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SOS3 Function in Plant Salt Tolerance Requires N-Myristoylation and Calcium Binding

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The salt tolerance gene *SOS3* (for salt overly sensitive3) of Arabidopsis is predicted to encode a calcium binding protein with an N-myristoylation signature sequence. Here, we examine the myristoylation and calcium binding properties of SOS3 and their functional significance in plant tolerance to salt. Treatment of young Arabidopsis seedlings with the myristoylation inhibitor 2-hydroxymyristic acid caused the swelling of root tips, mimicking the phenotype of the salt-hypersensitive mutant *sos3-1*. In vitro translation assays with reticulocyte showed that the SOS3 protein was myristoylated. Targeted mutagenesis of the N-terminal glycine-2 to alanine prevented the myristoylation of SOS3. The functional significance of SOS3 myristoylation was examined by expressing the wild-type myristoylated SOS3 and the mutated nonmyristoylated SOS3 in the *sos3-1* mutant. Expression of the myristoylated but not the nonmyristoylated SOS3 complemented the salt-hypersensitive phenotype of *sos3-1* plants. No significant difference in membrane association was observed between the myristoylated and nonmyristoylated SOS3. Gel mobility shift and $^{45}Ca^{2+}$ overlay assays demonstrated that SOS3 is a unique calcium binding protein and that the *sos3-1* mutation substantially reduced the capacity of SOS3 to bind calcium. The resulting mutant SOS3 protein was not able to interact with the SOS2 protein kinase and was less capable of activating it. Together, these results strongly suggest that both N-myristoylation and calcium binding are required for SOS3 function in plant salt tolerance.

INTRODUCTION

Post- and cotranslational modifications of proteins such as phosphorylation, glycosylation, and lipidation play important roles in determining protein function. Prenylation, myristoylation, and palmitoylation are the best-characterized types of protein modifications by lipids. Protein N-myristoylation refers to the covalent attachment of myristic acid by an amide bond to the N-terminal glycine residue of a nascent polypeptide. This cotranslational modification occurs on many proteins involved in signal transduction, including serine/threonine kinases, tyrosine kinases, kinase substrates, protein phosphatases, heterotrimeric G proteins, calcium binding proteins, and ADP ribosylation factors (reviewed in Johnson et al., 1994). In most cases, this modification is essential for protein function to mediate membrane association or protein–protein interaction. In some cases, however, no functional significance can be associated with myristoylation (Johnson et al., 1994).

By comparing the N-terminal sequences of known myristoylated proteins and by studying the substrate specificity of myristoyl transferases, Towler et al. (1988) identified a consensus signature sequence for myristoylation, MGXXXS/T(K). Although several genes in plants encode proteins that conform to such a consensus sequence and thus may be myristoylated, little is known if they are in fact myristoylated and whether such a modification is important for their function.

Recently, we cloned from Arabidopsis a major salt tolerance locus, *SOS3* (for salt overly sensitive3), which encodes a putative EF hand–type calcium binding protein with a consensus sequence for N-myristoylation (Liu and Zhu, 1998). The predicted amino acid sequence of SOS3 is similar to the B subunit of calcineurin (CNB) and to animal neuronal calcium sensors (NCS). Both CNB and NCS contain the myristoylation signature sequence and are known to be myristoylated (Dizhoor et al., 1993; Zhu et al., 1995). In the yeast *Saccharomyces cerevisiae*, CNB is also required for salt tolerance, and loss-of-function mutations in CNB cause increased sensitivity to salt stress (Nakamura et al., 1993; Mendoza et al., 1994). Myristoylation does not appear to be necessary for CNB function because yeast strains with nonmyristoylated forms of CNB exhibit normal salt tolerance (Zhu et al., 1995). In contrast, myristoylation plays essential roles in the functioning of NCS. Myristoylated but not nonmyristoylated recoverin, a member of the NCS family that functions in retinal rod cells, controls the lifetime of photoexcited rhodopsin by inhibiting rhodopsin kinase in a Ca^{2+}-dependent manner (Klenchin et al., 1995). Recoverin undergoes an interesting calcium-myristoyl switch (Dizhoor et al., 1993). When not bound to calcium (calcium-free

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state), the myristoyl group is embedded in a hydrophobic pocket. The binding of Ca^{2+} to recoverin induces conformational changes and exposes the myristoyl group, making it available to associate with disc membranes (Ames et al., 1997).

Here, we report evidence for an essential role of protein N-myristoylation in plant salt tolerance. Treating young Arabidopsis seedlings with the myristoylation inhibitor 2-hydroxymyristic acid (HMA) mimicked the phenotype of the *sos3-1* plants, which have reduced salt tolerance. A reticulocyte in vitro translation assay determined that SOS3 was myristoylated. Targeted mutagenesis of the N-terminal glycine-2 to alanine prevented myristoylation of SOS3. Myristoylated but not nonmyristoylated SOS3 was able to rescue the salt-hypersensitive phenotype of *sos3* mutant plants.

In addition, we provide experimental evidence that SOS3 is capable of binding calcium, although the binding is quite weak compared with typical EF-hand calcium binding proteins. The *sos3-1* mutation, which causes deletion of three conserved amino acids in a central EF-hand, substantially decreased the calcium binding of SOS3. We found that this mutation also disrupts the interaction between SOS3 and the SOS2 protein kinase. Taken together, these results reveal that both calcium binding and myristoylation are necessary for SOS3 function.

RESULTS

Inhibition of Myristoylation by HMA Mimics the *sos3* Mutant Phenotype

To determine whether protein N-myristoylation might be important for plant salt tolerance, we first tested the effect of HMA, a competitive inhibitor of N-myristoyltransferase (Paige et al., 1990; Nadler et al., 1993; Galbiati et al., 1996). One-week-old Arabidopsis seedlings were treated with 1 mM HMA for 1 day and then transferred to grow on a regular nutrient medium or on a medium supplemented with 100 mM NaCl. The HMA treatment did not affect the growth of either the wild-type or *sos3* mutant plants on regular nutrient medium (Figures 1A to 1D), but it did reduce the salt tolerance of Arabidopsis seedlings (Figure 1G). The decrease in salt tolerance is indicated by the swelling of the root tip. Wild-type seedlings not pretreated with HMA exhibited continued growth when transferred to 100 mM NaCl, and no swelling was observed at their root tips (Figure 1E). In contrast, even without HMA pretreatment, the *sos3* mutant showed increased sensitivity to NaCl inhibition and displayed swelling at the root tip (Figure 1F; Liu and Zhu, 1997). The results show that HMA treatment mimics the root tip swelling phenotype of the *sos3* mutant plants and suggest that protein N-myristoylation might be necessary for plant salt tolerance.

