The Arabidopsis Kinase-Associated Protein Phosphatase Regulates Adaptation to Na^+ Stress[C]

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The kinase-associated protein phosphatase (KAPP) is a regulator of the receptor-like kinase (RLK) signaling pathway. Loss-of-function mutations *rag1-1* (*root attenuated growth1-1*) and *rag1-2*, in the locus encoding KAPP, cause NaCl hypersensitivity in *Arabidopsis thaliana*. The NaCl hypersensitive phenotype exhibited by *rag1* seedlings includes reduced shoot and primary root growth, root tip swelling, and increased lateral root formation. The phenotype exhibited by *rag1-1* seedlings is associated with a specific response to Na^+ toxicity. The sensitivity to Na^+ is Ca^{2+} independent and is not due to altered intracellular K^+/Na^+. Analysis of the genetic interaction between *rag1-1* and *salt overly sensitive1* (*sos1-14*) revealed that KAPP is not a component of the SOS signal transduction pathway, the only Na^+ homeostasis signaling pathway identified so far in plants. All together, these results implicate KAPP as a functional component of the RLK signaling pathway, which also mediates adaptation to Na^+ stress. RLK pathway components, known to be modulated by NaCl at the messenger RNA level, are constitutively down-regulated in *rag1-1* mutant plants. The effect of NaCl on their expression is not altered by the *rag1-1* mutation.

Animal receptor Tyr kinases and receptor Ser/Thr kinases are cell surface enzyme-linked receptors that are activated by peptide ligands and initiate a diverse range of signal transduction pathways, including those that control cell growth, differentiation and survival, defensive responses. and metabolism (Holland and Holland, 2002). Receptor-like kinases (RLKs; a.k.a. plant receptor kinase; Cock et al., 2002) are animal receptor kinase orthologs in plants, so classified because of conserved structures that include an extracellular receptor, a transmembrane domain, and an intracellular kinase domain (Shiu and Li, 2004). The mechanisms by which RLKs activate and regulate downstream components of the signaling pathway resemble those of receptor Tyr kinases and receptor Ser/Thr kinases (Cock et al., 2002; Shiu and Li, 2004). RLK activation occurs upon binding of an extracellular ligand to the plasma membrane-localized heterodimeric receptor form (Morris and Walker, 2003; Tichtinsky et al., 2003; Torii, 2004). Subsequently, the RLK complex undergoes autotransphosphorylation to form an active complex (Trotochaud et al., 1999, 2000; Clark, 2001; Rojo et al., 2002). RLKs are also transcriptionally regulated (Becraft, 2002). Plant RLKs activate diverse signal transduction pathways, including those that control hormone responses (Li and Chory, 1997; Matsubayashi et al., 2002; Montoya et al., 2002; Scheer and Ryan, 2002; Yin et al., 2002; Szekeres, 2003), flower development (Williams et al., 1997; Stone et al., 1998), innate immunity against bacterial pathogens (Gomez-Gomez et al., 2001), self incompatibility (Braun et al., 1997), and root nodule formation (Downie and Walker, 1999; Endre et al., 2002; Krusell et al., 2002; Nishimura et al., 2002; Spaink, 2002; Stracke et al., 2002).

The kinase-associated protein phosphatase (KAPP; Stone et al., 1994) is a cytosolic-oriented, membrane-anchored type 2C protein phosphatase, which binds only to activated (i.e. phosphorylated) forms of RLK via its kinase interaction domain (Braun et al., 1997; Shah et al., 2002) and inactivates the RLKs through dephosphorylation (Tichtinsky et al., 2003). Genetic evidence indicates that KAPP may function as a negative regulator of RLK pathways (Williams et al., 1997; Stone et al., 1998; Gomez-Gomez et al., 2001). KAPP is also essential for RLK internalization via endocytosis (Shah et al., 2002; Vanoosthuyse et al., 2003). In animals, internalization of receptor kinases is an important step for signaling, which leads to degradation and recycling of receptor kinases (Shah et al., 2002). Although the RLK superfamily includes more than 600 members in Arabidopsis (*Arabidopsis thaliana*; Shiu et al., 2004), KAPP, which binds to many RLKs (Braun

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et al., 1997; Tichtinsky et al., 2003), is a single copy gene in Arabidopsis as well as in *Zea mays* (Stone et al., 1994; Braun et al., 1997). Based on these findings and considering its ubiquitous expression (Stone et al., 1994; Braun et al., 1997; Williams et al., 1997), KAPP is thought to play an important role in the RLK pathways. Another type 2C protein phosphatase, ABI1, was shown to negatively regulate abscisic acid-dependent gene repression (Sheen, 1998). Using KAPP overexpression lines, it was demonstrated that KAPP does not affect abscisic acid-dependent gene repression. Some RLKs and the RLK pathway components have been reported to be regulated by NaCl or other abiotic stresses (Piao et al., 2001; Kreps et al., 2002; Ozturk et al., 2002; Seki et al., 2002). However, direct evidence indicating a function of RLK pathway components in salt tolerance has so far not been demonstrated. In addition, plant genes with kinase interaction domain identity to the animal counterparts have not yet been isolated (Braun et al., 1997; Williams et al., 1997).

High salinity is one of the major abiotic stresses that limit land usage and reduce crop yield (Ward et al., 2003). Salinity is becoming more and more problematic for agriculture, as irrigation leads to salt accumulation in the soil (Ward et al., 2003). Recently, molecular genetic approaches aimed at unraveling the complexity of salt stress responses in plants have provided fundamental insights to understanding the biological processes involved in the perception and signal transduction of environmental stimuli (Zhu, 2002, 2003; Chinnusamy et al., 2004). The effects of high salinity on growth and viability of glycophytes are mainly associated with an increased osmolarity of the solution in contact with the roots, a specific Na^+ or Cl^- ion toxicity, and nutritional imbalance (Zhu, 2000). Sodium (Na) accumulation causes the primary damages due to ionic stress (Tester and Davenport, 2003; Zhu, 2003). Thus, maintenance of low intracellular Na^+ concentration is crucial for plant adaptation to saline stress. Toxic effects of Na^+ include inhibition of enzyme activity (Serrano et al., 1999; Hasegawa et al., 2000) and disruption of K^+ nutrient acquisition (Zhu, 2003). Ca^{2+} counteracts the negative effects of Na^+ by enhancing the selectivity of K^+ over Na^+ (Epstein, 1998; Zhu, 2002; Tester and Davenport, 2003).

