

The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3

Ursula Halfter, Manabu Ishitani, and Jian-Kang Zhu*

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721

Communicated by Winslow R. Briggs, Carnegie Institution of Washington, Stanford, CA, December 27, 1999 (received for review October 20, 1999)

The *Arabidopsis thaliana* SOS2 and SOS3 genes are required for intracellular Na⁺ and K⁺ homeostasis and plant tolerance to high Na⁺ and low K⁺ environments. SOS3 is an EF hand type calcium-binding protein having sequence similarities with animal neuronal calcium sensors and the yeast calcineurin B. SOS2 is a serine/threonine protein kinase in the SNF1/AMPK family. We report here that SOS3 physically interacts with and activates SOS2 protein kinase. Genetically, *sos2sos3* double mutant analysis indicates that SOS2 and SOS3 function in the same pathway. Biochemically, SOS2 interacts with SOS3 in the yeast two-hybrid system and *in vitro* binding assays. The interaction is mediated by the C-terminal regulatory domain of SOS2. SOS3 activates SOS2 protein kinase activity in a Ca²⁺-dependent manner. Therefore, SOS3 and SOS2 define a novel regulatory pathway important for the control of intracellular ion homeostasis and salt tolerance in plants.

The homeostasis of intracellular ion concentrations is a fundamental property of living cells. Eukaryotic cells employ primary active transport, mediated by P-type ATPases, and secondary transport, mediated by channels and cotransporters, to maintain characteristic high concentrations of K⁺ and low concentrations of Na⁺ in the cytosol (1). Intracellular K⁺ and Na⁺ homeostasis is important for the activities of many cytosolic enzymes and for maintaining membrane potential and appropriate osmoticum for cell volume regulation (1). In plants, maintenance of K⁺ and Na⁺ homeostasis is critical for salt tolerance, an important agronomic trait because salt stress significantly limits agricultural productivity in many parts of the world (2–5).

Research with *Saccharomyces cerevisiae* has been extremely valuable in dissecting the regulatory mechanisms of ion homeostasis and salt tolerance (6–8). The cellular mechanisms of plant salt tolerance are thought to be similar to those in yeast in which signaling through a protein phosphatase, calcineurin, modulates the expression and activities of Na⁺ and K⁺ transporters to maintain low Na⁺ and high K⁺ concentrations in the cytoplasm (6, 9, 10). The existence of calcineurin in plants has been indicated by several studies (9, 11–13). Recently, the *Arabidopsis thaliana* SOS3 gene product was found to share substantial sequence similarity with the regulatory subunit of yeast calcineurin (CNB) (10). Functionally, SOS3 is also similar to yeast CNB because, like the yeast *cnb* mutant (14–15), growth of *sos3* mutant plants is hypersensitive to Na⁺ and Li⁺ inhibition (16). Despite this evidence for SOS3 participation in a calcineurin-like pathway in mediating plant salt tolerance, we report in this paper that, instead of binding and activating a protein phosphatase, SOS3 physically interacts with and activates a protein kinase encoded by the *Arabidopsis* SOS2 gene.

Like SOS3, the SOS2 locus is required for intracellular Na⁺ and K⁺ homeostasis (17). Mutations in SOS2 cause hypersensitivity to high Na⁺ and Li⁺ stresses, as well as to low K⁺ stress (17). The similar phenotypes of the *sos2* and *sos3* mutant plants suggest that the two genes may function in the same pathway in regulating Na⁺ and K⁺ homeostasis. As part of this study, we constructed and analyzed *sos2sos3* double mutants and show that there is no additive effect when the two mutations are combined.

This provides genetic evidence that SOS3 and SOS2 function in the same regulatory pathway.

To identify other components in the regulatory pathway, we used SOS3 as a bait to select interacting proteins in a yeast two-hybrid screen. The screen identified a group of related protein kinases as SOS3-interacting proteins (SIPs). Strikingly, sequence analysis revealed that the SIP kinases are in the SOS2 subfamily of protein kinases. We show that SOS2 indeed interacts with SOS3 in the yeast two-hybrid system as well as in *in vitro* binding assays. Furthermore, SOS2 kinase activity is activated by SOS3 in a Ca²⁺-dependent manner. These results illustrate that plants have evolved a novel regulatory pathway for the control of intracellular Na⁺ and K⁺ homeostasis and salt tolerance.

Materials and Methods

Plant Growth Conditions. Conditions for plant growth in soil and in agar media were as described (17). Root growth measurement was carried out daily (16). Data presented are for growth after 1 week on the respective media.

Genetic Analysis. For double mutant analysis, *sos2-2* and *sos3-1* mutants were crossed, and progeny of F1 plants were collected. A root bending assay (18) was used to select salt-hypersensitive mutants from the F2 seedlings. Double mutants were identified from the mutants by PCR genotyping. Primer pairs used to distinguish *sos3-1* and its wild-type allele are 5'TCTCATGAATTTGCAGTTGC and 5'AAACTGTTTAATCTGGAGGG, resulting in 112- and 121-bp amplification products for the mutant and wild-type genes, respectively. To discern *sos2-2* from its wild-type allele, primer pairs 5'AATTTGGATGATATTCGTGCAGTTTTTG and 5'TTAACATTTAAATGGAATTGACC (for wild type, resulting in a 96-bp product), and 5'CAAATCAAGGAATCAAGAATTCGC and 5'TTAACATTTAAATGGAATTGACC (for *sos2-2*, yielding a 90-bp product) were used.

Yeast Two-Hybrid Screen and Interaction Assay. The SOS3 coding region was amplified by PCR with primers containing restriction sites and was cloned in frame between the *Nco*I and *Sal*I sites of pAS2, to form pAS-SOS3 as the bait to screen an *Arabidopsis* expression library in pACT1. The λ ACT cDNA expression library (19) was converted to a pACT plasmid library by infecting *Escherichia coli* BNN132 cells (20). pAS-SOS3 was transformed into yeast Y190, which was then analyzed by Western blotting using α -HA antibody (Babco, Berkeley, CA) for detection of SOS3 fusion protein expression. Transformation and screening were according to Bai and Elledge (21). Approximately 1.6×10^6

Abbreviation: SIP, SOS3-interacting protein.

