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A cellulose synthase-like protein is required for osmotic stress tolerance in *Arabidopsis*

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SUMMARY

Osmotic stress imposed by soil salinity and drought stress significantly affects plant growth and development, but osmotic stress sensing and tolerance mechanisms are not well understood. Forward genetic screens using a root-bending assay have previously identified *salt overly sensitive* (*sos*) mutants of Arabidopsis that fall into five loci, *SOS1* to *SOS5*. These loci are required for the regulation of ion homeostasis or cell expansion under salt stress, but do not play a major role in plant tolerance to the osmotic stress component of soil salinity or drought. Here we report an additional *sos* mutant, *sos6-1*, which defines a locus essential for osmotic stress tolerance. *sos6-1* plants are hypersensitive to salt stress and osmotic stress imposed by mannitol or polyethylene glycol in culture media or by water deficit in the soil. *SOS6* encodes a cellulose synthase-like protein, AtCSLD5. Only modest differences in cell wall chemical composition could be detected, but we found that *sos6-1* mutant plants accumulate high levels of reactive oxygen species (ROS) under osmotic stress and are hypersensitive to the oxidative stress reagent methyl viologen. The results suggest that SOS6/AtCSLD5 is not required for normal plant growth and development but has a critical role in osmotic stress tolerance and this function likely involves its regulation of ROS under stress.

Keywords

Arabidopsis; cell wall; cellulose synthase-like protein; osmotic stress tolerance; reactive oxygen species; SOS6

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INTRODUCTION

Salinity is a major abiotic stress that severely limits the productivity of crop plants world wide. An estimated 20% of cultivated land and approximately 40% of irrigated land are affected by salinity (Rhoades and Loveday, 1990). Understanding the molecular basis of salt stress signaling and tolerance mechanisms is critical for developing rational breeding and genetic engineering strategies for improving salt tolerance of crops.

Salinity causes ionic stress for plants. Plants have evolved mechanisms to maintain ion homeostasis in the cell cytoplasm by coordinating the activities of various ion transporters. Genetic, molecular and biochemical analysis has led to the identification of the Salt Overly Sensitive (SOS) pathway that regulates ion homeostasis in plants (Zhu, 2003). The calcium-binding protein, SOS3, is suggested to sense changes in cytosolic free calcium elicited by salt stress (Liu and Zhu, 1998; Ishitani *et al.*, 2000). SOS3 interacts with and activates the protein kinase SOS2 (Halfter *et al.*, 2000; Liu *et al.*, 2000; Guo *et al.*, 2001). The SOS3–SOS2 protein kinase complex regulates the expression and activity of a plasma membrane-localized Na^+/H^+ antiporter SOS1, which exports Na^+ to the apoplast (Shi *et al.*, 2000; Qiu *et al.*, 2002; Quintero *et al.*, 2002). Transgenic plants that overexpress *SOS1* showed improved tolerance to salt stress (Shi *et al.*, 2003a). Recent studies suggest that SOS2 also positively regulates the activities of tonoplast Na^+/H^+ anti-porters (Qiu *et al.*, 2004), which sequester Na^+ ions in the vacuole and of a vacuolar H^+/Ca^{2+}exchanger CAX1 (Cheng *et al.*, 2004). Forward genetic screens in tomato also led to the isolation of several salt-hypersensitive mutants, although the mutated genes have yet to be cloned (Borsani *et al.*, 2001a).

Salinity, as well as drought, also imposes osmotic stress on plants, resulting in cellular dehydration and growth inhibition. Salt and drought stress induces abscisic acid (ABA), which has a major role in various aspects of stress tolerance (Zhu, 2002). Besides ABA, little is known about the determinants of osmotic stress tolerance and forward genetic screens in Arabidopsis have thus far not yielded informative mutants. A large body of evidence shows that various protein kinases are activated by osmotic stress, although in many cases the function of the kinases in osmotic stress response pathways has yet to be defined (Zhu, 2002).

The cell wall is a unique feature which distinguishes plants from animals. The major polysaccharides in the primary cell wall consist of cellulose, hemicelluloses and pectins. The cellulose microfibrils are linked via hemicellulosic tethers to form the cellulose-hemicellulose network, which is embedded in the pectin matrix. Cellulose is a polysaccharide composed of 1,4-linked β-D-glucose residues accounting for 15% to 30% of the dry mass of primary cell wall and provides a rigid cellular environment. The most common hemicellulose in the primary cell wall is xyloglucan. In contrast, pectins are a mixture of heterogenous, branched and highly hydrated polysaccharides rich in D-galacturonic acid. A group of genes encoding cellulose synthases (*CesA*), which belong to the glycosyltransferase family, are responsible for the biosynthesis of cellulose in plants (Somerville, 2006). Report by Chen *et al.* (2005) has shown that mutation in one of the 10 Arabidopsis *CesAs, AtCesA8,* results in enhanced drought and osmotic stress tolerance. Plants also contain a large number of genes encoding cellulose synthase-like (CSL) proteins. The Arabidopsis genome encodes six subfamilies of CSL proteins (AtCSLA, AtCSLB, AtCSLC, AtCSLD, AtCSLE and AtCSLG) (Richmond and Somerville, 2000), most of which have not been assigned a physiological function. Two additional subfamilies (OsCSLF and OsCSLH) have been identified in rice (*Oryza sativa*). CSL proteins might function in the biosynthesis of non-cellulosic polysaccharides such as xylans, xyloglucans, galactans and mannans (Richmond and Somerville, 2001). This conjecture is supported by

recent findings. For example, the rice CSL proteins OsCSLFs were shown to function in (1,3;1,4)-β-D-glucan biosynthesis (Burton *et al.*, 2006). Liepman *et al.* (2005) reported that three members of the AtCSLA subfamily, AtCSLA2, AtCSLA7 and AtCSLA9, could function as β-mannan synthases when expressed in *Drosophila* Schneider 2 (S2) cells. Genetic characterization of an Arabidopsis mutant carrying a transposon insertion in the gene encoding AtCSLA7 implicated a physiological role for AtCSLA7 in pollen tube growth and embryogenesis (Goubet *et al.*, 2003).

Among all the CSL subfamilies, the CSLDs are the most similar to the CESAs, with sequence identity in the range of 6–42%. Thus far, no biochemical function has been assigned to any CSLD protein, although AtCSLD3 was found to have a role in root hair growth by forward genetic analysis (Favery *et al.*, 2001).

Here we report the isolation and characterization of a new *sos* mutant, *sos6-1*. *sos6-1* plants are hypersensitive to NaCl and KCl but not LiCl. Furthermore, the *sos6-1* plants are hypersensitive to general osmotic stress imposed by mannitol or polyethylene glycol (PEG). Map-based cloning revealed that *SOS6* encodes a cellulose synthase-like protein (AtCSLD5). *sos6-1* mutant plants accumulate higher levels of reactive oxygen species (ROS) under osmotic stress than the wild type and are hypersensitive to the oxidative stress reagent methyl viologen (MV). The results suggest that SOS6 and SOS6-dependent cell wall components may control osmotic stress tolerance in part by regulating stress-induced ROS levels in plant cells.