Plants maintain low cytosolic Na^+ concentration by controlling compartmentalization, influx, and efflux of Na^+ (Zhu, 2003). The Ca^{2+}-dependent SOS (salt overly sensitive) pathway is the only known Na^+-specific signal transduction pathway that regulates K^+/Na^+ homeostasis by controlling Na^+ efflux and K^+ acquisition. *SOS1* (Wu and Zhu, 1996), *SOS2* (Zhu et al., 1998), and *SOS3* (Liu and Zhu, 1997) were identified in a forward genetic screening (Zhu, 2002) for Na^+ hypersensitivity and growth defects under K^+ deficiency (Zhu, 2000). The SOS pathway is initiated by a salt stress-induced calcium signal that is sensed by the Ca^{2+}-binding protein SOS3. The activated SOS3 recruits the protein kinase SOS2 to the plasma membrane to form a complex. A conformational change that results from the SOS3-SOS2 complex formation relieves the autoinhibition of the SOS2 kinase activity. Subsequently, the SOS3-SOS2 complex regulates the expression and activity of SOS1, a plasma membrane-localized Na^+/H^+ exchanger that mediates Na^+ efflux across the plasma membrane (Halfter et al., 2000; Qiu et al., 2002; Quintero et al., 2002; Shi et al., 2002b; Zhu, 2003). Unidentified components of the SOS pathway include the phosphatase(s) that dephosphorylates SOS1 and SOS2 as part of the phosphorylation/dephosphorylation-based signal transduction (Ohta et al., 2003).

Here, we report the isolation and functional characterization of *root attenuated growth1* (*rag1*), a loss-of-function mutant of KAPP. *rag1* exhibits NaCl sensitivity and is not a component of the SOS pathway. The *rag1-1 sos1-14* double mutant exhibits an additive phenotype of both parental mutants, indicating that KAPP is a component of a novel Na^+ adaptation pathway, which may be related to the RLK pathway.

RESULTS

Characterization of *rag1-1* Salt-Sensitive Phenotype

rag1-1 mutant was isolated by screening of a T-DNA-tagged Arabidopsis population for salt tolerance phenotypes on NaCl-containing medium (Zhu et al., 2002; Koiwa et al., 2003). *rag1-1* exhibits substantially reduced primary root growth, root tip swelling, and enhanced lateral root formation on Murashige and Skoog (MS) salt agar medium supplemented with 160 mM NaCl (Fig. 1). However, no substantial differences were observed in the shoot of *rag1-1* compared to wild-type plants. In the absence of salt, wild-type and *rag1-1* roots were very similar also (Fig. 1A), exhibiting cells of rectangular shape uniformly distributed within the root tissue (Fig. 2, A and B).

In contrast, upon NaCl treatment, the diameter of *rag1-1* roots at the maximum swelling position was more than 2 times wider than wild-type roots (Fig. 2, C and D). The swollen region of the NaCl-treated *rag1-1* root showed enlarged, inconsistently sized, un-uniform, and round-shaped cells. Deformed cells were observed at the distal elongation zone and at the root cap but not at the differentiation zone (data not shown). One cell layer could not be distinguished from another in the epidermal, cortical, endodermal, and pericycular regions, since deformed cells intruded into each other and no longer formed clear cell layers (Fig. 2D). The boundary of the stele could be observed, but it was not clear if the cells in the stele were affected or not. From these observations, it was concluded that cell enlargement of NaCl-treated *rag1-1* appeared to be the primary cause of root swelling.

In wild type, cells of NaCl-treated roots were smaller than that of untreated roots; however, cells retained cell file organization (Fig. 2C). These observations suggest that proper maintenance of the cyto-

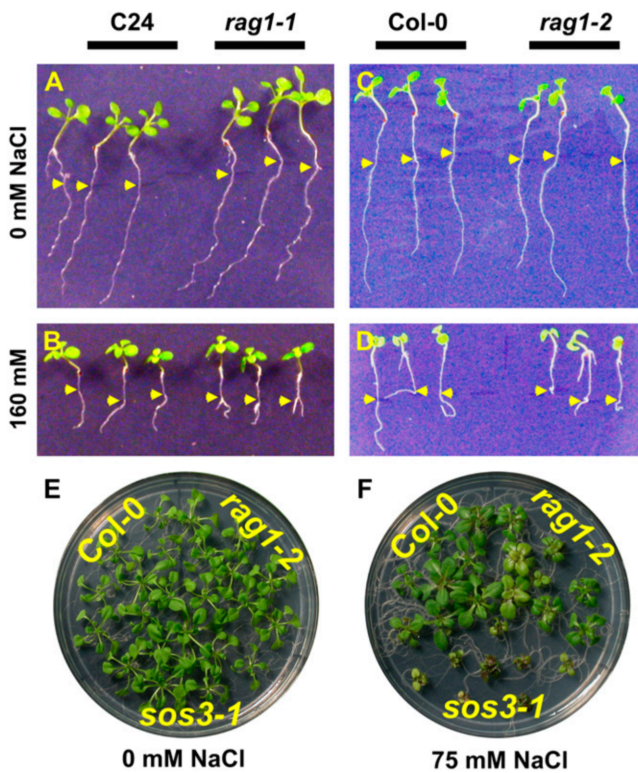

Figure 1. Growth of *rag1* plants. *rag1* is almost identical to wild type at optimal growth conditions and is hypersensitive to NaCl. A to D, Root growth reduction of *rag1* mutants under high NaCl. Four-day-old seedlings were transferred to MS-agar (1.5%) medium supplemented with 0 mM (A and C) or 160 mM (B and D) NaCl and were allowed to grow for 6 (A and B) or 9 (C and D) additional days. From left to right: wild-type (ecotype C24), *rag1-1*, wild-type (ecotype Col-0), and *rag1-2* seedlings. E and F, Shoot growth reduction of *rag1-2* under 75 mM NaCl. Four-day-old seedlings were transferred to MS-agar (0.8%) medium supplemented with 0 mM (E) or 75 mM (F) NaCl and were allowed to grow for 3 additional weeks.

skeleton is defective (Wasteneys and Galway, 2003) in *rag1-1* root but is maintained in the wild-type root after salt treatment.

Apparent differences between *rag1-1* and the wild type are only observed after NaCl treatment, indicating that the alteration in cell size and cell shape is a specific response to NaCl stress. Despite these morphological changes, however, the root meristem of some *rag1-1* retained the ability to regrow after up to 12 d of 160 mM NaCl treatment (data not shown), indicating that neither root tip swelling nor lateral root formation is induced by the death of the primary root meristem. The rescued root retained the already-deformed cells but produced normal-looking cells after being transferred back to control medium (data not shown).

Loss-of-Function Monogenic Mutation in *KAPP* Causes Salt Sensitivity

A T-DNA insertion was identified within the seventh exon (2,721 bp downstream of ATG translation start site) of *KAPP* (Stone et al., 1994) in *rag1-1* (Fig. 3A) by thermal asymmetric interlaced-PCR (Liu et al., 1995) analysis. NaCl-treated F_2 progenies (245 from 21 F_1 lines) derived from backcrossing with wild type segregated to wild-type:*rag1-1* phenotype at a 3:1 ratio (187:58; $\chi^2 = 0.23$; $P > 0.50$), indicating that *rag1-1* is a monogenic recessive mutation. Genotype was determined for 36 NaCl-sensitive F_2 progenies, and all of them were homozygous for the *rag1-1* mutation. *rag1-2* (SAIL_1255_D05), whose T-DNA insertion is located at the first intron (525 bp downstream of ATG) of *KAPP*, was isolated through reverse genetic in silico search from SAIL (formerly called GARLIC; Torrey Mesa Research Institute, San Diego; collection ecotype Columbia [Col-0]; http://www.nadii.com/pages/collaborations/garlic_files/GarlicDescription.html). These results indicate that the KAPP mutation is causing the NaCl-sensitive phenotype.