*To whom reprint requests should be addressed. E-mail: jkzhu@ag.arizona.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.0405776997. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.0405776997

transformants were plated and selected on SC agar medium, which lacks leucine, tryptophan, and histidine and contains 25 mM 3-amino-1,2,4-triazole (triple selection medium). Large colonies were streaked and subjected to a 5-bromo-4-chloro-3-indolyl β -D-galactoside filter assay. Seventy-eight clones showing blue color were chosen for further analysis by growing them in SC medium minus leucine, which favors the loss of the bait plasmid, and isolating the pACT-SIP plasmids for sequence determination. To confirm the interaction between putative SIP clones and SOS3, purified pACT-SIP plasmids were retransformed into the yeast Y190 harboring pAS-SOS3 and were subjected to growth on the triple selection medium and β -galactosidase assay. For control interactions with the identified prey proteins, empty vector pAS2, pAS-RB [maize retinoblastoma protein (22), a gift from B. Larkins, University of Arizona, Tucson, AZ] and pAS-p53 (21) (a gift from S. J. Elledge, Baylor College of Medicine, Houston, TX) were used as baits.

The N-terminal (residues 1–260) and C-terminal (residues 257–446) parts of *SOS2* were amplified by PCR with primers containing restriction sites and were cloned into *Bam*HI-*Eco*RI of pACT2 to test interaction with pAS-SOS3.

Protein Expression. GST-SOS2, GST-SOS2(K40N), and GST-SOS2(G197E) were produced as described by Liu *et al.* (23). To produce bacterially expressed GST-SOS3, the coding region of *SOS3* cDNA was amplified by PCR with primers harboring restriction sites, cloned in frame into *Bam*HI-*Eco*RI of pGEX-2TK (Amersham Pharmacia) and transformed into *E. coli* BL21 DE3 cells (Amersham Pharmacia). *E. coli* cultures were induced with 0.5 mM IPTG, and recombinant proteins were affinity-purified from bacterial lysates with glutathione-Sepharose beads (Amersham Pharmacia).

Kinase Assay. GST-fusion proteins were incubated in kinase buffer (20 mM Tris-HCl, pH 8.0/5 mM MgCl₂/1 mM CaCl₂/1 mM DTT). The kinase reaction was started by adding [γ -³²P] ATP and was transferred to 30°C for 30 min. The reaction was stopped by adding 4× SDS-sample buffer and was analyzed by SDS/PAGE and autoradiography. Oligopeptide phosphorylation was assayed in a final reaction volume of 25 μ l in the kinase buffer with 200 μ M oligopeptides and 1 μ g of GST-SOS2 and with or without 1 μ g of GST-SOS3. Peptides p1 (LRRASLG) and p2 (VRKRTLRL) were from Sigma. Peptide p3 (ALARAASAAALARRR) was custom synthesized by Research Genetics (Huntsville, AL). Incubation was for 30 min at 30°C. Fusion proteins (GST-SOS2 and GST-SOS3) bound to glutathione-Sepharose were pelleted by low speed centrifugation. Fifteen microliters of supernatant were used for detection of peptide phosphorylation according to Davies *et al.* (24). For Ca²⁺-free conditions, CaCl₂ was omitted, and 10 mM EGTA was included in the kinase buffer.

In Vitro Binding Assays. For pull-down assays, radiolabeled SOS3 protein was produced from pET14b-SOS3 (*Nco*I-*Bam*HI) by using an *in vitro* transcription and translation assay kit with ³H-leucine as the sole source of leucine (TNT Coupled Reticulocyte Lysate System, Promega), following the manufacturer's instructions. Equal amounts of ³H-leucine-SOS3 were incubated under constant rocking for 1 h at 4°C in 150 μ l of binding buffer (20 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM CaCl₂/0.1% Nonidet P-40) with 2 μ g each of GST-SOS2, GST-RB [GST fusion of the maize retinoblastoma protein (22)], or GST bound to glutathione-Sepharose beads. GST-RB and GST were used as controls in the binding assays. The Sepharose beads were pelleted and washed extensively with the binding buffer and were analyzed by SDS/PAGE and fluorography.

For gel blot overlay assays, the *SOS3* ORF was cloned into the pGEX-2TK vector to allow direct labeling of recombinant GST-SOS3 with ³²P because it contains the recognition sequence for

protein kinase A between the GST domain and the multiple cloning sites. The recombinant GST-SOS3 was purified by affinity chromatography on glutathione-Sepharose resin and was labeled by using protein kinase A and γ -³²P-ATP following published procedures (25). The GST was then cleaved from GST-SOS3 by using thrombin. GST-SOS2, GST-SOS2(K40N), GST-SOS2(G197E), and control proteins were fractionated by SDS/PAGE and were electroblotted onto nitrocellulose membrane. The blot was incubated with ³²P-labeled SOS3 in TBST [20 mM Tris-HCl, pH 7.8/0.5 M NaCl/0.15% (vol/vol) Tween 20], was washed with TBST, and was exposed to x-ray film.

Results

Genetic Interaction Between *sos2* and *sos3*. To determine genetically whether *SOS2* and *SOS3* function in the same pathway, we constructed *sos2sos3* double mutants by crossing the deletion alleles *sos2-2/sos2-2* and *sos3-1/sos3-1* and selecting *sos* mutants from the F2 generation. Because *SOS2* and *SOS3* are closely linked on chromosome V, we devised a PCR-based screen for the relatively rare occurrence of double mutants. Two lines of *sos2sos3* double mutants were identified from screening 86 *sos* mutants from the F2 population by the presence of *sos2-2*- and *sos3-1*-specific PCR products and the lack of PCR products corresponding to the respective wild-type alleles. The double mutants were confirmed by test crosses with the single mutants.

We tested the growth of the single mutants and *sos2sos3* double mutants on media containing 0, 50, or 75 mM NaCl. Consistent with previous results (17), both *sos2* and *sos3* mutants show hypersensitivity to NaCl stress, although *sos2* displays greater sensitivity than *sos3* (Fig. 1). The double mutants resembled *sos2-2* plants in appearance (e.g., dark curly leaves under 50 mM NaCl) and in shoot and root growth (Fig. 1A). Root growth measurements show that the double mutant was more severely inhibited than *sos3-1* but was indistinguishable from the *sos2-2* mutant under all conditions (Fig. 1B). The lack of additive effects of the mutations is consistent with *SOS2* and *SOS3* functioning in the same pathway.

