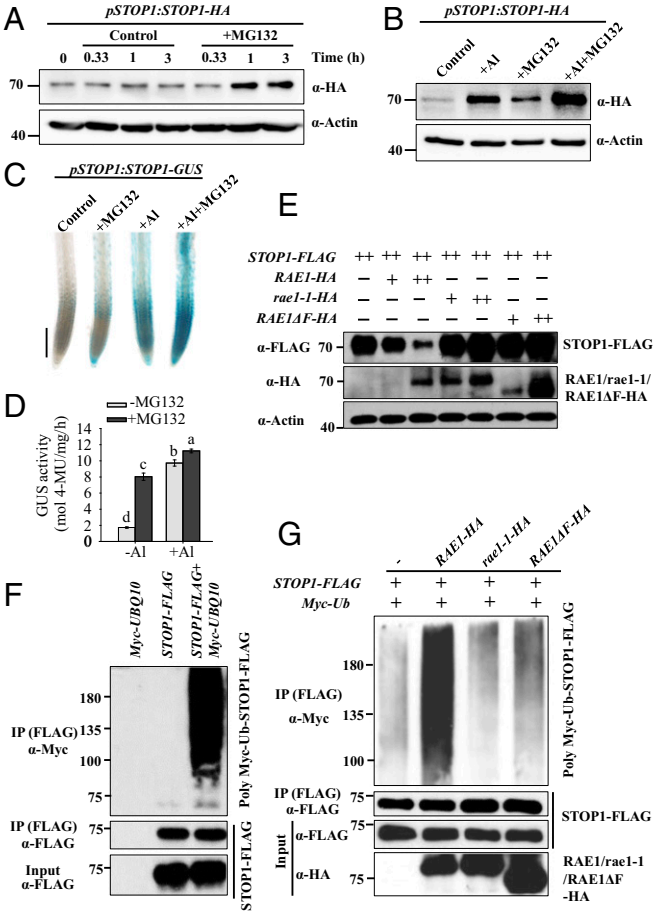


**Fig. 6.** RAE1 mediates STOP1 degradation through the ubiquitin-26S proteasome pathway. (*A*) Effect of MG132 on STOP1 accumulation. Roots of 4-wk-old *pSTOP1:STOP1-HA* plants were treated with 0 or 50 μM MG132 for 0.33, 1.0, or 3.0 h before STOP1-HA protein was detected using anti-HA antibody. (*B–D*) Effect of Al and MG132 on STOP1-HA accumulation (*B*) or STOP1-GUS accumulation (*C* and *D*). *pSTOP1:STOP1-HA* or *pSTOP1:STOP1-GUS* plants were treated with Al and/or MG132 for 12 h. Values are means ± SD ($n = 3$). Means with different letters are significantly different ($P < 0.05$, Duncan's multiple-range test). (Scale bar: 200 μm.) (*E*) RAE1 promotes STOP1 degradation. The *35S:STOP1-FLAG* was coexpressed with different amounts of *35S:RAE1-HA* or *35S:rae1-1-HA* or *35S:RAE1ΔF-HA* in *Arabidopsis* protoplasts. (*F* and *G*) RAE1 mediates ubiquitination of STOP1. (*F*) Ubiquitinated forms of STOP1 were detected in *Arabidopsis* protoplasts that coexpressed *35S:Myc-UBQ10* and *35S:STOP1-FLAG*. (*G*) RAE1 promotes ubiquitination of STOP1. Protoplasts were transfected with *35S:Myc-UBQ10*, *35S:STOP1-FLAG*, and with *35S:RAE1-HA*, *35S:rae1-1-HA*, or *35S:RAE1ΔF-HA*. The protoplasts were treated with 50 μM MG132 for 8 h before harvest. Crude lysates (input) were immunoprecipitated with anti-FLAG antibody and then the immunoprecipitates were detected with anti-Myc antibody.

**Overexpression of *RAE1* Reduces STOP1 Accumulation and *AtALMT1* Expression.** To further substantiate the role of RAE1 in the regulation of STOP1 stability, we generated transgenic lines overexpressing *RAE1* (Fig. 7*A*). The expression of the STOP1-regulated genes, *AtALMT1*, *AtMATE1*, and *ALS3*, was significantly reduced in the overexpression lines under both −Al and +Al conditions in comparison with that in the Col-0 WT (Fig. 7 *B–D*), whereas the expression of other Al-resistance genes *AtSTAR1*, *ALS1*, and *STOP1*, which are not regulated by STOP1, was not affected by the *RAE1* overexpression (*SI Appendix*, Fig. S10). These results suggest that STOP1 protein expression might be compromised in the overexpression lines. We utilized F1 plants derived from the crosses between *RAE1* overexpression lines and the *pSTOP1:STOP1-HA* line to examine the effect of *RAE1* overexpression on STOP1 levels. Compared with the Col-0/*pSTOP1:STOP1-HA* control, *RAE1-OX/pSTOP1:STOP1-HA* plants accumulated significantly lower levels of STOP1 under both −Al and +Al conditions (Fig. 7*E*), indicating that overexpression of *RAE1* reduces STOP1 levels.