Reverse transcription (RT)-PCR analysis revealed that mRNA accumulation of *KAPP* is diminished in plants with both mutant alleles to undetectable levels (Fig. 3B). Absence of a shorter transcript in *rag1-1* was confirmed using primer sets targeting the upstream region of T-DNA (data not shown). *rag1-2* (Fig. 1D) exhibits a root phenotype similar to that of *rag1-1* (Fig. 1B) in response to NaCl. However, shoot growth reduction after 3 weeks of 75 mM NaCl treatment was observed in *rag1-2* compared with the wild type (Fig. 1, E and F) but not in *rag1-1*. The phenotype difference between *rag1-1* and *rag1-2* in the shoot may be due to the ecotype background difference. Col-0 is more sensitive to NaCl treatment than C24,

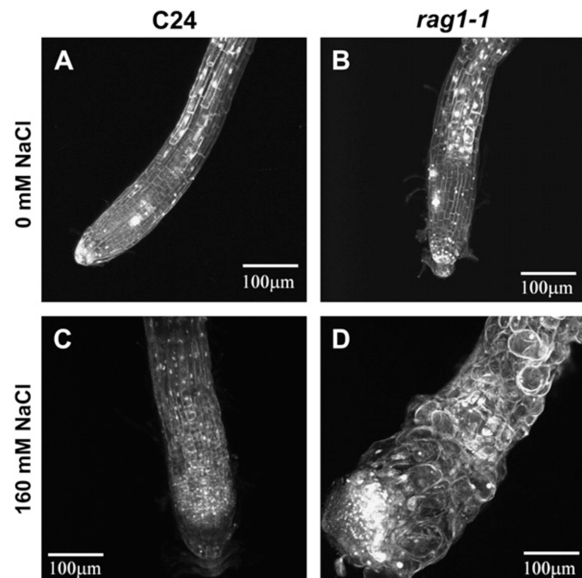

Figure 2. The root swelling phenotype of *rag1-1* is due to cell enlargement. Four-day-old seedlings were transferred to MS-agar medium supplemented with 0 (A and B) or 160 (C and D) mM NaCl and were allowed to grow for 9 additional days. Root tips of wild-type (ecotype C24; A and C) and *rag1-1* (B and D) seedlings were observed under a confocal microscope.

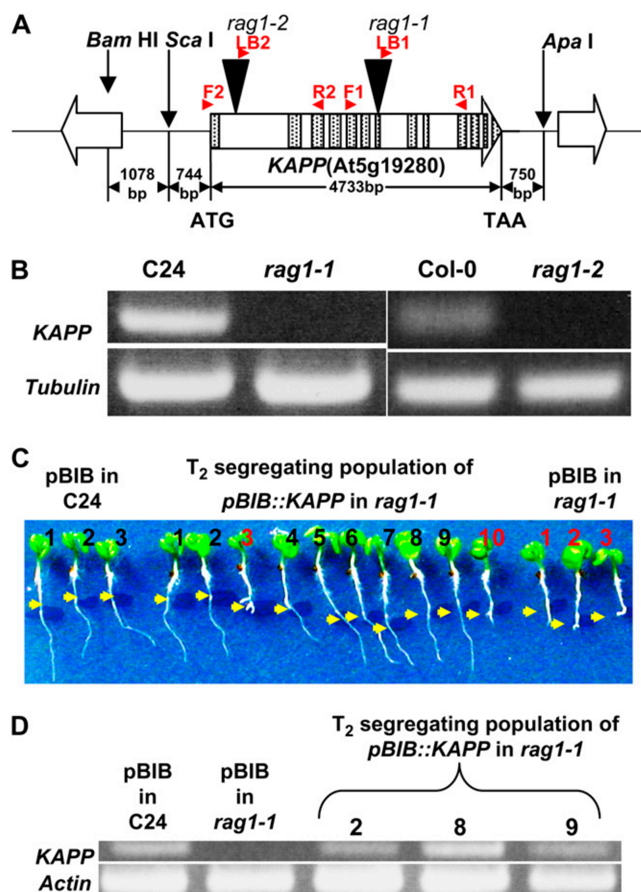

Figure 3. The *rag1-1* mutant has a T-DNA insertion within the seventh exon of KAPP. The insertion is homozygous and functionally disrupts the expression of the gene. A, Genetic structure of *KAPP* and its neighbor genes are shown as block arrows with arrowheads indicating the 3′ terminus. Exons and introns of *KAPP* are indicated as shaded boxes and the blank regions between them, respectively. T-DNA insertion sites of *rag1-1* (isolated by forward genetics, ecotype C24) and *rag1-2* (SAIL_1255_D05, Col-0) are shown as black triangles. The red arrowheads and the red letters above them indicate the forward (F1, CATGCACAGATAACATGGAACTCTAC; F2, TTGCTTCTCATCTCC-CTCATCA), reverse (R1, CAAGAGAACAGTAGCTGTACAAC; R2, CTTGGCAACATTCACATTGCCT), and left-border (LB1, TTGACCAT-CATACTCATTGCTG; LB2, TAGCATCTGAATTTCATAACCAATCTCGA-TACAC) primers used for diagnostic PCR (data not shown) and RT-PCR (B and D). B, mRNA expression of *KAPP* in 10-d-old C24, *rag1-1*, Col-0, and *rag1-2* seedlings (30 cycles of RT-PCR). *Tubulin* is used as control to show the equal amount of cDNA applied for RT-PCR. C, Phenotype complementation of three-quarters of T_2 progenies of *rag1-1*. Conditions of the treatment are the same as Figure 1. Red digits indicate the progenies showing the mutant phenotype. Yellow arrows indicate the position of root tips at the time of transfer. D, RT-PCR of the selected seedlings from the T_2 progeny. pBIB in C24 and pBIB in *rag1-1* seedlings were used as positive and negative control, respectively. DNA samples were extracted from the selected seedlings. 2, 8, and 9, The progenies located at the second, eighth, and ninth positions, respectively, from the left on C. *Actin* is used as control to show equal amount of cDNA applied for RT-PCR. [See online article for color version of this figure.]

with respect to root growth inhibition, shoot anthocyanin accumulation, and shoot growth reduction at lower salt concentrations (data not shown). A cross between *rag1-1* and *rag1-2* was made to perform a complementation test. As a result, all F_1 progenies (18 seedlings) exhibit a NaCl-sensitive phenotype, indicating that *rag1-1* and *rag1-2* are indeed allelic (data not shown). Those F_1 plants were tested by diagnostic PCR to confirm the heterozygous genotype for both insertions.