Two-Hybrid Screen for SOS3-Interacting Proteins Identifies a Family of Protein Kinases That Are Similar to SOS2. The entire *SOS3* ORF was fused in frame to the C terminus of the DNA-binding domain of GAL4 to create a bait in the plasmid pAS2 and was transformed into the yeast strain Y190 containing two reporter genes, *His3* and *LacZ* (21). The bait plasmid pAS-SOS3 by itself did not activate transcription of the two reporter genes. For the prey library, we used the λ -ACT cDNA library prepared from mRNA isolated from young *Arabidopsis* seedlings (19). The prey plasmids were excised and transformed into the yeast strain harboring pAS-SOS3. Of $\approx 1.6 \times 10^6$ transformants plated, 78 clones were confirmed positive for *His3* and *LacZ* expression. Prey plasmids were isolated from the clones, and sequence analysis revealed that 39 encode proteins with significant sequence similarities to serine/threonine protein kinases. Based on sequence alignments, the 39 SOS3-interacting (SIP) kinase clones define a family of seven proteins (i.e., SIP1 to SIP7) that are closely related to each other. At least one pACT-SIP plasmid representing each of the seven SIP kinases was reintroduced into the yeast strain Y190 to test for autoactivation of the reporter genes. They were also introduced into the yeast Y190 containing the control bait pAS-p53, pAS-RB, or the empty bait vector pAS2 to examine the specificity of the interactions. None of them was found to activate the reporter genes when expressed alone or in combination with pAS2 or the control baits.

Complete amino acid sequences were obtained for SIP1, SIP2, SIP3, and SIP4 by searching the GenBank database. SIP2 corresponds to a serine/threonine protein kinase-like sequence (26) previously identified through homology cloning. SIP1, SIP3, and SIP4 correspond to genomic sequences (GenBank accession

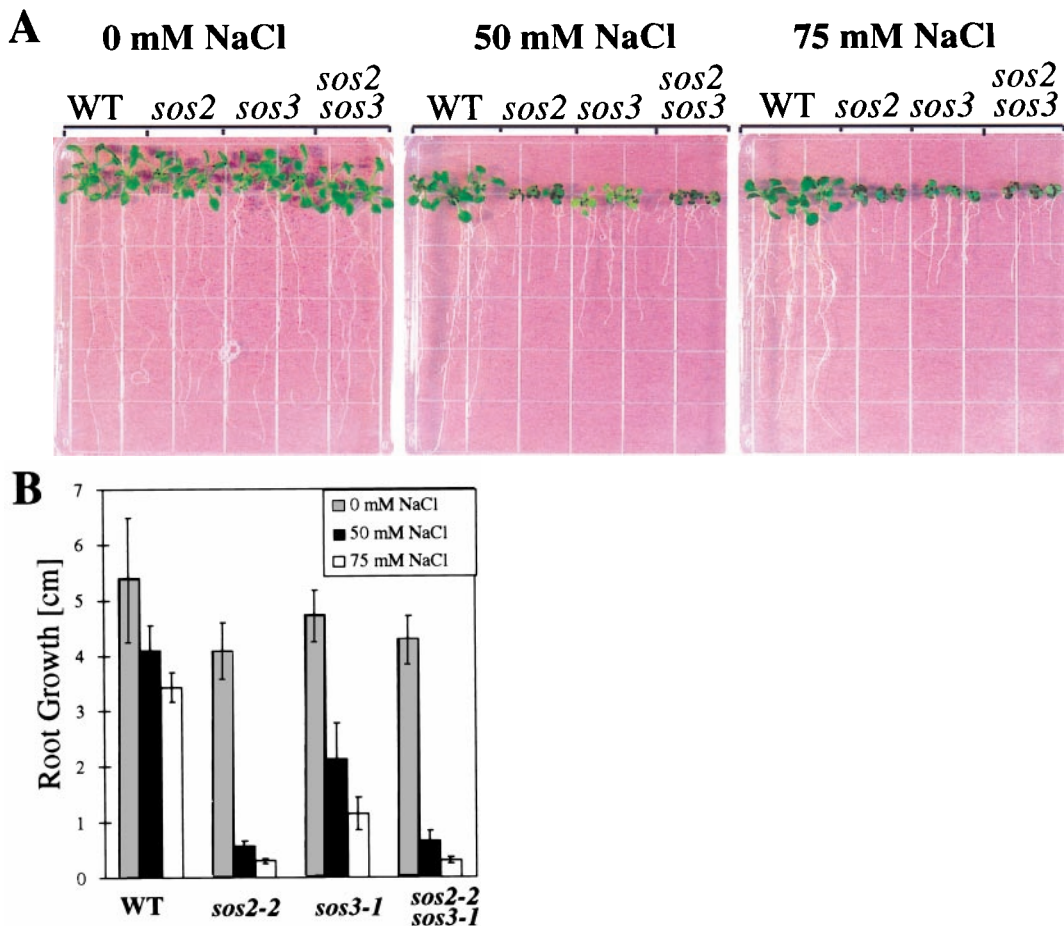


Fig. 1. Genetic interaction between *sos2* and *sos3*. (A) Seedling phenotypes of wild-type (WT) plants and *sos2-2*, *sos3-1*, and *sos2sos3* double mutants treated with various levels of NaCl. (B) Root growth measurement. Five-day-old seedlings were transferred from normal nutrient medium to media supplemented with different concentrations of NaCl, and root elongation after 7 days is presented. Error bars represent the standard deviation ($n = 12$). The pictures were taken 10 days after the NaCl treatments.

numbers AB019228, AL022198.1, and AC002338.1, respectively) released by the *Arabidopsis* Genome Initiative.