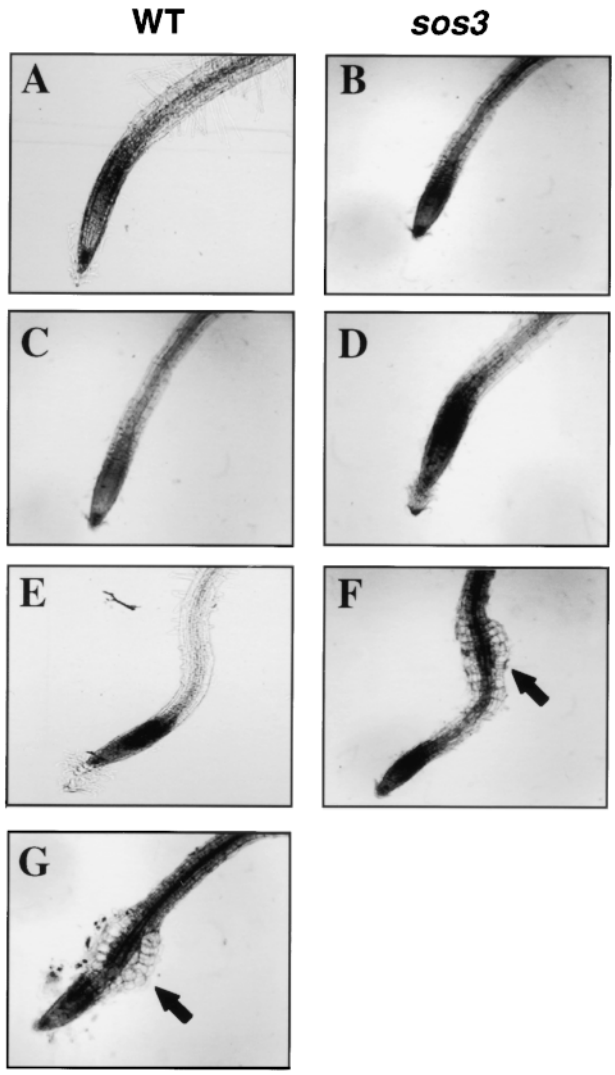

Figure 1. Treatment of Arabidopsis Seedlings with the Myristoylation Inhibitor HMA Causes Root Tip Swelling under NaCl Stress.

sos3 mutant plants also show root tip swelling when treated with NaCl stress. Arrows point to swelling cells.
(A) and **(B)** Root tips of wild-type (WT) and *sos3-1* seedlings, respectively, grown on Murashige and Skoog (MS) nutrient salts.
(C) and **(D)** Root tips of wild-type and *sos-3-1* seedlings, respectively, treated with MS plus 1 mM HMA.
(E) and **(F)** Root tips of wild-type and *sos3-1* seedlings, respectively, treated with MS plus 100 mM NaCl.
(G) Root tip of wild-type seedling treated with MS plus 1 mM HMA and 100 mM NaCl.

Synthesis and Myristoylation of SOS3 in Vitro

SOS3 mRNA was in vitro transcribed from the plasmid pET-SOS3. This mRNA was translated in a rabbit reticulocyte lysate, and the reaction products were analyzed by

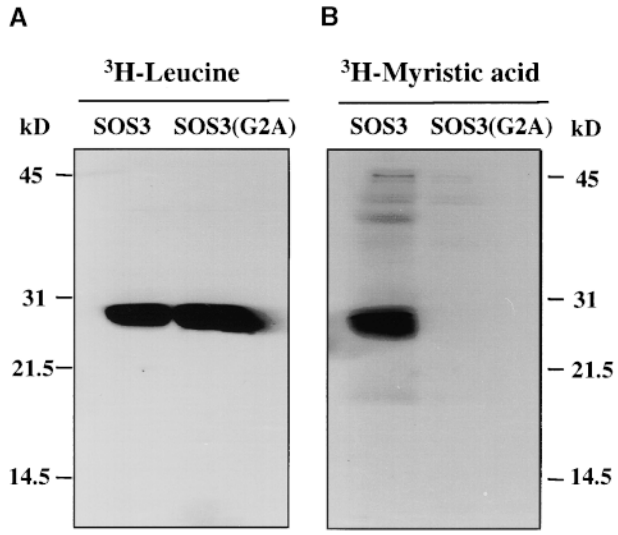

Figure 2. Incorporation of Myristic Acid into SOS3 but Not into SOS3(G2A) in a Rabbit Reticulocyte in Vitro Translation Assay.

(A) Control showing that [^{3}H]leucine was incorporated into both proteins.

(B) Incorporation of [^{3}H]myristic acid into SOS3 but not SOS3(G2A). Positions of molecular size markers are indicated at left and right in kilodaltons.

SDS-PAGE. In the presence of [^{3}H]leucine, a single polypeptide band was synthesized with the expected molecular size of 26 kD of SOS3 (Figure 2A). Similarly, SOS3(G2A), in which Gly-2 had been replaced with Ala, was synthesized and labeled with [^{3}H]leucine (Figure 2A). The Gly-2 to Ala alteration did not change the size of the labeled polypeptide. The first seven amino acid residues in SOS3 (MGCSVSK) conform to the consensus motif MGXXXS/T(K) recognized by *N*-myristoyl transferase (Towler et al., 1988), so we examined the incorporation of [^{3}H]myristic acid into SOS3 in vitro. Because reticulocyte lysates contain *N*-myristoyl transferase (Deichaite et al., 1988), myristic acid should also be incorporated into newly synthesized SOS3 in these lysates. Translation performed in the presence of [^{3}H]myristic acid resulted in radiolabel incorporation into the 26-kD band (Figure 2B). When the mutant *SOS3(G2A)* mRNA was translated in the presence of [^{3}H]myristic acid, no radiolabel incorporation could be detected (Figure 2B, lane 2). These results show that SOS3 can be myristoylated in vitro and that the myristoylation is mediated by its Gly-2 residue.

The G2A Mutation Abolishes SOS3 Function in Plant Salt Tolerance

To assess the in vivo function of SOS3 myristoylation, we expressed *SOS3(G2A)* in *sos3-1* plants under control of the cauliflower mosaic virus (CaMV) 35S promoter. We first tested whether expression of wild-type *SOS3* under the CaMV 35S promoter could suppress the *sos3-1* mutant phenotype. More than 20 independent transgenic lines were obtained and tested in the T_2 generation for growth on medium containing 100 mM NaCl. The lines segregated in the T_2 generation and all kanamycin-resistant plants showed salt tolerance of the wild type. Kanamycin-sensitive plants were determined to be hypersensitive to 100 mM NaCl, similar to the *sos3-1* plants (data not shown). *SOS3(G2A)* was similarly expressed in *sos3-1* plants under control of the CaMV 35S promoter, and $>$20 independent transgenic lines were tested in the T_2 generation. Although these lines also segregated for kanamycin resistance, all plants were like *sos3-1* plants under 100 mM NaCl treatment, and none showed salt tolerance of the wild type. Results for representative homozygous T_3 transgenic *sos3-1* seedlings expressing *35S–SOS3* and *35S–SOS3(G2A)*, respectively (Figure 3A), showed that *35S–SOS3(G2A)* cannot suppress the salt-hypersensitive phenotype of *sos3-1* plants. Because the G2A mutation disrupted the myristoylation but not the calcium binding (see below) of SOS3, we conclude that myristoylation is required for SOS3 function in plant salt tolerance.

