RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in *Arabidopsis*

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Soil salinity limits agricultural production and is a major obstacle for feeding the growing world population. We used natural genetic variation in salt tolerance among different *Arabidopsis* accessions to map a major quantitative trait locus (QTL) for salt tolerance and abscisic acid (ABA) sensitivity during seed germination and early seedling growth. A recombinant inbred population derived from Landsberg *erecta* (L*er*; salt and ABA sensitive) × Shakdara (Sha; salt and ABA resistant) was used for QTL mapping. High-resolution mapping and cloning of this QTL, *Response to ABA and Salt 1 (RAS1)*, revealed that it is an ABA- and salt stress-inducible gene and encodes a previously undescribed plant-specific protein. A premature stop codon results in a truncated RAS1 protein in Sha. Reducing the expression of *RAS1* by transfer-DNA insertion in Col or RNA interference in L*er* leads to decreased salt and ABA sensitivity, whereas overexpression of the L*er* allele but not the Sha allele causes increased salt and ABA sensitivity. Our results suggest that RAS1 functions as a negative regulator of salt tolerance during seed germination and early seedling growth by enhancing ABA sensitivity and that its loss of function contributes to the increased salt tolerance of Sha.

abscisic acid response | germination | natural variation | quantitative trait locus

About 20% of irrigated agricultural land is affected by salinity, and 10 million ha of the land is abandoned each year because of salinity (1). Despite this, crop production must be increased by 50% by 2025 to meet the demand of a burgeoning population (2). Soil salinity causes both ion toxicity and osmotic stress, and plants have evolved complex salt tolerance mechanisms (3). The salt overly sensitive (SOS) pathway of salt tolerance is crucial for maintaining ion homeostasis under salt stress. This pathway is conserved across diverse plant species, including rice (3, 4). The SOS2 ser/thr protein kinase regulates both SOS1-mediated Na^+ extrusion from the cytosol and vacuolar Na^+/H^+ antiporter-mediated Na^+ sequestration into the vacuole (5, 6). The high-affinity K^+ transporter (HKT) type transporters are also important for Na^+ homeostasis under salt stress. A genetic screen for second-site suppressor mutations of *sos3* led to the identification of the gene *AtHKT1;1*/*AtHKT1* (7). AtHKT1;1 was also identified from a genetic screen for mutants that overaccumulate Na^+ in the shoot (8), and it has been shown to contribute to natural variations for Na^+ accumulation in the shoot (9).

Natural variations in plants can help to unravel the molecular basis of agronomically important traits. These natural variations are often complex and require quantitative trait locus (QTL) analysis. Using natural variations among *Arabidopsis* accessions, QTLs have been cloned for a number of traits such as flowering time, seed dormancy, light response, biomass production, hybrid incompatibility, embryogenesis, root growth, low phosphate response, copper toxicity, sulfate assimilation, and molybdate accumulation (10). Natural variation has also been used to map salt tolerance QTLs in *Arabidopsis* (11, 12) and in some crop plants (13). However, the underlying genes for only a few salt tolerance QTLs have been identified. Ren et al. (14) cloned and characterized the rice *SKC1* QTL for salt tolerance. *SKC1* encodes an HKT-type Na^+-selective transporter involved in unloading Na^+ from the xylem (14). In durum wheat, Na^+ exclusion was linked to the *Nax1* (Na^+ exclusion 1) and *Nax2* loci, which likely correspond to the Na^+ transporters HKT1;4 (*HKT7*) and HKT1;5 (*HKT8*), respectively (15, 16).

The plant stress hormone abscisic acid (ABA) regulates many genes involved in plant salt tolerance (3). Although considerable progress has been made in identifying genes encoding components of ABA response pathways by molecular cloning of induced variations, little is known about QTLs controlling ABA sensitivity in plants.

The *Arabidopsis* accessions Landsberg *erecta* (L*er*, NW20) and Shakdara (Sha, N929) show substantial phenotypic difference in their seed dormancy and tolerance to heat, salt, and osmotic stress. A recombinant inbred line (RIL) population obtained from a cross between the L*er* and Sha accessions has been used to map QTLs for seed dormancy and for heat, salt, and osmotic stress tolerance during germination (12). In the present study, we mapped a major QTL conferring salt tolerance and ABA insensitivity in seedlings using an RIL population from L*er* × Sha. Positional cloning of this *Response to ABA and Salt 1* (*RAS1*) QTL revealed a previously undescribed gene that is induced by ABA and salt stress. Genetic and molecular data from our study demonstrate that RAS1 is a negative regulator of salt tolerance and an important modulator of ABA sensitivity.

