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Genetic Analysis of Salt Tolerance in Arabidopsis: Evidence for a Critical Role of Potassium Nutrition

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A large genetic screen for *sos* (for salt overly sensitive) mutants was performed in an attempt to isolate mutations in any gene with an *sos* phenotype. Our search yielded 28 new alleles of *sos1*, nine mutant alleles of a newly identified locus, *SOS2*, and one allele of a third salt tolerance locus, *SOS3.* The *sos2* mutations, which are recessive, were mapped to the lower arm of chromosome V, ~2.3 centimorgans away from the marker *PHYC.* Growth measurements demonstrated that *sos2* mutants are specifically hypersensitive to inhibition by Na^+ or Li^+ and not hypersensitive to general osmotic stresses. Interestingly, the *SOS2* locus is also necessary for K^+ nutrition because *sos2* mutants were unable to grow on a culture medium with a low level of K^+. The expression of several salt-inducible genes was superinduced in *sos2* plants. The salt tolerance of *sos1*, *sos2*, and *sos3* mutants correlated with their K^+ tissue content but not their Na^+ tissue content. Double mutant analysis indicated that the *SOS* genes function in the same pathway. Based on these results, a genetic model for salt tolerance mechanisms in Arabidopsis is presented in which *SOS1*, *SOS2*, and *SOS3* are postulated to encode regulatory components controlling plant K^+ nutrition that in turn is essential for salt tolerance.

INTRODUCTION

Excessive salt accumulation in soils affects the productivity of one-third of the world's limited arable land (Epstein et al., 1980). Much effort has been devoted toward understanding the mechanisms of plant salt tolerance with the eventual goal of improving the performance of crop plants in saline soils (Binzel and Reuveni, 1994). Equally important, these efforts continue to yield valuable knowledge about plant osmotic stress responses as well as cellular ion homeostasis (Bohnert et al., 1995; Niu et al., 1995; Zhu et al., 1997).

A widely used approach to unravel plant salt tolerance mechanisms has been to identify cellular processes and genes whose activity or expression is regulated by salt stress (reviewed in Hasegawa et al., 1987; Cushman et al., 1990; Skriver and Mundy, 1990; Bray, 1993; Bohnert et al., 1995; Zhu et al., 1997). The underlying assumption is that salt-regulated processes and genes likely function in salt tolerance. Although this correlative approach has contributed to our appreciation of the complex nature of plant responses to salinity, it has failed, to a large extent, to establish salt tolerance determinants (Zhu et al., 1997). There are numerous documented changes in cellular activities in higher plants in response to salt stress. Such changes include, for example, cell wall alterations (Jones and Turner, 1978; Iraki et al., 1989), declines in photosynthesis (Seemann and Critchley, 1985; Locy et al., 1996), protein synthesis (Singh et al., 1985; Hurkman and Tanaka, 1987), and potassium content (Rains, 1972; Greenway and Munns, 1980), and increases in Na^+ (Watad et al., 1983; Serrano and Gaxiola, 1994) and organic solutes, such as proline, glycinebetaine, and polyols (Greenway and Munns, 1980; Yancey et al., 1982; McCure and Hanson, 1990; Delauney and Verma, 1993). Although data correlating these cellular activities with salt tolerance abound, it has been difficult to ascertain which of the physiological and metabolic changes is required for salt tolerance. Similarly, despite the correlation of expression of numerous genes with salt stress (Cushman et al., 1990; Bray, 1993; Serrano and Gaxiola, 1994), very few genes are known to be essential for salt tolerance (Bray, 1993; Serrano and Gaxiola, 1994; Zhu et al., 1997). It has become evident that although some of these changes are adaptive and lead to salt tolerance, many are probably direct or indirect consequences of salt stress damage.

An alternative approach to identifying genes and cellular processes crucial for plant salt tolerance is to select for mutants with impaired salt tolerance (Wu et al., 1996). We have conducted a mutational analysis of plant responses to salt stress in the glycophyte Arabidopsis. Our initial efforts resulted in the isolation of several *sos* (for salt overly sensitive) mutants in one complementation group, *SOS1* (Wu et al., 1996). Analysis of these mutants showed that *sos1* is defective in high-affinity potassium uptake, suggesting that potassium acquisition is a cellular process essential for salt tolerance in glycophytic species (Wu et al., 1996). Under

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NaCl stress, *sos1* mutants accumulate more proline (Liu and Zhu, 1997a) but absorb less Na^+ as well as less K^+ (Ding and Zhu, 1997). Several salt-inducible genes show increased expression in *sos1* mutants under salt stress (Liu and Zhu, 1997a). We have since completed a large-scale screening (~260,000 plants) in an attempt to identify all genetic loci mutations in which can result an *sos* phenotype. Altogether, 42 *sos* mutants have been recovered. With these 42 *sos* mutants, it is now possible to address questions such as what other loci are also essential for plant salt tolerance and in what cellular processes do they function to control salt tolerance.

In this study, we summarize the screening results and present the characterization of mutants that define a new salt tolerance locus, *SOS2.* Analysis of all the *sos* mutants provided evidence suggesting that potassium nutrition, rather than sodium homeostasis or osmolyte accumulation, is most critical for salt tolerance in the glycophytic model plant Arabidopsis.

RESULTS

Identification of the *SOS2* Locus

Table 1 summarizes our screens for *sos* mutants. Altogether, 267,000 plants from either ethyl methanesulfonate– or fast-neutron–mutagenized M_2 seeds or from T-DNA insertion lines were screened using the root-bending assay (Wu et al., 1996; Figure 1). The more tedious single seedling transfer method for the root-bending assay was used because in our hands the rapid vertical-mesh-transfer method (Murphy and Taiz, 1995) was not as dependable. Approximately 156,000 seedlings were screened on 50 mM NaCl medium; the rest were screened using 75 mM NaCl. In total, 42 independent *sos* mutants were identified.

