Proline Accumulation and Salt-Stress-Induced Gene Expression in a Salt-Hypersensitive Mutant of Arabidopsis

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The sos1 mutant of Arabidopsis thaliana is more than 20 times more sensitive to NaCl stress than wild-type Arabidopsis. Because proline (Pro) is generally thought to have an important role in plant salt tolerance, the sos1 mutant and the wild type were compared with respect to their capacity to accumulate Pro under NaCl stress, and sos1 mutant plants accumulated more Pro than the wild type. The P5CS gene, which catalyzes the rate-limiting step in Pro biosynthesis, is induced by salt stress to a higher level in sos1 than in the wild type. Although a defective high-affinity K uptake system in sos1 causes K deficiency and inhibits growth in NaCl-treated plants, this decrease is not a sufficient signal for Pro accumulation and P5CS gene expression. Not all salt-stress-induced genes have a higher level of expression in sos1. The expression levels of Atplc and RD29A, which encode a phospholipase C homolog and a putative protective protein, respectively, are the same in sos1 as in the wild type. However, the expression of AtMYB, which encodes a putative transcriptional factor, is induced to a much higher level by salt stress in sos1. Thus, the SOST gene product serves as a negative regulator for the expression of P5CS and AtMYB, but has no effect on Atplc and RD29A expression.

In higher plants either the glutamate or the Orn pathway is used for Pro biosynthesis (Adams and Frank, 1980; Delauney and Verma, 1993). Under osmotic stress, Pro accumulation is mainly a result of de novo synthesis from glutamate (Delauney and Verma, 1993). Genes encoding Pro biosynthesis enzymes have recently been cloned from higher plants (Delauney and Verma, 1990; Hu et al., 1992). The first two steps beginning from glutamate are catalyzed by P5CS (Adams and Frank, 1980), which is the rate-limiting enzyme (Hu et al., 1992; Zhang et al., 1995). Expression of P5CS has been found to be induced by salt stress, dehydration, and ABA (Hu et al., 1992; Yoshioka et al., 1995).

The mechanism by which osmotic stress induces P5CS gene expression and Pro accumulation is not understood. The gram-negative bacteria Escherichia coli and Salmonella typhimurium also accumulate osmolytes such as Pro and betaine in response to osmotic stress (Csonka and Hanson, 1991). The proU gene, which encodes a high-affinity betaine transport system, is induced by osmotic stress at the transcriptional level (Sutherland et al., 1986). This induction has been found to depend not on changes in turgor elicited by osmotic stress, but on the intracellular concentration of K+ ions (Sutherland et al., 1986). Therefore, when bacterial cells are osmotically stressed by NaCl, the consequent changes in cell turgor lead to an increase in intracellular K+ concentrations, which then induces proU expression. In salt-stressed plant cells, while the concentration of osmolytes such as Pro increases, the intracellular K+ concentration decreases (Greenway and Munns, 1980). It is possible that intracellular K+ depletion serves as an intermediate signal that mediates salt-stress-induced Pro accumulation in higher plants.

In addition to P5CS, many other genes are induced by salt stress in Arabidopsis. Some of the salt-stress-induced genes such as RD29A encode putative protective proteins (Yamaguchi-Shinozaki and Shinozaki, 1993), whereas others, such as AtMYB and Atplc, encode proteins that share sequence identities to transcription factors and signaling components (Urao et al., 1993; Hirayama et al., 1995). The expression patterns of these genes have been extensively analyzed. However, no cellular component that positively or negatively regulates their expression has been identified.

Our laboratory has recently isolated an Arabidopsis mutant, sos1, which exhibits growth that is hypersensitive to...
NaCl inhibition (Wu et al., 1996). Availability of this single-gene mutant provides an excellent opportunity to address whether Pro accumulation is obligatory for salt tolerance. We report here that sos1 mutant plants accumulate more Pro than wild-type plants in response to salt stress. The P5CS gene is found to be overexpressed in salt-stressed sos1 plants. Our results demonstrate that more Pro production does not necessarily lead to increased salt tolerance. Furthermore, we found that salt stress induced overaccumulation of Pro and the P5CS message is not mediated by an intracellular depletion of K⁺. Several other salt-stress-induced genes were also examined in the sos1 mutant, and the results suggest that the SOS1 gene plays a negative role in the expression of some of these genes.

**MATERIALS AND METHODS**

*Arabidopsis thaliana* (ecotype Columbia) carrying the homozygous recessive *glabrous (gl1)* marker (Koornneef et al., 1982) was used as the wild-type control. The sos1 mutant used for this work was the sos1–1 allele (Wu et al., 1996). Seeds were surface-sterilized, suspended in sterile, 0.3% (w/v), low-melting-point agarose, and germinated in vertical agar plates containing MS salt (Murashige and Skoog, 1962), 3% (w/v) Suc, and 1.2% (w/v) agar, pH 5.7. Plants were grown at 22 to 24°C with continuous cool-fluorescent illumination.