RESULTS

Identification of the *sos6-1* mutant

An Arabidopsis salt hypersensitive mutant, designated as *sos6-1* (*salt overly sensitive 6*), was isolated from an ethyl methanesulfonate (EMS)-mutagenized M_2 population using the previously reported root-bending assay (Wu *et al.*, 1996) on medium containing 100 mM NaCl. The *sos6-1* seedlings grow relatively normally in control medium containing MS salts and 3% sucrose (Figure 1). On medium supplemented with 50 mM NaCl, growth of both roots and shoots of *sos6-1* seedlings was substantially more inhibited than that of the wild type (Figure 1a). *sos6-1* mutant plants displayed an even greater decrease in shoot growth and development after prolonged exposure (e.g. 1 month) to media containing various levels of NaCl (Figure 1b).

sos6-1 mutant plants were backcrossed with wild type plants. All the F_1 plants displayed a wild type phenotype and about three-quarters of the F_2 progeny from self-pollinated F_1 showed a wild type phenotype (data not shown). The result suggests that *sos6-1* is a recessive mutation in a single nuclear gene.

sos6-1 plants are hypersensitive to NaCl and KCl but not LiCl

We tested whether the salt hypersensitivity of *sos6-1* plants is specific to certain salts by measuring the response of root growth. Root growth of *sos6-1* plants is hypersensitive to NaCl and KCl (Figure 1c,d). We also examined the effect of *sos6-1* mutation on sensitivity to a more toxic analog of Na^+, Li^+, which can be used at a low concentration that does not cause significant osmotic stress. However, root growth of both the wild type and *sos6-1* plants was similarly inhibited by LiCl (Figure 1e). This result indicates that *sos6-1* is likely not hypersensitive to ionic stress. Consistent with this notion, ion absorbance spectrometry analysis (Zhu *et al.*, 2002) revealed that the *sos6-1* mutant plants accumulated essentially the same levels of Na^+ or K^+ as the wild type with or without NaCl treatment (Figure 1f,g). These results indicate that the salt hypersensitivity of *sos6-1* plants was not due to disrupted Na^+ homeostasis or impaired K^+ acquisition.

We also tested the effect of *sos6-1* mutation on seed germination under salt stress. Germination of *sos6-1* seeds was also more susceptible to NaCl inhibition than was the wild type (Figure 1h) suggesting that salt hypersensitivity of *sos6-1* plants does not depend on developmental stage.

sos6-1 plants are hypersensitive to mannitol and polyethylene glycol

The increased sensitivity of *sos6-1* plants to NaCl and KCl but not LiCl raises the possibility that *sos6-1* mutation mainly affects tolerance to general osmotic stress. Indeed, we tested and found that *sos6-1* plants are hypersensitive to osmotic stress imposed by mannitol. The increased inhibition by mannitol is especially obvious in the mutant shoot (Figure 2a,b). We then tested the response of *sos6-1* to another osmotic stress agent, polyethylene glycol (PEG). When germinated in medium with low water potential imposed by PEG, *sos6-1* seedlings were severely impaired in development and had significantly less fresh weight as compared with the wild type (Figure 2c,d). Thus, we conclude that the *sos6-1* mutation confers hypersensitivity to general osmotic stress in Arabidopsis.

sos6-1 plants accumulate more soluble sugars and proline under stress conditions

To investigate the physiological basis for the osmotic stress hypersensitivity in *sos6-1* mutant plants, we determined the levels of compatible osmolytes such as soluble sugars and proline in *sos6-1* and wild type plants. Under normal growth conditions, *sos6-1* plants accumulated a similar level of total soluble sugars as the wild type (Figure 3a). When treated with 150 mM NaCl, total soluble sugar content was much higher in *sos6-1* plants than in the wild type (Figure 3a). High pressure liquid chromatography (HPLC) analysis of soluble sugar content further revealed that *sos6-1* plants accumulated a similar amount of glucose and fructose (data not shown) but much higher levels of raffinose and sucrose as compared with wild type plants (Figure 3b,c). Under the low water potential condition imposed by PEG (−1.2 MPa), the level of proline was higher in the *sos6-1* mutant than in the wild type (Figure 3d). Clearly, the osmotic stress hypersensitivity of the mutant cannot be attributed to a deficiency in compatible osmolytes.

sos6-1 plants are hypersensitive to salt and drought stress in soil

Without stress, the growth and development of *sos6-1* plants were not distinguishable from those of the wild type (Figure 4a,c). When irrigated with 150 mM NaCl for 4 weeks, *sos6-1* plants were more damaged, their growth was more severely inhibited and their survival was much more decreased than that of wild type plants, which continued to grow and develop to maturity (Figure 4a,b). At the seedling stage, *sos6-1* plants showed no difference compared with wild type to growth inhibition by ABA in the media (Figure S1c,d). Both wild type and *sos6-1* plants became wilty when water was withheld from soil for 20 days (Figure 4c,e). However, *sos6-1* plants accumulated more anthocyanin under this dehydration condition (Figure 4c). When watering was resumed, most of the wilted *sos6-1* plants failed to recover, whereas more than 50% of the wilted wild type plants survived (Figure 4d,e).

sos6-1 plants overaccumulate ROS and are hypersensitive to oxidative stress

Salt and drought stress treatments can lead to the accumulation of ROS and cause oxidative stress (Price and Hendry, 1991; Borsani *et al.*, 2001b). We examined the effect of *sos6-1* mutation on ROS levels and the response to oxidative stress. Without stress, *sos6-1* and wild type plants showed similar basal levels of ROS (Figure 5a,b). When treated with NaCl or mannitol, *sos6-1* plants accumulated much higher amounts of total ROS (Figure 5a). Leaf staining with a superoxide-specific dye indicated that superoxide accumulated to higher levels in *sos6-1* mutant plants compared with the wild type (Figure 5b). We quantified the levels of hydrogen peroxide (H_2O_2) using the Amplex red reagent (10-acetyl-3,7-

dihydrophenoxazine). As shown in Figure 5c, under salt stress, *sos6-1* plants accumulated a substantially higher amount of H_2O_2 than the wild type. Furthermore, we found that *sos6-1* plants were hypersensitive to methyl viologen (mv), a chemical that generates superoxide radicals in chloroplasts, as indicated by substantial loss of chlorophyll in the mutant (Figure 5d,e). Taken together, these results suggest that SOS6 is an important regulator of ROS accumulation in plants under osmotic stress.