Protein expression in the *35S–SOS3* and *35S–SOS3(G2A)* transgenic plants was determined with anti-SOS3 antisera. In both transgenic plants, the protein was detected in soluble as well as in membrane fractions (Figure 3B). We sought to determine the subcellular localization of SOS3 and SOS3(G2A) by using the green fluorescent protein (GFP) as a marker. Because of the low expression of SOS3 under control of its own promoter, it was necessary to express SOS3–GFP fusion protein under the CaMV 35S promoter to obtain enough protein to be detected. The GFP marker protein was fused to the C terminus rather than the N terminus of SOS3 because the N-terminal fusion would disrupt myristoylation. Wild-type SOS3–GFP fusion protein expression was able to suppress the NaCl-hypersensitive phenotype of *sos3* plants, whereas the nonmyristoylated mutant form, SOS3(G2A)–GFP, could not (Figure 4A). Because the results were identical to that obtained with the SOS3 and SOS3 (G2A) expression without GFP fusion, we concluded that GFP fusion did not adversely affect SOS3 function.

Immunoblot analysis with anti-SOS3 antisera detected a polypeptide band at 53 kD, the expected size of SOS3–GFP (Figure 4B). The fusion protein is present in both the soluble and the membrane fractions but in greater amounts in the soluble fraction (Figure 4B). Similar results can be seen with SOS3(G2A)–GFP. It is present in both the soluble and the membrane fractions, and the soluble fraction contains more than the membrane fraction (Figure 4B). The antisera did not detect any endogenous SOS3 protein (26 kD), because of the very low abundance of the endogenous protein.

The same protein blot was probed with commercial anti-GFP antibodies (Figure 4B). The antibodies reacted with the SOS3–GFP and SOS3(G2A)–GFP fusion proteins as well

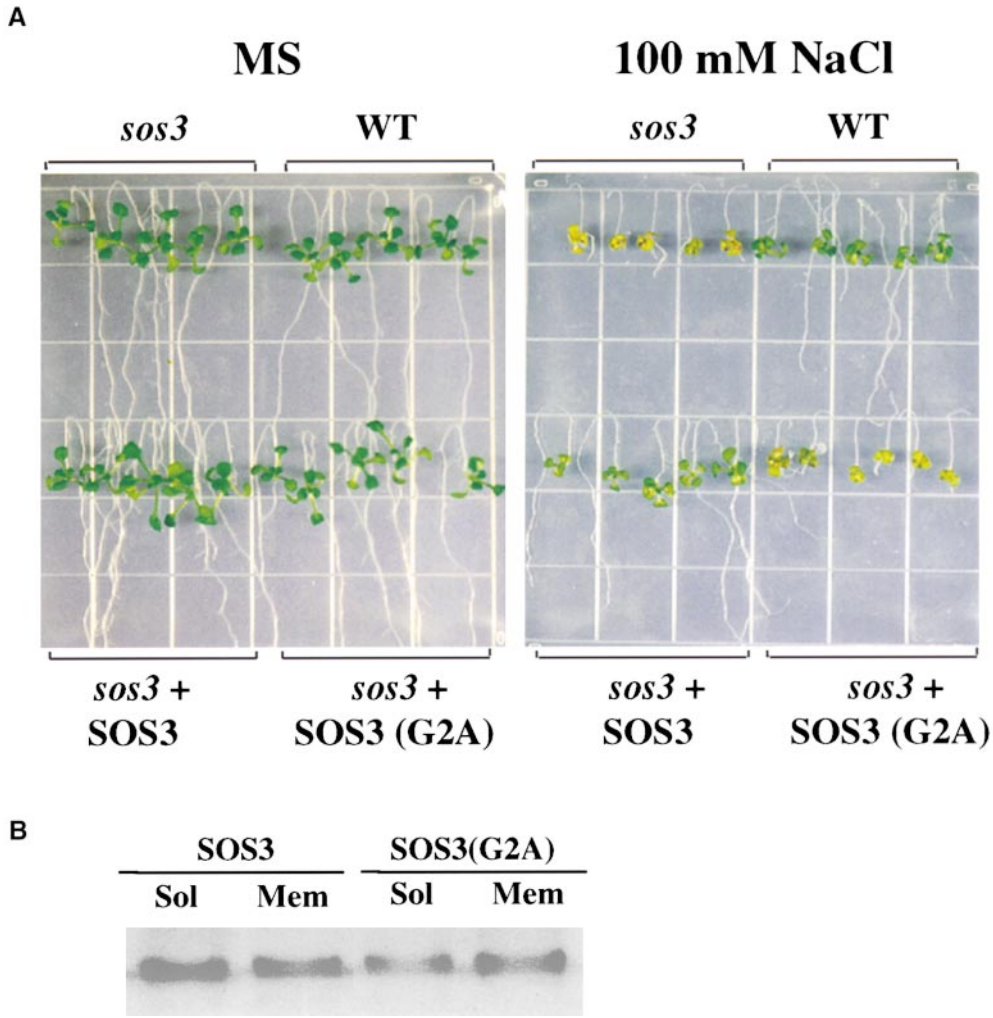

Figure 3. Expression of SOS3 but Not SOS3(G2A) Suppresses the Salt-Hypersensitive Phenotype of *sos3-1* Plants.

(A) Examples of T_3 plants that are homozygous for the respective transgenes. WT, wild-type parent of *sos3* mutant plants. MS, Murashige and Skoog nutrient medium; 100 mM NaCl, MS supplemented with 100 mM NaCl.
(B) Protein expression in *35S–SOS3* and *35S–SOS3(G2A)* transgenic plants, as detected with anti-SOS3 antisera. Sol, soluble proteins; Mem, microsomal membrane proteins.

as nonspecifically with other polypeptides. Again, the fusion proteins were found in both the soluble and membrane fractions, with greater amounts in the soluble fractions (Figure 4B).

We attempted to determine the subcellular localization of SOS3–GFP by green fluorescence imaging. Green fluorescence in the cytosol was evident in both SOS3–GFP and SOS3(G2A)–GFP plants. There were no detectable differences between the localization of myristoylated and nonmyristoylated SOS3–GFP fusion proteins (data not shown).