Results

Mapping of Salt Tolerance QTLs Using L*er* × Sha RILs. We evaluated the salt tolerance of the *Arabidopsis* accessions L*er* and Sha in Murashige–Skoog (MS) agar medium supplemented with NaCl and in soil irrigated with NaCl. Sha is more tolerant to NaCl stress than L*er* under both salt-stress conditions (Fig. 1*A*). To dissect this natural variation for salt tolerance by using a QTL approach, we employed L*er* × Sha RILs (12). Salt tolerance was measured in terms of two phenotypic traits, namely, the percentage of green seedlings (GSs) and root length (RL); these traits were measured on MS agar medium containing 120 mM NaCl. For the GS trait, one major QTL was detected on chromosome 1 (Table S1). The *GS1* QTL on chromosome 1 explained 76.6% of the total pheno-

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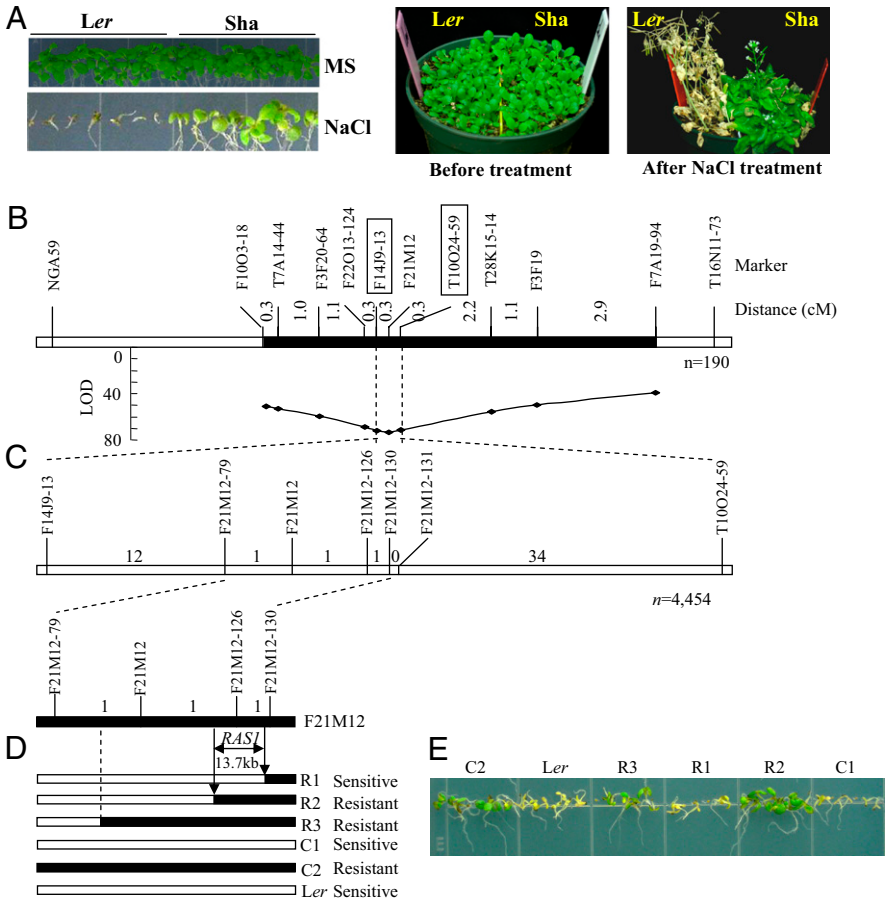


Fig. 1. Map-based cloning of *RAS1*. (*A*) Salt tolerance of the parents, L*er* and Sha accessions. Seedlings were germinated and grown on vertical MS agar medium (control) or MS supplemented with 120 mM NaCl for 2 weeks, or 2-week-old seedlings grown in soil (before treatment) were irrigated with 50 mM NaCl for 4 days, 150 mM NaCl for the next 3 days, and then 200 mM NaCl for 19 days. (*B*) Map location of *RAS1* in a small region (filled bar) on *Arabidopsis* chromosome 1 based on 190 BC_2F_2 plants. (*C*) High-resolution linkage map of the *RAS1* region produced with 4,454 BC_2F_2 plants. The number of recombinants between the adjacent markers is indicated above the linkage map. Filled bar shows a part of BAC clone F21M12. (*D*) Progeny testing of fixed recombinant plants (BC_2F_3) narrowed the *RAS1* locus to the region between markers F21M12 and F21M12-130. Two recombinant lines (R2 and R3) and control 2 (C2) homozygous for Sha in the target region were resistant to 120 mM NaCl. In contrast, one recombinant line (R1) and control 1 (C1) homozygous for L*er* in the target region were sensitive to 120 mM NaCl. Filled and open bars represent homozygous chromosomal segments for Sha and L*er*, respectively. (*E*) Salt-stress response phenotype of the recombinant lines on MS medium containing 120 mM NaCl.