SOS6 encodes a cellulose synthase-like protein

sos6-1 plants were crossed with *Landsberg erecta* wild type plants for genetic mapping of the *SOS6* gene. *SOS6* was mapped by using simple sequence length polymorphism (SSLP) markers. The gene was localized between BAC clones T7I23 and F21B7 on chromosome 1. The genomic region between T14P4–46K and F22D16–93K markers was sequenced and a mutation was identified in the gene *At1g02730.* This gene encodes a cellulose synthase-like protein (CSL) and is classified as AtCSLD5 (Figure S2). There is a duplication of 158 nucleotides (2626–2783 numbered from translation start codon ATG) in the mutant (Figure 6a). This mutation would insert three new amino acid (aa) residues followed by a premature stop codon, resulting in a truncated 933 amino acid polypeptide, as compared with a 1181 aa polypeptide in the wild type. The truncated residues are part of a putative cellulose synthase activity domain (aa 302–1181 in the wild type).

To confirm that *At1g02730* is *SOS6,* a genomic fragment containing the wild type *At1g02730* gene was cloned and introduced into the *sos6-1* mutant. Transformants in the T_2 population showed wild type phenotypes, which indicates that *At1g02730* is indeed *SOS6* (Figure 6b,c). Plants of a second allele of *At1g02730,* designated as *sos6-2,* were also hypersensitive to NaCl or mannitol treatment compared with *Col-0* plants (background of *sos6-2*) (Figure 6b,d). These results demonstrate that SOS6 is AtCSLD5.

The *SOS6* promoter region was fused to a GUS reporter gene. *SOS6::GUS* expression was observed in all the tissues examined, with stronger expression in root tips, leaves, inflorescent stems and male and female organs of the flower (Figure 6e). Real-time RT-PCR analysis of *SOS6* expression in different tissues (Figure 6f) suggested a similar pattern as *SOS6::GUS* expression. Real-time RT-PCR and northern blot analysis also indicated that *SOS6* is constitutively expressed and not induced by salt stress and its transcript is more abundant in shoots than roots (Figure 6f).

The salt tolerance function is specific for CSLD5 in the CSLD subfamily

Among the five loci encoding additional members in the CSLD subfamily (*CLSD1-4 and CSLD6*), *CSLD1-3* genes are induced by NaCl stress while *CSLD4* and *CSLD6* are essentially not responsive to salt stress (Figure S3). The *sos6-1* mutation seems to have no effect on expression of these genes with or without NaCl stress (Figure S3) except that *CSLD3* is more abundant in the untreated *sos6-1* plants than wild type. We determined whether other members in the CSLD subfamily are involved in salt stress responses. For this purpose, homozygous T-DNA insertion lines with reduced expression levels in respective *CSLD* genes were identified and their ability to grow in the presence of NaCl or mannitol was measured (Figures S4–S8). None of other *csld* loss-of-function mutants displayed enhanced or decreased tolerance to NaCl or mannitol stress conditions. These results implicate that SOS6 in the CSLD subfamily is specifically required for osmotic stress tolerance.

Effect of the *sos6-1*/*csld5* mutation on cell wall composition

We determined the glycosyl composition of the whole cell wall from shoots of *sos6-1* and wild type plants, as an indication of their wall structures. Despite extensive analysis, we

found only modest differences between the wild type and *sos6-1* (Table 1). Under normal growth conditions, no statistically significant differences were detected between the cell wall from wild type and *sos6-1* plants. Under 200 mM NaCl treatment, the mutant showed reduced levels of Ara and Rha and an increased level of Fuc relative to the wild type. Although these differences were statistically significant, together they represented only about 2 mol% of the glycosyl content of the cell wall. Larger change was detected when plants grown under 200 mM NaCl treatment were contrasted with plants grown under normal conditions. The mol% of GalU in the cell wall was significantly lower when *sos6-1* plants were grown under 200 mM NaCl treatment than under normal conditions, while the analogous change in wild type plants was not statistically significant. To test for this change on an absolute, rather than mol%, basis, we performed a colorimetric assay of the total uronic acid (GalU + GlcU) content of the cell wall. Although seemingly in the same direction, differences between total uronic acids in the normal and NaCl conditions were not statistically significant (Table 1).

To examine the effect of *sos6-1* mutation on cell wall in detail, we sequentially extracted different fractions of the cell wall based on their solubility in imidazole or NaOH and subsequently determined the glycosyl composition of each wall fraction. Among the three different cell wall fractions, main alterations between *sos6-1* and wild type were found in the imidazole-soluble fraction (Table 2, principally pectic polysaccharides). Without stress, the levels of Rha, Fuc and Gal are significantly less in the *sos6-1* mutant plants than wild type whereas Glc is more abundant in the mutant (Table 2). Under 200 mM NaCl treatment, the *sos6-1* mutant displayed increased amount of Rha, reduced level of Xyl compared with wild type (Table 2). Although the treatment of salt stress in *sos6-1* also causes increased level of Ara and reduced amounts of GalU and Glc (Table 2), there is no significant difference between wild type and *sos6-1* for these three sugar residues. There are a few notable changes in the NaOH-soluble cell wall fraction (hemicelluloses and residual pectic polysaccharides) between *sos6-1* and wild type plants (Table 3). We reasoned that this might be due to the incomplete extraction of pectic polysaccharides in the previous step (imidazole extraction) during the preparation of the cell wall fractions. Essentially there is no significant difference between *sos6-1* and wild type in the NaOH-insoluble/cellulose fraction (Table 4).

Effect of *sos6-1* mutation on gene expression

Since the *sos6-1* plants carry a homozygous firefly luciferase report gene driven by the stress-inducible *RD29A* promoter (*RD29A::LUC*), we examined the expression of *RD29A::LUC* under different stress conditions (Figure S1a,b). These data indicate that *sos6-1* mutation does not affect *RD29A:: LUC* expression.

We then performed Affymetrix near-full genome Gene-Chip analysis to determine the effect of *sos6-1* mutation on gene expression. Statistical analysis of the microarray data revealed that after 150 mM NaCl treatment, there were only 23 genes that showed reduced levels of expression in roots of *sos6-1* by at least two fold compared with the wild type (Table S1), whereas the transcript of only one gene was significantly increased in roots of *sos6-1* (Table S2). Under the NaCl stress, we did not identify any gene with a significant reduction in transcript level in the shoot of *sos6-1*, whereas four genes showed increased transcript levels in the shoot of *sos6-1* compared with wild type (Table S3). Real-time PCR analysis of transcript levels of six randomly selected genes from the Tables S1–S3 confirmed the microarray results (Figure S9). Consistent with the increased growth inhibition by salt stress in the mutant, several of the genes with reduced expression in salt stress-treated *sos6-1* compared with the wild type (Table S1) have putative functions in cell expansion, e.g. the delta-TIP (Barkla *et al.*, 1999), arabinogalactan-protein (Shi *et al.*, 2003b), xyloglucan endotransglycosylase (Campbell and Braam, 1999) and expansin (Cosgrove, 1999).