All mutants were backcrossed to their respective wild-type backgrounds. Analysis of the F_1 progeny showed that all of the mutations are recessive. The F_2 progeny resulting from the backcrosses showed approximately a 3:1 segregation ratio of wild type to mutant (χ^2 test, $P > 0.05$). Thus, all of the mutants are caused by single nuclear mutations. Allelism tests showed that the mutants fall into three complementation groups. Of the 42 *sos* mutants, 32, including four that were reported earlier (Wu et al., 1996), were found to be alleles of *SOS1* (data not shown). Except that one is not as sensitive to NaCl as *sos1-1*, the *sos* phenotype of all new *sos1* alleles appears as strong as *sos1-1.* One mutant defines the *SOS3* locus (Liu and Zhu, 1997b). The remaining nine alleles define a new locus, *SOS2.* There is no substantial difference in NaCl sensitivity among the nine *sos2* alleles. The recessivity and complementation results of *sos2* mutants are presented in Table 2. In the allelism tests, all nine *sos2* alleles were crossed to *sos1-1* and *sos3-1*, although only data on a representative allele (i.e., *sos2-1*) are presented in Table 2. For the many *sos1* alleles, in most cases only the representative *sos1-1* mutant was used to determine their allelism.

All subsequent physiological and phenotypic studies were performed on mutants that had been backcrossed to the wild type at least once. Figure 1 compares the phenotypes of *sos1-1*, *sos2-1*, and *sos3-1* seedlings in the root-bending assay. All three mutants as well as wild-type seedlings grew relatively well and appeared healthy on control Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) without NaCl supplementation (Figure 1A). On 50 mM NaCl, the growth of *sos1-1* and *sos2-1* was substantially inhibited. In comparison, the growth of wild-type and *sos3-1* seedlings was much less inhibited by 50 mM NaCl (Figure 1B). On 100 mM NaCl, wild-type seedlings still grew, albeit not very well.

Table 1. Summary of *sos* Mutant Screening

Mutagen	No. of M_1 or T_1 Lines Screened	No. of M_2 or T_4 Plants Screened	[NaCl] in Screening Media (mM)	No. of Mutants	Types of Mutants	Reference
EMS[a]	6,500	50,000	50	4	4 *sos1*	Wu et al. (1996)
	5,000	30,000	50	14	9 *sos1* 5 *sos2*	This study
	3,000	25,000	75	7	4 *sos1* 3 *sos2*	This study
Fast neutron	2,000	16,000	50	6	5 *sos1* 1 *sos2*	This study
	2,000	16,000	75	7	6 *sos1* 1 *sos3*	This study; Liu and Zhu (1997b)
T-DNA	6,000	60,000	50	1	1 *sos1*	This study
	5,000	70,000	75	3	3 *sos1*	This study

[a] EMS, ethyl methanesulfonate.

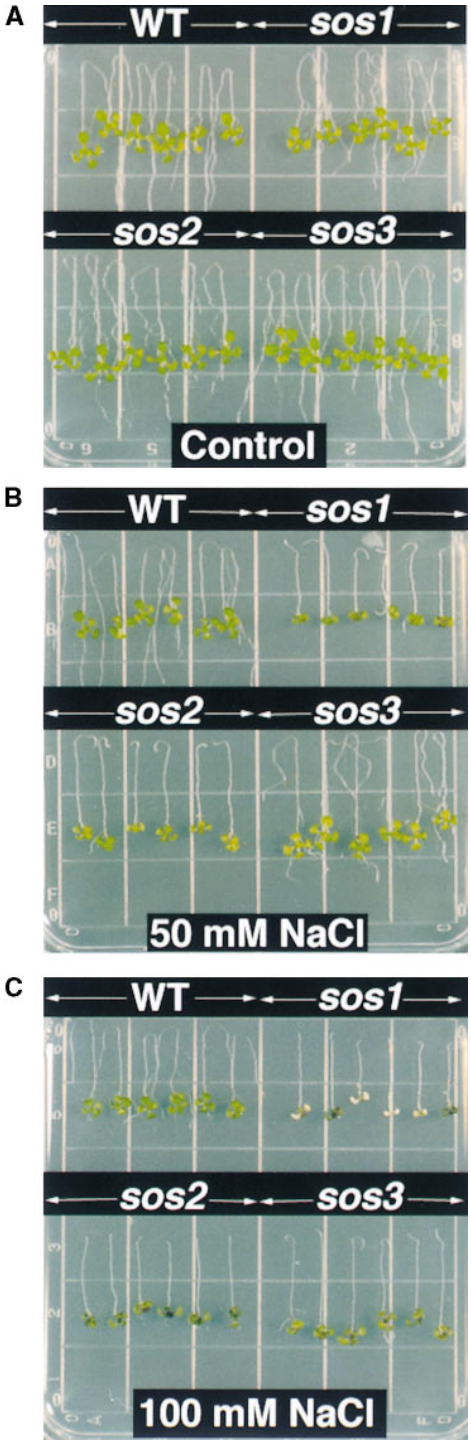

Figure 1. Comparison of Salt Sensitivity in *sos* Mutants on Vertical Plates by Using the Root-Bending Assay.

Five-day-old seedlings were transferred from normal MS medium to MS media supplemented with different concentrations of NaCl, and the seedlings (with roots upside down) were allowed to grow for 7 days. WT, wild type.

(A) Control (no NaCl).
(B) 50 mM NaCl.
(C) 100 mM NaCl.

However, 100 mM NaCl exhibited complete growth inhibition on *sos2-1* and near-complete growth inhibition on *sos3-1*, whereas *sos1-1* seedlings were killed by this high level of NaCl stress (Figure 1C).