Remarkably, when SOS2 was cloned recently (23), we found that the deduced amino acid sequence of *SOS2* is very similar to the SIP kinases. The alignment of the *SOS2* amino acid sequence with four of the SIP kinases is shown in Fig. 2. Like *SOS2*, the putative kinase catalytic domain of the SIPs resides in the N-terminal portion of the proteins and is most similar to the yeast SNF1 and mammalian AMPK kinases (27, 28) (alignments not shown). Similarities between *SOS2* and the SIP kinases are found in not only the N-terminal catalytic domain but also in the C-terminal regulatory domain.

SOS2 Interacts with SOS3 in the Yeast Two-Hybrid System. Although *SOS2* was not among the SIP kinases identified by the yeast two-hybrid screen, its high sequence similarity with the SIP kinases indicates that it may interact with *SOS3*. To test this hypothesis, *SOS2* was cloned in frame in the pACT2 vector and was introduced into the yeast strain Y190 containing the bait pAS-*SOS3*, or pAS2 vector or pAS-p53 as controls. Indeed, *SOS2* interacted with *SOS3* in the yeast two-hybrid system, and its interaction appeared stronger than that of the other SIP kinases, as suggested by the faster and stronger color appearance in β -galactosidase assays (Fig. 3). Expression of pACT-*SOS2* alone (not shown) or in combination with pAS2 bait vector or with the control bait pAS-p53 did not activate the reporter gene

expression (Fig. 3). Thus, *SOS2* specifically interacts with *SOS3* in the yeast two-hybrid system. Because the *SOS2* gene is expressed at a very low level, it would not be unexpected that *SOS2* message would be underrepresented or even absent in the *Arabidopsis* λ -ACT cDNA expression library used for yeast two-hybrid screening. Indeed, PCR amplification using *SOS2*-specific primers on plasmid DNA of this library yielded no product whereas using SIP-clone specific primers resulted in good amplification (not shown).

SOS3 Interacts with the C-Terminal Regulatory Domain of SOS2. All SIP kinase clones encoded only truncated proteins, and the positions of N-terminal truncations for SIP1, SIP2, SIP3, and SIP4 clones are shown in Table 1. It appears that protein kinase activity is not required for interaction with *SOS3* because many of the interacting clones have their putative catalytic domain truncated (Table 1). To determine whether *SOS3* interacts with only the regulatory domain of *SOS2*, the N-terminal kinase catalytic domain (residues 1–260) and the C-terminal regulatory domain (257–446) were each cloned in frame into the prey vector pACT2, and their interaction with *SOS3* was determined by using the yeast two-hybrid assay. Expression of the preys in yeast cells were confirmed by using antibodies against the HA-tag, which is part of the prey fusion proteins (21). Fig. 3 shows that *SOS3* interacted with the C-terminal but not the N-terminal domain of *SOS2*.

SOS2 Interacts with SOS3 in Vitro. To corroborate the interaction between *SOS2* and *SOS3* observed in the yeast two-hybrid

SIP1 1 MEKSS--PS--VITDKYDGRLLGGTFKAVYHGGHINOSVAIKMIEKRV
SIP2 1 MEKSS--PS--VITDKYDGRLLGGTFKAVYHGGHINOSVAIKMIEKRV
SIP3 1 MVGAKPVNGSDGGSSTGLHGRYELGRLLGGTFKAVYHGGHINOSVAIKMIEKRV
SIP4 1 MPETELIAGSSDNDNDHDKYELGLLGGTFKAVYHGGHINOSVAIKMIEKRV
SOS2 1 ITN-----MRRVSKYEVGRTGGTFKAVYHGGHINOSVAIKMIEKRV

SIP1 49 MKVGLLEQIKREISVNRIRRHPPVVEIHEVMAKTRIVFMEYKGGELFNKYV--SKL
SIP2 49 MKVGLLEQIKREISVNRIRRHPPVVEIHEVMAKTRIVFMEYKGGELFNKYV--SKL
SIP3 61 MKVGLLEQIKREISVNRIRRHPPVVEIHEVMAKTRIVFMEYKGGELFNKYV--SKL
SIP4 58 MTNPAIANNIKREISVNRIRRHPPVVEIHEVMAKTRIVFMEYKGGELFNKYV--SKL
SOS2 48 MKVGLLEQIKREISVNRIRRHPPVVEIHEVMAKTRIVFMEYKGGELFNKYV--SKL

SIP1 107 RDDVAIKYEQQLNAVDFCHSRGVYHRDLKPNLLLDNGLKVSDFGLSALPCKRQDG
SIP2 107 RDDVAIKYEQQLNAVDFCHSRGVYHRDLKPNLLLDNGLKVSDFGLSALPCKRQDG
SIP3 119 RDDVAIKYEQQLNAVDFCHSRGVYHRDLKPNLLLDNGLKVSDFGLSALPCKRQDG
SIP4 118 SEDLSRRYEQQLNAVDFCHSRGVYHRDLKPNLLLDNGLKVSDFGLSALPCKRQDG
SOS2 107 RDDVAIKYEQQLNAVDFCHSRGVYHRDLKPNLLLDNGLKVSDFGLSALPCKRQDG

SIP1 167 LHTTCGTTPAYVAPEVIRKGYDCAKADINSCGVILFVLLAGYLFPEDNINMYRKIKH
SIP2 167 LHTTCGTTPAYVAPEVIRKGYDCAKADINSCGVILFVLLAGYLFPEDNINMYRKIKH
SIP3 179 LHTTCGTTPAYVAPEVIRKGYDCAKADINSCGVILFVLLAGYLFPEDNINMYRKIKH
SIP4 178 LHTTCGTTPAYVAPEVIRKGYDCAKADINSCGVILFVLLAGYLFPEDNINMYRKIKH
SOS2 165 LHTTCGTTPAYVAPEVIRKGYDCAKADINSCGVILFVLLAGYLFPEDNINMYRKIKH

SIP1 227 ADFKADKAPAEVRRLLKMLDPPNPTITITIAIRRESNFRKGLHMKQKQK--RVK
SIP2 227 ADFKADKAPAEVRRLLKMLDPPNPTITITIAIRRESNFRKGLHMKQKQK--RVK
SIP3 239 GDFKADKAPAEVRRLLKMLDPPNPTITITIAIRRESNFRKGLHMKQKQK--RVK
SIP4 238 GDFKADKAPAEVRRLLKMLDPPNPTITITIAIRRESNFRKGLHMKQKQK--RVK
SOS2 225 ADFKADKAPAEVRRLLKMLDPPNPTITITIAIRRESNFRKGLHMKQKQK--RVK