typic variance (PVE). The additive effect of the Sha allele at this locus was very large for GSs and contributed to increasing GSs. For the RL trait, four QTLs were found on three chromosomes. Two *RL* QTLs were located on chromosome 1 and one each on chromosomes 3 and 4, respectively (Table S1). The PVE values of these QTLs were consistent with the coefficient of multiple determination (R^2) from ANOVA, suggesting that the putative QTLs were mapped close to specific markers (Table S1). The *RL1* QTL on chromosome 1 was localized at the same region as the *GS1* QTL. The additive effect of the Sha allele at the *RL1* locus was the highest and contributed to increasing RL. The results suggest that *GS1* and *RL1* may be the same QTLs involved in salt tolerance of *Arabidopsis*. Later experiments revealed that salt tolerance and ABA insensitivity cosegregate with the *GS1* locus; hence, the locus was renamed *RAS1*. We confirmed the salt tolerance function of *RAS1* by QTL mapping of the GS trait under 120 mM NaCl treatment in another RIL population from a Bay-0 × Sha cross (17). In this population, the *RAS1* QTL explained 89.4% of the total PVE, with a logarithm of odds (LOD) value of 45.5 (Fig. S1*A*).

Map-Based Cloning of the *RAS1* QTL. Because the *RAS1* QTL was identified as a major QTL conferring salt tolerance to *Arabidopsis* in both the L*er* × Sha and Bay-0 × Sha RIL populations, we chose this QTL for map-based cloning. To perform fine mapping, CS24560, an RIL derived from the L*er* × Sha cross, was selected. This line possessed the L*er* genetic background, except for chromosome 1 and a ≈9-cM region of the lower arm of chromosome 3 from Sha (Fig. S1*B*). Fine mapping in the F2 population derived from the CS24560 × L*er* cross placed the *RAS1* locus between the markers F14J9-13 and T10O24-59 on chromosome 1, where the QTL was mapped originally (Fig. 1*B*). Further high-resolution mapping of *RAS1* was carried out using 4,454 BC_2F_2 plants (Fig. 1*C*). *RAS1* was localized to a high-resolution linkage map by progeny testing of BC_2F_4 homozygous recombinant plants (Fig. 1 *D* and *E*). By sequencing two key recombinant plants, the *RAS1* locus was narrowed down to a 13.7-kb region between markers F21M12 and F21M12-130 (Fig. 1*D*). This region contains four predicted ORFs, namely, At1g09932 (phosphoglycerate/bisphosphoglycerate mutase-related), At1g09935 (phosphoglycerate/bisphosphoglycerate mutase family protein), At1g09940 (glutamyl-tRNA reductase), and At1g09950 (transcription factor-related). We sequenced this region from L*er* and Sha and then identified At1g09950 as the candidate gene for *RAS1*. Comparison of the nucleotide sequences of the Col-0, L*er*, and Sha alleles of *RAS1* revealed a premature stop codon in the C-terminal region of the Sha *RAS1* allele. In addition to the premature stop codon, the L*er*

and Sha alleles differ at 16 nucleotide positions, including 14 silent nucleotide substitutions and two nucleotide substitutions that result in two amino acid variations (Fig. S2*A*).

***RAS1* Near-Isogenic Line Exhibits Salt Tolerance and ABA Insensitivity.** To investigate the effects of *RAS1* on salt tolerance, we bred a near-isogenic line, NIL(*RAS1*), in the L*er* genetic background with a 4.9-cM region between markers F21M12 and F7A19-94 that contains the *RAS1* genomic region from Sha. NIL(*RAS1*) exhibited substantially higher salt tolerance and ABA insensitivity than the L*er* isogenic control (Fig. 2*A* and Fig. S2 *B* and *C*).