**Salt Stress and Low-K Treatments**

Four-day-old seedlings from vertical plates were transferred onto vertical agar plates containing either the MS medium supplemented with different levels of NaCl or low-K (200 μM) medium. Low-K medium was prepared as described by Wu et al. (1996). After 5 to 7 d, the transferred plants were collected for Pro analysis. For RNA extraction, approximately 100 4-d-old seedlings from vertical plates were transferred to 250-mL flasks with 75 mL of medium containing one-half-strength MS salts and 2% (w/v) Suc, and 2% (w/v) NaCl, pH 5.5. The flasks were shaken at 120 rpm under constant light. After 2 d, the solution in the flasks was replaced with the same solution supplemented with different concentrations of NaCl or with the low-K solution. The plants were treated for 12 h before being harvested for RNA extraction, or were treated for 2 d in the case of Pro analysis.

**RNA-Blot Analysis**

Seedlings were harvested from the flasks, blotted dry with paper towels, and immediately frozen in liquid nitrogen. The samples were ground in liquid nitrogen and extracted on ice with 4.5 mL of extraction buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM auriurinicotricarbonylic acid, and 10 mM β-mercaptoethanol. After the addition of 0.7 mL of cold 3 M KCl, the mixture was incubated on ice for 15 min and then centrifuged at 9000 g for 20 min. RNA was precipitated from the supernatant by the addition of 2 mL of 8 M LiCl and incubation at 4°C overnight. The precipitate was pelleted by centrifugation at 9000 g for 20 min and then resuspended in 2 mL of water. The suspension was extracted with phenol and chloroform and RNA was precipitated from the aqueous phase with ethanol. The pellet was washed with 80% ethanol, dried, and resuspended in 200 μL of water. RNA was separated on formaldehyde-agarose gels and blotted onto nylon membrane. The membrane was first stained with methylene blue to verify equal loading and transfer. Blots were then hybridized with fragments of RD29A, P5CS, AtMYB, or AtPLC that were labeled with ³²P by random primer labeling. The P5CS probe was a 1.6-kb cDNA fragment showing sequence identity to the P5CS gene reported in Yoshiha et al. (1995). RD29A (Yamaguchi-Shinozaki and Shinozaki, 1993), AtMYB (Urao et al., 1993), and AtPLC (Hirayama et al., 1995) probes were cloned from genomic DNA of wild-type Columbia plants by PCR. The PCR primer pairs used for the amplification of RD29A, AtMYB, and AtPLC were 5′ CCC GGA TCC TTT TCT GAT GTT GCC 3′ and 5′ GCC CTC GAG CCG AAC AAT TTA TTA ACC 3′, respectively. The RD29A fragment was a gene-specific probe of 0.5 kb from the 3′ noncoding region. Hybridizations were carried out at 55°C. Blots were washed at 55°C in 3× saline sodium citrate (Ausubel et al., 1987) plus 0.1% SDS.

mRNA bands on radiographic films were scanned with a densitometer (model 300A, Molecular Dynamics, Sunnyvale, CA). rRNA bands probed with labeled 18s rRNA were also scanned as controls. mRNA intensities were normalized against the corresponding rRNA levels.

**Pro Analysis**

Seedlings were harvested, frozen in liquid nitrogen, and then dried by lyophilization. Approximately 50 mg of dry seedling tissue was ground in 3% sulfosalicylic acid to extract free Pro. Pro concentration was determined as described by Bates et al. (1973).

**RESULTS**

**sos1 Plants Overaccumulate Pro under Salt Stress**

In terms of growth inhibition, the sos1 mutant is more than 20 times more sensitive to salt stress than the wild type (Wu et al., 1996). Because Pro accumulation has been suggested to be important for salt tolerance, we were interested in determining the level of Pro in sos1 plants. Free Pro in wild-type and sos1 seedlings exposed to different levels of NaCl were extracted and measured. Figure 1A shows that Pro levels increased in both wild-type and sos1 plants in response to 50 mM NaCl treatment for 2 d in solution culture. However, the Pro level in sos1 was nearly twice as much as that in the wild type after 50 mM NaCl treatment (Fig. 1A). Without NaCl treatment, sos1 contained slightly more Pro than the wild type (Fig. 1A). Because most of the growth measurements and estimates of NaCl sensitivity sos1 were carried out on plants that were treated on NaCl-containing agar plates (Wu et al., 1996), the Pro contents of wild-type and sos1 seedlings treated for 5 d on plates containing 0 or 50 mM NaCl were determined (Fig. 1B). Similarly, Pro levels increased in both wild-type...
Gene is induced by salt stress and P5CS. In Arabidopsis, the P5CS enzyme catalyzes the synthesis of proline from glutamate (Hu et al., 1992; Zhang et al., 1995). In different levels of NaCl, is expressed at a higher level in sos1 plants under salt stress. Wild-type and sos1 plants were treated with NaCl. Figure 1C presents results from a quantitative analysis of the steady-state levels of P5CS mRNA in both wild-type and sos1 plants as a function of external NaCl concentration. However, the increase was higher in sos1 plants (Fig. 2). A quantitative analysis of the results presented in Figure 3 showed that the P5CS message abundance is approximately 1 to 2 times higher in sos1 plants than in the wild-type plants. For example, both sos1 and the wild type had maximal levels of P5CS expression at 150 mM NaCl. However, the abundance in sos1 is about 2.5 times higher than in wild-type plants (Fig. 3). The results suggest that Pro overaccumulation in sos1 is due at least partially to increased P5CS expression.