We then investigated the phenotype of Al resistance and low Pi response in the *RAE1* overexpression lines. In accordance with reduced expression of STOP1, the overexpression lines showed increased sensitivity to Al (Fig. 7 *F* and *G*) and decreased sensitivity to low Pi-induced inhibition of root growth compared with the Col-0 WT (Fig. 7 *H* and *I*). These results indicate that overexpression of *RAE1* reduces Al resistance and enhances the tolerance of root growth to low Pi.

## Discussion

Unlike wheat *TaALMT1* whose expression is relatively constant (9), *AtALMT1* expression is regulated by multiple stresses, including Al (10). Our *pAtALMT1:LUC* reporter gene system enabled us to identify upstream factors involved in the regulation of *AtALMT1* expression. With this screening system, we identified a number of mutants in which the expression of LUC is altered. Among these mutants, *rae1* mutants were overrepresented, highlighting the important role of *RAE1* in the regulation of *AtALMT1* expression. *RAE1* encodes an uncharacterized F-box protein that is presumed to be involved in the ubiquitin-26S proteasome pathway (15). Based on the following evidence, we propose that RAE1 mediates STOP1 degradation via ubiquitination: (*i*) RAE1 can interact with STOP1 and *Arabidopsis* SKP1 of SCF-type E3 ligases; (*ii*) coexpression of *RAE1* with *STOP1* promotes STOP1 ubiquitination and degradation, and inhibition of 26S proteasome function can stabilize STOP1; (*iii*) the STOP1 protein level is increased in *rae1* mutants and decreased in *RAE1* overexpression lines; and (*iv*) mutation of *RAE1* specifically induces the expression of STOP1-downstream genes.

We demonstrated that an F-box protein is important for the regulation of metal stress resistance. RAE1 belongs to the LRR-type F-box subfamily, whose C-terminal LRR domains are involved in the interaction with substrates (16). Unexpectedly, we