Relevant to the defect in ROS management and increased ROS sensitivity, oxidative stress related genes such as the putative glutathione transferase and peroxidase genes (Table S1) also showed reduced expression in salt stress-treated *sos6-1* mutant plants compared with the wild type plants. Interestingly, a number of cytochrome P450 genes showed altered expression in the mutant (Tables S1 and S3). Cytochrome P450s are responsible for the biosynthesis and catabolism of a wide variety of compounds in plants including phytohormones such as ABA (Kushiro *et al.*, 2004). It is possible that the altered level of one or more of these cytochrome P450s may contribute to the osmotic stress hypersensitivity of the *sos6-1* mutant.

DISCUSSION

Forward genetic screening in Arabidopsis has been a powerful tool that helped identify several key loci important for the ion homeostasis aspect of plant salt tolerance (Zhu, 2003). However, mutants affected in the osmotic stress response pathway(s) have been difficult to isolate. The *sos6-1* mutant isolated in this study showed a strong hypersensitive phenotype under osmotic stress imposed by salts, mannitol or PEG. Thus, *SOS6* is a major locus for hyperosmotic stress tolerance in Arabidopsis. A few other mutants have been reported previously to be affected in their osmotic stress responses (Zhu *et al.*, 2002; Koiwa *et al.*, 2003), but their effects on osmotic stress tolerance are much smaller than that of *sos6-1*. Positional cloning led to the identification of the *SOS6* gene as *AtCSLD5 and* this is confirmed by complementation of the *sos6-1* mutant with the wild type *AtCSLD5* gene (Figure 6). The results implicate AtCSLD5 and/or AtCSLD5-mediated cell wall components as critical for osmotic stress tolerance.

We analyzed the glycosyl composition of the whole cell wall and different cell wall fractions and found only moderate effects of *sos6-1* mutation (Tables 1–4). No significant differences were detected between the wild type and *sos6-1* under normal conditions using the whole cell wall fractions while four residues showed differences (decreases in Rha, Fuc, Gal; increase in Glc) in abundance in *sos6-1* relative to wild type in the imidazole-soluble cell wall fraction (Table 2). Under NaCl stress, three residues – Ara, Rha and Fuc – differed slightly in abundance between the genotypes in the whole cell wall extracts (Table 1) and Rha and Xyl residues displayed altered levels between the genotypes in the imidazole-soluble cell wall fraction (Table 2). The two types of sugar analyses employed on the whole cell wall or different cell wall fractions complemented each other. Contrasting between normal and NaCl stress conditions within a genotype revealed that *sos6-1* had significantly less GalU under NaCl stress (Tables 1 and 2). Likely relevant to this observation is the report of Zablackis *et al.* (1995) who found that 0.1–0.5 M potassium phosphate (pH 7) extracted remarkably more pectic polysaccharides from Arabidopsis leaves than from leaves of other plants. The lower level of GalU (the major residue in pectins) in the cell wall fraction and the lower levels of Ara and Rha (also found in pectins) in *sos6-1* than in the wild type under NaCl stress together suggest a role for SOS6/AtCSLD5 in the incorporation of pectic polysaccharides into the cell wall. Overall, however, SOS6 does not appear to have a major role in controlling cell wall structure, consistent with the relatively normal growth and development of the *sos6-1* mutant plants under unstressed conditions. A recent study of the T-DNA knockout lines of *AtCSLD5* revealed that a null allele of *AtCSLD5* shows a growth reduction under the growth conditions used (Bernal *et al.*, 2007). Immunocytochemical analysis between wild type and the null allele of *AtCSLD*5 further indicates a role in xylan and homogalacturonan synthesis (Bernal *et al.*, 2007). Our sugar analysis data also indicate that *sos6-1* mutation has similar effects (Tables 1 and 2). Earlier evidence obtained by gene expression profiling and immunocytochemical analysis using isoxaben-habituated Arabidopsis suspension culture cells revealed that *AtCLSD5* was highly induced and may contribute to the biosynthesis of the pectic component of the habituated

cells (Manfield *et al.*, 2004). Again, our data presented in this study are consistent with the previous observations.

In contrast to the apparent lack of function or minor function in unstressed conditions, SOS6 is required for plant growth under salt and drought stresses. What might be the mechanism of SOS6/AtCSLD5 function in osmotic stress tolerance? As the first line of defense of the cell, the cell wall and the plasma membrane attached to it are expected to be involved in the sensing and early signaling of osmotic stress (Zhu *et al.*, 1997). CesA and possibly CSL proteins as well are transmembrane proteins that link with both wall polymers and microtubules and thus are part of the wall-membrane-cytoskeleton continuum, which is hypothesized to be important for turgor sensing (Zhu *et al.*, 1993, 1997). It is conceivable that SOS6/AtCSLD5 may even have a specific role in osmotic stress sensing.

Alternatively, SOS6 may function in the synthesis of certain component(s) of the wall and this function may be related to ROS generation or scavenging. SOS6/AtCSLD5 itself or through the wall polymer it synthesizes may interact with and regulate proteins that generate ROS at the cell surface, such as plasma membrane NADPH oxidases, cell wall polyamine oxidases and peroxidases (Keller *et al.*, 1998; Mittler, 2002; Sagi and Fluhr, 2006; Yoda *et al.*, 2006). Upon osmotic stress, ROS accumulates to higher levels in the *sos6-1* mutant (Figure 5), which indicates that SOS6 and/or related cell-wall components are indeed required for maintenance of ROS levels. Furthermore, the expression of a putative peroxidase (At2g39040) was less in *sos6-1* relative to the wild type after stress treatment, as revealed by microarray analysis and validated by real-time PCR analysis (Figure S9 and Table S1). On the other hand, a germin-like protein (At5g39100) showed an elevated transcript level in *sos6-1* upon salt stress (Figure S9 and Table S1). Germin and germin-like proteins have been shown to have superoxide dismutase activity (Yamahara *et al.*, 1999; Carter and Thornburg, 2000; Christensen *et al.*, 2004). Notwithstanding the unknown mechanism of function, our results open up new questions for salt and drought stress research and suggest an important role of SOS6/AtCSLD5 and related cell-wall components in regulating stress-induced ROS levels and osmotic stress tolerance.

EXPERIMENTAL PROCEDURES

Plant materials

A firefly luciferase reporter gene driven by the stress-responsive *RD29A* promoter (Ishitani *et al.*, 1997) was introduced into Arabidopsis plants in the Columbia *glabrous1* (*gl1*) background. Seeds from one homozygous line (referred to as the wild type) were mutagenized with ethyl methanesulfonate (EMS) and M_2 seeds were used to screen for high throughput mutants with altered *RD29A::LUC* gene expression, for example STABILIZE 1 (Lee *et al.*, 2006) and this same batch of seeds was also used to screen for hypersensitive mutants in the presence of 100 mM NaCl in this study with use of a root-bending assay (Wu *et al.*, 1996).