Genetic complementation with genomic DNA fragments under the control of the natural promoter further confirmed that a loss-of-function mutation of *KAPP* caused the salt-sensitive phenotype in *rag1* mutants. Two different genomic DNA fragments (6,227-bp *Sca*I-*Apa*I and 7,305-bp *Bam*HI-*Apa*I fragments; Fig. 3A) containing the *KAPP* open reading frame were digested from the bacterial artificial chromosome F7K24. Interestingly, only the *Bam*HI-*Apa*I fragment complemented the NaCl-sensitive phenotype (Fig. 3C). This result suggests that there are essential regulatory factors at the region −744 to −1,822 of the *KAPP* gene. T_2 progenies of plants transformed with the *Bam*HI-*Apa*I fragment exhibited a 3:1 (38:14; $\chi^2 = 0.10$; $P > 0.70$) segregation ratio for NaCl-sensitive phenotype (Fig. 3C). All seedlings shown in Figure 3C were examined for hygromycin sensitivity after the phenotype scoring. Both of two NaCl-sensitive progenies were killed by hygromycin treatment, whereas all of eight NaCl-resistant progenies survived, indicating a tight linkage between recovery of wild-type phenotype and hygromycin resistance. mRNA expression level of *KAPP* was also recovered in those progenies that survived (Fig. 3D). These results confirmed that the recessive loss-of-function mutation of *KAPP* is the cause of the NaCl-sensitive phenotype.

rag1-1 NaCl Sensitivity Is Specific to Na^+ Ion Toxicity

The function of KAPP in ion/osmotic adaptation was further assessed by examining the effects of nonionic and ionic osmotic solutes on root growth and development of wild-type and *rag1-1* seedlings. In addition, *sos1-14* (Koiwa et al., 2003), *osm1-1* (Zhu et al., 2002), and *npct1-1* (Y. Nakagawa, B. Cubero, F. Li, K.G. Raghothama, J.M. Pardo, R.A. Bressan, and P.M. Hasegawa, unpublished data) were used as specific controls due to their sensitivity to Na^+ and Li^+ ions, osmotic stress, and Cl^- ion, respectively. Normal root growth of *rag1-1* seedlings was inhibited by NaCl (Fig. 4A), but not by mannitol (Fig. 4B) or by KCl (Fig. 4D). These results indicate that *rag1-1* seedlings are sensitive to Na^+ but not to osmotic stress or Cl^- ions. Root growth of *rag1-1* was slightly inhibited by LiCl (Fig. 4C). Li^+ is a more toxic analog of Na^+, with which it presumably shares a transport system and a mechanism of toxicity (Serrano et al., 1999). Small yet statistically significant differences were observed in terms of primary root length. In addition to the primary root growth inhibition, characteristic swollen root tips and lateral root formation (Fig. 1B) were observed only on high NaCl-containing medium and not on high LiCl-containing medium.

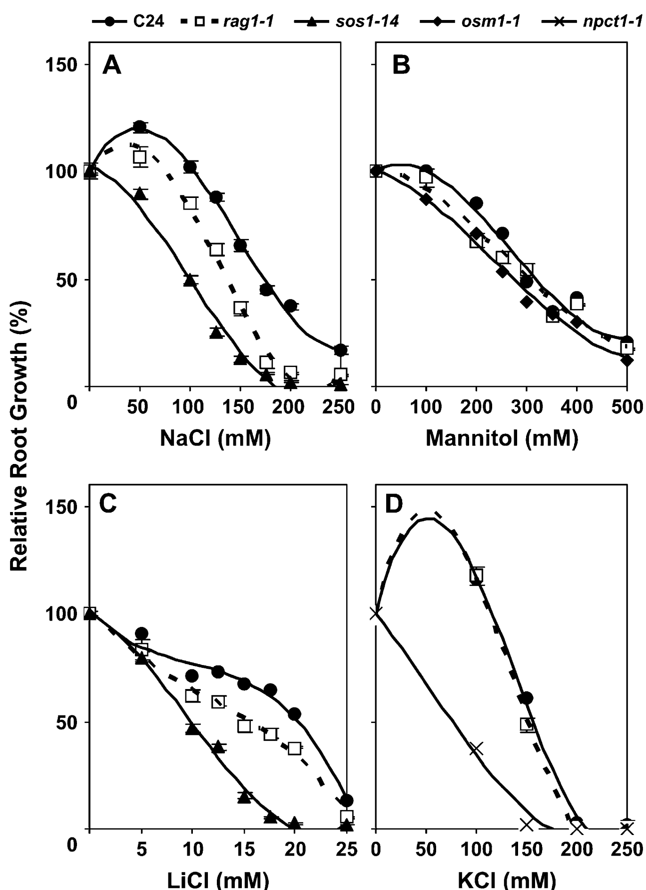

Figure 4. Root growth of the *rag1-1* is hypersensitive to Na^+. Four-day-old seedlings were transferred onto 1× MS-agar (1.5% agar) plates supplemented with various concentration of NaCl (A), mannitol (B), LiCl (C), and KCl (D) and allowed to grow for 6 additional days ($n = 12$). Then the plates were scanned, and the length of primary root growth after the transfer was measured by National Institutes of Health's Scion Frame Grabber (Buer et al., 2000). The root growth is shown as relative root length to that of nontreated seedlings. The error bars indicate the SE.

These results indicate that KAPP functions in salt adaptation through the control of Na^+ homeostasis. The next question arose as to whether this Na^+ sensitivity was associated with altered K^+ uptake. NaCl-sensitive mutants have been reported to be sensitive to low K^+ as well as to high Na^+ conditions (Zhu, 2003). There was no substantial difference detected between primary root growth of *rag1* and wild-type seedlings treated on 1/20 MS-agar (1.2% agar, 0.15 mM Ca^{2+}) medium supplemented with 0 to 10 (one-half-strength of standard MS medium) mM KCl (Rus et al., 2001), whereas the primary root growth of the positive controls, *sos1-14* and *sos3-2*, was greatly inhibited in low K^+ (up to 0.2 mM) medium. When we compared *rag1-2*, *sos3-2*, and their relative controls (Col-0 and *gl-1*), only *sos3-2* exhibited substantially more severe growth reduction (about 30%) at lower concentrations of KCl. There was no substantial growth difference among these genotypes at 10 mM KCl. This result indicates that, unlike other known salt-sensitive mutants, the NaCl sensitivity of *rag1* is not associated with K^+ deficiency. It further suggests that the KAPP-RLK sodium adaptation pathway regulates Na^+ toxicity independent of K^+ deficiency.