SIP1 285 NSVAGTATINENGAGPSGAGPSGDRVTESNHTDPTNNAFDLALSAFDFLAGI
SIP2 282 TEPDAEEN-----ASAEKKKRCINLNAFETISLSGDFDLSGL
SIP3 295 TTTSEVDFL-----VHSKSTETLNAFETISLSGDFDLSGL
SIP4 290 DEEDDQ-----KVSLSLAVKSLNAFETISLSGDFDLSGL
SOS2 285 NSVAGTATINENGAGPSGAGPSGDRVTESNHTDPTNNAFDLALSAFDFLAGI

SIP1 345 GDD---DKKRSRPSQDPASVITSKLEVAORPKISRRRERAGLKLRIKESRGTIT
SIP2 322 EKG---DEEDKRRPSQDPASVITSKLEVAORPKISRRRERAGLKLRIKESRGTIT
SIP3 333 EKKRDEKRRPSQDPASVITSKLEVAORPKISRRRERAGLKLRIKESRGTIT
SIP4 325 GAGCSNDSERPSQDPASVITSKLEVAORPKISRRRERAGLKLRIKESRGTIT
SOS2 328 EKKRDEKRRPSQDPASVITSKLEVAORPKISRRRERAGLKLRIKESRGTIT

SIP1 401 SMDAETPVDNPHVEVKKSGDGTLEQLVAEDLRPAISDIVVWQGEDELTSQOET
SIP2 374 VVPAEVEEAPSVHVMVKKSGDGTLEQLVAEDLRPAISDIVVWQGEDELTSQOET
SIP3 392 AVEAEIVVAPSVHVMVKKSGDGTLEQLVAEDLRPAISDIVVWQGEDELTSQOET
SIP4 382 GQICSRNDLLVVEAPSVHVMVKKSGDGTLEQLVAEDLRPAISDIVVWQGEDELTSQOET
SOS2 386 AVEAEIVVAPSVHVMVKKSGDGTLEQLVAEDLRPAISDIVVWQGEDELTSQOET

SIP1 461 EYQQQQQQEQEQEPLKTF
SOS2 445 TF

Fig. 2. Amino acid alignment of SOS2 with SIP1, SIP2, SIP3, and SIP4. Amino acids identical in SOS2 and at least another two proteins are highlighted in black and conservative substitutions in gray.

system, we tested whether the two proteins bind to each other *in vitro*. In a pull-down assay, [³H]leucine-labeled SOS3 protein was first synthesized by *in vitro* transcription and translation, and then was incubated with GST-SOS2, GST-RB, or GST that were bound to glutathione-Sepharose beads. The beads were pelleted and washed extensively, and the bound proteins were resolved by SDS/PAGE and were detected by fluorography. SOS3 was found to bind to GST-SOS2 but not to the control GST or GST-RB (Fig. 4A).

In addition, we conducted a gel blot overlay assay in which ³²P-labeled SOS3 was used to probe a Western blot containing GST-SOS2 and, as controls, GST, GST-RB, and protein size markers (Fig. 4B). Again, SOS3 was found to bind to GST-SOS2 but not to GST, GST-RB, or the molecular markers (Fig. 4B). To determine whether protein kinase activity is required for SOS2 interaction with SOS3, recombinant proteins of the two inactive SOS2 mutants, GST-SOS2(K40N) and GST-SOS2(G197E) (23), were tested in the overlay assay. SOS3 was able to bind to the inactive SOS2 mutants (Fig. 4B), demonstrating that protein kinase activity is not required for SOS2-SOS3 interaction. The binding between SOS3 and SOS2 *in vitro* does not seem to require Ca²⁺ because, when the pull-down assay was conducted in the absence of Ca²⁺ and in the presence of 5 mM EGTA, identical results were obtained (results not shown).

SOS3 Activates SOS2 Kinase Activity. We tested various proteins and peptides as substrates for SOS2 in kinase assays. SOS2 did not phosphorylate histone H1 or casein (results not shown). However, several synthetic peptides based on the recognition sequences of protein kinase C or SNF1/AMPK are strongly phosphorylated by SOS2 (Fig. 5A). SOS2 can phosphorylate either a serine or threonine residue because both the serine-containing (p1: LRRASLG and p3: ALARAASAAALARRR) and threonine-containing (p2: VRKRTLRL) peptides are recognized as substrates (Fig. 5A). Phosphorylation of the peptides by SOS2 depends on the presence of SOS3. Without SOS3, little phosphorylation of the peptides was detected (Fig. 5A).

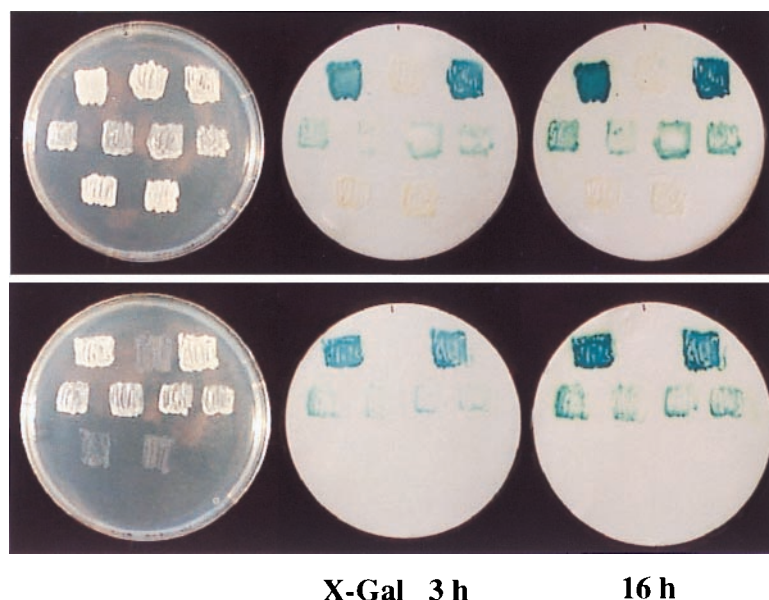
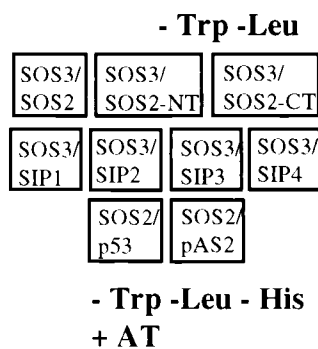