**Low K Inhibits the Growth of sos1 Plants, But Has No Effect on Pro Production and P5CS Gene Expression**

The sos1 mutants have a defective high-affinity K uptake system, which results in intracellular K deficiency under salt stress (Wu et al., 1996). Since increased K content during salt stress serves as a signal for pro and betaine accumulation in bacteria (Sutherland et al., 1986), we hypothesized that a decreased level of K in salt-stressed plants could be a signal for Pro accumulation in plants as well. Thus, the increased Pro content and P5CS expression in sos1 could be due to a more pronounced decrease in K concentration (Wu et al., 1996) when the mutant is treated with NaCl. Phenotypically, low K+ and high NaCl both inhibit the growth of sos1 plants (Wu et al., 1996). sos1 and wild-type plants grown on MS media were transferred to medium containing either 20 mM K+ in MS medium, 100 mM NaCl in MS medium, or 200 μM K+ for 5 d. As shown in Figure 4A, 100 mM NaCl induced Pro accumulation. However, neither wild-type nor sos1 plants treated with 200 μM K+ had significantly increased Pro levels (Fig. 4A), even though 200 μM K+ and 100 mM NaCl caused similar growth inhibition in sos1 plants (Fig. 4B). RNA-blot analysis showed that P5CS expression was not induced by low-K treatment in either wild-type or sos1 plants (Fig. 4C). These data do not support our original hypothesis, and instead suggest that Pro accumulation and P5CS expression are other forms of osmotic stress (Yoshiba et al., 1995). To investigate if the overaccumulation of Pro in sos1 plants under salt stress could be due to changes in P5CS gene expression, northern analysis was performed on RNA extracted from seedlings treated with different concentrations of NaCl for 12 h. Figure 2 shows that the steady-state levels of P5CS mRNA increased in both wild-type and sos1 plants as a function of external NaCl concentration. However, the increase was higher in sos1 plants (Fig. 2). A quantitative analysis of the results presented in Figure 3 showed that the P5CS message abundance is approximately 1 to 2 times higher in sos1 plants than in the wild-type plants. For example, both sos1 and the wild type had maximal levels of P5CS expression at 150 mM NaCl. However, the abundance in sos1 is about 2.5 times higher than in wild-type plants (Fig. 3). The results suggest that Pro overaccumulation in sos1 is due at least partially to increased P5CS expression.

**P5CS, a Gene Encoding a Key Enzyme in Pro Biosynthesis, Is Expressed at a Higher Level in sos1 Plants under Salt Stress**

The rate-limiting step in pro biosynthesis from glutamate is catalyzed by P5CS (Hu et al., 1992; Zhang et al., 1995). In Arabidopsis, the P5CS gene is induced under salt stress. The levels were approximately three to four times higher in plants treated in the agar plates compared with plants from the solution culture (Fig. 1). Again, the Pro level in sos1 was about twice as much as that in wild-type plants when treated with 50 mM NaCl. Pro content in plants treated with different concentrations of NaCl was also measured. Although the absolute values varied 2- to 3-fold among different experimental runs, the trend was always that sos1 contained at least twice as much Pro as the wild type when they were both treated with NaCl. Figure 1C presents results from a typical experiment in which the mutant and the wild-type seedlings were treated for 7 d on agar plates containing different levels of NaCl.

Figure 1. sos1 plants produce higher levels of Pro than the wild type in response to salt stress. A, Pro levels in seedlings treated in solution culture for 2 d (n = 3); B, Pro levels in seedlings treated in agar plates for 5 d (n = 3); C, Pro content as a function of external NaCl concentration. The plants were treated in agar plates for 7 d. Open bar, Wild type; shaded bar, sos1; O, wild type; •, sos1; dw, dry weight.

