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Mutants of *Arabidopsis thaliana* Capable of Germination under Saline Conditions[1]

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Three mutant strains of *Arabidopsis thaliana* var Columbia were selected for their ability to germinate in elevated concentrations of NaCl. They were not more tolerant than wild type at subsequent development stages. Wild-type strains could not germinate at concentrations >125 mM NaCl. Two of the mutant strains, RS17 and RS20, could withstand up to 225 mM, whereas RS19 was resistant to 175 mM. The RS mutants could also germinate under even lower osmotic potentials imposed by high concentrations of exogenous mannitol (550 mM), whereas the effects of elevated levels of KCl, K_2SO_4, and LiCl were similar among the mutants and wild type. Therefore, the mutants are primarily osmotolerant, but they also possess a degree of ionic tolerance for sodium. Sodium and potassium contents of seeds exposed to high salinities indicated that the NaCl-tolerant mutants absorbed more of these respective cations during imbibition. These higher internal concentrations of potassium and sodium could contribute to the osmotic adjustment of the germinating seeds to the low osmotic potential of the external medium. Genetic analysis of F_1 and F_2 progeny of outcrosses suggest that the salt-tolerant mutations are recessive and that they define three complementation groups.

A better understanding of the underlying mechanisms involved in the plant response to salinity is essential to confront this agronomic problem. It is known that the detrimental effects of salinity (mostly but not exclusively attributable to NaCl) occur because of (a) osmotic stress, (b) interruption of metabolic activities by ionic excesses and imbalances, and (c) interference of salt ions on the uptake of essential macro- and micronutrients (Pasternak, 1987). These adverse effects are manifested in the inhibition of germination, reduction of growth, and disturbance of development (Levitt, 1980).

Plants vary greatly in their tolerances to salt. Halophytes can complete their life cycles under saline conditions (Flowers et al., 1986), but glycophytes, although generally more sensitive to saline stress, range widely in their tolerances between species and even among varieties (Greenway and Munns, 1980; Flowers and Yao, 1987). The fact that pertinent mechanisms, as a consequence, must involve many gene products emphasizes the importance of genetic analysis. Descriptions of single genes that contribute to salt tolerance are few, but they include those responsible for Cl^- exclusion in certain varieties of soybean (Abel, 1969), Pro accumulation in mutant barley (Kueh and Bright, 1982), and mutants of the fern *Ceratopteris ricardii* (Warne and Hickok, 1987; Hickok et al., 1991), in which the physiological mechanisms have yet to be determined.

The study of stage-specific variabilities in response to saline stress may result in the identification of the heritable components of salt resistance (Fooland and Jones, 1991). Isolation and characterization of mutants with stable salt-resistant properties at given growth stages should provide valuable insight into salt-tolerant systems and ultimately into the characterization of relevant genes.

Studies of germination performance have indicated that the major effects of a saline environment on germination are the prevention of imbibition and ionic toxicity (Torres-Schumann et al., 1989). The present study characterizes mutants of *Arabidopsis thaliana* that are able to germinate on high NaCl concentrations.

MATERIALS AND METHODS

Plant Material

Seeds of *Arabidopsis thaliana* var Colombia were mutagenized for 16 h in 0.2% ethyl methyl sulfonate under ambient laboratory conditions (Haughn and Somerville, 1986). Seeds were then washed thoroughly with distilled water and grown on artificial soil (Sunshine Mix No. 1, Fisons Horticulture, Inc., Vancouver, Canada) to produce M_2 seeds. Seeds were grown in a controlled environment growth chamber at 21°C under constant illumination (220 $\mu E\ m^{-2}\ s^{-1}$).

Approximately 0.5×10^6 M_2 seeds were screened at high seed density (25 replicates of approximately 20,000 seeds per 150- × 15-mm plates) on medium containing 250 mM NaCl. Seeds were considered to be germinated only after they produced fully expanded cotyledons. Selected seedings were propagated in soil to obtain M_3 progeny for further study.