We also assessed the effect of Ca^{2+} on *rag1* NaCl sensitivity. Ca^{2+} is known to affect Na^+ uptake (Pardo and Quintero, 2002); therefore, NaCl sensitivity of some NaCl-hypersensitive mutants (such as *sos1* to *sos3* and *hkt1*) can be altered by Ca^{2+} concentration. Up to 10 mM $CaCl_2$ (1× MS medium contains 3 mM Ca^{2+}) did not affect the NaCl sensitivity of *rag1-1* at 100 mM NaCl, while NaCl sensitivity of *sos1-14* and *sos3* control plants was rescued by high Ca^{2+} concentration (data not shown).

To further determine if *KAPP* is involved in Na^+/K^+ homeostasis, intracellular contents of Na^+ and K^+ ions were measured (Fig. 5) as described by Rus et al. (2001). Whole seedlings of *rag1-1*, a positive control, *sos1-14*, and wild type were treated with 100 mM NaCl. There was no substantial difference in Na^+ and K^+ content among genotypes before the treatment (K^+, 53 mg/g dry weight; Na^+, 1.3 mg/g dry weight). After the treatment, *rag1-1* (K^+, 31.5 ± 0.3 mg/g dry weight; Na^+, 36.7 ± 3.0 mg/g dry weight) and wild-type seedlings (K^+, 28.9 ± 1.9 mg/g dry weight; Na^+, 35.0 ± 4.0 mg/g dry weight) exhibited equivalent contents for both Na^+ and K^+ ions, while *sos1-14* (K^+, 8.65 ± 0.4 mg/g dry weight; Na^+, 37.5 ± 0.5 mg/g dry weight) exhibited much lower K^+ content (Fig. 5). Ion contents were also measured on leaves of seedlings treated with 0 to 75 mM NaCl. No substantial differences in Na^+ and K^+ levels were detected (data not shown).

Taken together, these results indicate that *KAPP* is not directly involved in Na^+/K^+ uptake, yet it may be responsible for sensing high intracellular Na^+ concentration or for regulating subcellular Na^+ localization. An additional feature of *rag1-1* is that it contains higher Ca^{2+} compared to its relative wild type in the absence of NaCl treatment (D.E. Salt, personal communication).

KAPP Is Involved in a Novel Salt Stress-Responsive Pathway

sos1, *sos2*, and *sos3* mutants exhibit both Na^+ and Li^+ ion-specific sensitivity. Therefore, we generated *rag1-1 sos1-14* double mutants to determine the genetic interaction between *KAPP* and *SOS1* and, ultimately, verify whether or not KAPP functions as a part of the SOS pathway. *rag1-1 sos1-14* double mutants showed an additive NaCl-sensitive phenotype (Fig. 6). The seedlings of *rag1-1 sos1-14* look identical to the wild type at 0 mM NaCl; however, the primary root growth of *rag1-1 sos1-14* seedlings was substantially reduced compared to *sos1-14* at 25 mM to 75 mM NaCl. In addition to primary root growth inhibition, root morphological alteration (i.e. root tip swelling and lateral root formation) was detectable in *rag1-1 sos1-14* double

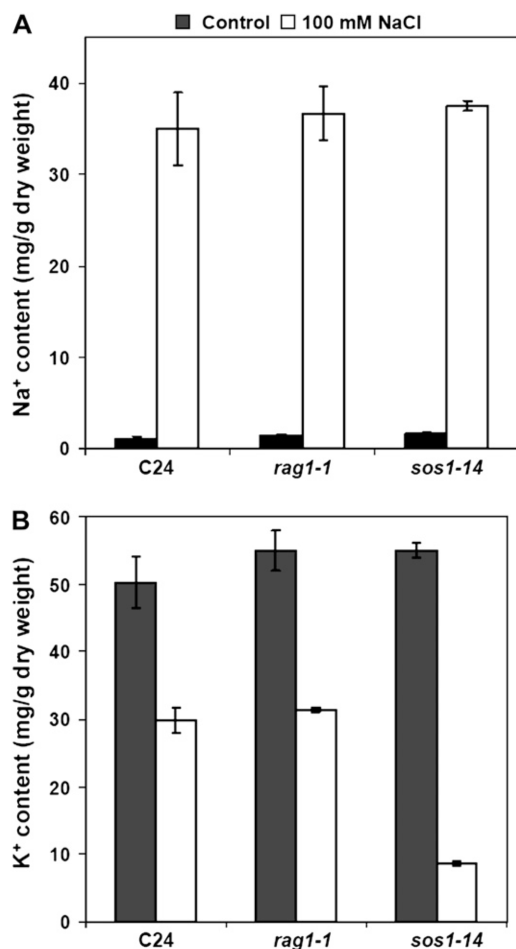

Figure 5. K^+ and Na^+ contents in *rag1-1* seedlings exposed to salt stress. Ten-day-old seedlings were transferred into liquid medium 2 d for pretreatment. Then the liquid medium was supplemented with 0 or 100 mM NaCl for 2 additional days of treatment. There was no significant difference observed between wild type and *rag1-1* either in presence or in absence of NaCl, while K^+ content of the Na^+ ion homeostasis mutant, *sos1-14*, was much lower after NaCl treatment. The value is the average measurement of three individually treated flasks, and error bars indicate SE.

mutants at 50 mM NaCl (Fig. 6), whereas the same phenotype was detectable in *rag1-1* only at a much higher concentration (>150 mM NaCl; Fig. 1). This result also supports the interpretation that the phenotype of the *rag1-1* mutant is Na^+ ion specific. Either a higher Na^+ content or an increased Na^+ to K^+ ratio of *rag1-1 sos1-14* double mutant, compared to *rag1-1*, may have acted as an early trigger for swelling and lateral root formation, which are both typical of the *rag1-1* mutation. Additional evidence suggests that *KAPP* is not involved in the SOS pathway: (1) *sos* mutants are more sensitive to Li^+ ions than Na^+ ions, while *rag1* is more sensitive to Na^+ ions; (2) *sos* mutants do not exhibit the root tip swelling and lateral root formation phenotype characteristic of *rag1*; (3) unlike *sos* mutants, *rag1* mutants are not sensitive to K^+ deficiency; and (4) unlike *sos* mutants, the NaCl-sensitive phenotype of *rag1* is not affected by Ca^{2+} availability. These phenotypic differences combined with the genetic analysis lead us to the conclusion that *KAPP* functions in a novel Na^+-responsive pathway.

rag1-1 Exhibits Partial De-Etiolation and Root Branching

Both the shoots and roots of *rag1-1* seedlings were identical to wild type under optimal growth conditions (1× MS, 3% Suc, and 1.5% agarose), indicating that *KAPP* is required primarily during salt adaptation (Fig. 1A). Mature *rag1-1* plants did not exhibit any of the extreme abnormal growth and development phenotypes associated with RLK pathway mutants (data not shown), such as enlarged siliques (*clv1-3*; for review, see Clark, 2001), dwarfism, or male sterility (*bri1* and *bin2*; for review, see Clouse, 2002).