SOS3 Is a Calcium Binding Protein and the *sos3-1* Mutation Reduces Calcium Binding

The primary sequence of SOS3 predicted three EF-hand calcium binding domains (Liu and Zhu, 1998). To determine whether SOS3 is indeed capable of binding calcium, recombinant His-tagged SOS3 protein was obtained by expression of pET-His-SOS3 in *Escherichia coli* and purified by nickel column affinity chromatography. His–SOS3 and control proteins were fractionated by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and then incubated with

$^{45}Ca^{2+}$. Figure 5 shows that His–SOS3 was capable of binding $^{45}Ca^{2+}$. The binding was much weaker than that of caltractin (Zhu et al., 1996) or calmodulin (data not shown). The weak nature of the binding was also reflected in the failure of His–SOS3 to show a mobility shift when subjected to SDS-PAGE in the presence of calcium or EGTA in the sample buffer (Figure 6A). Most EF-hand calcium binding proteins such as calmodulin or caltractin (Figure 6A) show obvious mobility shifts (i.e., faster migration in the presence of calcium). Garrigos et al. (1991) reported that the calcium-induced mobility shift is greater if calcium is included in the SDS–polyacrylamide gel. Indeed, when calmodulin was subjected to SDS-PAGE with either 5 mM $CaCl_2$ or 5 mM EGTA in the gel, a drastic change in its apparent molecular size was observed (Figure 6B). However, no mobility shift was observed for His–SOS3 or Glutathione S-transferase (GST)–SOS3, even in such a very sensitive mobility shift system (Figure 6B). No mobility shift was observed when other

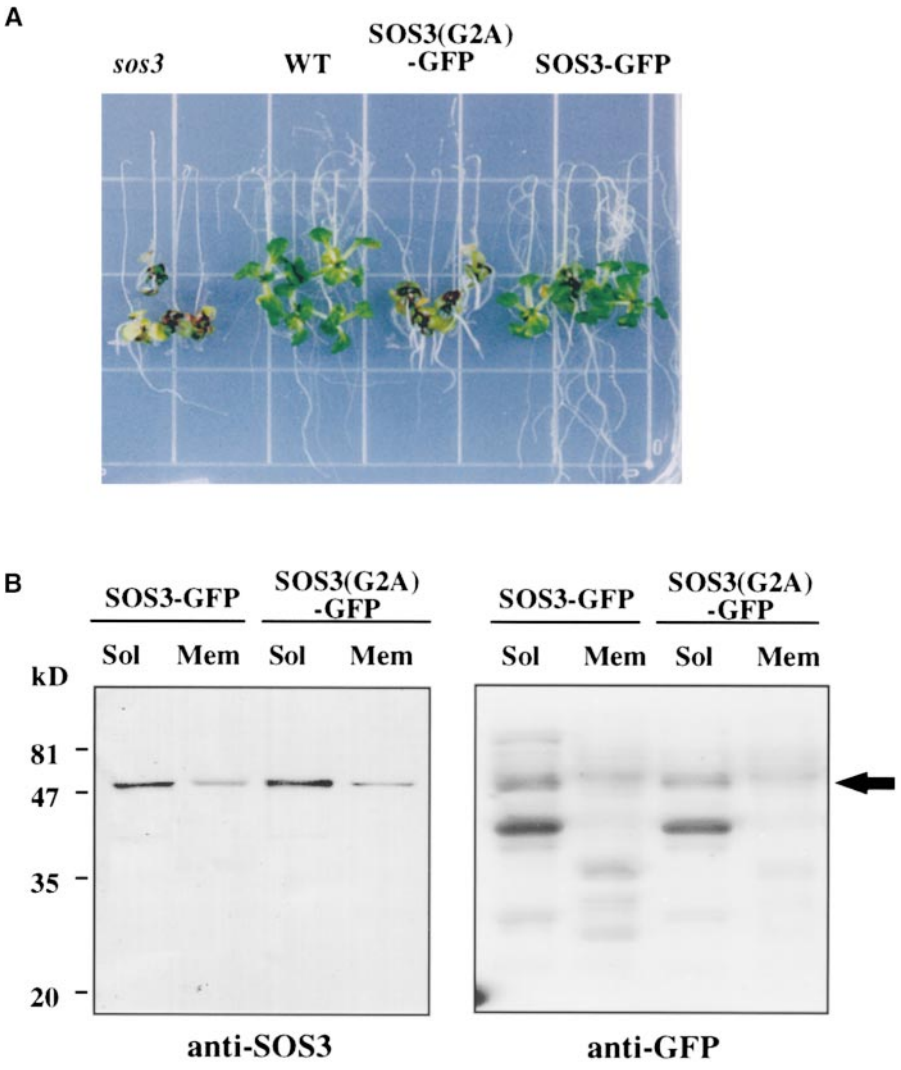

Figure 4. Expression of SOS3–GFP but not SOS3(G2A)–GFP Suppresses the Salt-Hypersensitive Phenotype of *sos3-1* Plants.

(A) *sos3-1* and wild-type (WT) seedlings, and examples of T_3 plants that are homozygous for *35S–SOS3–GFP* or *35S–SOS3(G2A)–GFP*, after growth for 2 weeks on medium containing 100 mM NaCl.

(B) Distribution of SOS3–GFP and SOS3(G2A)–GFP proteins in soluble versus microsomal membrane fractions. *sos3-1* seedlings expressing 35S–SOS3–GFP or 35S–SOS3(G2A)–GFP were grown in liquid culture, treated with 75 mM NaCl, and fractionated as described in Methods. Sol, soluble proteins; Mem, microsomal membranes. Arrow indicates the position of SOS3–GFP or SOS3(G2A)–GFP. Molecular size markers are indicated at left in kilodaltons.

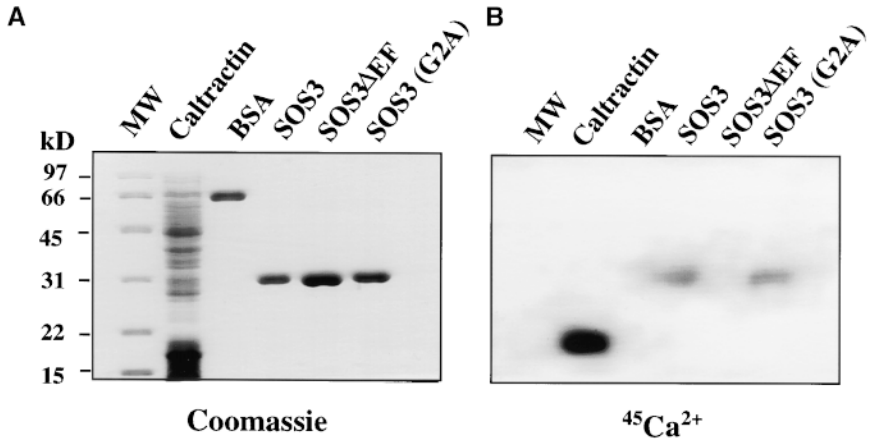

Figure 5. SOS3 Binds $^{45}Ca^{2+}$.