In all experiments, seeds were surface sterilized in 30% (v/v) commercial bleach containing 0.02% Triton X-100 for 10 min, followed by six washes with sterile distilled water before plating. Plates were sealed with Parafilm and placed in a growth chamber set at 22°C.

The solid medium was composed of MS basal salts (Murashige and Skoog, 1962) and 2% Suc, solidified with 1% agar (Difco) and pH adjusted to 5.8 before autoclaving. Different concentrations of salt and organic compounds were made by adding appropriate amounts of reagents to the basal medium.

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Abbreviations: MS, Murashige-Skoog; WT, wild type.

Low Seed Density Screening of M_3 Seeds on Various Osmotica

Putative NaCl-resistant mutants were tested at low seed density (up to 400 seeds per 100-mm Petri plates) on various concentrations of NaCl (0–250 mM), KCl (0–200 mM), K_2SO_4 and Na_2SO_4 (0–110 mM), LiCl (0–30 mM), and mannitol (0–550 mM). In this type of screening, the area of a 100- × 15-mm plate was divided into quadrants with each sector containing 50 to 100 seeds that were evenly spaced. Each experiment was performed four times. The percentage of germinated seeds was calculated from the number of seedlings that reached the cotyledon stage at 14 d.

Genetic Analysis

Crosses between mutant lines and WT were performed by transferring pollen to the stigmas of emasculated flowers. F_2 seeds were produced through self-fertilization of F_1 plants. F_1 and F_2 seeds were tested by plating at low density on medium containing 150 mM NaCl. This concentration of NaCl prevents germination of WT. Germination was determined 14 d after plating.

Osmolality Measurements

Osmotic potentials of growth media containing various concentrations of different osmotica were determined before the addition of agar via the freezing point depression technique using an automatic osmometer (Osmette A; Precision Systems, Inc., Natick, MA).

Measurement of the Na and K Contents of Seeds

Na and K contents of WT and mutant seeds were measured by atomic absorption spectrophotometry. Four replicates of 20 mg of seeds were plated on medium containing either 100 or 150 mM NaCl for 0, 24, 48, 72, 96, or 120 h. Samples were collected from the surface, washed briefly with 50 mL of distilled water, and dried at 60°C for 48 h on Whatman No. 1 filter paper. These were then weighed before digestion with 2 mL of 70% nitric acid and dilution with distilled H_2O to a final volume of 10 mL. Na and K contents of these solutions were measured using a Unicam SP 90A spectrophotometer.

RESULTS

Recognition of Three Salt-Tolerant Mutants

Large-scale screening of approximately 0.5×10^6 M_2 seeds identified 24 individuals capable of germination on media with 250 mM NaCl. The seedlings were transferred to soil to obtain M_3 seeds that were tested for resistance to NaCl at low seed density. The germination capacity of each type of putative mutant was compared directly to that of coplated WT seeds. Of the 24 putative mutants, only three could germinate on media with NaCl concentrations higher than 125 mM. These three NaCl-tolerant mutants, RS17, RS19, and RS20, were further characterized.

Germination of Mutants on Media Containing Various Concentrations of NaCl

The mutants differed in their ability to germinate at high NaCl concentrations. RS17 and RS20 could germinate on media containing up to and including 225 mM NaCl (Fig. 1A). RS19, on the other hand, could not germinate on concentrations >175 mM NaCl. The salt-tolerant mutants were viable under these saline conditions for at least 8 weeks.

Growth of the germinated seedlings was adversely affected by NaCl concentrations >75 mM NaCl. Yellowing of foliage accompanied growth retardation. With NaCl concentrations >150, >125, and >150 mM for RS17, RS19, and RS20, respectively, seeds germinated and produced two cotyledons, and the seedlings grew to the size of 7-d-old WT seedlings grown in the absence of salt. Growth inhibition imposed on these plants was alleviated when seeds were transferred to the regular MS medium or to soil. In addition, inflorescence emergence did not occur in the mutants under saline conditions, and, as such, although these mutants germinated and survived elevated salinity, they could not complete their normal life cycle and reproduce under these harsh conditions.