To genetically map the *sos2* mutation, we crossed homozygous *sos2-2* plants in the Columbia *glabrous1* (*gl1*) background to plants of the Landsberg *erecta* background. The *sos2-2* allele was chosen for mapping because it is a fast-neutron allele and virtually identical to *sos2-1* in phenotype. We selected 624 *sos* mutants from the resulting F_2 population and extracted genomic DNA from each of these plants. By using the mapping methods of Bell and Ecker (1994), *sos2* was found to be linked to the simple sequence length polymorphisms (SSLP) marker *nga76* on chromosome V. It was further determined that *sos2* is linked more tightly to the cleaved amplified polymorphic sequence marker *PHYC* (Konieczny and Ausubel, 1993) on the lower arm of chromosome V. Of the 1248 chromosomes surveyed, only 29 recombinants were found, indicating that the *sos2* mutation is ~2.3 centimorgans away from *PHYC* (Figure 2). In comparison, the *sos3* mutation was mapped to a different segment of chromosome V near the SSLP marker *nga139* (Liu and Zhu, 1997b), and the *sos1* mutation was mapped to chromosome II (Wu et al., 1996).

sos2 Plants Are Hypersensitive to Na^+ and Li^+ but Not to Cs^+ and Mannitol Stresses

The responses of *sos2-1* seedlings to various salts and mannitol were analyzed to determine whether *sos2-1* is hypersensitive to general osmotic stress or to specific ions. Figures 3A and 3B show that *sos2-1* mutant plants are hypersensitive to NaCl but not to KCl. The concentration of NaCl that decreased the root growth rate by 50% relative to medium without salt (I_{50}) was estimated. The I_{50} concentrations for *sos2-1* seedlings and the wild-type seedlings were ~10 and 100 mM, respectively. In comparison, the I_{50} values for *sos1-1* and *sos3-1* are ~4 mM (Wu et al., 1996) and 40 mM (Liu and Zhu, 1997b), respectively. This difference in sensitivity to NaCl inhibition among *sos* mutants is also seen in Figure 1. Growth analysis of *sos2-1* on LiCl indicated that *sos2-1* was also hypersensitive to Li^+, a toxic cation closely related to Na^+ (Figure 3C). Interestingly, although Cs^+ is also a toxic cation related to Na^+, *sos2-1* was not hypersensitive to Cs^+ stress (Figure 3D).

Figure 4 shows that *sos2-1* was not hypersensitive to osmotic stress caused by mannitol. In contrast, *sos1-1* exhibited hypersensitivity to mannitol stress at low concentrations (<150 mM; Figure 4). At higher mannitol concentrations (150 to

Table 2. Genetic Analysis of *sos2* Mutants

Crosses	Generation	Total Seedlings Tested	Resistant[a]	Sensitive[a]
SOS2-1/SOS2-1 × *sos2-1/sos2-1*[b]	F_1	120	120	0
	F_2	429	322	107
sos2-1/sos2-1 × *sos1-1/sos1-1*[b]	F_1	105	105	0
sos2-1/sos2-1 × *sos3-1/sos3-1*[b]	F_1	126	126	0
sos2-2/sos2-2 × *sos2-1/sos2-1*	F_1	101	0	101
sos2-3/sos2-3 × *sos2-1/sos2-1*	F_1	127	0	127
sos2-4/sos2-4 × *sos2-3/sos2-3*	F_1	38	0	38
sos2-5/sos2-5 × *sos2-3/sos2-3*	F_1	61	0	61
sos2-6/sos2-6 × *sos2-2/sos2-2*	F_1	31	0	31
sos2-7/sos2-7 × *sos2-2/sos2-2*	F_1	51	0	51
sos2-8/sos2-8 × *sos2-2/sos2-2*	F_1	30	0	30
sos2-9/sos2-9 × *sos2-2/sos2-2*	F_1	10	0	10

[a] Resistance or sensitivity was determined in the root-bending assay by using 100 mM NaCl.
[b] Similar results were obtained when all other *sos2* alleles were crossed with the wild type, *sos1-1*, and *sos3-1*.

500 mM), *sos1-1* was also not hypersensitive (Figure 4). Taken together, the results in Figures 3 and 4 indicate that the *sos2-1* mutation does not cause a defective osmotic stress response. Rather, the defect is restricted to Na^+ and Li^+ tolerance. Therefore, *sos2-1* is likely to be defective in ion homeostasis.

sos2-1 Seedlings Are Unable to Grow on Low-Potassium Culture Media

Because *sos1* and *sos3* mutants are unable to grow on culture media containing low levels of potassium, we determined whether *sos2-1* plants are similarly affected. Wild-type and *sos2-1* seedlings grown on MS medium (~20 mM K^+) were transferred to modified MS media containing various levels of K^+ (Liu and Zhu, 1997b). Root growth was measured 1 week after the transfer. Root growth in the first day was not included so that the effect of residual K^+ carried from the seedlings transferred from the MS medium was minimized. Figure 5 shows that wild-type and *sos2-1* mutant seedlings had very different K^+ requirements for maximal growth. Despite their poor growth in the absence of added K^+, inclusion of 100 μM K^+ in the medium was enough to produce near-maximal growth for wild-type seedlings (Figure 5). In contrast, *sos2-1* seedlings required substantially more K^+ for growth; 10 mM K^+ was necessary for *sos2-1* to achieve 80% of maximal growth (Figure 5). For comparison, *sos3-1* seedlings require 1 mM K^+ for 80% of maximal growth (Liu and Zhu, 1997b), and *sos1-1* seedlings require ⩾20 mM K^+ for normal growth (Wu et al., 1996). At least 50 mM K^+ was necessary for maximal growth for both *sos2-1* (Figure 5) and *sos1-1* seedlings (Liu and Zhu, 1997b). The growth data on high concentrations of K^+ (Figure 5) differ somewhat from those in Figure 3B because the base growth media used in this case were not based on MS but its modified version (Liu and Zhu, 1997b), which contains potassium-free 1/20 strength MS major salts and 1 × MS minor salts.

$^{86}Rb^+$ tracer uptake experiments demonstrated that *sos1-1* seedlings have a defect in K^+ uptake (Wu et al., 1996). Similar measurements have failed to detect any substantial difference in K^+ uptake between wild-type and *sos2-1* (data not shown) or *sos3-1* (Liu and Zhu, 1997b) seedlings or excised roots. The growth defect of *sos3-1* but not *sos1-1* seedlings on low K^+ can be rescued by increased external Ca^{2+} (Liu and Zhu, 1997b). High external Ca^{2+} levels up to 10 mM were not able to rescue the growth of *sos2-1* seedlings on low K^+ (data not shown).