(A) and **(B)** SOS3 and other protein samples were separated by SDS-PAGE and **(A)** stained with Coomassie blue or **(B)** electroblotted onto a nitrocellulose membrane, overlayed with $^{45}Ca^{2+}$, and autoradiographed. MW, molecular size markers shown at left in kilodaltons; caltractin, bacterial lysate containing recombinant caltractin as a positive control for $^{45}Ca^{2+}$ binding; BSA, negative control for $^{45}Ca^{2+}$ binding; SOS3, His–SOS3; SOS3ΔEF, His-tagged SOS3 containing the *sos3-1* mutation; SOS3(G2A), His–SOS3(G2A).

divalent ions such as Mg^{2+} or Mn^{2+} were tested (data not shown).

We also expressed the *sos3-1* allele in *E. coli* as a His-tagged fusion protein and determined whether it binds calcium. The *sos3-1* allele has a three–amino acid deletion in the second EF-hand (Liu and Zhu, 1998). Therefore, the mutant protein is referred to as SOS3ΔEF. Figure 5 shows that SOS3ΔEF did not bind $^{45}Ca^{2+}$ as strongly as the wild-type SOS3 protein. After prolonged exposure, a very faint signal for $^{45}Ca^{2+}$ binding could be detected for SOS3ΔEF (data not shown). The result indicates that the *sos3-1* mutation decreases the calcium binding of SOS3.

To test the effect of the Gly-2 to Ala mutation on the calcium binding of SOS3, His–SOS3(G2A) was obtained by expression in *E. coli* and affinity-purified. Figure 5 shows that this myristoylation mutation did not affect the calcium binding of SOS3.

The *sos3-1* Mutation Disrupts the Interaction between SOS3 and SOS2

Recently, SOS3 has been found to interact with SOS2 (Halfter et al., 2000). SOS2 is a serine/threonine protein kinase that functions in the same pathway as SOS3 to control plant salt tolerance (Halfter et al., 2000; Liu et al., 2000). To determine whether the *sos3-1* mutation affects SOS3 interaction with SOS2, we conducted a yeast two-hybrid assay, using the mutant SOS3ΔEF as the bait and pACT-SOS2 as the prey. Figure 7 shows that unlike the wild-type SOS3, SOS3ΔEF was not capable of interacting with SOS2. This result indicates that the central EF-hand of SOS3 is necessary for interaction with SOS2. To determine whether other structural elements of SOS3 are also required for its interaction with SOS2, the N-terminal or C-terminal region of SOS3 was deleted (Figure 7A) and then the truncated SOS3 was tested in the yeast two-hybrid system. Both N-terminal and C-terminal truncations substantially reduced the binding between SOS3 and SOS2 (Figures 7B and 7C). These results indicate that SOS3 N-terminal and C-terminal regions also contribute to the interaction with SOS2.

The *sos3-1* Mutation Renders SOS3 Incapable of Activating SOS2 Kinase Activity

Having determined that the *sos3-1* mutation reduces the capacity of SOS3 to bind calcium and disrupts the interaction between SOS3 and SOS2, we then assessed whether the mutation affects SOS3 activation of SOS2 kinase activity. SOS2 kinase activity was measured in a peptide phosphorylation assay (Halfter et al., 2000). Figure 8 shows that the *sos3-1* mutation substantially reduces the capacity of SOS3 to activate SOS2.

DISCUSSION

SOS3 was identified as a genetic locus essential for plant salt tolerance (Liu and Zhu, 1998). Two motifs were recognized in its predicted amino acid sequence. One motif is a signature sequence for N-myristoylation, and the other consists of three EF-hands for calcium binding (Liu and Zhu,

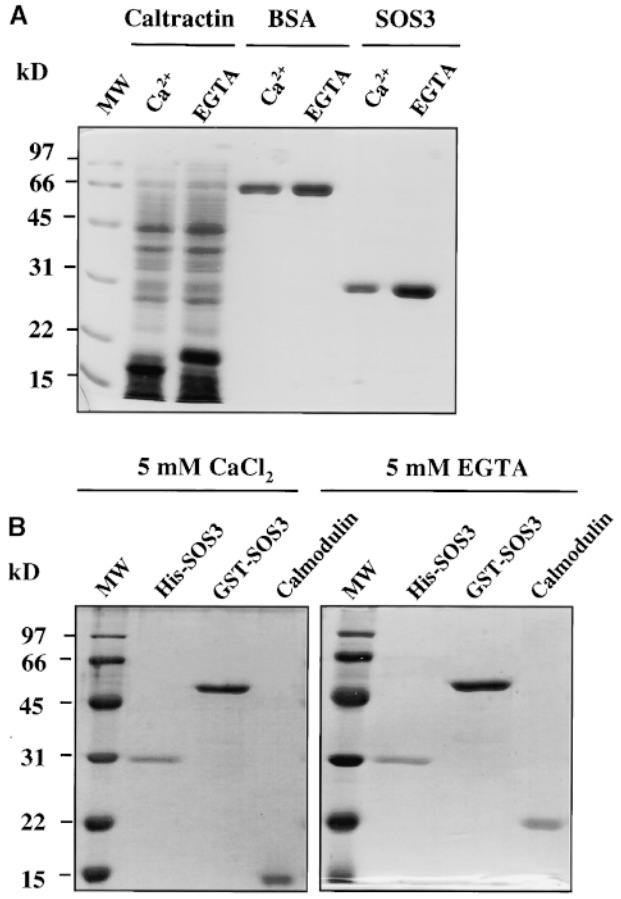

Figure 6. SOS3 Does Not Exhibit a Calcium- or EGTA-Induced Mobility Shift on SDS-PAGE.

(A) His–SOS3 does not show a mobility shift when calcium or EGTA is included in the sample buffer.
(B) Neither His–SOS3 nor GST–SOS3 shows a mobility shift even when calcium or EGTA is included in the polyacrylamide gels.
MW, molecular size markers, shown at left in kilodaltons; caltractin (in bacterial lysate) and calmodulin, positive controls for the mobility shift assays; BSA, negative control.

1998). In this article, we provide experimental evidence that SOS3 is indeed myristoylated and capable of binding calcium. More importantly, we show that myristoylation and calcium binding are essential for SOS3 function in plant salt tolerance. Mutations that disrupt either myristoylation (G2A) or calcium binding (*sos3-1*) render Arabidopsis plants hypersensitive to NaCl stress.