Genetic Analysis

F_1 and F_2 progeny of crosses between WT and the mutants were analyzed on medium containing 150 mM NaCl (Tables I and II). All mutations were recessive because F_1 progenies of all crosses were incapable of germination on 150 mM NaCl. Complementation analyses between the mutants indicated that the traits could be explained by distinct genes in each mutant line.

Statistical analysis of the number of germinated F_2 seeds from self-fertilized F_1 plants was used to test the single recessive non-allelic model. Theoretically, one-fourth of the F_2 progenies of either of RS17\WT, RS19\WT, or RS20\WT hybrids are expected to be homozygous recessive and, therefore, salt tolerant. Moreover, it is expected that 7/16ths of tested seeds from F_2 progeny of RS17\RS19, RS17\RS20, or RS19\RS20 hybrids would be salt tolerant. The result of χ^2 testing supports the above-mentioned hypothesis for RS17 and RS20 mutant lines, but not for RS19.

The response of the F_2 progenies of crosses between the RS19 mutant line and other strains was problematic. The null hypothesis was rejected at the 1% confidence level for any of the F_2 progenies of RS19 hybrids with WT, RS17, or RS20. The observed numbers for germinated seeds were higher than expected because only 30% of RS19 seeds were capable of germination on medium containing 150 mM NaCl (Fig. 1A). The ratios of germinated: nongerminated seeds did not fit into any simple form of epistasis. Null hypotheses for any of the χ^2 values calculated for different genomic possibilities for RS19 were rejected. Furthermore, data did not fit any simple model of a second gene segregating in the background.

Germination Performance of WT and Mutants Exposed to Salt and Osmotic Stress

Mutant lines were tested on media containing various concentrations of KCl, LiCl, K_2SO_4, Na_2SO_4, and mannitol to