Complementation Analysis. To confirm that the *RAS1* gene is responsible for the GS phenotype under salt and ABA treatment, we performed complementation experiments. The F1 plants from a cross between L*er* and NIL(*RAS1*) exhibited NaCl and ABA sensitivity similar to that of L*er* (Fig. S3). This result suggests that the *RAS1* allele from L*er* is dominant. We cloned a 2.7-kb genomic fragment from L*er*, which contains the promoter, ORF, and 3′UTR of the *RAS1* gene, and introduced it into NIL(*RAS1*), Sha, and L*er*. Northern blot analysis showed greater *RAS1* expression in transgenic plants than in their respective untransformed parental genotypes (Fig. S4). The transgenic plants expressing L*er* *RAS1* in NIL (*RAS1*), Sha, and L*er* backgrounds showed hypersensitivity to NaCl and ABA relative to the nontransgenic control plants (Fig. 2 *B*–*D*).

ABA and NaCl Induce *RAS1* Expression. Northern blot analysis showed that *RAS1* expression was virtually undetectable under control conditions, but the expression was induced transiently by NaCl or ABA treatment (Fig. 3*A*). Although the *RAS1* expression level was somewhat lower in L*er*, the expression kinetics were similar among L*er*, NIL(*RAS1*), and Sha during the time course of NaCl or ABA treatment (Fig. 3*A*). The results suggest that the salt tolerance of NIL(*RAS1*) and Sha is probably not attributable to any difference in the expression level of *RAS1* but rather to differences in the RAS1 protein between L*er* and Sha (Fig. 2 and Fig. S2).

RAS1 Is a Negative Regulator of Salt Tolerance. Because the salt-tolerant Sha allele has a premature stop codon, we predicted that a loss of function, reduction, or knockdown of *RAS1* may increase the salt tolerance of *Arabidopsis*. To test this hypothesis, we obtained a transfer-DNA (T-DNA) insertion mutant (Salk_058470) for the *RAS1* candidate gene At1g09950 in the Col-0 background. The T-DNA insertion was found in the 5′ region at 113 bp upstream of the start codon of At1g09950. The T-DNA insertion substantially reduced *RAS1* expression (Fig. 3*B*). The T-DNA mutant showed enhanced salt tolerance and ABA-resistant seedling growth compared with Col-0 (Fig. 3*C*). This phenotype is similar to that of NIL(*RAS1*).

Results from the analyses of NIL(*RAS1*), the T-DNA mutant, and *RAS1* complementation lines suggested that *RAS1* is a negative regulator of salt tolerance and a positive regulator of ABA sensitivity. To confirm this further, we generated RNA interference (RNAi) lines of *RAS1* in L*er*, Sha, and NIL(*RAS1*). Northern blot analysis showed that *RAS1* expression was reduced in the RNAi lines (Fig. S5 *A*, *C*, and *E*). L*er* *RAS1* RNAi lines exhibited enhanced salt tolerance and

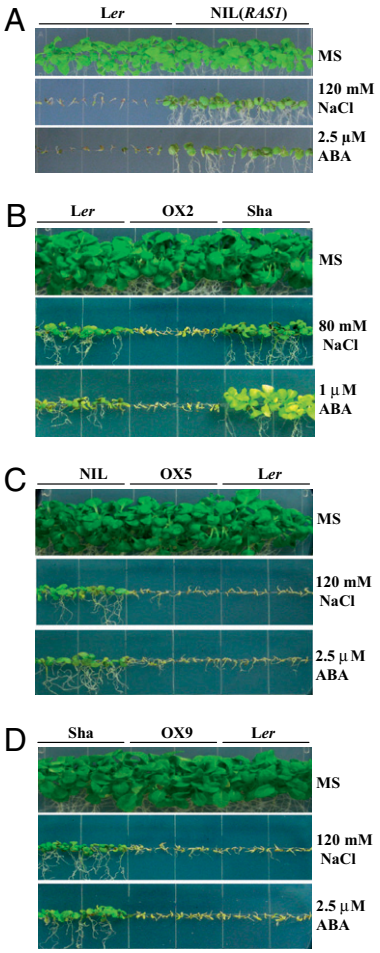


Fig. 2. Salt and ABA sensitivity of NIL(*RAS1*) and transgenic complementation lines. (*A*) Germination and seedling growth phenotype of NIL(*RAS1*) and L*er* on MS medium supplemented with 120 mM NaCl or 2.5 μM ABA after 14 and 45 days, respectively. Effect of ABA and NaCl on germination and early seedling growth of a representative transgenic (OX) L*er* (*B*), NIL(*RAS1*) (*C*), or Sha (*D*) line transformed with the L*er* *RAS1* genomic DNA (L*er*RAS1*g*).