Fig. 3. Interaction between SOS3 and SOS2, SOS2 N-terminal (SOS2-NT) and C-terminal (SOS2-CT) domains, and SIP1, SIP2, SIP3, or SIP4 in the yeast two-hybrid assay. Yeast strains containing pAS-SOS3 and pACT-SOS2, pACT-SOS2-NT, pACT-SOS2-CT, pACT-SIP1-1, pACT-SIP2-1, pACT-SIP3-1, pACT-SIP4-1, respectively, were assayed for LacZ expression. pAS-p53 and pAS2 were used as negative controls in combination with pACT-SOS2. (Upper Right) Yeast grown on SC medium minus tryptophan and leucine to select for both the bait and prey proteins. (Lower Right) SC medium minus tryptophan, leucine, histidine, and plus 25 mM 3-amino-1,2,4-triazole (AT) to allow the growth of only positively interacting clones.

Table 1. Positions of N-terminal truncations of SIP1, SIP2, SIP3, and SIP4 kinase clones

Clone no.	1	2	3	4	5	6
SIP1	Δ276	Δ283	Δ288*			
SIP2	Δ4	Δ237	Δ251†			
SIP3	Δ223	Δ300‡				
SIP4	Δ86	Δ186§	Δ219	Δ221	Δ293	Δ300

Given are the number of amino acids missing from the N-terminal part of the protein.
 *Clones represented three times.
 †Clones represented three times.
 ‡Clones represented two times.
 §Clones represented four times.

SOS3 Activation of SOS2 Kinase Depends on Calcium. The predicted amino acid sequence of SOS3 suggested that it is a calcium-binding protein (10). ⁴⁵Ca²⁺-overlay assays confirmed that SOS3 is capable of binding Ca²⁺ (J.-K.Z., unpublished results). We tested whether SOS3 activation of SOS2 kinase depends on Ca²⁺. Ca²⁺-free conditions in the kinase assay were achieved by the presence of a large excess of EGTA (10 mM). Without free Ca²⁺, phosphorylation of p3 was substantially reduced (Fig. 5*B*), suggesting that Ca²⁺ is required for SOS3 activation of SOS2 kinase.

Discussion

We employed two different approaches to identify proteins that function in the SOS3 pathway for intracellular ion homeostasis. In one approach, SOS3 was used as a bait in the yeast two-hybrid

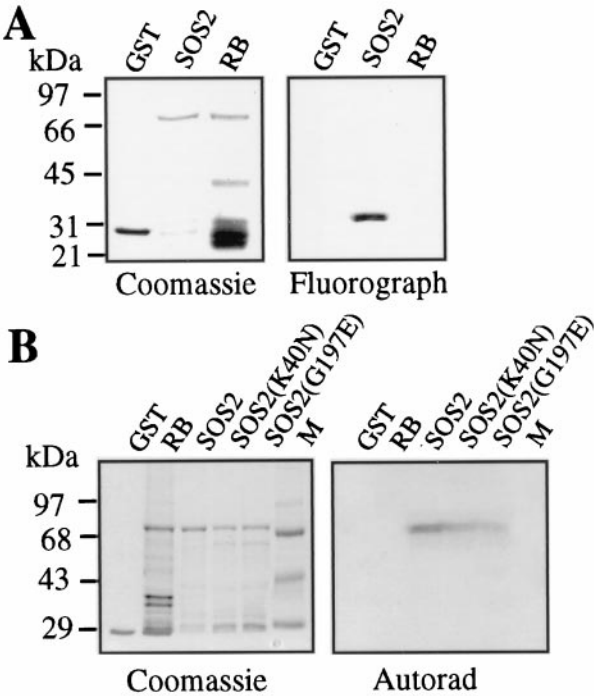


Fig. 4. SOS2 interacts with SOS3 *in vitro*. (A) Interaction between SOS3 and SOS2 in a pull-down assay. [³H]leucine-labeled SOS3 was incubated with glutathione-Sepharose immobilized GST, GST fusion proteins of SOS2, or RB. Proteins bound to the Sepharose beads were pelleted, washed thoroughly, electrophoresed, and detected by fluorography. (B) Interaction between SOS3 and SOS2 in a gel blot overlay assay. GST, partially purified GST-RB, GST-SOS2, GST-SOS2(K40N), GST-SOS2(G197E), and protein size markers (M) were separated by SDS/PAGE. Two identical gels were run; one was stained with Coomassie blue (Left), and the other was electroblotted and probed with [³²P]labeled SOS3 (Right).

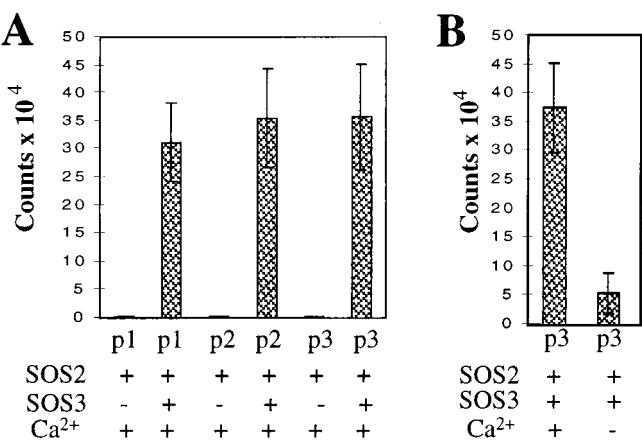


Fig. 5. SOS2 kinase activity is activated by SOS3 in a Ca²⁺-dependent manner. (A) SOS2 phosphorylation of peptide substrates is activated by SOS3. Oligopeptides p1, p2, and p3 were incubated in the presence of [^γ-³²P] ATP and Ca²⁺ in a kinase buffer with GST-SOS2, and with or without GST-SOS3. ^γ-³²P-incorporation was measured by scintillation counting. (B) SOS3 activation of SOS2 is Ca²⁺-dependent. Oligopeptide p3 was phosphorylated by GST-SOS2 in the presence of GST-SOS3, and with or without free Ca²⁺. Error bars represent the standard deviation (*n* = 3).