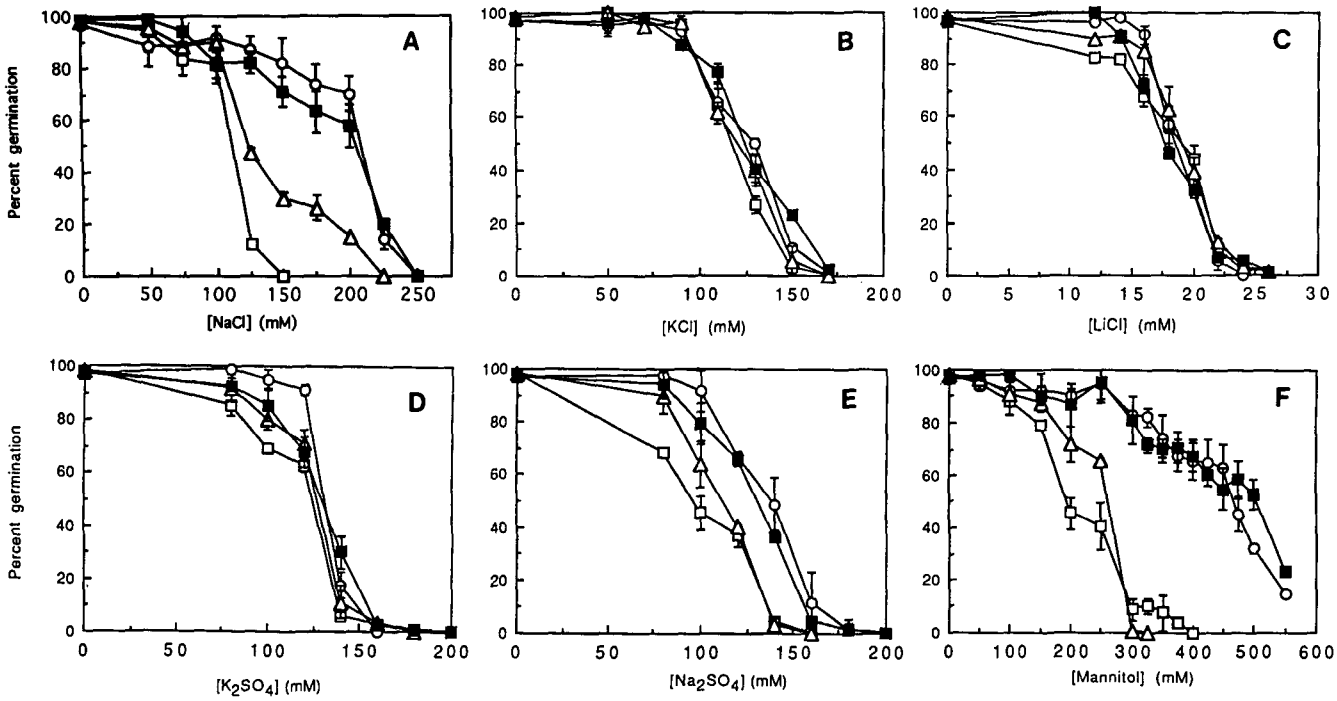

Figure 1. Germination pattern of WT (□) and mutant lines RS17 (○), RS19 (△), and RS20 (■) on various concentrations of NaCl (A), KCl (B), LiCl (C), K_2SO_4 (D), Na_2SO_4 (E), and mannitol (F). Percentage of germination was calculated from the number of seedlings with aerial organs (cotyledons) within 2 weeks from the beginning of the experiment. Data are means of four replicates of 50 to 100 seeds. Bar indicates SE. $n = 4$.

characterize the physiological differences between WT and mutant seeds (Fig. 1, B–F). Li^+ was included in the study because its accumulation by plant cells is similar to that of Na^+, but it is much more toxic (Lefebvre, 1989). Unlike NaCl, the increasing concentrations of KCl, LiCl, and K_2SO_4 had similar effects on the germination of WT and mutant lines. RS17 and RS20 seeds, compared to WT seeds, were capable of germination on media with higher concentrations of Na_2SO_4. Resistance to high concentrations of NaCl and, to a lesser extent, to Na_2SO_4, but not to KCl and K_2SO_4, suggests that these mutant lines are particularly resistant to the Na^+ ion. The comparison of salt tolerances of WT and mutant lines was based on the highest concentration of a given salt that permitted a 50% germination level (Fig. 2). These values were extrapolated from the relevant graphs in Figure 1.

Germination at Low Osmotic Potential

RS17 and RS20 mutants could germinate at lower osmotic potentials than WT seeds when mannitol was used as osmoticum (Fig. 1F). Figure 3 summarizes the results of comparative studies on the osmotic potentials of the media allowing 50% germination. It is evident that 50% germination of RS17 and RS20 seeds is accomplished under lower osmotic potentials imposed by elevated levels of mannitol or NaCl compared with that of KCl or K_2SO_4. These mutants, therefore, can tolerate low osmotic potentials caused by addition of high concentrations of both NaCl and mannitol, although not to the same extent.

Capacity to Tolerate NaCl Depends on Developmental Stage

Mutant and WT seeds were first plated on MS media, and, after 1 week, the newly germinated seedlings were transferred to plates containing different concentrations of NaCl. Newly germinated seedlings of all RS mutant lines and WT reacted similarly to the high concentrations of NaCl (data not given). No difference was observed in the survival pattern of these seedlings, suggesting that the mutant lines were more tolerant to saline stress only at the germination stage.

NaCl Effects on WT Seeds Are Reversible

Germination of WT seeds resumed after removal of saline stress. Ungerminated WT seeds were capable of germination on regular MS medium after being exposed to 200 and 250 mM NaCl concentrations for 7 d. Normal performance of the seeds was seen after only 2 d of exposure to the low NaCl medium.