Several plant proteins contain a putative N-myristoylation motif (Lindzen and Choi, 1995; Rommens et al., 1995; Loh et al., 1998; Ellard-Ivey et al., 1999). A calcium-dependent protein kinase (CDPK) from zucchini has been shown to be myristoylated in vitro (Ellard-Ivey et al., 1999), although whether the myristoylation is necessary for its function is unknown. The tomato Pto kinase and its homolog Fen both contain a putative N-myristoylation signal. Mutation of the putative myristoylation site of Pto does not affect Pto function in plant resistance to bacterial speck disease (Loh et al., 1998), whereas a putative myristoylation mutation renders the Fen protein inactive (Rommens et al., 1995). By using a rabbit reticulocyte in vitro translation system, we found that SOS3 can be myristoylated (Figure 2). The myristyl group is probably linked to Gly-2 of SOS3 because, when Gly-2 is mutated to Ala, no myristoylation was detected. The Gly-2 to Ala mutation does not affect the calcium binding of SOS3 (Figure 5).

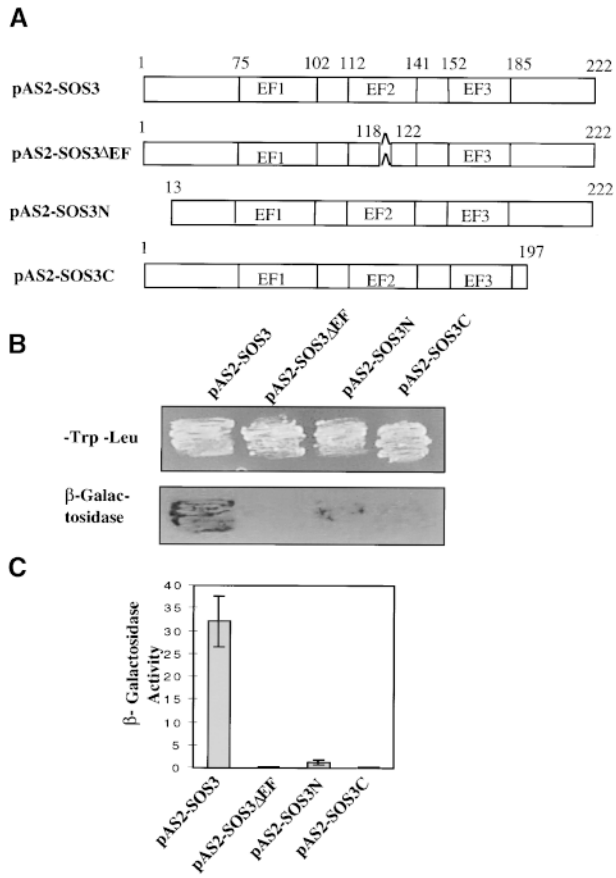

Figure 7. The *sos3-1* Mutation as Well as N- or C-Terminal Truncations in SOS3 Disrupts Its Interaction with SOS2 in the Yeast Two-Hybrid System.

(A) Diagram of the structures of the bait proteins. The *sos3-1* mutation is represented by SOS3ΔEF. pAS2-SOS3N, N-terminal truncation of SOS3; pAS2-SOS3C, C-terminal truncation of SOS3.
(B) Yeast growth and two-hybrid interactions. Upper panel, growth of yeast cells harboring the pACT-SOS2 prey and various baits. Lower panel, β-galactosidase assay.
(C) Quantitative results of the β-galactosidase assay with pACT-SOS2 as prey. Error bars indicate SD ($n = 3$).

Expression of the nonmyristoylated SOS3(G2A) in *sos3-1* plants was not able to suppress the salt-hypersensitive mutant phenotype. Similarly, expression of SOS3(G2A)–GFP under control of the CaMV 35S promoter did not suppress the mutant phenotype. In contrast, expression of SOS3 or SOS3–GFP under control of the 35S promoter fully rescued the *sos3-1* mutant phenotype. Immunoblot analysis showed that SOS3(G2A) as well as SOS3(G2A)–GFP was expressed in the mutant plants (Figures 3B and 4B), suggesting an essential role of myristoylation for SOS3 function in plant salt tolerance. This is consistent with our observation that treatment with a myristoylation inhibitor on young Arabidopsis seedlings caused swelling of root tips specifically under NaCl stress, partially mimicking the *sos3-1* mutant phenotype. Together, these results strongly suggest that SOS3 is myristoylated and that this modification is necessary for SOS3 to function in salt tolerance, although our study does not demonstrate directly myristoylation of SOS3 in vivo.

SOS3 has sequence as well as functional similarities with yeast CNB (Liu and Zhu, 1998). Like SOS3, yeast CNB is also myristoylated (Zhu et al., 1995). However, myristoylation is not functionally essential for CNB function (Zhu et al., 1995). Differences between SOS3 and yeast CNB are not limited to the myristoylation requirement. Recently, we have discovered that unlike yeast CNB, which activates the protein phosphatase CNA, SOS3 physically interacts with and activates a protein kinase encoded by SOS2 (Halfter et al., 2000).

Why myristoylation is necessary for SOS3 function remains a mystery. We attempted to determine whether nonmyristoylated SOS3 has less capacity to associate with cellular membranes but were unable to find clear differences between the membrane association of myristoylated and nonmyristoylated SOS3. Perhaps, myristoylation may not be important for SOS3 to associate with membranes. However, a definitive conclusion cannot be drawn at this time for the following reasons. First, small or subtle differences between the two protein forms may exist but are difficult to detect with our experimental techniques. Second, our experimental design has some limitations. The very low abundance of SOS3 necessitated the use of the strong 35S promoter to express enough SOS3 and SOS3(G2A) for detection. However, the strong expression could cause mislocalization of the proteins. Because the 35S promoter–driven expression of SOS3 did fully suppress the *sos3-1* mutant phenotype, at least some of the overexpressed protein apparently is targeted properly. The use of epitope tags such as HA is unlikely to be sufficient to detect the protein expression driven by the SOS3 promoter, as evidenced by the fact that our very high titer anti-SOS3 sera failed to detect any endogenous SOS3. The very low abundance of SOS3 largely reflects its restricted expression in specific plant tissues (J.-K. Zhu, unpublished observation). Future in planta subcellular immunolocalization with the anti-SOS3 antibodies may permit precise determination of SOS3 localization in the few SOS3-expressing cells.

For some myristoylated proteins, the N-myristoylation is important in protein–protein interactions (Johnson et al., 1994). However, the interaction between SOS3 and SOS2 does not seem to require SOS3 myristoylation; for example, recombinant SOS3 produced in bacteria is not myristoylated but is capable of interacting with SOS2 (Halfter et al., 2000). Nevertheless, this does not exclude the possibility that myristoylated SOS3 may be more efficient in interacting with and activating SOS2. The role of myristoylation in SOS3 function could also be a subtle one, as it is for the catalytic subunit of bovine protein kinase A (Yonemoto et al., 1993). Disruption of myristoylation does not seem to alter protein kinase A enzyme activity or its ability to interact with other proteins; nonetheless, nonmyristoylated protein kinase A is less stable and more susceptible to thermal denaturation than is the myristoylated form (Yonemoto et al., 1993).