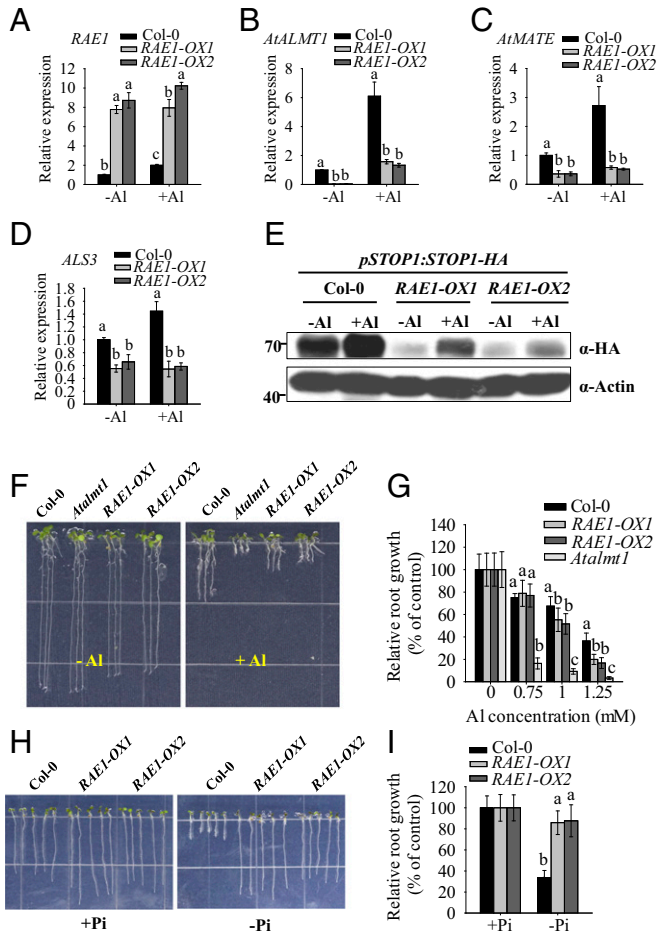


**Fig. 7.** Phenotypic analysis of *RAE1* overexpression lines. (*A–D*) Expression analysis of *RAE1* (*A*), *AtALMT1* (*B*), *AtMATE* (*C*), and *ALS3* (*D*) in roots of Col-0 wild-type and two *RAE1* overexpression lines under both −Al and +Al conditions. Values are means ± SD ($n = 3$). (*E*) Effect of *RAE1* overexpression on STOP1 accumulation. Roots of F1 plants from Col-0/*pSTOP1:STOP1-HA*, *RAE1-OX1*/*pSTOP1:STOP1-HA*, and *RAE1-OX2*/*pSTOP1:STOP1-HA* treated with or without Al were subjected to Western blot analysis of STOP1-HA. Actin protein was used as an internal control. (*F* and *G*) Images (*F*) and quantitative data (*G*) of decreased Al resistance in the two *RAE1* overexpression lines. Values are means ± SD ($n = 20–25$). (*H* and *I*) Images (*H*) and quantitative data (*I*) of reduced root growth inhibition in response to low Pi in the two *RAE1* overexpression lines. Values are means ± SD (*A–D*, $n = 3$; *G* and *I*, $n = 15–20$). Means with different letters are significantly different ($P < 0.05$, Tukey test).

found that mutation in the LRR domains of RAE1 in *rae1-1* did not influence its interaction with the substrate STOP1 but instead affected the ubiquitination of STOP1, suggesting that LRR domains might be important for the transfer of ubiquitin to substrates. Like STOP1, which has orthologs in all land plants (28), RAE1 has putative orthologs in dicots, monocots, and mosses. Given that the functioning of STOP1 orthologs in the control of Al resistance is evolutionarily conserved in plants (28), it is likely that RAE1 orthologs might have a conserved function in the regulation of Al resistance via the modulation of the stability of STOP1 orthologs.

We provide evidence that Al stress triggers STOP1 protein accumulation. The question remains, how do plant cells perceive the Al stress signal and then stabilize STOP1? Although the components involved in the perception of Al stress signal are unknown, we propose two possible explanations for how Al stress regulates STOP1 stability: Al-dependent signaling events may directly inhibit RAE1-mediated STOP1 degradation, or the signaling events phosphorylate or otherwise modify the STOP1 protein so that it is not degraded by RAE1 (Fig. 8). Because Al stress induces *RAE1* expression, it is unlikely that Al stress promotes STOP1 accumulation through the regulation of *RAE1* expression. In addition, Al stress and the 26S proteasome inhibitor MG132 additively increase STOP1 accumulation. We therefore doubt that Al stress directly inhibits RAE1-mediated STOP1 degradation. Phosphorylation-dependent signaling cascades are well documented to modulate the stability of transcription factors involved in cold tolerance (29–31). Future research should determine whether Al stress initiates a phosphorylation-dependent cascade to regulate STOP1 phosphorylation and stability.

In addition to the regulation of Al resistance, we discovered that RAE1 also regulates low Pi response. Low Pi availability is a common abiotic stress that limits plant growth and yield production in many natural and agricultural ecosystems. In response to Pi deficiency, plants can modify root system structure, i.e., the reduction of primary root growth and the increase of the number and length of lateral roots, to enhance Pi uptake (32–34). Recently, malate release mediated by the STOP1-ALMT1 pathway has been demonstrated to promote primary root growth inhibition via the facilitation of iron (Fe) accumulation into the apoplast of root tip regions under Pi-deficient conditions (22, 23). The accumulation of apoplastic Fe triggers peroxide-dependent cell wall stiffening and consequently inhibits root growth. Consistently, the increased malate release in *rae1* mutants due to the elevated expression of *AtALMT1* might facilitate the accumulation of apoplastic Fe, which then enhances the inhibition of root growth