Na and K Content of Seeds Exposed to Different Levels of NaCl

Na and K contents of seeds were measured during increasing periods of exposure to 100 and 150 mM NaCl. At 100 mM NaCl, WT germination performance was similar to that of RS17 and RS20 (Fig. 1A), whereas 150 mM NaCl suppressed WT but allowed germination of a high percentage of RS17 and RS20.

Table I. *Genetic analysis of the F_1 progeny of crosses between mutant lines and WT screened on 150 mM NaCl*

The genotypes of the RS17, RS19, and RS20 are represented by AA, BB, and CC, respectively. The WT allele for each mutant gene is represented by ++.

Cross	Germinated		Nongerminated		Total	χ^2	Significance
	Observed	Expected	Observed	Expected			
RS17 × WT AA × ++	0	0	58	58	58	0	NS
RS19 × WT BB × ++	0	0	38	38	38	0	NS
RS20 × WT CC × ++	0	0	21	21	21	0	NS
RS17 × RS19 AA × BB	0	0	14	14	14	0	NS
RS17 × RS20 AA × CC	0	0	60	60	60	0	NS
RS19 × RS20 BB × CC	0	0	28	28	28	0	NS

Maximum Na content of all seed lines occurred during the first 24 h of salt exposure, apparently because of a sudden inward flood of Na^+ from the medium (Fig. 4, A and B). These increases were followed by relatively rapid decreases in Na contents. After 2 d, all lines in 100 mM NaCl started to reaccumulate Na^+ ions, whereas only RS17 and RS20 did so in 150 mM NaCl.

The effect of 100 and 150 mM NaCl on the K content of seeds was also determined (Fig. 4, C and D). It rapidly declined during the first 48 h of exposure to 150 mM NaCl when there was less than one-third of the original K in the seed of all lines. K accumulation increased only in RS17 and RS20 during the fifth day. Under the less severe 100 mM NaCl conditions, germinating seeds of all lines possessed similar K leakage properties. However, under these conditions all seeds began to reabsorb K^+ from the environment immediately after the second day. All lines were more efficient in K uptake from medium containing 100 mM NaCl than from that containing 150 mM. These seeds could recover their initial K losses by the fourth (RS20) or fifth day (WT, RS17, and RS19) of exposure to 100 mM NaCl.

The relative K:Na content of seeds shows a rapid decline during the first 24 h of exposure to both 100 and 150 mM NaCl (Fig. 4, E and F). In this respect, during this period and subsequent days, there was little difference among the lines.

DISCUSSION

Three NaCl-tolerant mutants, RS17, RS19, and RS20, of *A. thaliana* were isolated by screening mutagenized seeds

Table II. χ^2 *testing of the null hypothesis for single recessive nonallelic mutations in mutant lines*

The data are presented for the F_2 progeny of self-fertilized F_1 hybrids. The mutant genes in RS17, RS19, and RS20 are represented by uppercase letters A, B, and C, respectively. The WT allele for each gene is represented by +.

Cross	Germinated		Nongerminated		Total	χ^2	Significance
	Observed	Expected	Observed	Expected			
RS17/WT self-cross A+ × A+	580	551.5	1626	1655	2206	1.96	NS
RS20/WT self-cross C+ × C+	161	180	559	540	720	2.65	NS
RS17/RS20 self-cross A+ × A+ +C +C	270	249	298	319	568	3.31	NS
RS19/WT self-cross B+ × B+	1410	1594	4965	4781	6375	28.2	**[a]
RS17/RS19 self-cross A+ × A+ +B +B	506	405	419	520	925	45.1	**
RS19/RS20 self-cross B+ × B+ +C +C	334	379	533	488	867	9.6	**

[a] **, $P < 0.01$.