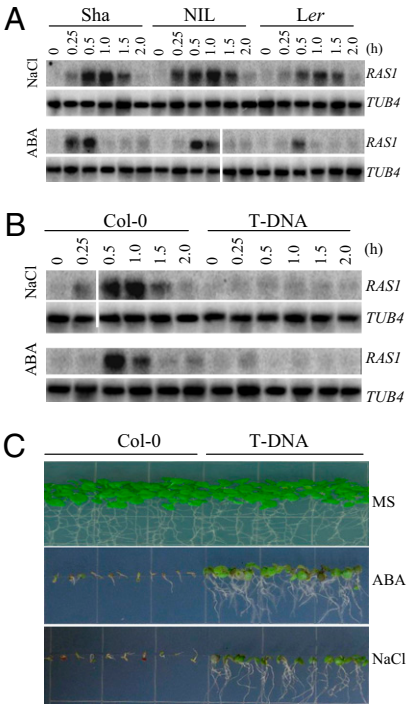
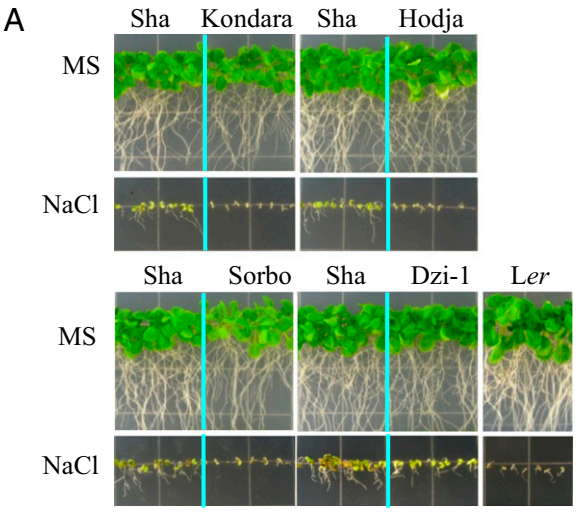


Fig. 3. Characterization of *RAS1* expression and T-DNA insertion mutant. (*A*) *RAS1* expression in Sha, NIL, and L*er*. (*B*) *RAS1* expression in the T-DNA line and its WT. Seedlings were treated with 150 mM NaCl or 100 μM ABA for 0–2 h. *TUB4* expression serves as the RNA loading control. (*C*) Germination and seedling growth of the T-DNA insertion mutant of *RAS1* on MS medium supplemented with 150 mM NaCl or 2.5 μM ABA.

reduced ABA sensitivity compared with WT L*er* plants (Fig. S5*B*). This phenotype of the L*er* RNAi line is consistent with that of the T-DNA knockdown mutant in the Col-0 background (Fig. 3). *RAS1* RNAi lines of NIL(*RAS1*) and Sha did not differ from their respective WTs in salt or ABA sensitivity (Fig. S5 *D* and *F*). In addition, L*er*, NIL (*RAS1*), Sha, the T-DNA mutant, and Col-0 plants were transformed with a construct in which the L*er RAS1* cDNA was driven by the strong CaMV 35S promoter. Overexpression of full-length L*er RAS1* conferred salt and ABA sensitivity to NIL(*RAS1*), Sha, and the T-DNA mutant plants. The salt and ABA sensitivity of Col-0 and L*er* was further increased by the *RAS1* overexpression (Fig. S6). In contrast, CaMV 35S promoter-driven overexpression of the C-terminally truncated Sha allele of *RAS1* or a mutated C-terminally truncated version of L*er* RAS1 (209 amino acids) in L*er*, Col, Salk_058470, and NIL(*RAS1*) did not affect salt and ABA responses (Fig. S7).

RAS1 Effects Require a Functional ABA Signaling Pathway. *RAS1* loss of function reduced ABA sensitivity, whereas its overexpression increased ABA sensitivity (Figs. 2 and 3 and Figs. S5, S6, and S7), suggesting that RAS1 is a positive regulator of ABA responses. The dominant *abi1-1* mutation blocks ABA signaling by disrupting the interaction between ABSCISIC ACID INSENSITIVE 1 (ABI1) and ABA receptors PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-like (PYLs) (18). To probe the genetic interaction between *abi1-1* and *RAS1*, we made reciprocal crosses between *abi1-1* and the ABA hypersensitive *RAS1*-overexpression line of L*er*. F1 plants of the *abi1-1* × L*er* reciprocal crosses exhibited NaCl and ABA responses similar to those of *abi1-1* (Fig. S8*A*). F1 plants obtained from reciprocal crosses between *abi1-1* and the *RAS1*-overexpression line also showed ABA insensitivity (Fig. S8*B*), suggesting that a functional ABA signaling pathway is required for the effects of the overexpressed RAS1.