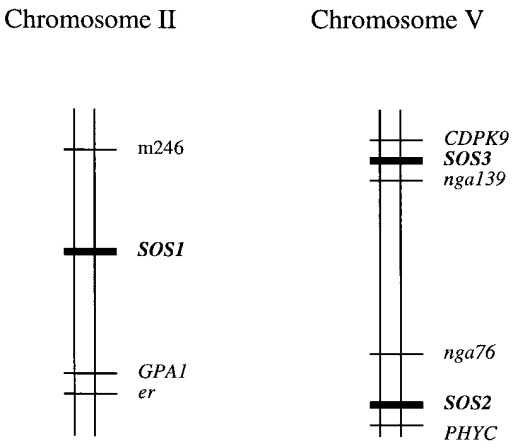

Figure 2. Map Positions of *SOS* Loci.

SOS2 and *SOS3* (Liu and Zhu, 1997b) are both mapped on chromosome V; *SOS1* (Wu et al., 1996) is located on chromosome II.

NaCl Sensitivity of *sos* Mutants Correlates with Cellular K^+ but Not Na^+ Content

The K^+ and Na^+ contents of wild-type, *sos1-1*, *sos2-1*, and *sos3-1* seedlings were measured after being treated with 50 mM NaCl for 24 hr. Consistent with previous observations (Wu et al., 1996; Ding and Zhu, 1997; Liu and Zhu, 1997b), *sos1-1* seedlings treated with NaCl were found to have lower Na^+ as well as K^+ content, whereas *sos3-1* seedlings accumulated less K^+ and more Na^+ than did the wild type (Figures 6A and 6B). *sos2-1* seedlings treated with NaCl also had lower K^+ levels than did the wild type (Figure 6A). Interestingly, the K^+ level in NaCl-treated wild-type and *sos* seedlings (Figure 6A) appears to correlate with their salt tolerance (Figure 6C).

Na^+ content in *sos2-1* seedlings treated with 50 mM NaCl was higher than that in *sos1-1*, *sos3-1*, or the wild type (Figure 6B). The results indicate that salt sensitivity of the *sos* mutants is not correlated with their cellular Na^+ content.

sos2 Mutations Cause Selective Superinduction of Salt-Induced Gene Expression

Because the *SOS2* locus is essential for salt tolerance, we were interested in determining the role of this locus in the regulation of gene expression by salt stress. Four salt-inducible genes were chosen for this purpose. The *RD29A* gene encodes a protein with potential protective function during desiccation (Yamaguchi-Shinozaki and Shinozaki, 1993). The *P5CS* gene encodes the enzyme Δ^1-pyrroline-5-carboxylate synthetase involved in proline biosynthesis (Delauney and Verma, 1990; Hu et al., 1992). *AtMYB* and *AtPLC*, which encode a MYB-related transcription factor and phospholipase C, respectively, have been proposed to play roles in osmotic signal transduction (Urao et al., 1993; Hirayama et al., 1995). Expression of each of the four genes was induced by NaCl stress in both wild-type and *sos2-1* seedlings (Figure 7). However, the induction of *AtMYB* and *AtPLC* expression in *sos2-1* was much

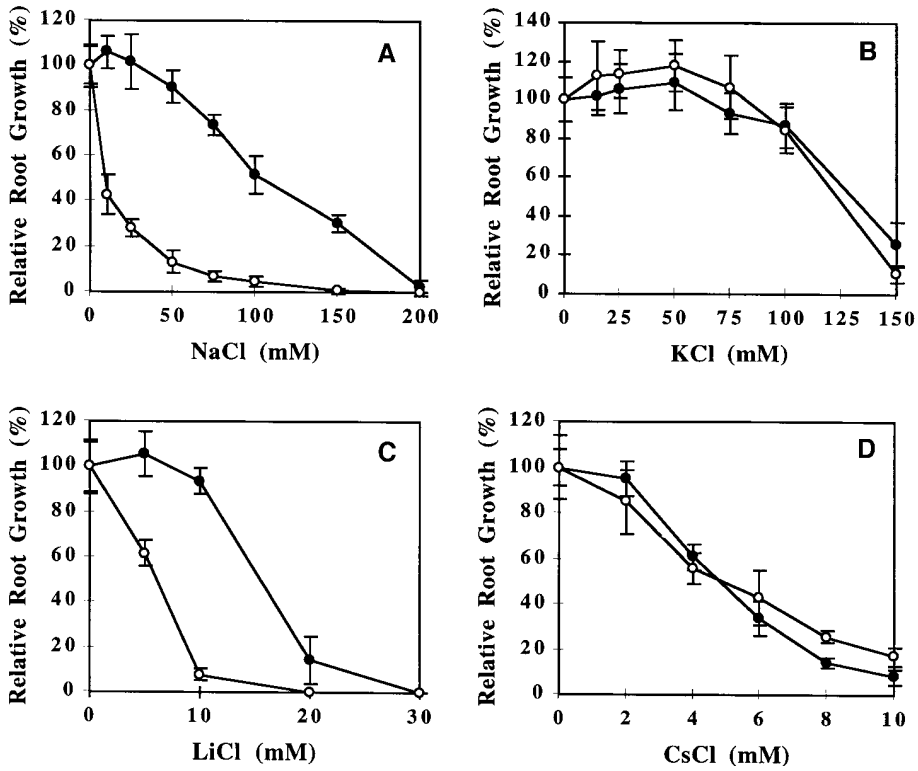

Figure 3. Sensitivity of *sos2* Seedlings to Various Salts.

Four-day-old seedlings were transferred to MS medium or MS media supplemented with various concentrations of NaCl, KCl, LiCl, or CsCl. Root elongation after 7 days is presented as a percentage relative to elongation on MS medium. Filled circles, wild type; open circles, *sos2*. Error bars represent the standard deviation ($n = 15$).

(A) Sensitivity to NaCl.

(B) Senstivity to KCl.

(C) Sensitivity to LiCl.

(D) Sensitivity to CsCl.

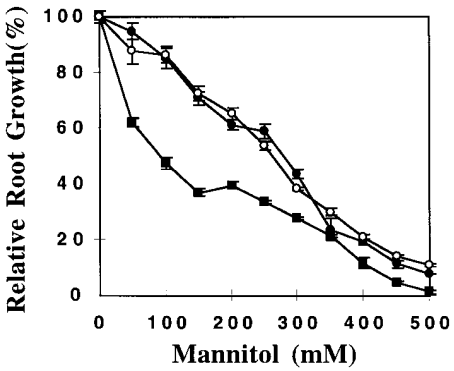

Figure 4. *sos1* Mutants but Not *sos2* Mutants Are Hypersensitive to Mannitol Stress.

