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). Despite the correlation of expression of numerous genes with salt stress (Frey 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; McCune 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
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 (\(\chi^2\) 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>
<thead>
<tr>
<th>Mutagen</th>
<th>No. of M(_1) or T(_1) Lines Screened</th>
<th>No. of M(_2) or T(_4) Plants Screened</th>
<th>[NaCl] in Screening Media (mM)</th>
<th>No. of Mutants</th>
<th>Types of Mutants</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS(^a)</td>
<td>6,500</td>
<td>50,000</td>
<td>50</td>
<td>4</td>
<td>4 sos1</td>
<td>Wu et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>30,000</td>
<td>50</td>
<td>14</td>
<td>9 sos1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 sos2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>25,000</td>
<td>75</td>
<td>7</td>
<td>4 sos1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 sos2</td>
<td>This study</td>
</tr>
<tr>
<td>Fast neutron</td>
<td>2,000</td>
<td>16,000</td>
<td>50</td>
<td>6</td>
<td>5 sos1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 sos2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>16,000</td>
<td>75</td>
<td>7</td>
<td>6 sos1</td>
<td>This study; Liu and Zhu (1997b)</td>
</tr>
<tr>
<td>T-DNA</td>
<td>6,000</td>
<td>60,000</td>
<td>50</td>
<td>1</td>
<td>1 sos1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>70,000</td>
<td>75</td>
<td>3</td>
<td>3 sos1</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\)EMS, ethyl methanesulfonate.
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 F2 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 (I50) was estimated. The I50 concentrations for sos2-1 seedlings and the wild-type seedlings were ~10 and 100 mM, respectively. In comparison, the I50 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...
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 mM 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 1/3 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).

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**Table 2. Genetic Analysis of sos2 Mutants**

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Generation</th>
<th>Total Seedlings Tested</th>
<th>Resistant(^a)</th>
<th>Sensitive(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS2-1/SOS2-1 × sos2-1/sos2-1(^a)</td>
<td>F(_1)</td>
<td>120</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>429</td>
<td>322</td>
<td>107</td>
</tr>
<tr>
<td>sos2-1/sos2-1 × sos2-1/sos2-1(^a)</td>
<td>F(_1)</td>
<td>105</td>
<td>105</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>126</td>
<td>126</td>
<td>0</td>
</tr>
<tr>
<td>sos2-2/sos2-2 × sos2-1/sos2-1</td>
<td>F(_1)</td>
<td>101</td>
<td>0</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>127</td>
<td>0</td>
<td>127</td>
</tr>
<tr>
<td>sos2-3/sos2-3 × sos2-1/sos2-1</td>
<td>F(_1)</td>
<td>38</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>61</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>sos2-4/sos2-4 × sos2-1/sos2-2</td>
<td>F(_1)</td>
<td>31</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>51</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>sos2-5/sos2-5 × sos2-1/sos2-2</td>
<td>F(_1)</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F(_2)</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^a\) 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.
Salt Tolerance Loci of Arabidopsis

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 \(\Delta^1\)-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) Sensitivity to KCl.
(C) Sensitivity to LiCl.
(D) Sensitivity to CsCl.
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.
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

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**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).
The Plant Cell

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.

**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.

**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; Moffah 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 accompanied 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, 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 86Rb⁺ 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 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 Ca²⁺ sensor, which regulates K⁺ nutrition (Liu and Zhu, 1997b). SOS1 is likely to be a general regulator of K⁺ 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 (L. 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 M₂ Arabidopsis thaliana seeds were obtained from Lehle Seeds (Round Rock, TX) and were from ecotype Columbia carrying the homozygous recessive glabrous mutation (Koomneef 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°C ± 2°C. Light by cool-white fluorescent bulbs was 50 to 70 μE m⁻² sec⁻¹ (constant) for seedlings in agar plates and ~100 μE 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). F₂ and F₃ 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 F₂ 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 F₂ 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 F1 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 HNO3, 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 NaCl concentration, and the seedlings were allowed to continue growing for another 48 hr. The seedlings were then hybridized, rinsed briefly with distilled water, and dried at 65°C for 24 hr and weighed. The samples were digested with HNO3, and the K+ and Na+ concentrations were determined with an atomic absorption spectrophotometer (model 560; Perkin-Elmer, Norwalk, CT).

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