Seeds of *sos6-2* (SALK_002118), *csld1* (SALK_097300), *csld2* (SALK_119808), *csld4* (SALK_059059) and *csld6* (SALK_095234) were obtained from Arabidopsis Biological Resource Center (ABRC). Verification of insertions was performed using primers listed in Table S4. Homozygous *csld3–1* (seed stock number CS899) was obtained from ABRC as described (Wang *et al.*, 2001).

Genetic analysis and genetic mapping of *sos6-1* locus

sos6-1 plants were backcrossed with the wild type. F_1 and F_2 seedlings were scored for salt sensitivity by the root-bending assay (Wu *et al.*, 1996). For mapping of the *sos6-1* locus, homozygous *sos6-1* plants were crossed to wild type plants of Landsberg *erecta*. From the

segregating F_2 population, 771 homozygous *sos6-1* mutants were selected and DNA was extracted from each of these plants for mapping with SSLP markers between Columbia and Landsberg *erecta*.

Complementation of *sos6-1* mutant

The genomic DNA fragment of At1g02730 was amplified by PCR with F22D16 BAC clone used as a template with the primers listed in Table S4. The PCR product was cloned into pCAMBIA1200 between *Kpn*I and *Xba*I sites and sequenced. The resulting binary vector was transferred to *sos6-1* through *Agrobacterium* (strain GV3101)-mediated transformation. The transgenic plants (T_1) were selected on hygromycin (25 mg/L) and transferred to soil to maturity. T_2 seedlings were examined for sensitivity to 100 mM NaCl and 100 mM mannitol, respectively.

SOS6 promoter::GUS construct

The DNA fragment containing the *SOS6* promoter was amplified by PCR with BAC F22D16 clone DNA used as a template with the primers listed in Table S4. The PCR product was cloned into the binary vector pCAMBIA1381Z between *Eco*RI and *Pst*I sites and sequenced. The resulting binary vector was transferred to wild-type (Columbia) plants through an Agrobacterium (strain GV3101)-mediated transformation by a floral dip method. Seedlings or tissues from the T_1 and T_2 populations were first emerged in 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) solution (2 mM X-Gluc, 100 mM sodium phosphate buffer (pH 7.5), 0.5% Triton X-100, 2 mm $K_3[Fe(CN)_6]$, 2 mM $K_4[Fe(CN)_6]$, 0.02% NaN_3) and vacuum infiltrated for 10 min and then incubated at 37°C for at least 12 h in dark, followed by incubation in 70% ethanol to remove chlorophyll as described (Jefferson *et al.*, 1987).

RNA gel analysis, microarray analysis and real-time PCR analysis

Wild type and *sos6-1* seedlings were grown on separate halves of the same 0.6% agar MS medium for 14 days and then left untreated or treated with NaCl. Total RNA was extracted from whole seedlings and RNA gel analysis was conducted as described (Zhu *et al.*, 2007a).

Total RNA (20 μg) extracted with use of the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from 28-day-old wild type and *sos6-1* seedlings grown on 1.2% agar MS medium after salt treatment (150 mM NaCl for 24 h) was used to make biotin-labeled cRNA targets. Affymetrix GeneChip arrays were performed as described (Chinnusamy *et al.*, 2003). Statistical analysis of the microarray data were performed as described (Li and Wong, 2001; Tusher *et al.*, 2001; Wu *et al.*, 2004; Johnson *et al.*, 2007). Data were adjusted for batch effect (due to the fact that experiments were done at two different locations: University of Arizona and University of California-Riverside) and compared by the same statistical method. Fold change indicates relative expression level in *sos6-1* (value <0 indicates downregulation in *sos6-1;* value >0 indicates upregulation in *sos6-1*). Q-value (%) stands for chance of false positive. We used a threshold q-value of 0.05 to select the genes whose expression levels are statistically significantly changed in *sos6-1*.

Total RNA isolation, first strand cDNA synthesis and real-time PCR analysis were carried out as previously described (Zhu *et al.*, 2007b). The primers used for real-time PCR reactions were listed in Table S4.

Soluble sugar measurement

Ground fresh tissues (0.5 g) from 1-month-old wild-type and *sos6-1* plants grown in soil treated or untreated with 150 mM NaCl for 10 days were extracted with 10 ml 80% ethanol and incubated in a 80°C water bath for 30 min. The extract was centrifuged for 5 min at 17

300 ***g*** (Beckman, J2-MC) and the supernatant was dried in a speed vacuum concentrator (Savant, Farmingdale, NY, USA). The dry samples were then extracted with 5.8 ml of methanol/water/chloroform (1:0.9:1) and the extract was then dried in the Savant speed vacuum concentrator. The extract was suspended in 1 ml deionized water and deionized by passage through 1 ml anion and cation resin columns (AG1-X8/formate and AG50W-X8/H^+, BIORAD). Sugars were eluted with 10 ml water, dried and redissolved in 200 μl water and filtered (0.45 μm pore size). Then 20 μl of the soluble sugar extract was analyzed with HPLC by use of a Sugar-pak (Waters, Milford, MA, USA) column as described by Liu *et al.* (1999). Total soluble sugar was also assayed with the remaining soluble sugar extract as described (Dubois *et al.*, 1956).

Determination of ROS levels

One-week-old seedlings grown on 1.2% agar MS medium were used to determine ROS levels as described (Zhu *et al.*, 2007a).

Glycosyl composition analysis of cell wall

One-month-old plants grown in soil under long day photoperiod (16 h light, 8 h dark) were treated with 0 or 200 mM NaCl for 10 day. Preparation of the cell wall was performed as described (Fry, 2000). Briefly, 2 g of fresh shoots were harvested and ground into fine powder in liquid N_2. Proteins and starch were removed by extracting twice with phenol-acetic acid-water (approximately first 1:2:0.5 and then 1:2:1) and twice with 90% DMSO, respectively. Residual lipids were extracted twice with 2:1 (v/v) chloroform-methanol and twice with acetone. The cell wall fraction was then dried in air and again in a vacuum desiccator over P_2O_5. The dry cell wall fraction was then analyzed by methanolysis and trimethylsilylation combined with gas liquid chromatography (GC) for glycosyl composition of non-cellulosic polysaccharides as described (Komalavilas *et al.*, 1990). For analysis including cellulosic polysaccharides, an aliquot of the cell-wall fraction was first hydrolyzed by H_2SO_4 (Fry, 2000) and then subjected to methanolysis and trimethylsilylation followed by GC. Different cell wall fractions were also sequentially extracted from total cell wall prepared with procedures described above. Briefly, two grams of dry cell wall were extracted twice in 500 mM imidazole-HCl, pH 7.0 (40 ml each time) and stirred overnight. The pooled supernatant after centrifugation was filtered through GF/A glass fiber filter (Whatman, 3.7 cm), dialyzed against distilled water and freeze dried. The imidazole-insoluble residue was further extracted twice in 30 ml each time of 6 M NaOH (with addition of 1% (w/v) $NaBH_4$) and stirred overnight. The pooled supernatant after centrifugation was filtered through GF/A glass fiber filter (3.7 cm), titrated to pH 6 by addition of acetic acid, dialyzed against distilled water and freeze-dried. The NaOH-insoluble residue was repeatedly washed by centrifugation with distilled water until the pH was neutral and then freeze-dried. The dry cell wall fractions (imidazole soluble and NaOH soluble) were analyzed by methanolysis and trimethylsilylation combined with gas liquid chromatography (GC) for glycosyl composition as described (Komalavilas *et al.*, 1990). The NaOH-insoluble cell-wall fraction was first hydrolyzed by H_2SO_4 (Fry, 2000) and then subjected to methanolysis and trimethylsilylation followed by GC analysis.