However, dark-grown *rag1-1* seedlings exhibit a partial de-etiolation phenotype manifested with short and radially thickened hypocotyls and increased cotyledon size typical of brassinosteroid-deficient or -insensitive

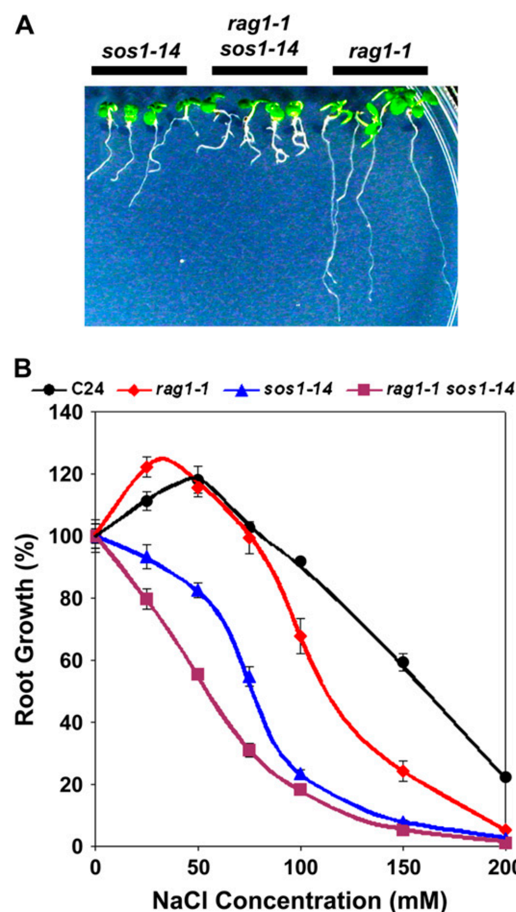

Figure 6. *rag1-1 sos1-14* double mutant shows additive phenotype relative to parental monogenic mutants. A, From left to right, *sos1-14*, *rag1-1 sos1-14*, and *rag1-1* seedlings treated with 75 mM NaCl. B, Primary root growth of *rag1-1 sos1-14* along with other relative controls (C24, *rag1-1*, *sos1-14*) were measured as reported for Figure 4.

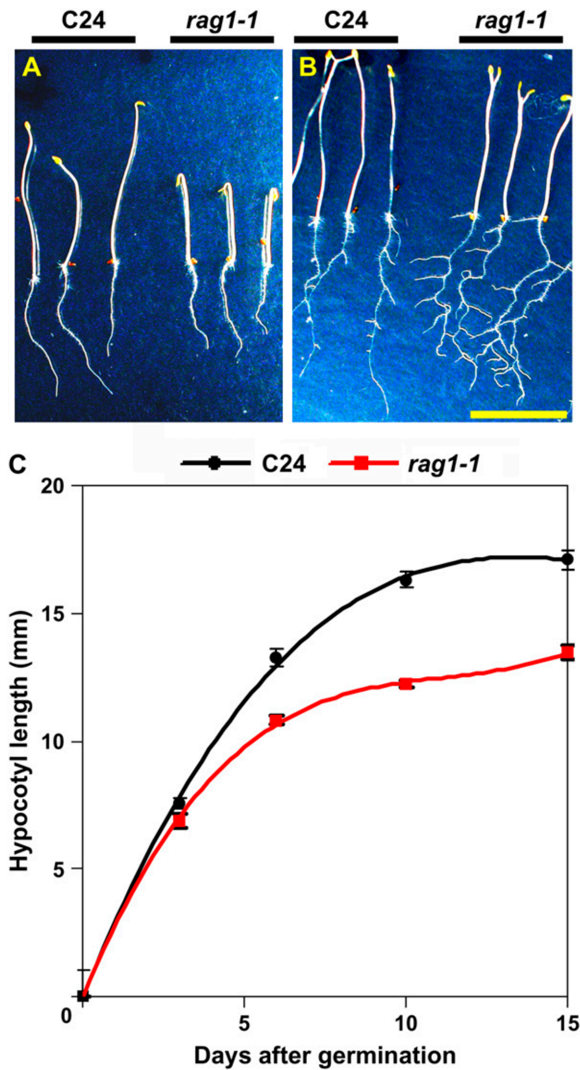

Figure 7. Dark-grown *rag1-1* seedlings exhibit enhanced lateral root formation and slightly thicker hypocotyls. A, Dark-grown 7-d-old wild-type (left, ecotype C24) and *rag1-1* (right) seedlings. Dark-grown seedlings were exposed to light for the first day and then covered with aluminum foil and allowed to grow for additional 6 d on 1× MS (3% Suc, 1.2% agar medium). B, Dark-grown 14-d-old wild-type (left, ecotype C24) and *rag1-1* (right) seedlings. C, Hypocotyl length of dark-grown seedlings 3, 6, 10, and 15 d after germination.

mutants (Li et al., 1996; Chory, 1997; Chory and Li, 1997; Bishop et al., 1999; Symons and Reid, 2003; Fig. 7). In addition, hypocotyls of *rag1-1* grow more slowly compared to wild-type seedlings (Fig. 7C). These results indicate that the mutation does not arrest growth but may interfere with the regulation of directional cell expansion. Moreover, dark-grown *rag1-1* seedlings exhibit greater root branching (Fig. 7B), a phenotype that has not been reported for brassinosteroid-deficient or -insensitive mutants at later growth stages. This difference was most apparent in 12-d-old seedlings. The magnified dark-grown *rag1-1* root is deformed and widened compared to wild-type roots (data not shown). These results suggest a possible conditional function of *KAPP* in brassinosteroid perception.

The KAPP-RLK Pathway Regulates a Novel Na^+-Responsive Pathway

The results so far presented strongly indicate that KAPP is involved in salt adaptation. To confirm this conclusion and to identify possible interactions between stress adaptation pathways, several characterized RLK and RLK pathway components were tested for salt sensitivity and transcriptional regulation. Candidate genes were selected based on their established differential transcriptional regulation in response to salt treatment (Becraft, 2002). *LecRK1* (Herve et al., 1996) and *LRK1* (Kreps et al., 2002) were tested for transcriptional abundance in *rag1* mutants in the absence or presence of NaCl. The level of expression of *LecRK1*, *LRK1*, and two other genes included as controls, *RD29A* and *RD22*, was constitutively reduced in nonsalinized *rag1-1* seedlings, indicating that *RAG1* may positively regulate the basal level of these stress-induced genes (Fig. 8). However, upon salt treatment (150 mM NaCl), wild-type and mutant seedlings had similar levels of expression, suggesting that transcription of these genes was possibly induced through a RAG1-independent pathway. Indeed, transcriptional activation of AtLecRK2 in response to salt stress has been reported to be regulated by the ethylene signaling pathway (He et al., 2004). In addition, salt sensitivity of candidate T-DNA mutants was also tested (data not shown). Among the T-DNA mutants tested, only SALK_005054 and SALK_008611 showed altered sensitivity to NaCl in shoot growth but not in root growth (data not shown). Both SALK_005054 and SALK_008611 had a T-DNA insertion in RPK1. These results indicate that RPK1 is possibly involved in this RLK-mediated NaCl adaptation pathway.