Natural Variation in Salt Tolerance and *RAS1* Sequence Polymorphism. To investigate the association between *RAS1* sequence polymorphism and natural variation in salt tolerance among *Arabidopsis* accessions, we evaluated the salt tolerance of 38 accessions and compared their *RAS1* sequences. Phenotypic analysis showed that 5 of the accessions (i.e., Sha, Chi-0, CO-1, Litva, Wil-1) were tolerant to NaCl (Fig. S9). However, only the *RAS1* of Sha encodes a truncated protein, whereas the *RAS1* in the remaining 37 *Arabidopsis* accessions encodes a full protein with 230 amino acid residues. Because the Sha accession was from Tadjikistan, we evaluated the salt tolerance and RAS1 sequence polymorphism from 4 additional accessions from Tadjikistan [i.e., Kondara, Hodja-Obi-Garm (Hodja), Sorbo, Dzhiland (Dzi-1)]. Of these, only Dzi-1 exhibited a salt-tolerant phenotype similar to that of Sha (Fig. 4*A*). Sequence comparisons of *RAS1* from the Tadjikistan accessions revealed that like Sha, Dzi-1 *RAS1* had a premature stop codon that causes a truncated RAS1 protein with 209 amino acid residues (Fig. 4*B*). Interestingly, all 5 Tadjikistan accessions differed from L*er* at two amino acid residues (amino acid 117 and amino acid 130) (Fig. 4*B*). These results further support that the premature stop codon rather than the two amino acid substitutions is responsible for the salt tolerance of Sha.



B

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Dzi-1    QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQKDRI 120
Shah     QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQKDRI 120
Sorbo    QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQKDRI 120
Hodja    QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQKDRI 120
Kondara  QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQKDRI 120
Ler      QEKFASVSLAGDNVFTFFCPPWFNSYAKLILWVGDFKPSLVFKLTEVSVADLTRHQRDRI 120
         ************************************************************:***

Dzi-1    SSLKSETRRREREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
Shah     SSLKSETRRREREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
Sorbo    SSLKSETRRREREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
Hodja    SSLKSETRRREREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
Kondara  SSLKSETRRREREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
Ler      SSLKSETRRKEREVMRDFALVQQSVADPPVMLAARRVGAVGMVDGEETDLEEAMEVLKAG 180
         *********:**************************************************

Dzi-1    MAAAMNNADQLRCSTVGKVVEILTPPQAI---------------------- 209
Shah     MAAAMNNADQLRCSTVGKVVEILTPPQAI---------------------- 209
Sorbo    MAAAMNNADQLRCSTVGKVVEILTPPQAIKVLRTIGQLHLRLRDRDQERA 230
Hodja    MAAAMNNADQLRCSTVGKVVEILTPPQAIKVLRTIGQLHLRLRDRDQERA 230
Kondara  MAAAMNNADQLRCSTVGKVVEILTPPQAIKVLRTIGQLHLRLRDRDQERA 230
Ler      MAAAMNNADQLRCSTVGKVVEILTPPQAIKVLRTIGQLHLRLRDRDQERA 230
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Fig. 4. Salt tolerance and RAS1 sequence polymorphism of *Arabidopsis* accessions from Tadjikistan. (*A*) Germination and early seedling growth of the Tadjikistan accessions as compared with L*er*. Seeds were sown on MS medium or MS medium supplemented with 120 mM NaCl and allowed to grow for 2 weeks. The Arabidopsis Biological Resource Center seed stock numbers for Kondara, Hodja, Sorbo, and Dzi-1 are CS916, CS922, CS931, and CS28980, respectively. (*B*) Alignment of RAS1 amino acid sequences from the Tadjikistan accessions and L*er*. The two residues with polymorphisms are colored in red.