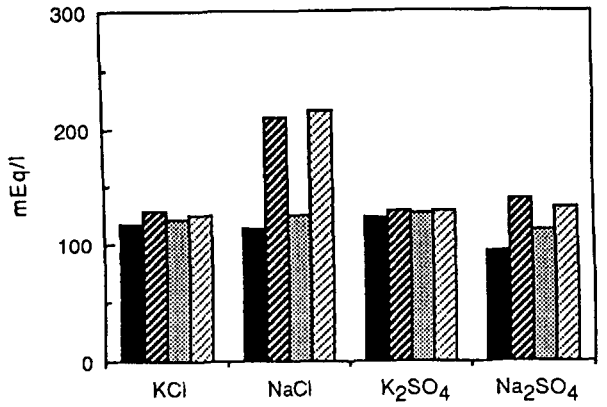

Figure 2. Comparison of the highest concentration of different salts allowing 50% germination of WT (■) and mutant lines RS17 (▨), RS19 (▩), RS20 (▨). The values were extrapolated from the relevant graphs presented in Figure 1.

under restrictive germination conditions for WT. The mutations represent distinct complemention groups, and those of RS17 and RS20 were single-gene traits that were recessive to WT alleles. Genetic attributes of RS19, a less salt-tolerant line, could not be clearly defined. The mutant plants tolerated both NaCl and, to a lesser degree, elevated Na_2SO_4 while remaining as sensitive as WT to toxic levels of LiCl, KCl, and K_2SO_4. Although the elevated toxicity of Li^+ could contribute to the similarity of response among the lines, the mutations, nevertheless, appear to possess an ionic specificity in their tolerance mechanisms. However, because the mutants demonstrated an even greater resistance to osmotic stress imposed by the organic solute mannitol, they are primarily osmotolerant. The additional suppression of germination by salinity over and above the osmotic inhibition by a relatively neutral solute such as mannitol can be explained by ionic interference of metabolic activities (Stumpf et al., 1986), and in the RS mutants the effect of Na^+ is much less than that of K^+. An innate ability to sequester Na^+ in the vacuole to a greater extent than K^+ could account for no significant improvement in tolerances for salts of the latter ion, especially if Cl^- partitioning follows suit. K salts have been shown to be more toxic than Na ones in WT cell cultures of *Brassica napus* (Lefebvre, 1989). A mutant of this cell line was resistant to elevated NaCl and KCl concentrations because of an increased ability to accumulate K^+, presumably in the vacuole. This is clearly not the case in the RS mutants of *A. thaliana*.

The performance of WT seeds exposed to permissive (100 mM NaCl) and restrictive (150 mM NaCl) germination conditions demonstrated a positive relationship between the ability to germinate and the accumulation of Na and K. Under permissive germination conditions all lines began to absorb these cations after 48 h, whereas under restrictive concentrations WT and RS19 failed to do so. Moreover, the higher levels of Na and K in RS17 and RS20 seeds compared with those of WT and RS19 seeds points to the possible relationship between tolerance capacity and the ability to take up Na^+ and, perhaps, K^+ as osmotica. The accumulation of Na^+ would certainly permit water absorption, although this should be accompanied by ionic sequestration in the vacuole to prevent metabolic toxicity, as well as the accumulation of compatible solutes within the cytoplasm (Reuveni et al., 1991). These are possible attributes of the RS mutants, although both an elevated production of cytoplasmically compatible solutes and an increased transport capacity for Na^+ and its counter-ion, Cl^-, into the vacuole are mechanisms that are most easily reconciled as genetically dominant processes. The mutant alleles in RS17 and RS20 are recessive.