One-way Analysis of Variance (ANOVA) of the cell wall glycosyl composition data was performed using InStat (version 2.0, GraphPad Software, San Diego, CA, USA) and the same software was used to perform the Tukey–Kramer multiple comparison post-test when ANOVA indicated the existence of statistically significantly differences.

Total uronic acid analysis was performed as described (Blumenkrantz and Asboe-Hansen, 1973). Briefly, an aliquot of the dry cell-wall fraction (~300 μg) was suspended in 1 ml H_2O in a glass tube and 6 ml of 0.0125 M sodium tetraborate in concentrated H_2SO_4 were added.

The mixture in the glass tube was boiled in a water bath for 7 min and then cooled in an ice-H_2O bath. One hundred μl of 0.15% (w/v) meta-hydroxydiphenyl reagent in 0.5% NaOH were added and uronic acid was quantified by measuring the absorbance at 520 nm, with D-galacturonic acid used as a standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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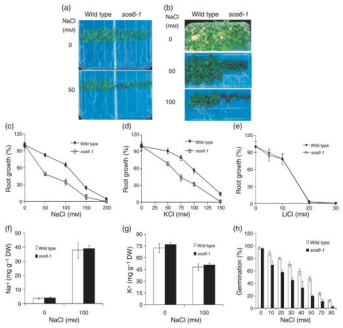

Figure 1. Response of *sos6-1* plants to different salts and ion content measurement

(a) Four-day-old wild type and *sos6-1* seedlings grown on germination media were transferred to media containing 0 or 50 mM NaCl and allowed to grow an additional 8 days. (b) Four-day-old wild type and *sos6-1* seedlings grown on germination media were transferred to media containing 0, 50 or 100 mM NaCl and allowed to grow additional 30 days. (c) Response of *sos6-1* plants to various levels of NaCl. The root growth was represented as proportion of growth compared with growth without NaCl (100%). (d) Response of *sos6-1* plants to different levels of KCl. (e) Response of *sos6-1* plants to various concentrations of LiCl. Error bars in (c–e) indicate standard deviation (n = 18). (f) Na^+ content in *sos6-1* plants as described (Zhu *et al.*, 2007a). (g) K^+ content in *sos6-1* plants as described (Zhu *et al.*, 2007a). (h) Sensitivity of *sos6-1* seed germination to NaCl as described (Zhu *et al.*, 2002). Error bars in (f–h) indicate standard deviation (n = 6). All experiments presented here and in the subsequent figures were performed at least three times and similar results were obtained.

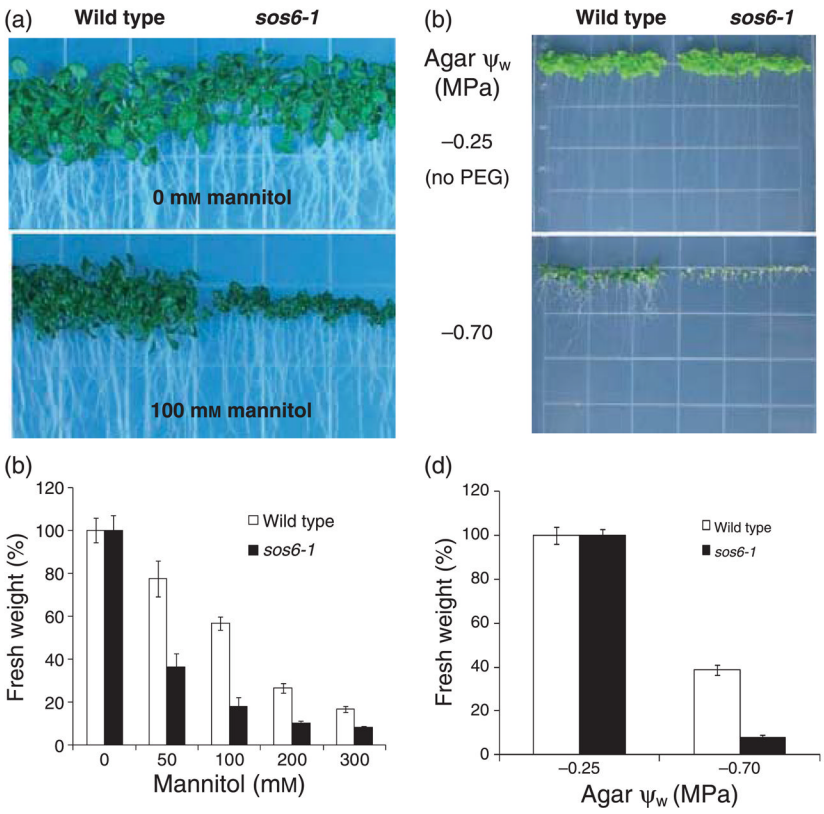

Figure 2. *sos6-1* plants are hypersensitive to general osmotic stress

(a) Growth of wild type and *sos6-1* plants in the presence of 0 or 100 mM mannitol for 21 days. The seeds were first germinated on media containing 0 mM mannitol and then transferred to media containing 0 or 100 mM mannitol. (b) Quantification of growth (at the end of day 21; indicated by fresh weight) of wild type and *sos6-1* in response to different levels of mannitol. (c) Seed germination and seedling development of wild-type and *sos6-1* plants in media with low ψ_W imposed by PEG for 21 days. Seeds were directly germinated in agar plates with low ψ_W. (d) Quantification of growth of wild type and *sos6-1* as shown in (c). Error bars in (b) and (d) indicate standard deviation (n = 60–80).