Discussion

In this study, we have identified the *RAS1* locus involved in salt tolerance and ABA responses in *Arabidopsis* by QTL mapping of progenies from a cross between L*er* × Sha. By analyzing the QTLs in the Bay-0 × Sha RIL population and by studying NIL(*RAS1*) in the L*er* background, we confirmed the involvement of the *RAS1* QTL in salt tolerance and ABA insensitivity at germination and early seedling stages. We mapped and cloned *RAS1* and found that it encodes a previously undescribed plant-specific protein with a high similarity to At1g58330, a predicted transcription factor-related protein (www.arabidopsis.org). In the salt-sensitive L*er*, *RAS1* encodes a polypeptide with 230 amino acid residues. In Sha, a premature stop codon led to a truncated RAS1 with 209 amino acid residues. Northern blot analysis showed a fast and transient induction of *RAS1* expression in response to NaCl and ABA treatments. The inducible expression is consistent with a role for RAS1 in salt stress and ABA responses. L*er*, Sha, and NIL(*RAS1*) plants genetically transformed with the L*er RAS1* genomic sequence showed NaCl and ABA hypersensitivity. Genetic crosses and transgenic complementation analysis showed that the full-length RAS1 is dominant over the C-terminally truncated RAS1 in the response of *Arabidopsis* to salt and ABA. Consistent with these findings, our results with the T-DNA knockdown mutant of *RAS1* in the Col-0 genetic background and with *RAS1* RNAi lines in L*er*, Sha, and NIL (*RAS1*) backgrounds confirmed that the knockdown or loss of *RAS1* leads to enhanced salt tolerance and ABA insensitivity. Furthermore, CaMV 35S promoter-driven overexpression of full-length but not truncated *RAS1* conferred NaCl and ABA hypersensitivity to transgenic plants. Importantly, our analysis of the salt tolerance and RAS1 sequences of *Arabidopsis* accessions from Tadjikistan revealed that the premature stop codon but not the two amino acid substitutions at residues 117 and 130 is associated with salt tolerance and ABA insensitivity. Together, our results suggest that a loss of function of RAS1 attributable to the C-terminal truncation is responsible for the decrease in ABA sensitivity and increase in salt tolerance of Sha. On the other hand, it is possible that the function of the Sha allele may depend on interactions with the

Sha alleles of other genes involved in salt and ABA responses. The truncated protein may have evolved a function that is beneficial for salt tolerance specifically in Sha.

Salt stress induces ABA accumulation, which, in turn, inhibits germination and seedling growth (19). Previous genetic studies have shown that recessive mutations in positive regulators of ABA signaling, such as *abi3*, *abi4*, and *abi5*, confer ABA insensitivity and NaCl-resistant germination capacity to *Arabidopsis* (20). The results from our QTL analysis are consistent with the findings from mutational studies. Genetic analysis showed that the *abi1-1* mutation, which disrupts ABA signaling, can suppress the salt and ABA sensitivity conferred by *RAS1* overexpression. This suggests that the function of RAS1 requires the core ABA signaling pathway. ABI1 and its related type 2C protein phosphatase family members interact with the ABA receptors, and negatively regulate ABA signaling during germination (18, 21). Our results suggest that although RAS1 is not essential for ABA signaling, it plays an important role in enhancing ABA sensitivity. The induction of *RAS1* by ABA suggests that RAS1 is part of a feed-forward loop for modulating ABA sensitivity.

Our finding of RAS1 as a previously undescribed regulator of NaCl and ABA responses is an initial step toward determining the molecular basis of this natural variation in stress responses. Future studies will clarify the molecular mechanisms of RAS1 in modulating ABA sensitivity and salt-stress tolerance. Abiotic stresses such as salinity reduce germination and seedling establishment, resulting in poor crop establishment and yield. Further studies on RAS1 will increase our understanding of the cellular basis for regulation of seed germination and seedling establishment under salt stress.

Materials and Methods

Plant Materials. RILs for QTL mapping were from a cross between the Sha (salt-tolerant) and L*er* (salt-sensitive) accessions. One of the RILs, CS24560, was crossed with L*er*. The resultant F1 seeds were selfed to produce F2 seeds for fine mapping and were back-crossed with L*er* to produce BC_1F_1 seeds. By repetitive back-crossing and marker-assisted foreground and background selection, we selected several plants in which the region around *RAS1* was heterozygous, although the rest of the genomic regions were homozygous for L*er*, so as to develop the segregating populations for high-resolution mapping of *RAS1*. From the BC_2F_2 generation, we developed NIL(*RAS1*), which contains a very small Sha chromosomal region containing the *RAS1* locus in the L*er* genetic background. The Bay-0 × Sha RIL population (17) was obtained from the Arabidopsis Biological Resource Center (stock no. CS57921).

Evaluation of Physiological Traits for Salt Tolerance. The parents, Sha and L*er*, and 114 F9 RILs (16–20 plants in each line) were used to evaluate salt tolerance. The seeds were sterilized for 5 min with 4% (wt/vol) sodium hypochlorite in 0.01% (wt/vol) Triton × 100 and were sown on MS nutrient medium supplemented with 120 mM NaCl. After stratification at 4 °C for 4 days, the seeds on the agar plates were placed in a growth chamber with 16-h light/8-h dark conditions at 23 °C for 16 days. Resistance to salt stress was measured as the percentage of GS and RL for QTL mapping.