Four-day-old seedlings were transferred to MS medium or media supplemented with various concentrations of mannitol. Root elongation after 7 days is presented as the percentage relative to that on MS medium. Filled circles, wild type; filled squares, *sos1*; open circles, *sos2.* Error bars represent the standard deviation ($n = 15$).

higher than in the wild type (Figure 7). The expression of *P5CS* was slightly higher in *sos2-1* compared with that in the wild type. No substantial difference in the expression of *RD29A* was found between *sos2-1* and the wild type (Figure 7).

The expression of *AtMYB* and *RD29A* in *sos2-1* is similar to that seen in *sos1-1* plants (Liu and Zhu, 1997a). *P5CS* has a higher level of expression in *sos1-1* compared with *sos2-1* and the wild type. The greatest difference between *sos2-1* and *sos1-1* mutants is in the expression of *AtPLC* because *sos1-1* and the wild-type plants have similar levels of *AtPLC* expression. The data suggest that the *SOS2* locus plays a negative role in the expression of *AtMYB*, *AtPLC*, and *P5CS* but not of *RD29A.*

Genetic Interaction between *sos2* and *sos1* Mutations

sos1, *sos2*, and *sos3* mutants exhibited similar phenotypes in that they are all hypersensitive to Na^+ and Li^+ and are not capable of growing on low-K^+ culture media. Thus, the three *SOS* genes may function in the same pathway that regulates K^+ acquisition or utilization and salt tolerance. Double mutant analysis indicated that *sos1* is epistatic to *sos3* (Liu and Zhu, 1997b). To determine the epistatic relationship between *sos1* and *sos2* mutations, we constructed *sos1 sos2* double mutants.

The response of the *sos1 sos2* double mutant to NaCl inhibition was similar to that of *sos1.* This can be demonstrated conveniently when *sos1-1*, *sos2-1*, and *sos1 sos2* seedlings were exposed for 1 week to an agar medium containing 100 mM NaCl (Figure 8). Only ~38% of *sos1* seedlings survived the treatment, whereas none of the *sos2* seedlings were killed. Approximately 42% of *sos1 sos2* double mutants survived the same treatment. Furthermore, the growth of *sos1 sos2* double mutants on 50 or 100 mM NaCl was virtually identical to that seen in *sos1* (data not shown). The data indicate that the two mutations are not additive and support the notion that the *SOS* genes function in a linear pathway.

DISCUSSION

SOS2 Locus Is Required for Both Salt Tolerance and Potassium Nutrition

The *SOS2* locus is defined by nine independent *sos* mutants. The nine *sos2* alleles are all recessive and confer salt hypersensitivity when present in the homozygous state. The salt hypersensitivity phenotype of *sos2* is a basic cellular trait and is displayed by the mutants at every developmental stage (data not shown). In fact, calli derived from *sos2* seeds are also hypersensitive to NaCl (data not shown). *sos2* seedlings were not able to grow on low-K^+ culture medium, which reveals an essential role of the *SOS2* gene in potassium nutrition.

sos2 mutants are similar to *sos1* and *sos3* mutants in that they are all hypersensitive to Na^+ and Li^+ stresses and incapable of growing on low-K^+ culture medium. However, *sos2* mutants are also different from *sos1* and *sos3* in several ways. *sos2* plants were not as sensitive to Na^+ as were *sos1*

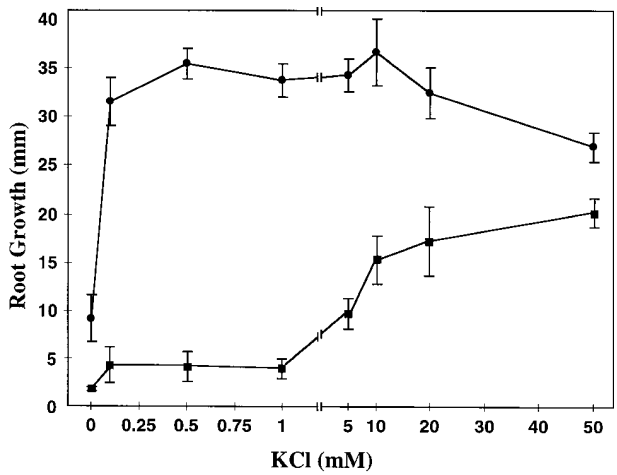

Figure 5. Optimal Growth of *sos2* Requires Increased External K^+ in the Culture Media.

Root elongation 7 days after the transfer of seedlings to culture media containing various levels of K^+ was measured. Wild-type seedlings (filled circles) reached near-maximal growth at a very low K^+ concentration (100 μM K). *sos2* seedlings (filled squares) required as high as 50 mM K for maximal growth. Error bars represent the standard deviation ($n = 15$).

plants (Figures 1, 6C, and 8). *sos2* plants were not hypersensitive to mannitol stress, whereas *sos1* plants were hypersensitive to low to moderate levels (<150 mM) of mannitol (Figure 4). Growth of *sos1* mutants had a higher requirement for K^+ than did *sos2* (Figure 5). K^+ deficiency in *sos2* mutants was not as severe as in *sos1* under the same level of NaCl stress (Figure 6A). Although the Na^+ content in NaCl-treated *sos1* seedlings was lower than that of the wild type, *sos2* had a slightly higher Na^+ content than did the wild type. Finally, *SOS2* but not *SOS1* is a negative regulator of *AtPLC* gene expression.

Compared with *sos3*, *sos2* is more sensitive to Na^+ (Figure 1). Growth of *sos2* seedlings required more K^+ than did *sos3* plants. *sos2* also had a lower K^+ content than did *sos3* when treated with NaCl. Most notably, high Ca^{2+} concentration cannot restore growth of *sos2* seedlings on low-K^+ culture medium as it does for *sos3.*

It is interesting that the *sos2* mutation also resulted in the superinduction of some salt-regulated genes. This appears not to be a simple consequence of more severe growth inhibition and damage caused by NaCl stress in the mutant. Increased inducibility was seen mainly in the *AtPLC* and *AtMYB* genes, which have been proposed to be intermediate components in stress signaling pathways. It is possible that *SOS2* encodes a regulatory component that cross-talks with signaling pathways that control the expression of *AtPLC*, *AtMYB*, and *P5CS.*

Which Is More Detrimental to Plants under Salt Stress: Higher Tissue Na^+ Content or Lower K^+ Content?