Although the SOS3 sequence has three predicted EF-hands for calcium binding, its ability to bind calcium was initially unclear. First, the EF-hands of SOS3 are very unique in that the second acidic amino acid consensus residues (D or N) in all three calcium binding loops are replaced by basic residues (K or R) (Liu and Zhu, 1998). Second, recombinant SOS3 protein does not exhibit a calcium- or EGTA-induced mobility shift on SDS-PAGE (Figure 6). Despite these unusual properties of SOS3, $^{45}Ca^{2+}$ overlay assays showed that the protein is capable of binding calcium (Figure 5). Calcium has been proposed as a second messenger for salt stress responses (Lynch et al., 1989; Knight et al., 1997; Liu and Zhu, 1998; Halfter et al., 2000). The novel calcium binding properties of SOS3 are likely to be important in determining the specificity of calcium signaling under Na^+ stress.

The *sos3-1* mutation substantially reduces the capacity of SOS3 to bind calcium (Figure 5), an observation consistent with the fact that the mutation is a deletion of three conserved amino acids in the central calcium-binding EF-hand (Liu and Zhu, 1998). *sos3-1* plants are hypersensitive to Na^+ inhibition and cannot grow on culture media depleted of K^+ (Liu and Zhu, 1997). Increased external calcium can fully rescue the K^+ starvation defect of *sos3-1* plants and partially suppress their Na^+-hypersensitive phenotype (Liu and Zhu, 1997). Perhaps, the increased external calcium raises internal calcium to a concentration that masks the reduced calcium binding defect of *sos3-1* mutant protein. And perhaps myristoylation might enable some SOS3 protein to associate with the plasma membrane, perhaps near a calcium influx transporter, where the local calcium concentration could be very high. It is also possible that in the presence of increased external calcium, the SOS3 pathway is bypassed and an alternative pathway for Na^+ tolerance is activated.

The *sos3-1* mutation abolishes SOS3 interaction with SOS2 (Figure 7). The mutation markedly diminishes the efficacy of SOS3 to activate SOS2 kinase activity (Figure 8). Aside from the calcium binding domains, the N- and C-ter-

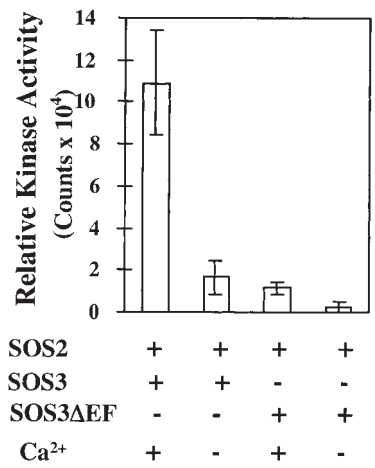

Figure 8. The *sos3-1* Mutation Reduces the Efficacy of SOS3 in the Activation of SOS2 Kinase Activity.

The *sos3-1* mutation is represented by SOS3ΔEF. Phosphorylation assays were performed by using the p3 peptide as a substrate (see Methods). (+) and (−) indicate presence or absence, respectively, of the protein or Ca^{2+} ions. Error bars indicate SD ($n = 3$).

minal regions of SOS3 are also required for interaction with SOS2 (Figure 7). Apparently, the SOS3 protein is folded in a way that its different parts come together to form an SOS2 binding structure.

METHODS

Treatment with 2-Hydroxymyristic Acid

Wild-type and *sos3-1 Arabidopsis thaliana* seeds were germinated on Murashige and Skoog (MS) nutrient agar media as described in Wu et al. (1996). Five-day-old seedlings of the wild type and *sos3-1* were treated for 2 days. The treatments were performed by placing seedlings on filter paper (Schleicher and Schuell, Keene, NH) soaked in one of the following solutions: MS solution; MS + 1 mM 2-hydroxymyristic acid (HMA); MS + 100 mM NaCl; or MS + 100 mM NaCl + 1 mM HMA.

Expression of SOS3, SOS3ΔEF, and SOS3(G2A) in *Escherichia coli*

SOS3 (wild type) and *sos3-1* (containing a 9-bp deletion in a calcium binding domain) cDNAs were obtained by reverse transcription–polymerase chain reaction (RT-PCR) from wild-type and *sos3-1* plants, respectively. The RT-PCR products were cloned into pBluescript SK− (Stratagene, La Jolla, CA) to obtain pSOS3 and psos3-1, respectively. The constructs were sequenced to ensure that no serendipitous mutations were introduced during cloning.

To express His-tagged SOS3 and SOS3ΔEF proteins in bacteria, the corresponding cDNAs were isolated from pSOS3 and psos3-1, respectively, by PCR with the sense primer 5′-GGCCATATGGGCTGCTCTGTATCGA and the antisense primer 5′-ACGCTCGAGTACACAAGGCAAGT. The PCR products were digested and cloned into the NdeI and XhoI sites of pET14b (Novagen, Madison, WI) to yield pET-His-SOS3 and pET-His-SOS3ΔEF, respectively. To change Gly-2 of SOS3 to Ala, the forward primer was changed to 5′-GGCCATATGGCCTGCTCTGTATCGA (G2A mismatch mutation is underlined), yielding the construct pET-His-SOS3-G2A. The plasmids were transformed into the *E. coli* strain BL21(DE3) for protein expression. Aliquots (0.5 mL) of overnight cultures were subcultured into 5 mL of fresh LB (Luria-Bertani) medium (10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl) plus carbenicillin (100 mg/mL) and allowed to grow for 1.5 hr at 37°C. Protein expression was then induced for 2.5 hr by adding isopropyl-β-D-thiogalactopyranoside to 0.4 mM. The recombinant proteins were purified by affinity chromatography on a nickel column (Novagen, Madison, WI) according to the manufacturer's instructions.

For in vitro transcription/translation assays, pET-SOS3 and pET-SOS3-G2A were obtained by PCR from pSOS3 and cloned into the NcoI and XhoI sites of pET14b. The forward PCR primers were 5′-TGACCCGGGATCCATGGGCTGCTCTGTATCGAA and 5′-GGATCCATGGCCTGCTCTGTATCGAA (G2A mismatch mutation is underlined), respectively, for pET-SOS3 and pET-SOS3-G2A. The reverse primers were both 3′-ACGCTCGAGTACACAAGGCAAGT.

In Vitro Labeling and Myristoylation

In vitro transcription and translation were performed by using the TNT Quick coupled transcription/translation systems (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, pET-SOS3 and pET-SOS3-G2A were transcribed and translated in a TNT Quick master mix containing rabbit reticulocyte lysate. Radiolabeling was performed with 0.1 μCi/μL [^{3}H]leucine in the presence of 20 μM amino acids (minus leucine) or with 0.5 μCi/μL [^{3}H]myristic acid that had been dried under nitrogen gas and suspended in distilled water. Reactions were performed at 30°C for 90 min. Aliquots of reaction products were analyzed by SDS-PAGE, followed by fluorography.