system to isolate interacting proteins (i.e., SIPs). In an alternative approach, we isolated the *SOS2* gene through positional cloning (23). The two approaches converged when we found that *SOS2* encodes a protein kinase highly similar to a group of SOS3-interacting proteins identified in the two-hybrid screen. *SOS2* interacts with SOS3 *in vitro* as well as in the yeast two-hybrid assay. *SOS2* was not identified in the original yeast two-hybrid screening because it did not appear to be represented in the prey library, which is likely attributable to its very low level of expression (23). The C-terminal regulatory domain of *SOS2* is responsible for interaction with SOS3, and the interaction does not depend on *SOS2* kinase activity or Ca²⁺. In the presence of Ca²⁺, SOS3 activates *SOS2* kinase activity. Double mutant analysis indicates that the *sos2* and *sos3* mutations are not additive. Together, these results strongly suggest that *SOS2* kinase acts as a downstream component in the SOS3 pathway leading to K⁺ and Na⁺ homeostasis and plant salt tolerance.

Many plant ion transporters have recently been cloned and characterized. Knowledge of the regulatory mechanisms of transporter abundance and activities in response to environmental, hormonal, and developmental signals is essential for understanding plant growth and development. SOS3 is the first regulatory protein

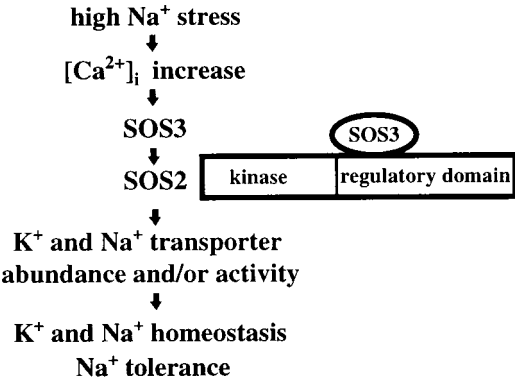


Fig. 6. Proposed regulatory pathway for intracellular Na⁺ and K⁺ homeostasis and Na⁺ tolerance in plants.

known to control intracellular K^+ and Na^+ homeostasis and salt tolerance in plants. It is a myristoylated (J.-K.Z., unpublished data) calcium-binding protein with sequence similarities to CNB and animal neuronal calcium sensors. Because of the sequence and functional similarities of SOS3 with yeast CNB, a calcineurin-like pathway had been anticipated to mediate plant salt tolerance (6, 10). However, as yet, no protein phosphatase has been found to interact with SOS3. Instead, SOS3 was found to interact with and activate a protein kinase encoded by SOS2. The strong and stable binding between SOS2 and SOS3 suggests the interesting possibility that the two proteins form a novel protein kinase complex. Such a complex would be reminiscent of multisubunit protein kinases such as calmodulin-dependent kinases that consist of calmodulin as a regulatory subunit plus a kinase catalytic subunit, although very little sequence similarity exists between components of the two types of kinase complexes. The SOS3/SOS2 kinase complex is also different from calmodulin-dependent kinases in that the binding between calmodulin and the kinase is calcium-dependent. The phenotypes of the *sos* mutants suggest that the SOS3/SOS2 kinase complex functions specifically in the regulation of intracellular Na^+ and K^+ homeostasis and salt tolerance.

It is unlikely that SOS3 also activates a calcineurin A (CNA)-like protein phosphatase to mediate plant salt tolerance. Despite pharmacological evidence supporting the existence of calcineurin in plants (11, 12), no genes similar to *CNA* have been identified from plants, yet. Our results imply that plant salt tolerance may be mediated not by a calcineurin-like protein phosphatase but, rather, by the SOS3/SOS2 kinase complex. Nevertheless, the possible existence and involvement of calcineurin-like phosphatases in other plant processes cannot be ruled out. Recently, another CNB-like protein from *Arabidopsis* was characterized (13). Its induction by cold, drought, and wounding stresses suggested that it does not function like yeast CNB or *Arabidopsis* SOS3 in regulating intracellular Na^+ and K^+ homeostasis, although it was able to mediate Ca^{2+} signaling through the rat calcineurin A phosphatase when both were expressed in yeast. Previous pharmacological studies (11, 12) indicated the existence of calcineurin in guard cell regulation but did not address a role in salt tolerance. Pardo *et al.* (9) observed improved salt tolerance caused by ectopic expression of a constitutively active yeast calcineurin in tobacco plants. Because the mode of action of the yeast protein phosphatase in plant cells is not known, it may function by a mechanism other than ion homeostasis.

Although SOS3 also shares sequence similarity with animal neuronal calcium sensors (10), they too appear to function differently. Neuronal calcium sensor 1 can replace calmodulin in the activation of calcineurin (29). Another member of the neuronal calcium sensor superfamily, recoverin, functions by inhibiting rhodopsin kinase (30, 31).

The physical interaction between SOS3 and SOS2 is consistent with genetic evidence that suggests that the *SOS* genes function in the same pathway (Fig. 1). We propose that, in response to a cytosolic Ca^{2+} signal generated by Na^+ stress (32), SOS3 activates SOS2 kinase, which then phosphorylates downstream effectors of salt tolerance (Fig. 6). SOS3 activation, rather than inhibition, of SOS2 kinase (Fig. 5) is consistent with the observation that both SOS3 and SOS2 are positive regulators of salt tolerance (10, 17) and with the indication from analyses of the *sos2-5* mutant allele that an active SOS2 kinase is required for its function as a positive regulator (23). The physiological substrates of the SOS2 kinase are not yet known but may include certain Na^+ and K^+ transporters (4, 33–36) and transcriptional factor(s) that mediate the expression of these transporters under salt stress.