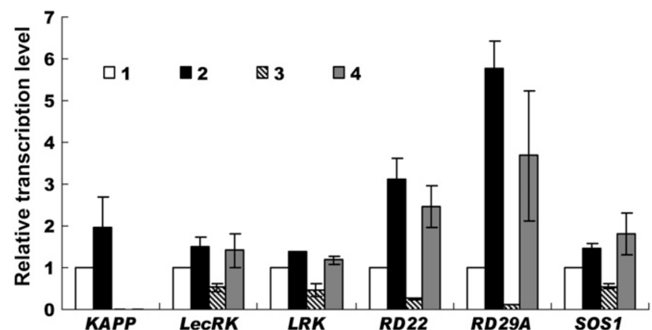

Figure 8. KAPP transcriptionally regulates RLKs and other stress-induced genes. Relative transcription levels of *KAPP*, *LecRK*, *LRK*, *RD22*, *RD29A*, and *SOS1* in wild-type (1 and 2) and *rag1-1* (3 and 4) plants were determined using quantitative RT-PCR. Total RNA was isolated from the untreated seedlings (1 and 3) or the seedlings treated with 150 mM NaCl for 3 h (2 and 4). The gene transcript levels were normalized for the expression of tubulin measured in the same RNA samples. Data are means ± SD of three independent experiments.

DISCUSSION

We report the isolation of two allelic loss-of-function mutations (*rag1-1*, ecotype C24; and *rag1-2*, ecotype Col-0) of KAPP as salt-sensitive mutants by forward and reverse genetic identification. Functional characterization revealed that *KAPP* functions in adaptation to NaCl stress. A unique feature of the *rag1* mutant is its specific Na^+ hypersensitivity. Salt sensitivity of *hkt1* is also Na^+ ion specific (Berthomieu et al., 2003). However, there are distinctive phenotypic differences associated to mutations at these two loci. For instance, *hkt1* accumulates more sodium into the shoot compared to the wild type, whereas *rag1* does not. In addition, *hkt1* does not have a seedling root growth attenuation phenotype as seen in *rag1* (Fig. 1).

The salt sensitivity of *rag1-1* is less severe compared to other reported salt-sensitive mutants isolated through a root-bending assay (Wu and Zhu, 1996; Liu and Zhu, 1997; Zhu et al., 1998; Shi et al., 2002a, 2002b; Zhu et al., 2002; Koiwa et al., 2003). This may explain why *rag1* has not been isolated in previous extensive screenings (Zhu et al., 1998; Shi et al., 2003). In this respect, *rag1-2* (Col-0 background), for instance, does not exhibit a clear phenotype at 50, 75, or 100 mM NaCl, the concentrations used to isolate the *sos* mutant series. In addition, the severe *sos1*, *sos2*, and *sos3* salt-sensitive phenotypes are associated with K^+ imbalance. In contrast, Na^+-specific hypersensitive mutants such as *rag1* may show a less dramatic salt-sensitive phenotype.

The NaCl-Induced Root Tip Swelling and the Dark-Grown Phenotype of *rag1* Are Similar to Mutants with Impaired Cellulose Biosynthesis

Constitutive root swelling has been reported for several other salt-sensitive mutants (Liu and Zhu, 1997; Shi et al., 2002a, 2003; Koiwa et al., 2003). Nevertheless, all the mutants showing this phenotype also present additional distinctive features with respect to other disrupted/altered functions associated with the salt-sensitive phenotype. Unlike *sos5*, swelling and lateral root formation in *rag1* was not observed upon an extended period of culture (data not shown; Shi et al., 2003). The swollen root tip observed in *rag1*, as well as in some other salt-sensitive mutants, resembles the temperature-sensitive phenotype of *rsw* mutants (Baskin and Wilson, 1997; Wiedemeier et al., 2002), which have defects in cellulose synthesis or microtubule organization. These results indicate that KAPP may contribute to the regulation of cellulose synthesis and/or microtubule organization only under high Na^+ concentration.

Relationship between KAPP and the SOS Pathway

Analysis of the double mutant, *rag1-1 sos1-14*, revealed that *KAPP* is not a component of the *SOS* pathway. Unlike *sos* mutants, the *rag1-1* mutant is only slightly more sensitive to Li^+, which is thought to share transport systems and toxicity target with Na^+ (Serrano et al., 1999). Two possible explanations may be provided for the hypersensitivity to Na^+ observed in *rag1-1 sos1-14* double mutant. The first possibility is that a diffusive signal produced in the shoot, due to an altered higher Na^+ to K^+ ratio resulting from the *sos1-14* mutation, is transported to the root where it initiates an RLK pathway, which in turn leads to the branching phenotype. The second possibility is that either the higher Na^+ concentration or any other damage caused by the *sos1* mutation in the root induces the branching phenotype.

The Function of KAPP in Planta

Because KAPP is predicted to have a promiscuous function in down-regulating multiple RLK pathways (Braun et al., 1997; Williams et al., 1997), it was surprising to find that *rag1-1* did not have an apparent phenotype at optimum growth conditions. Although overexpression of KAPP is shown to mimic the phenotype of CLV and FLS (Williams et al., 1997; Gomez-Gomez et al., 2001), the lack of a developmental phenotype in *rag1-1* indicates that KAPP may not be a central component of these pathways in planta. Stone et al. (1998) showed that inhibition of *KAPP* could suppress the clv-like phenotype. Gene suppression may cause a cosuppression of other genes, and, consequently, the resulting phenotype may diverge from that of a single gene knockout. Our preliminary results indicate that loss of function of KAPP does not suppress the clv phenotype of either *clv2-1* or *clv3-2* (data not shown).

One possible explanation is that there is another functionally redundant gene(s), which is not similar in the overall structure but has partial homology, such as POLTERGEIST (Yu et al., 2003). Another possibility is that KAPP becomes active only in response to certain environmental cues such as salinity or lack of light. In this case it is likely that, in addition to the described salt and light sensitivity of *rag1*, other undetermined phenotypes related to RLK may exist, which can only be observed in certain conditions or in response to certain stimuli.

RLK pathways regulate a broad range of signaling involved in either development or defense (Dievart and Clark, 2004). Those pathways involved in development regulate the balance between cell division and cell expansion or differentiation. *rag1* mutants have a defect in maintaining the correct ratio of cell division and expansion under NaCl-stressed or dark-grown conditions. Therefore, *rag1* exhibits a radially expanded root or shoot under those conditions. Shah et al. (2002) proposed that KAPP is an integral part of the endocytosis mechanism of RLKs. In this respect, KAPP may function as a positive regulator by mediating salt-induced endocytosis. This could be an important function of KAPP in the regulation of salt adaptation.