Leakage of endogenous K^+ during imbibition of *Arabidopsis* seeds (Fig. 4, C and D) can be attributed to the loss of normal membrane integrity in the dried state (Simon, 1974, 1978). After 48 h the seeds began to accumulate Na^+ and K^+ under conditions permissive for germination. It is, therefore, reasonable to assume that the commitment to germinate occurs within this initial 48-h period. A defect in such a regulatory mechanism, designed to prevent germination under unfavorable conditions for subsequent growth and development, could result in the RS phenotypes. This is supported by the finding that WT seeds retain their capacity to germinate and develop to maturity upon removal of the restrictive saline conditions. The implication is that, although the osmotic potential is conducive to germination, its control is governed by a sensory mechanism that may act through detection of the cellular hydration level. This, by necessity, must be distinct from the threshold physical level of hydration required for germination to proceed (Bliss et al., 1986). Such an altered signaling system could also account for the distinct effects of mannitol, NaCl, and KCl, which could then be simply attributed to normal cellular processes as described above, i.e. the mutants need to be altered only in their signaling. An alternative to a defective signaling system that permits osmotolerant germination is an altered metabolic sensitivity to the hydration state. This is far less likely than a signaling defect because of the biochemical complexity of germinal processes. Furthermore, a defect in control agrees with the recessive nature of the mutant traits.

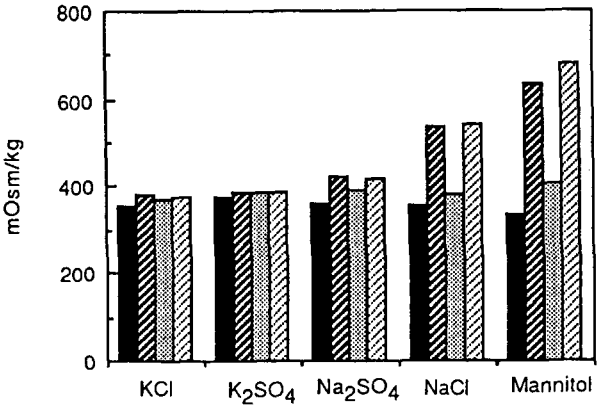

Figure 3. The effect of osmolality of media on the performance of seeds. The osmotic potentials of different media allowing 50% germination of seeds were used as a basis for this comparison. RS17 and RS20 mutant lines can tolerate lower osmotic potentials than WT in the presence of NaCl and mannitol. WT (■), Mutant lines RS17 (▨), RS19 (▩), RS20 (▨).