A key factor limiting plant growth in the case of salt stress, besides decreased water potential, is excessive Na^+, which is a harmful element not required by most glycophytes for normal growth (Niu et al., 1995). High Na^+ tissue content is therefore often considered as the most critical factor responsible for salt toxicity in non-halophytes (Greenway and Munns, 1980; Niu et al., 1995). Our results with the *sos* mutants, however, indicated that salt sensitivity in Arabidopsis is not closely related with Na^+ tissue content (Figure 6). *sos1* plants take up less Na^+ and consequently have a lower Na^+ content than do wild-type plants (Ding and Zhu, 1997), yet the lower Na^+ level is not associated with a reduced salt sensitivity in this mutant. In the *rrs* mutant of Arabidopsis that exhibits less sensitivity to Na^+ during germination, the Na^+ content is higher than in wild-type plants exposed to high external NaCl (Werner and Finkelstein, 1995). These observations do not support the notion that excess Na^+ is the primary cause of salt sensitivity in non-halophytes (Greenway and Munns, 1980).

In contrast, the results presented in Figure 6 indicate that the level of salt tolerance as measured by root growth correlates closely with K^+ content. Because of the critical role of K^+ homeostasis, relatively small variations in cellular K^+ content are expected to cause large differences in plant

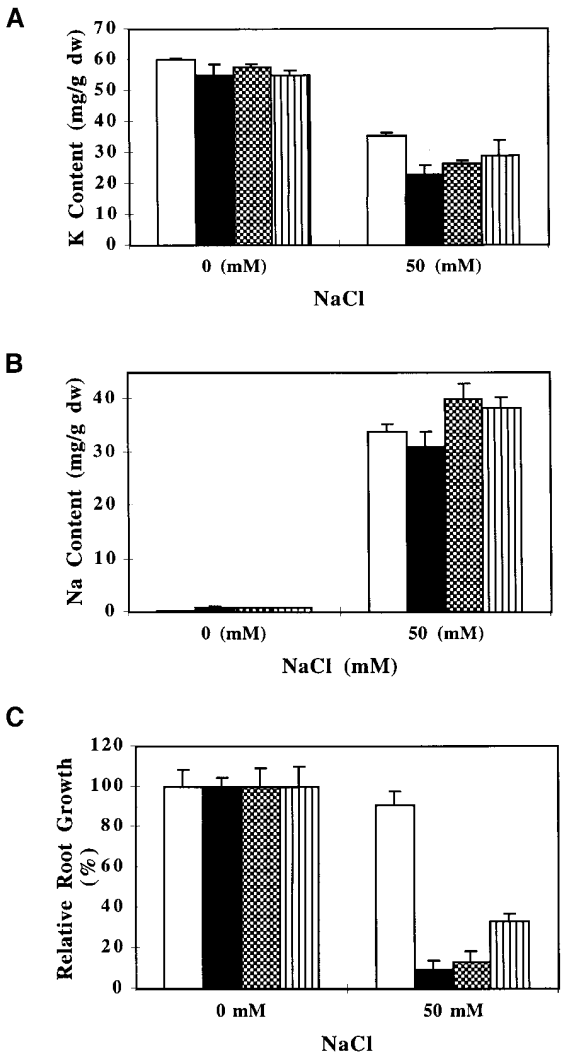

Figure 6. Salt Tolerance Correlates with K^+ Content but Not with Na^+ Content in Seedlings.

Five-day-old seedlings on MS agar plates were transferred to media (agar plates for root elongation measurement and liquid culture for K^+ and Na^+ assay) with or without 50 mM NaCl and allowed to grow for 48 hr. Open bars, wild type; black bars, *sos1-1*; stippled bars, *sos2-1*; striped bars, *sos3-1*; dw, dry weight.

(A) K^+ content in whole seedlings. Results are the average from three independent replicates. Error bars represent the standard deviation.

(B) Na^+ content in whole seedlings. Results are the average from three independent replicates. Error bars represent the standard deviation.

(C) Relative root growth. Error bars represent the standard deviation ($n = 15$).

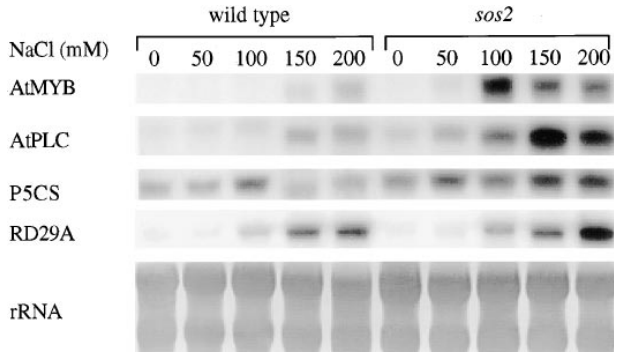

Figure 7. Salt Stress–Induced Gene Expression in Wild-Type and *sos2-1* Seedlings Subjected to NaCl Treatment.

Expression of *AtMYB* and *AtPLC* genes was superinduced in *sos2* seedlings.

growth. Because these plants are single-gene mutants and specifically sensitive to Na^+, the results highlight the important role of K^+ nutrition in salt tolerance of this glycophytic plant. Several reports from studies with plant cell cultures and yeast cells have also suggested the importance of K^+ nutrition in salt tolerance (Gorham et al., 1991; Watad et al., 1991; Gaxiola et al., 1992; Haro et al., 1993).

Mechanistically, K^+ nutrition is critical for Na^+ tolerance because Na^+ and K^+ are chemically very similar. High concentrations of external Na^+ inhibit K^+ absorption by plant roots, particularly through the low-affinity systems (Epstein, 1972). K^+ is an essential element that plays vital roles in various aspects of plant cell growth and metabolism and is needed in large quantities. Therefore, maintaining cellular K^+ content above certain threshold levels in the presence of excess external Na^+ is critical for plant growth and salt tolerance.