Calcium Binding Assays

For calcium-induced mobility shift assays, 5 mM $CaCl_2$ or 5 mM EGTA was included in either the sample buffer or the polyacrylamide gels. For $^{45}Ca^{2+}$ overlay assays, proteins were separated by SDS-PAGE and transferred onto nitrocellulose by electroblotting. After transfer, the membrane was soaked in a solution containing 10 mM imidazole-HCl, pH 6.8, 60 mM KCl, and 5 mM $MgCl_2$, exchanging the buffer three times during a 1.5-hr period. The membrane was then incubated in the same buffer plus 37 kBq/mL $^{45}Ca^{2+}$ for 10 min. The membrane was rinsed with distilled water for 30 min, air-dried, and then exposed to x-ray film for 3 days.

Expression of SOS3 and SOS3(G2A) in *sos3-1* Plants

pSOS3 was used as the template to obtain *SOS3* and *SOS3(G2A)* cDNAs by PCR. A mismatch nucleotide was introduced to the sense primer of *SOS3(G2A)* to change Gly-2 to Ala. The PCR products were digested and inserted between the XbaI and SstI sites of the binary vector pBI121 (Clontech, Palo Alto, CA). The constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101 and transformed into *sos3-1* plants by flower bud infiltration.

35S–SOS3–GFP and *35S–SOS3(G2A)–GFP* Constructs and Plant Expression

Wild-type *SOS3* cDNA was amplified from pSOS3 by PCR with the sense primer 5′-ACGATCTAGAATGGGCTGCTCTGTATCGAA and the antisense primer 5′-CCTTAGATCTCGTTTTGCAATTCCATTTCT. To obtain the *SOS3(G2A)* cDNA, 5′-AGTCTCTAGAAGAAGGGTGTGTTTGTATGGCCTGC (mismatch mutation is underlined) was used as the sense primer. The PCR products were digested with XbaI and BglII and cloned into the XbaI and BamHI sites of a green fluorescent protein (GFP) translational fusion vector (CD3-326 from the Arabidopsis Biological Resource Center, Columbus, OH). These as well as all the above constructs were sequenced to ensure that no serendipitous mutations were introduced during cloning. The constructs were transformed into *sos3-1* and wild-type plants.

Cell Fractionation and Immunoblot Analysis

Seeds of *35S–SOS3*, *35S–SOS3(G2A), 35S–SOS3–GFP,* and *35S–SOS3(G2A)–GFP* transgenic plants were surface-sterilized, germinated, and grown in liquid culture in the dark as described in Liu and Zhu (1997). For salt treatment, nutrient solution was drained and fresh nutrient solution supplemented with 75 mM NaCl was added. After 12 hr of treatment, the seedlings were harvested, cut into small pieces with scissors, and homogenized in a buffer (250 mM Tris-HCl, pH 8.5, containing 290 mM sucrose, 2 mM EDTA, 76 mM β-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride) by grinding with a mortar and pestle at 4°C. The homogenate was filtered through cheesecloth and centrifuged at 5000*g* for 5 min at 4°C. The supernatant was then centrifuged at 100,000*g* for 1 hr to pellet the microsomal membranes. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, and 10% glycerol. To obtain soluble proteins, the supernatant was precipitated by adding cold acetone to 80% (v/v) and incubated overnight at −20°C. The precipitated protein was collected by centrifugation at 12,000*g* for 15 min, washed twice with cold acetone, and then resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, and 10% glycerol.

The samples were separated by SDS-PAGE and then electroblotted onto a nitrocellulose membrane. The membrane was blocked for 1 hr with 1% powdered milk in buffer consisting of 20 mM Tris, pH 7.5, 500 mM NaCl, and 0.15% Tween-20 (TBS-T buffer). Then the membrane was incubated for 4 hr at room temperature with anti-GFP antiserum (MBL, Nagoya, Japan) at a dilution of 1:4000 or anti-SOS3 antiserum at a dilution of 1:1000. Nickel column affinity-purified His–SOS3 protein was used as an antigen to generate polyclonal antisera in rabbits. Secondary antibodies (horseradish peroxidase–conjugated goat anti–rabbit) were used diluted 1:2000 in TBS-T for 1 hr at room temperature. The membrane was washed three times in TBS-T, incubated in Amersham ECL Reagent (Amersham, Piscataway, NJ) for 1 min, and then exposed to x-ray film for 5 min.

Yeast Two-Hybrid Assays

To contruct bait plasmids containing SOS3, SOS3ΔEF (*SOS3* with the *sos3-1* mutation), or SOS3 with N-terminal (SOS3N) or C-terminal (SOS3C) truncation, *SOS3* or *sos3-1* cDNA was amplified with the following primers, digested with NcoI and PstI, and then ligated into the pAS2 bait vector. All constructs were sequenced completely to ensure no PCR or cloning errors. The primer sequences used were as follows: for both pAS2-SOS3 (*SOS3* cDNA as template) and pAS2-SOS3ΔEF (*sos3-1* cDNA as template), 5′-ATGGCCATGGGCTGCTCTGTATCGAAGAAGA and 5′-ATGGCCATGGGCTGCTCTGTATCGAAGAAGA; for pAS2-SOS3N, 5′-TGGCCATGGCAATGCGACCACCGGGATATGAGGATCC and 5′-AACTGCAGGGCTTATATTAGGAAGATACGTTTTGC; and for pAS2-SOS3C, 5′-ATGGCCATGGGCTGCTCTGTATCGAAGAAGA and 5′-AACTGCAGTATATTATAGATATGGCAAAGTCATGTTCTTGATG.

The bait plasmids were introduced into the yeast containing pACT2-SOS2 as the prey (Halfter et al., 2000). Filter interaction assays were conducted as described in Halfter et al. (2000). For quantitative assays, we used a procedure published at http://www.fhcrc.org/labs/gottschling/Bgal.sht. β-Galactosidase activity (U) was calculated as $U = 1000 \times [(OD_{420}) - (1.75 \times OD_{550})]/[(Time[min]) \times (Vol[ml]) \times OD_{600}]$.

Peptide Phosphorylation Assays

Phosphorylation assays using GST–SOS2 as kinase and peptide p3 (ALARAASAAALARRR) as substrate were as described in Halfter et al. (2000). The kinase buffer includes 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM DTT. For Ca^{2+}-free conditions, $CaCl_2$ was omitted and 10 mM EGTA was included in the kinase buffer. The data reported were obtained by subtracting the background activity (SOS2 kinase without SOS3) from the scintillation counts measured.

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