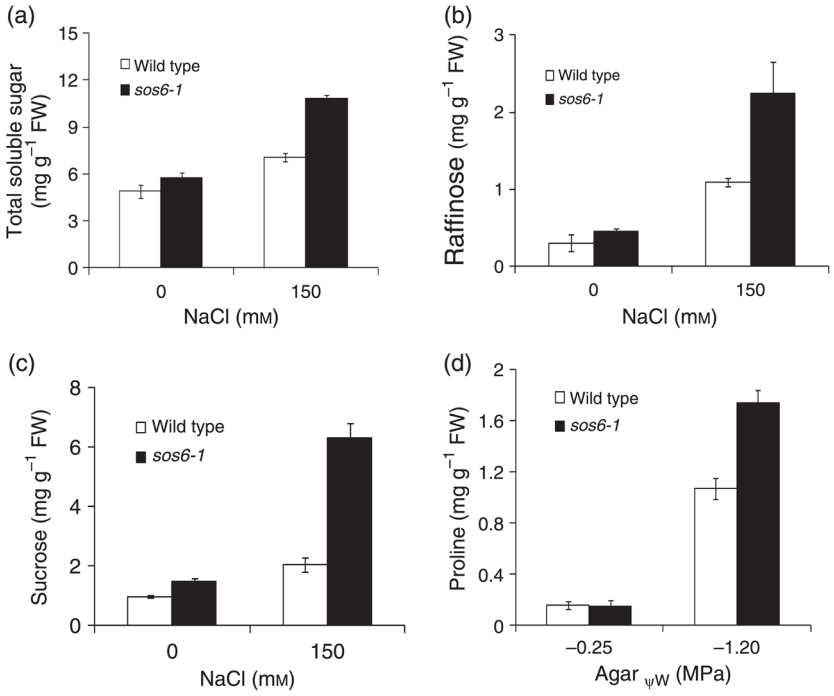

Figure 3. Content of soluble sugars and proline in *sos6-1*.

(a) Effect of *sos6-1* mutation on soluble sugar levels. (b) Raffinose content of wild type and *sos6-1* plants. (c) Sucrose content of wild type and *sos6-1* plants. Error bars in (a–c) represent standard deviation (n = 16). (d) Effect of *sos6-1* mutation on proline levels under low water-potential condition. Proline content was quantified as described (Verslues and Bray, 2004). Error bars represent standard deviation (n = 8).

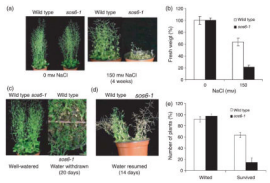

Figure 4. Soil-grown *sos6-1* plants are hypersensitive to NaCl treatment and dehydration conditions

(a) Two-week-old wild type and *sos6-1* plants were treated with 0 or 150 mM NaCl and allowed to grow for additional 28 days. Plants were watered with solution containing 0 or 150 mM NaCl every 3 days. (b) Quantification of growth of wild type and *sos6-1* at the end of salt treatment. Error bars represent standard deviation (n = 80–96). (c) Two-week-old wild type and *sos6-1* plants grown in soil in the same pots were either well-watered or deprived of water for an additional 20 days. (d) Wild type and *sos6-1* plants grown for 14 days when water was resumed. (e) Quantification of plants that were wilted when water was withdrawn (Wilted) and wilted plants that survived again when water was resumed (Survived). Error bars represent standard deviation (n = 74–90).

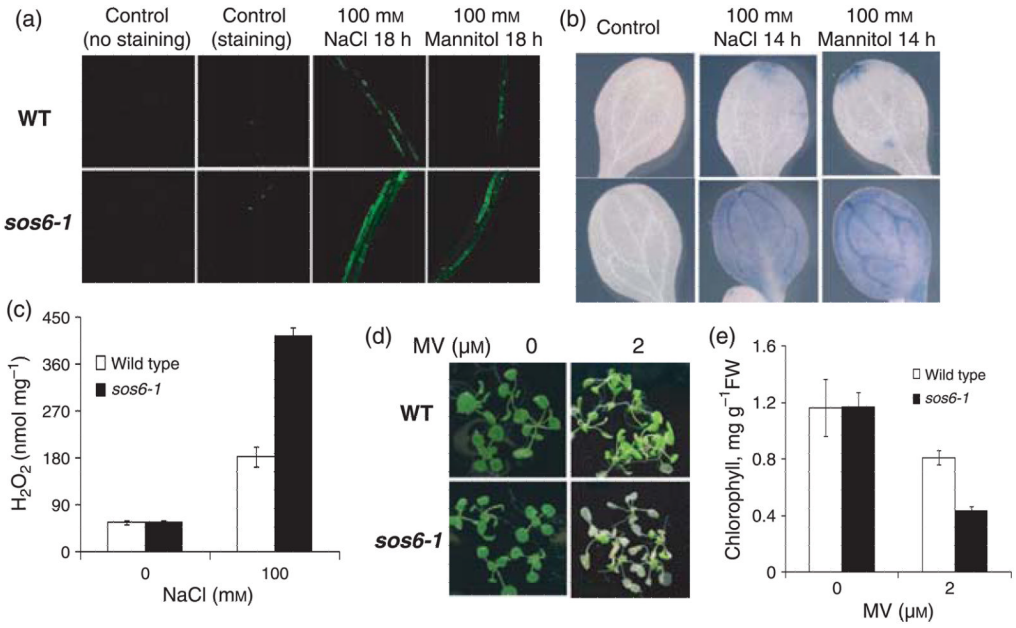

Figure 5. Detection of ROS and oxidative stress tolerance

(a) Detection of total ROS accumulation in seedlings with or without stress treatment. Fluorescence indicates the presence of ROS. (b) Nitroblue tetrazolium staining for superoxide radical. Blue color indicates the presence of superoxide. (c) Quantitative measurement of H_2O_2 levels in *sos6-1* and wild type plants. Error bars indicate standard deviation (n = 12). (d) Tolerance of *sos6-1* and wild type plants to methyl viologen as indicated by chlorophyll content. (e) Chlorophyll content in plants as shown in (d) determined as described (Lichtenthaler and Wellburn, 1983). Error bars represent standard deviation (n = 10).

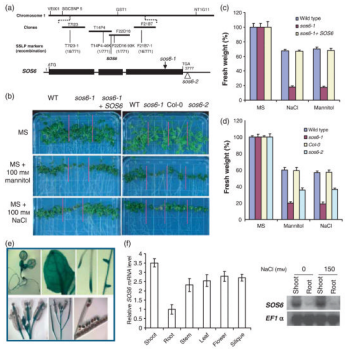

Figure 6. Molecular cloning of *SOS6*.