Why Were Only Three *SOS* Loci Identified, and Why Are They All Involved in Potassium Nutrition?

The large number of plants (267,000) and primary mutagenized lines (29,500) that we screened suggests that the *sos* mutant screening was nearly saturated. Altogether, 32 alleles of *sos1* and nine alleles of *sos2* were found. The large numbers of *sos1* and *sos2* alleles support the notion that the screening was close to saturation under the conditions used. The screening method is very effective in selecting mutants with great reduction in salt tolerance. However, mutants that are slightly more sensitive than the wild type are difficult to select with the present root-bending assay. This is probably why fewer *sos3* alleles were selected, because *sos3* is the least sensitive to NaCl among the three *sos* mutants.

Another constraint we put on the *sos* screening is that the mutants have to display relatively normal root bending (hence normal growth) in the absence of salt stress. Therefore, genes that are essential for both salt tolerance and normal plant growth were purposely excluded from our search. The constraint is necessary at the present time because it is still very difficult to distinguish between a gene essential only for normal growth and a gene essential for both normal growth and salt tolerance. Mutations in many housekeeping genes that have little to do with salt tolerance may result in decreased salt tolerance by reducing plant vigor.

Even considering these constraints, it is still surprising to find that the 42 *sos* mutants identified to date from near-saturated screening fall into only three complementation groups. Thus, only three *SOS* genes were found necessary for salt tolerance in Arabidopsis. Strictly speaking, only three genes were found that make large contributions to salt tolerance.

Even more surprisingly, all three *SOS* genes identified thus far turned out to be involved in potassium nutrition as well. We had expected to obtain mutants defective in Na^+ extrusion or compartmentation, proline synthesis, expression of some salt-inducible genes, or osmotic signaling. These latter processes probably operate primarily at high concentrations of NaCl. The accumulation of compatible osmolytes is often considered to be a universal protective mechanism used by many plants under salt stress. Whether proline accumulation is an adaptive process or a response to salt stress injury remains open to question (Bar-Nun and Poljakoff-Mayer, 1977; Hanson et al., 1979; Richards and Thurling, 1979; Moftah and Michel, 1987). The salt-hypersensitive *sos1* mutant in fact accumulates more proline than does the wild type (Liu and Zhu, 1997a). It appears that at the level of NaCl stress seriously inhibitory for a sensitive

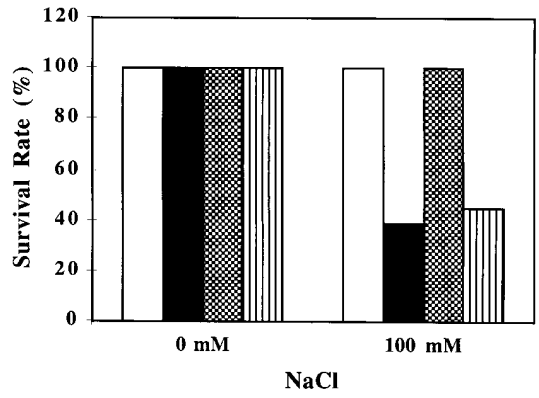

Figure 8. Comparison of Salt Sensitivity among *sos1*, *sos2*, and *sos1 sos2* Double Mutants.

The *sos1 sos2* double mutant resembles *sos1* in salt tolerance. Seedlings were subjected to 100 mM NaCl treatment for 7 days, and the percentage of seedlings that survived the stress is presented. Seedling death was scored as complete bleaching of cotyledons and leaves. Fifty-six each of the wild-type and *sos1* seedlings, 59 *sos2* seedlings, and 63 *sos1 sos2* double mutant seedlings were tested. Open bars, wild type; black bars, *sos1*; stippled bars, *sos2*; striped bars, *sos1 sos2* double mutant.

species like Arabidopsis (<100 mM NaCl), intracellular Na^+ toxicity or osmotic stress is still not a significant limiting factor. However, ≤100 mM NaCl does pose a serious problem for potassium nutrition, which is crucial for active growth. The fact that only these *sos* mutants were recovered suggests that although other processes such as Na^+ extrusion, Na^+ compartmentation, proline accumulation, and osmotic signaling may also be important, K^+ nutrition is the most critical process for salt tolerance in Arabidopsis.

We have shown here that impaired potassium nutrition accompanies increased salt sensitivity. Several reports have shown that improved K^+ nutrition correlates with increased salt tolerance (Gorham et al., 1991; Watad et al., 1991; Dvorak and Gorham, 1992; Gaxiola et al., 1992; Rubio et al., 1995). Perhaps under NaCl stress, only when K^+ nutrition is assured, will problems with other processes, such as maintaining a low cytoplasmic Na^+ content, accumulation of compatible solutes, and protective stress proteins, subsequently become more prominent. In this context, it is interesting to speculate that salt-tolerant plant species must possess very effective K^+ nutrition systems that are not disrupted by exposure to high external Na^+.

A Genetic Model of Salt Tolerance Mechanisms in Glycophytes

Based on the results from the analysis of *sos* mutants, a genetic model for salt tolerance mechanisms in glycophytes can be outlined. In this model, K^+ nutrition is credited as a crucial cellular process and a key element for salt tolerance in Arabidopsis. The term K^+ nutrition is used here to refer loosely to a collection of K^+-related activities, including influx, efflux, and utilization. Tracer uptake experiments using $^{86}Rb^+$ have shown that *sos1* mutants clearly have a defect in K^+ uptake (Wu et al., 1996; Ding and Zhu, 1997). Similar experiments have failed to detect substantial differences in K^+ uptake between *sos3* and wild-type seedlings or excised roots (Liu and Zhu, 1997b) or between *sos2* and wild-type seedlings or excised roots (data not shown). It is unclear whether *sos2* and *sos3* plants are really not defective in K^+ uptake or whether the results simply reflect the technical limitation of the uptake methodology. Notwithstanding, *sos2* and *sos3* plants, like *sos1*, do exhibit K^+ deficiency under NaCl stress (Figure 6).