Figure 2. Expression of the P5CS gene is induced to higher levels by salt stress in sos1 than in the wild type. Wild-type and sos1 seedlings were treated with various levels of NaCl for 12 h in solution culture. The lower panel shows rRNA stained with methylene blue as a control for loading and transfer.
The sos1 Mutation Differentially Affects Salt-Induced Gene Expression

A large number of plant genes are induced under salt-stress conditions (Bray, 1993; Serrano and Gaxiola, 1994). Because the sos1 mutation increases the salt-stress-induced expression of P5CS, we examined several other salt-induced genes to determine the effect of the mutation on their expression. All of the three genes (RD29A, AtMYB, and AtPLC) examined here are known to be induced by salt-stress conditions in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1993; Urao et al., 1993; Hirayama et al., 1995). RD29A encodes a putative protective protein (Yamaguchi-Shinozaki and Shinozaki, 1993). AtMYB encodes a putative transcriptional factor related to MYB protein (Urao et al., 1993). AtPLC is a gene coding for phosphatidylinositol-specific phospholipase C, which functions in signal transduction (Hirayama et al., 1995). Neither the mechanisms of salt-stress regulation nor the exact functions of these genes in salt tolerance are known.

As shown in Figure 5, all of the three genes are induced by NaCl treatments. Expression levels of AtPLC and RD29A are not significantly different between sos1 and wild-type plants. The level of AtMYB expression is substantially higher in sos1 compared with the wild type. At 100 mM NaCl, AtMYB mRNA abundance in wild-type plants did not increase significantly compared with the control treatment (0 mM NaCl). However, 100 mM NaCl strongly increased AtMYB expression in sos1. Figure 5B also shows that low-K treatment did not induce the expression of RD29A.

**DISCUSSION**

In this paper we examined Pro accumulation and salt-stress-induced gene expression in the salt-hypersensitive Arabidopsis mutant, sos1. The fact that a mutant more sensitive to salt stress contains more Pro suggests that Pro accumulation is not a factor limiting salt tolerance in this plant. The data strongly support the notion that Pro accumulation is a symptom of stress injury rather than an indicator of stress tolerance.

The overaccumulation of Pro in this single-gene mutant also provides an opportunity to study how Pro increases under salt stress. We hypothesized that overaccumulation of Pro could be due to a much lower cellular K+ content in salt-stressed sos1. The hypothesis was based on the observation that salt stress leads to more K in bacterial cells, which in turn serves as a signal for betaine accumulation (Sutherland et al., 1986). Salt stress results in decreased K content in plant cells. Despite a good correlation between Pro accumulation and K deficiency in salt-stressed sos1 plants, our results indicate that decreased K content is not an intermediate signal for salt regulation of Pro accumulation or of P5CS gene expression. What, then, is the signal for Pro accumulation and P5CS gene expression? Apparently, Pro production is not simply a consequence of growth inhibition. This is because, although low-K treatment...
ment caused near complete inhibition of sos1 growth (Fig. 4), it did not significantly induce Pro accumulation. One possibility is that the signal could be an increased Na
 content in salt-treated plants. However, we found that sos1 has a significantly lower Na
 content than the wild type when treated with NaCl (Ding and Zhu, 1996). We suggest that turgor reduction is an intermediate signal for Pro accumulation in salt-stressed plant cells. When treated with NaCl stress, sos1 plants contain less K
 and less Na
 compared with wild-type plants. Because Pro and other organic osmolytes only increase slightly (Ishitani et al., 1996), K
 and Na
 are the predominant osmolytes in salt-stressed Arabidopsis. Therefore, less cellular K
 and Na
 in sos1 plants probably leads to higher cellular osmotic potential and, consequently, more pronounced turgor reduction. The greater turgor reduction could then cause higher P5CS gene expression and Pro production. In this regard, increased Pro production in sos1 may partially compensate its deficiency in inorganic osmolytes (i.e. K
 and Na
). Future investigations into the water relations in the sos1 mutant might provide clues regarding this speculation.

It is intriguing that the level of AtMYB expression is higher in sos1. Because AtMYB encodes a putative transcriptional factor, its increased expression may result in higher levels of expression of a group of its target genes. None of the genes regulated by AtMYB has been identified. It is possible that P5CS is one such gene, because it also has a higher level of expression in sos1.

One would expect that the level of RD29A expression would be higher in sos1 because the RD29A gene product is generally considered a stress protein (although its function is unknown). It is surprising that this stress gene is not expressed at a higher level in salt-treated sos1, even though this mutant is much more sensitive to salt stress than the wild type (Fig. 5B), which might indicate that RD29A expression is caused by a stress factor that is not preferentially activated in sos1. In any case, our results suggest that the SOS1 gene is a negative regulator for the salt-stress-regulated expression of P5CS and AtMYB, but not AtPLC or RD29A. The results also imply that the signal transduction pathways for the salt induction of P5CS and AtMYB are different from those used by AtPLC and RD29A.

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