Table I. *Primers used for quantitative RT-PCR analysis (Fig. 8)*

Primer	Sequence
KAPP-F	CAAAATTGGTGTGGCCTCAGA
KAPP-R	AAGCGGCCACTTGTAATGACA
SOS1-f	CCAAAATTGAGCGACATGATCA
SOS1-r	GTGACACCACGCAGTTTCAT
Tubulin-f	AGGCAAAATGAGCACGAAAGA
Tubulin-r	TCAGACCTGTTGGTGGAATGTCAC
LRK-f	GTTTCGCATGAGTCTCGTCAAG
LRK-r	GAAGCTCACCTCTCCTACGACAA
LecRK1-f	TAGGTGTGTTGTGTTCGCATCA
LecRK1-r	TCCATTGATGTCTCAGGCCA
RD22-f	TACCCATTCGCGGTGTTCTACT
RD22-r	CTTTAGCTCGCATCCCGTTCT
RD29A-f	GAGACCCCGATAACGTTGGA
RD29A-r	CAATCTCCGGTACTCCTCCA

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) C24*RD29A:LUC* was transformed (mutagenized) with pSKI015 (Weigel et al., 2000). The T_2 T-DNA population was screened for NaCl-sensitive root growth (Zhu et al., 2002; Koiwa et al., 2003). *rag1-2*, *sos1-14*, *osm1-1*, and *npct1-1* were identified through this screen. *rag1-2* (Col-0) was identified through an in silico search of the SAIL T-DNA insertional mutant collection (Sessions et al., 2002).

For dose response experiments and histochemistry, seeds were sown on cellophane membranes and grown for 4 d as described previously (Zhu et al., 2002). Four-day-old seedlings were transferred to treatment medium (1× MS, pH 5.7, 1.5% agar, 3% Suc) supplemented with various concentrations of salts or osmolyte and allowed to grow for 6 or 9 additional days. Treatment media for low potassium and low calcium contained (1/20× MS macro elements, 1× MS micro elements) as described previously (Liu and Zhu, 1997; Rus et al., 2001). Seedlings grown on plates were placed in a controlled environment (16 h of light at 22°C and 8 h of darkness at 18°C), and plants grown on soil were placed in the greenhouse.

Growth Measurements

To measure root growth, the position of the root tip was marked at the bottom of petri dishes at the time of transfer and scanned by a flatbed scanner (Epson Perfection 1200U) at 300 pixels per inch after the treatments. The scanned images were saved as TIF format and were measured using the National Institutes of Health's Scion Frame Grabber as described (Buer et al., 2000).

Genetic Analysis of *rag1* T-DNA Insertion Alleles

Genomic sequence flanking T-DNA in *rag1-1* was determined using thermal asymmetric interlaced-PCR as described (Koiwa et al., 2003). To confirm the cosegregation of T-DNA with the salt-sensitive phenotype, homozygous *rag1-1* plants were backcrossed to wild type (C24 × *rag1-1*), and F_2 progenies were tested for salt sensitivity. DNA was extracted from F_2 progenies exhibiting a salt-sensitive phenotype. Then, diagnostic PCR was performed using primers F1, R1, and LB1 (shown in Figure 3) as described previously (Koiwa et al., 2003).

T-DNA insertion of *rag1-2* was confirmed by diagnostic PCR using primers F2, R2, and LB2 (shown in Fig. 3).

RNA was isolated from 10-d-old seedlings that were grown on cellophane membrane and treated on filter paper soaked with one-half-strength MS medium, pH 5.7, supplemented with 0 mM (control) or 175 mM (NaCl treatment) using the RNAeasy total RNA isolation kit (Qiagen) as described (Yokoi et al., 2002). First-strand cDNA was synthesized using the Superscript III kit (Gibco BRL) from total RNA (2 mg) as recommended in the manual protocol. The PCR reaction was carried out using the primers described in Figure 3A.

Genetic Complementation

Two different genomic DNA fragments (6,227-bp *Sca*I-*Apa*I and 7,305-bp *Bam*HI-*Apa*I fragments; Figure 3A) containing the *KAPP* open reading frame were digested from the bacterial artificial chromosome F7K24. The *Sca*I-*Apa*I fragment contains 740 bp of the 5′-untranslated region, and the *Bam*HI-*Apa*I fragment includes a sequence region of the putative 3′-untranslated region of the next upstream open reading frame. These fragments were subcloned into a shuttle vector, pBluescript SK+ (Stratagene); the 7,305-bp fragment into the *Xba*I site, and the 6,227-bp fragment blunt-end ligated into the *Hin*dIII site. Both fragments were cloned subsequently into the *Kpn*I site of the pBIB binary vector that contains a gene for hygromycin resistance in planta as selection marker (Becker, 1990). The *pBIB:KAPP* and pBIB (without insert as a control) vectors were introduced into *Agrobacterium* GV3101 to transform *rag1-1* and wild type (C24) using the floral infiltration method (Bechtold et al., 1993) as modified by Koiwa et al. (2002).

The progeny of segregating T_2 populations derived from hygromycin-resistant T_1 lines (plants obtained from seed of plants directly after floral transformation) were evaluated for cosegregation of *KAPP* expression-dependent salt tolerance and hygromycin resistance (χ^2 analysis for one or multiple insertions).

Histochemical Analysis

Seedling roots, treated in the same way as described for the dose response tests, were stained with 10 μg/mL propidium-iodide for 10 min to visualize cell walls and washed with distilled water. Then samples were imaged in distilled water using a confocal laser microscope (Bio-Rad MRC1024; Bio-Rad Laboratories). Illumination was provided at 568-nm wavelength and red emission was collected for 5 min. The figures are projection of 52 to 69 optimal dissections (Fig. 2).

Quantitative RT-PCR Analysis

The expression of the related genes was analyzed by real-time quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green and ABI PRISM 7000 Real-Time system. Tubulin was used as a standard control in the RT-PCR reactions. A two-step RT-PCR procedure was performed in all experiments. Total RNA was isolated from seedlings treated by one-half-strength MS or one-half-strength MS supplemented with 150 mM NaCl. The cDNA were used as template in real-time PCR reactions with gene-specific primers (Table I). The RNA RT and real-time PCR reaction were performed using SYBR PrimeScript RT-PCR kit (TaKaRa) according to manufacturer's instruction. PCR amplification was done in two steps: DNA denaturation at 95°C for 10 s and elongation at 60°C for 40 s. Fluorescence was evaluated at the end of the elongation. PCR reactions were maintained for 40 cycles. The amplification of the target genes was monitored for every cycle by SYBR-Green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene.

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