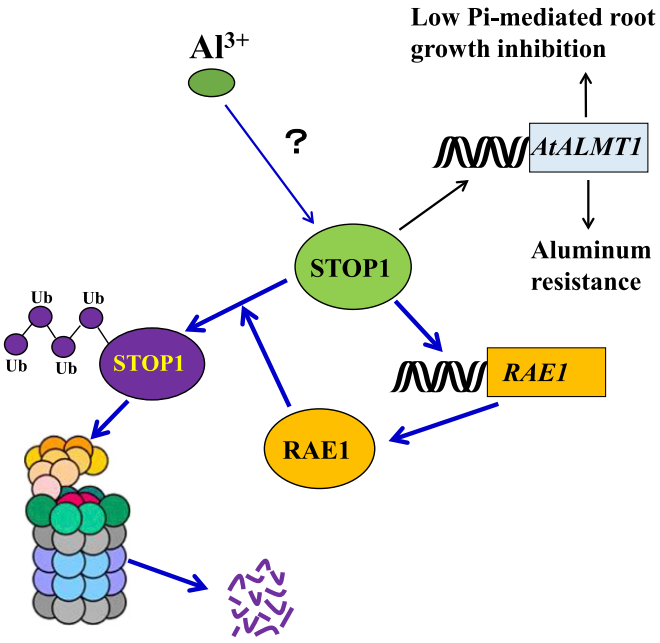


**Fig. 8.** Model for regulation of STOP1 stability by RAE1 and Al stress. Al ($Al^{3+}$) in acid soils stabilizes STOP1, which regulates Al resistance and low Pi-induced root growth inhibition mainly by regulating *AtALMT1* expression. AtALMT1 mediates Al resistance and low Pi response through the secretion of malate to chelate and detoxify Al. The F-box protein RAE1 interacts with STOP1 and promotes STOP1 ubiquitination (Ub) and the ubiquitinated STOP1 is then degraded by the 26S proteasome system. Because STOP1 also promotes *RAE1* transcription by directly binding to the *RAE1* promoter, there is a negative feedback loop between STOP1 and RAE1. Al stress increases STOP1 accumulation by unidentified mechanisms (?). The increases in STOP1 accumulation might be responsible for Al-triggered *AtALMT1* and *RAE1* expression. Blue arrows indicate the processes discovered in the current research, while black arrows indicate the processes determined by previous studies.

under Pi-deficient conditions. Conversely, reduced *AtALMT1* expression in the *RAE1* overexpression lines might reduce apoplastic Fe accumulation and consequently decrease the sensitivity to low Pi. Our results thus demonstrate that RAE1 is also important for the modulation of low Pi response.

*RAE1* expression, which is induced by Al stress, is dependent on STOP1. We found that STOP1 directly controls *RAE1* transcription. Therefore, STOP1 and RAE1 form a negative feedback loop to regulate STOP1 accumulation (Fig. 8). This feedback loop might be important for maintaining STOP1 homeostasis. The increased accumulation of RAE1 under Al stress enables plants to rapidly degrade overaccumulated STOP1 and to attenuate Al-resistance responses once the Al stress is removed. A similar negative-feedback, regulatory mechanism has been well documented in plant hormone signaling pathways (17, 35–37). Finally, because mutation of *RAE1* increases Al resistance in *Arabidopsis*, it would be interesting to investigate whether mutation of *RAE1* homologs can enhance Al resistance in crops.

## Materials and Methods

**Plant Materials, Mutant Library Construction, and Screening.** The *AtALMT1* promoter was fused to a *LUC* and then transformed into *Arabidopsis* ecotype Col-0 to generate the *pAtALMT1:LUC* reporter line. The T-DNA insertion line *stop1-2* (SALK_114108) was obtained from the *Arabidopsis* Biological Resource Center. Detailed information on mutant library construct and screening is provided in *SI Appendix, SI Materials and Methods*.

**Evaluation of Sensitivity to Al and Low Pi.** A soaked gel medium was used to evaluate Al sensitivity. An agar medium with or without the addition of Pi was used to evaluate low Pi-induced root growth inhibition. See *SI Appendix, SI Materials and Methods*.

**Determination of Malate Secretion and Al Content.** The malate concentration in the root exudates was determined by the NAD/NADH enzymatic cycling method and the concentration of Al was determined by inductively coupled plasma mass spectrometry. See *SI Appendix, SI Materials and Methods*.

**Cloning of *RAE1*.** *RAE1* was cloned by the mapping-by-sequencing method combined with marker linkage analysis and by complementation test. See *SI Appendix, SI Materials and Methods*.

**Phylogenetic Analysis.** Phylogenetic analysis was carried out with the MEGA5.0 program (megasoftware.net). The neighbor-joining method was used to construct the phylogenetic tree with 1,000 bootstrap trials by MEGA5.0.

**RNA Isolation and Quantitative RT-PCR.** Standard protocols for RNA isolation and qRT-PCR were performed. See *SI Appendix, SI Materials and Methods*.

**Subcellular Localization Analysis and BiFC Assay.** Vector construct and gene expression in *Arabidopsis* protoplasts for subcellular localization and BiFC assays are described in detail in *SI Appendix, SI Materials and Methods*.

**GUS Analysis.** Vector construct and GUS analysis for *pAtALMT1:GUS*, *pRAE1:GUS*, *pSTOP1:GUS*, or *pSTOP1:STOP1-GUS* transgenic lines are described in detail in *SI Appendix, SI Materials and Methods*.

**Determination of Protein Accumulation in Roots.** Western blot analysis was used to detect the STOP1-3×HA protein level. See *SI Appendix, SI Materials and Methods*.

**EMSA Assay.** Details on EMSA assay are provided in *SI Appendix, SI Materials and Methods*.

**Pull-Down Assay.** Details on vector construct and pull-down assay are provided in *SI Appendix, SI Materials and Methods*.

**Split-LUC Complementation Assays.** Details on vector construct and gene transformation in *Nicotiana benthamiana* leaves are provided in *SI Appendix, SI Materials and Methods*.

**Transient Expression in Protoplasts.** The assays for STOP1 regulation on RAE1 expression, Co-IP, and ubiquitination of STOP1 are described in detail in *SI Appendix, SI Materials and Methods*.

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