The physiological significance of the interaction between SOS3 and other SIP kinases observed in the yeast two-hybrid system is still unclear. The interaction between SOS3 and the other SIP kinases is much weaker compared with the interaction with SOS2 (Fig. 2). In addition, possible differences in the temporal and spatial expression between SOS3 and the other SIP kinases may preclude their interaction *in planta*. The natural interaction partners of some of the other SIP kinases might be SOS3-like calcium-binding proteins (13). We propose that SOS3-like calcium-binding proteins each interact with certain members of the SIP family of protein kinases to form specific kinase complexes that may perform a broad range of functions in mediating Ca^{2+} signaling and the regulation of ion transport or other processes in various plant cells. For example, SOS3 and SOS2-like proteins (i.e., SIPs) expressed in guard cells may interact to mediate Ca^{2+} signaling in stomatal regulation.

We sincerely thank R. T. Leonard, R. A. Bressan, H. Bohnert, and J. Lasswell for critical reading of the manuscript; B. A. Larkins for the gift of GST-RB1 and pAS-RB1; and B. Dilkes, R. Dante, and P. Hughes for helpful discussions. This work was supported by National Institutes of Health Grant R01GM59138 and U.S. Department of Agriculture/National Research Initiative Grant 9801270.

- Stein, W. D. (1990) *Channels, Carriers and Pumps: An Introduction to Membrane Transport* (Academic, New York).
- Apse, M. P., Aharon, G. S., Snedden, W. A. & Blumwald, E. (1999) *Science* **285**, 1256–1258.
- Niu, X., Bressan, R. A., Hasegawa, P. M. & Pardo, P. M. (1995) *Plant Physiol.* **109**, 735–742.
- Rubio, F., Gassmann, W. & Schroeder, J. I. (1995) *Science* **270**, 1660–1663.
- Epstein, E., Norlyn, J. D., Rush, D. W., Kingsbury, R. W., Kelley, D. B., Cunningham, G. A. & Wrona, A. F. (1980) *Science* **210**, 399–404.
- Bressan, R. A., Pardo, J. M. & Hasegawa, P. M. (1998) *Trends Plant Sci.* **3**, 411–412.
- Serrano, R. & Gaxiola, R. (1994) *Crit. Rev. Plant Sci.* **13**, 121–138.
- Mulet, J. M., Leube, M. P., Kron, S. J., Rios, G., Fink, G. R. & Serrano, R. (1999) *Mol. Cell. Biol.* **19**, 3328–3337.
- Pardo, J. M., Reddy, M. P., Yang, S., Maggio, A., Huh, G.-H., Matsumoto, T., Coca, M. A., Pardo-D'Urzo, M., Koiwa, H., Uan, D.-J., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 9681–9686.
- Liu, J. & Zhu, J.-K. (1998) *Science* **280**, 1943–1945.
- Luan, S., Li, W., Rusnak, F., Assmann, S. M. & Schreiber, S. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2202–2206.
- Allen, G. J. & Sanders, D. (1995) *Plant Cell* **7**, 1473–1483.
- Kudla, J., Xu, Q., Harter, K., Gruissem, W. & Luan, S. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 4718–4723.
- Nakamura, T. Y., Liu, Y., Hirata, D., Namba, H., Harada, S., Hirokawa, T. & Miyakawa, T. (1993) *EMBO J.* **12**, 4063–4071.
- Mendoza, I., Rubio, F., Rodriguez-Navarro, A. & Pardo, J. M. (1994) *J. Biol. Chem.* **269**, 8792–8796.
- Liu, J. & Zhu, J.-K. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 14960–14964.
- Zhu, J.-K., Liu, J. & Xiong, L. (1998) *Plant Cell* **10**, 1181–1192.
- Wu, S.-J., Ding, L. & Zhu, J.-K. (1996) *Plant Cell* **8**, 617–627.
- Kim, J., Harter, K. & Theologis, A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 11786–11791.
- Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H. & Elledge, S. J. (1993) *Genes Dev.* **7**, 555–569.
- Bai, C. & Elledge, S. J. (1996) *Methods Enzymol.* **273**, 331–347.
- Graf, G., Burnett, R. J., Helentjaris, T., Larkins, B. A., DeCaprio, J. A., Sellers, W. R. & Kaelin, W. G., Jr. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 8962–8967.
- Liu, J., Ishitani, M., Halfter, U., Kim, C.-S. & Zhu, J.-K. (March 21, 2000) *Proc. Natl. Acad. Sci. USA*. 10.1073/pnas.060034197. <http://www.pnas.org/cgi/doi/10.1073/pnas.060034197>
- Davies, S. P., Carling, D. & Hardie, D. G. (1989) *Eur. J. Biochem.* **186**, 123–128.
- Kaelin, W. G., Jr., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J. & Blumberg, P. M. (1992) *Cell* **70**, 351–364.
- Mizoguchi, T., Hayashida, N., Yamaguchi-Shinikawa, K., Kamada, H. & Shinosaki, K. (1994) *Plant Physiol.* **106**, 1229–1230.
- Celenza, J. L. & Carlson, M. (1986) *Science* **233**, 1175–1180.
- Mitchell, K. L., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L. A. & Kemp, B. E. (1994) *J. Biol. Chem.* **269**, 2361–2364.
- Schaad, N. K., De Castro, E., Nef, S., Hegi, S., Hinrichsen, R., Martone, M. E., Ellisman, M. H., Sikkink, R., Rusnak, F., Sygusch, J. & Nef, P. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 9253–9258.
- Klenchin, V. A., Calvert, P. D. & Bownds, M. D. (1995) *J. Biol. Chem.* **270**, 16147–16152.
- Chen, C. K., Inglese, J., Lefkowitz, R. J. & Hurley, J. B. (1995) *J. Biol. Chem.* **270**, 18060–18066.
- Lynch, J., Polito, V. S. & Lauchli, A. (1989) *Plant Physiol.* **90**, 1271–1274.
- Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J. & Gaber, R. F. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 3736–3740.
- Hirsch, R. E., Lewis, B. D., Spadling, E. P. & Sussman, M. R. (1998) *Science* **280**, 918–921.
- Kim, E. J., Kwak, J. M., Uozumi, N. & Schroeder, J. I. (1998) *Plant Cell* **10**, 51–62.
- Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L. & Fink, G. R. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 1480–1485.