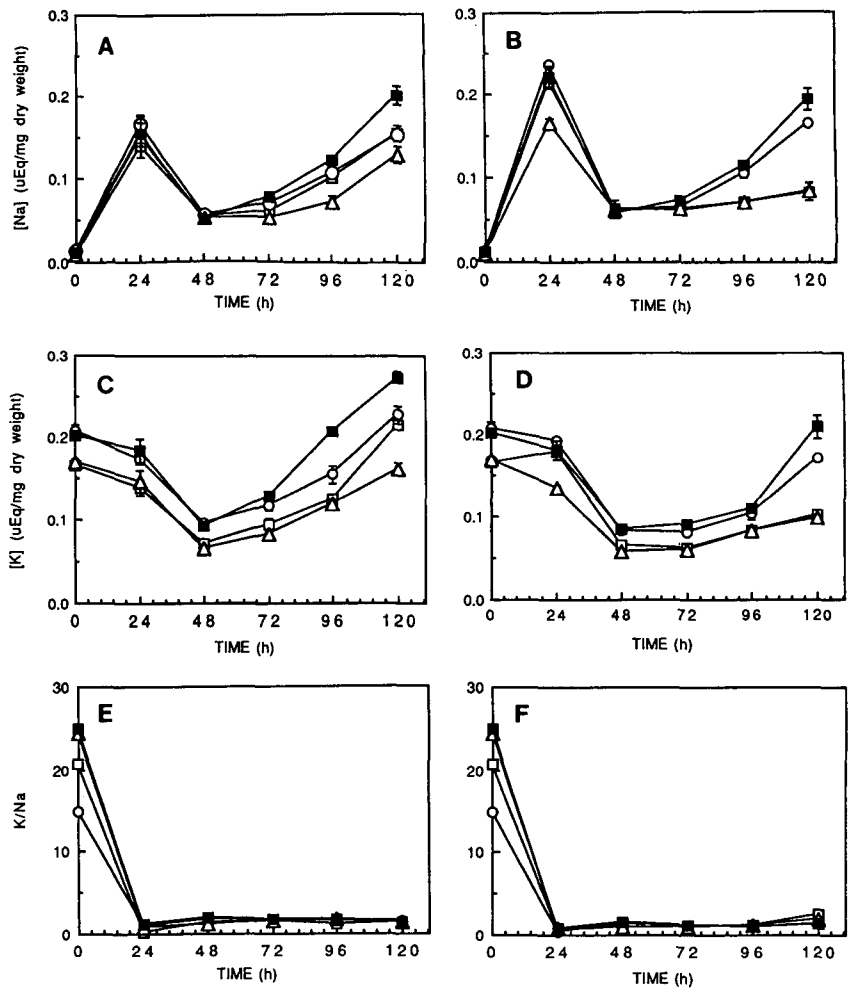

Figure 4. Na contents (μEq mg^{-1} dry weight) of WT (□) and mutant RS17 (○), RS19 (△), and RS20 (■) seeds exposed to growth medium containing 100 (A) and 150 (B) mM NaCl. C and D show K contents of seeds exposed to 100 and 150 mM NaCl, respectively. Bars indicate SE. n = 4. The relative K/Na contents after exposure to 100 mM (E) and 150 mM (F) NaCl are also presented.

Because there was no difference between the subsequent performance of mutant and WT seedlings under saline conditions after they had germinated in the absence of salt, the tolerance to low osmotic potentials is restricted to the germination stage. The disassociation between tolerance at germination and tolerance during growth and subsequent development was anticipated. Radicle emergence coincides with the inception of pronounced salt sensitivity in most crop plants, and the seedling stage of development is often the most salt sensitive in both glycophytic and halophytic plants (West and Taylor, 1981; Kayani et al., 1990).

Although the RS17 and RS20 mutant alleles are recessive to WT, the data obtained for the less-tolerant strain, RS19, do not fit into any simple single- or double-gene pattern of inheritance. At the phenotypic scoring conditions of 150 mM NaCl, RS17, RS19, and RS20 show an average of 80, 30, and 70% germination, respectively. The F_2 segregation data fit a model of single-gene inheritance for RS17 and RS20. If adjusted for the average germination capacity, however, the χ^2 is significant, and the simple model is rejected. Furthermore, in the case of RS19, adjustment does not help resolve the inheritance pattern. Because of seed shortage, we tested F_2 progenies only once at the restrictive NaCl concentration for WT germination. This does not allow for the calculation of means or variation between different trials; nor does it provide an accurate assessment of degrees of penetrance for these particular genotypes, which would require replicated response curves for germination among the different F_1 and F_2 progenies.

It has been shown that weakening of the physical restraints imposed by surrounding endosperm tissue on embryos can play an important role in the germination of tomato seeds (Haigh and Barlow, 1987). Groot and colleagues (1987, 1988) suggested that radicle protrusion across the seed coat relies on weakening the endosperm cell wall by hydrolysis. The genetic analysis applied to mutant strains in the present study does not address the question of whether salt tolerance is a trait conferred by an embryo, endosperm, or maternal genotype. Fooland and Jones (1990) studied the effects of these three components on trait segregation in tomato. Their results indicate that endosperm additive and testa dominance effects were highly significant and that the embryonic genotype played no significant role in germination performance under saline stress. It is possible that the RS mutations of *Arabidopsis* are involved in similar tissue-specific processes.

More extensive genetic analysis of salt tolerance in *A. thaliana* will provide us with a measure of its complexity and of the potential for manipulation of this trait in this geneti-

cally relatively simple glycophytic plant. In addition to tolerance during germination, the RS mutants now provide us with the opportunity to select for additional mutations that confer salt tolerance at subsequent developmental stages.

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