(a) *SOS6* was identified through map-based cloning. *SOS6* is located on bacterial artificial chromosome clone F22D16. The numbers of recombinant and total chromosomes tested (in parentheses) and developed sslp markers are indicated. The structure of *SOS6* and the positions of *sos6-1* and *sos6-2* mutations are also indicated. Positions are relative to the translation initiation codon. Closed boxes indicate the open reading frame and lines between boxes indicate introns. (b) Gene complementation of *sos6-1* and tolerance of *sos6–2* to NaCl and mannitol. (c) Quantification of seedling development as shown in (b). (d) Quantification of growth of wild type, *sos6-1*, Col-0 and *sos6-2* as shown in (b). Error bars in (c) and (d) represent standard deviation (n = 90–110). (e) Tissue-specific expression of *SOS6::GUS*. (f) Validation of *SOS6::GUS* expression patterns by real-time PCR analysis (left panel) and *SOS6* is not regulated by salt stress as revealed by northern blot analysis (right panel). *EF1α* gene was used as loading controls. Error bars indicate standard deviation (n = 8).

Table 1

Glycosyl composition (mol%) of the whole cell wall fraction from shoots of wild type and *sos6-1* plants treated with 0 or 0.2 M NaCl

	Glycosyl composition (mol%) (treatments/genotypes)			
	0 M NaCl		0.2 M NaCl	
Residue[a]	**Wild type**	***sos6-1***	**Wild type**	***sos6-1***
Ara	3.51[d]	3.47[d]	5.33[e]	4.08[d]
Rha	3.77[d]	3.48[d]	4.31[e]	3.64[d]
Fuc	0.79[d,e]	0.79[d,e]	0.69[d]	0.87[e]
Gal	5.43[d]	5.59[d]	7.00[d]	7.26[d]
Xyl	5.38[d]	5.69[d]	8.10[e]	10.12[e]
Xyl-SA[b]	1.46[d]	0.95[d]	1.16[d]	2.00[d]
Man	1.25[d]	1.26[d]	1.29[d]	1.26[d]
Man-SA[b]	1.20[d]	1.07[d]	1.26[d]	1.00[d]
GlcU	3.97[d]	3.73[d]	4.11[d]	5.53[d]
GalU	35.44[d]	35.44[d]	31.04[d,e]	23.93[e]
Glc	5.09[d]	5.06[d]	2.91[e]	3.19[e]
Glc-SA[b]	32.73[d]	33.48[d]	32.81[d]	37.10[d]
Uronics[c] (weight%)	25.53[d]	25.78[d]	24.22[d]	23.07[d]

Each entry is the mean of $n = 5$ collections of plants.

[a] From gas chromatographic analysis of trimethylsilyl ethers of methyl glycosides.

[b] Additional amounts of glycosyl residues detected when the samples were subjected to H_2SO_4 (SA) swelling and hydrolysis, for cleavage of cellulose and tightly associated polysaccharides, prior to methanolysis.

[c] From colorimetric analysis of total uronic acids.

[d,e] Within each row, entries not followed by the same letter are statistically different by ANOVA ($P < 0.05$).

Table 2

Glycosyl composition of the imidazole-soluble cell wall fraction from shoots of wild type and *sos6-1* plants treated with 0 or 0.2 M NaCl

	Glycosyl composition (mol%) (treatments/genotypes)			
	0 M NaCl		0.2 M NaCl	
Residue[a]	Wild type	*sos6-1*	Wild type	*sos6-1*
Ara	7.98[b]	9.16[b]	10.85[b,c]	13.96[c]
Rha	9.60[b]	7.60[c]	9.28[b,c]	11.66[d]
Fuc	3.40[b]	1.63[c]	3.04[b,c]	3.58[b]
Gal	17.98[b]	11.24[c]	17.47[b]	18.83[b]
Xyl	10.12[b]	8.93[b]	10.98[b]	8.24[c]
Man	1.91[b]	1.46[b]	2.36[b]	2.47[b]
GlcU	10.12[b]	8.94[b]	10.98[b]	8.26[b]
GlaU	28.97[b,c]	24.97[b]	25.96[b,c]	18.34[c]
Glc	16.48[b]	31.72[c]	16.07[b]	16.24[b]

Each entry is the mean from $n = 5$ collections of plants.

[a] From gas chromatographic analysis of trimethylsilyl ethers of methyl glycosides.

[b–d] Within each row, entries not followed by the same letter are statistically different by ANOVA ($P < 0.05$).

Table 3

Glycosyl composition of the NaOH-soluble cell wall fraction from shoots of wild type and *sos6-1* plants treated with 0 or 0.2 M NaCl

	Glycosyl composition (mol%) (treatments/genotypes)			
	0 M NaCl		0.2 M NaCl	
Residue[a]	Wild type	*sos6-1*	Wild type	*sos6-1*
Ara	8.53[b]	10.60[b]	8.86[b]	9.44[b]
Rha	8.43[b]	9.29[b,c]	10.17[c]	14.82[d]
Fuc	2.16[b]	2.72[b]	2.27[b]	3.06[b]
Gal	13.51[b]	13.35[b]	17.36[c]	13.81[b]
Xyl	20.48[b]	23.21[c]	22.08[b,c]	30.84[d]
Man	5.47[b]	4.01[b]	4.12[b]	4.64[b]
GlcU	4.05[b]	3.53[b]	3.77[b]	3.97[b]
GlaU	26.85[b]	24.20[b]	18.99[c]	5.06[d]
Glc	10.52[b]	9.09[b]	12.39[b,c]	14.36[c]

Each entry is the mean from $n = 5$ collections of plants.

[a] From gas chromatographic analysis of trimethylsilyl ethers of methyl glycosides.

[b–d] Within each row, entries not followed by the same letter are statistically different by ANOVA ($P < 0.05$).

Table 4

Glycosyl composition of the NaOH-insoluble cell wall fraction from shoots of wild type and *sos6-1* plants treated with 0 or 0.2 M NaCl

	Glycosyl composition (mol%) (treatments/genotypes)			
	0 M NaCl		0.2 M NaCl	
Residue[a]	**Wild type**	***sos6-1***	**Wild type**	***sos6-1***
Ara	0.79[b]	1.54[c]	0.51[b]	0.78[b]
Rha	1.03[b]	0.95[b]	1.07[b]	0.88[b]
Fuc	0.42[b]	0.43[b]	0.92[c]	1.21[d]
Gal	1.65[b]	2.62[b]	2.35[b]	2.42[b]
Xyl	2.32[b]	2.81[b]	2.39[b]	3.20[b]
Man	0.67[b]	0.65[b]	0.72[b]	0.52[b]
GlcU	1.13[b]	1.07[b]	1.20[b]	1.50[b]
GlaU	1.88[b]	1.02[b]	0.63[b]	0.80[b]
Glc	90.10[b]	88.92[b]	90.21[b]	88.68[b]

Each entry is the mean from $n = 5$ collections of plants.

[a] From the NaOH-insoluble cell-wall fraction, which was first hydrolyzed by H_2SO_4 and then subjected to methanolysis and trimethylsilylation followed by gas chromatographic analysis.

[b] Within each row, entries not followed by the same letter are statistically different by ANOVA ($P < 0.05$).