One possibility is that all three *SOS* genes encode regulatory components and not transporters. An Arabidopsis homolog of the wheat high-affinity K^+/Na^+ cotransporter (Rubio et al., 1995) has been mapped recently. Evidence indicates that it is a single gene, and its map location does not correspond to any of the *SOS* genes (E. Kim and J. Schroeder, personal communication). Double mutant analysis (Figure 8; Liu and Zhu, 1997b) showed that the three *SOS* genes function in a linear pathway, although the epistatic relationship among the *sos* mutations cannot be ascertained because it is not known whether the mutant alleles are nulls. *SOS3* likely encodes a Ca^{2+} sensor, which regulates K^+ nutrition (Liu and Zhu, 1997b). *SOS1* is likely to be a general regulator of ion uptake because it has been found to play a role in Na^+ as well as K^+ uptake (Ding and Zhu, 1997) and perhaps the uptake of several other cations (J. Liu and J.-K. Zhu, unpublished data). The proposed model is probably most relevant for low levels of salt stress (≤100 mM NaCl). It does not exclude the functioning of additional mechanisms (e.g., Na^+ extrusion and compartmentation) that may become important at higher levels of stress.

METHODS

Plant Materials and Growth Conditions

The ethyl methanesulfonate– or fast-neutron–mutagenized M_2 *Arabidopsis thaliana* seeds were obtained from Lehle Seeds (Round Rock, TX) and were from ecotype Columbia carrying the homozygous recessive *glabrous* mutation (Koornneef et al., 1982). *Agrobacterium tumefaciens*–transformed lines (ecotype Wassilewskija) were obtained from K. Feldmann (Feldmann, 1991). The wild-type Arabidopsis Landsberg *erecta* ecotype used for genetic mapping was from the Arabidopsis Stock Center (Columbus, OH).

Seeds were surface-sterilized in a solution of Clorox plus 0.1% Triton X-100 for 10 min and rinsed five times with sterile water. The seeds were resuspended in sterile 0.4% [w/v] low-melting-point agarose before being sown in rows onto agar plates for germination. The agar medium contained Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with 3% [w/v] sucrose and 1.2% [w/v] agar, pH 5.7. After 48 hr at 4°C to improve germination uniformity, the plates were placed vertically in a growth room for germination. Mutant screening was conducted as previously described (Wu et al., 1996). When appropriate, seedlings (10 to 20 days old) were transferred to soil (Metro-Mix; Grace Sierra Horticultural Products Co., Milpitas, CA) in pots and grown to maturity. The plants were watered twice a week with one-quarter strength Hoagland solution. Growth room temperature was 23° ± 2°C. Light by cool-white fluorescent bulbs was 50 to 70 $\mu E\ m^{-2}\ sec^{-1}$ (constant) for seedlings in agar plates and ~100 $\mu E\ m^{-2}\ sec^{-1}$ (16 hr of light and 8 hr of dark) for potted plants.

Genetic Analysis

Backcrosses of *sos* mutants to the wild type and crosses among *sos* mutants were performed as described by Wu et al. (1996). F_1 and F_2 seedlings were scored for salt sensitivity by the root-bending assay (Wu et al., 1996). For mapping of the *SOS2* locus, homozygous *sos2-2* plants in the Columbia *glabrous1* (*gl1*) background were crossed to wild-type plants of the Landsberg *erecta* background. From the segregating F_2 generation, 624 homozygous *sos* mutants were selected, and DNA was extracted from each of these plants for mapping with molecular markers that are polymorphic between Columbia and Landsberg *erecta.*

To obtain a *sos1 sos2* double mutant, 35 *sos* mutants were selected from the selfed F_2 progeny of a cross between *sos2-1 sos2-1* and *sos1-1 sos1-1.* To identify a double mutant, these *sos* mutants were each testcrossed to *sos2-1 sos2-1* and *sos1-1 sos1-1.* One

double mutant line was identified because the F_1 progenies from both of the testcrosses showed a NaCl-hypersensitive phenotype.

Growth Measurements

For growth measurements, 4-day-old seedlings from vertical agar plates were transferred to MS agar plates supplemented with various salts. The treatment plates were placed vertically with seedlings in the upright position. Three replicates were run for each treatment. Increases in root length were measured with a ruler every day for 7 days.

Determination of K^+ and Na^+ Content

Four-day-old seedlings on MS agar plates were transferred to 250-mL flasks containing 50 mL of medium (half-strength MS salts and 2% sucrose, pH 5.5). The flasks were shaken at 120 rpm at 22°C with continuous cool fluorescent light illumination. After 4 days, the appropriate amount of 5 M NaCl was added to give the desired NaCl concentration, and the seedlings were allowed to continue growing for another 48 hr. The seedlings were then collected, rinsed briefly with distilled water, and dried at 65°C for 24 hr and weighed. The samples were digested with HNO_3, and the K^+ and Na^+ concentrations were determined with an atomic absorption spectrophotometer (model 560; Perkin-Elmer, Norwalk, CT).

RNA Gel Blot Analysis

Approximately 100 5-day-old seedlings were transferred from vertical MS plates to 250-mL flasks containing 50 mL of half-strength MS solution and 2% [w/v] sucrose, pH 5.5. The flasks were shaken at 120 rpm under cool fluorescent light. After 2 days, an appropriate volume of 5 M NaCl was added to give the desired Na^+ concentration. Twelve hours later, the seedlings were collected from the flasks and frozen immediately in liquid nitrogen. The samples were ground in liquid nitrogen, and then RNA was extracted as described by Liu and Zhu (1997b). RNA was separated on formaldehyde–agarose gels and blotted onto a nylon membrane. The membrane was first stained with methylene blue to verify equal loading and transfer. Blots were then hybridized with ^{32}P-labeled fragments of *RD29A*, *P5CS*, *AtMYB*, or *AtPLC*. The *P5CS* probe was a 1.6-kb fragment showing sequence identity to the *P5CS* gene reported in Yoshiba et al. (1995). *RD29A*, *AtMYB*, and *AtPLC* probes were cloned from genomic DNA of wild-type Columbia plants by polymerase chain reaction. The *RD29A* fragment was a gene-specific 0.5-kb probe from the 3′ noncoding region. Hybridizations were performed at 55°C. Blots were washed at 55°C in 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) (Ausubel et al., 1987) plus 0.1% SDS.

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