Construction of a Linkage Map and QTL Analysis. The genotype of each plant in the Ler × Sha RIL population was determined by a set of 66 markers covering the whole *Arabidopsis* genome. These data were kindly provided by Maarten Koornneef (Wageningen, The Netherlands). A linkage map was established using the MAPMAKER/EXP 3.0 program (Whitehead Institute, Cambridge, MA) based on the genotype data of the F2 population. Map distances between marker loci were presented in centimorgans, which were derived using the Kosambi function of the program. A LOD score of 2.0 was used to determine both the linkage groups and the order of markers. The MAPMAKER/QTL program (22) was used to identify QTLs affecting salt tolerance on the basis of interval analysis. The proportion of phenotypic variation explained by individual marker loci associated with specific QTLs was also determined by ANOVA (R^2) (23). A LOD score of 2.0 was used to determine the presence of putative QTLs in a given genomic region. The percentages of total phenotypic variation explained by each QTL, and the additive effect, were estimated by MAPMAKER/QTL (22).

Fine Mapping and High-Resolution Mapping. To perform fine mapping of the *RAS1* QTL, we used the GS percentage calculated for 190 F2 plants growing on MS agar medium with 120 mM NaCl, 12 markers in a target region containing *RAS1*, and 13 markers in other regions. The molecular markers F14G9-13 and T10O24-59, which flank *RAS1*, were used to detect recombinants in 4,454 BC_2F_2 plants. To determine the location of the recombination nearest to *RAS1*, we developed markers on the basis of the sequence of BAC clone F21M12 and determined genotypes of the recombinants with these markers. The BC_2F_3 progeny derived from recombinant plants were used to screen for homozygous recombination products. We used fixed recombinant plants (BC_2F_4) to test the salt tolerance and determine the *RAS1* genotypes. The chromosomal region between markers F21M12 and F21M12-130 from the recombinants R1 and R2 were sequenced to identify the fine position of recombination.

Complementation Test. We amplified the genome region of *RAS1*, including the 2.72-kb promoter region upstream of the ATG start codon and the 0.32-kb region downstream of the stop codon, from L*er* genomic DNA by PCR with PfuUltra High-Fidelity DNA Polymerase (Stratagene) and the following primers: RAS1gF, GAGCTCTTTCTCGCTTCACAGTCGTC, and RAS1gR, TCTAGAGCGACCACATCATAAGCTAC. The PCR product was digested with *Sac*I and *Xba*I (restriction sites underlined in the primer sequences) and cloned into the plant binary vector pCAMBIA1305.1. This construct was introduced into *Agrobacterium tumefaciens* stain GV3101 and used for transformation of L*er*, NIL(*RAS1*), and Sha. NIL(*RAS1*) was also crossed with L*er*, and the salt tolerance of F1 plants was evaluated.

We amplified the 630-bp truncated coding sequence (CDS) from Sha or L*er* with the following primers: RAS1cdsF, CACCATGCCAAACACTAGCAGCTC, and RAS1truncatedR, TTAAATCGCTTGCGGCGGAG. The CDS was cloned into pMDC32 by Gateway Technology (Invitrogen), under the transcriptional control of the *CaMV 35S* promoter. The constructs were used for *Agrobacterium*-mediated transformation of L*er*, NIL(*RAS1*), Col, and Salk_058470. The 693-bp full-length CDS from L*er* was amplified with the primers RAS1cdsF and RAS1cdsR (TTAAGCTCTTTCTTGGTCTCTG) and cloned into pMDC32. The construct was used for *Agrobacterium*-mediated transformation of L*er*, NIL(*RAS1*), Col, Salk_058470, and Sha.

RNAi of *RAS1*. A 374-bp *RAS1* fragment was amplified by PCR with the following primers: RAS1cdsF and RAS1RNAiR, GACTTAAGACTCGAGATCCG. This fragment was cloned into pANDA35HK using Gateway Technology.

RNA Analysis. Seedlings (2-week-old) were treated with 150 mM NaCl or 100 μM ABA for the indicated durations. Total RNA was extracted using TRIZOL Reagent (Invitrogen) according to the manufacturer's protocol. Ten micrograms of total RNA was fractionated on 1.2% (wt/vol) formaldehyde agarose gel and transferred onto a Hybond N^+-Nylon membrane (GE Healthcare). After transfer, RNA was fixed by UV cross-linking and was hybridized with [α-^{32}P]dCTP-labeled *RAS1* probe. Blots were rehybridized using [α-^{32}P]dCTP-labeled *TUB4* as a control for RNA loading.

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