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An Autophosphorylation Site of the Protein Kinase SOS2 Is Important for Salt Tolerance in *Arabidopsis*

Hiroaki Fujii and **Jian-Kang Zhu**[1]

Department of Botany and Plant Sciences, 2150 Batchelor Hall, University of California, Riverside, CA 92521, USA

ABSTRACT The protein kinase SOS2 (Salt Overly Sensitive 2) is essential for salt-stress signaling and tolerance in *Arabidopsis*. SOS2 is known to be activated by calcium-SOS3 and by phosphorylation at its activation loop. SOS2 is autophosphorylated *in vitro*, but the autophosphorylation site and its role in salt tolerance are not known. In this study, we identified an autophosphorylation site in SOS2 and analyzed its role in the responses of *Arabidopsis* to salt stress. Mass spectrometry analysis showed that Ser 228 of SOS2 is autophosphorylated. When this site was mutated to Ala, the autophosphorylation rate of SOS2 decreased. The substrate phosphorylation by the mutated SOS2 was also less than that by the wild-type SOS2. In contrast, changing Ser228 to Asp to mimic the autophosphorylation enhanced substrate phosphorylation by SOS2. Complementation tests in a *sos2* mutant showed that the S228A but not the S228D mutation partially disrupted the function of SOS2 in salt tolerance. We also show that activation loop phosphorylation at Thr168 and autophosphorylation at Ser228 cannot substitute for each other, suggesting that both are required for salt tolerance. Our results indicate that Ser 228 of SOS2 is autophosphorylated and that this autophosphorylation is important for SOS2 function under salt stress.

Key words: SOS2; autophosphorylation; salt tolerance; protein kinase; *Arabidopsis*.

INTRODUCTION

Because soil salinity is a serious environmental stress limiting plant productivity, comprehension of cell signaling pathways under salt stress is important. In *Arabidopsis thaliana*, the SOS (Salt Overly Sensitive) pathway is a major signaling pathway required for salt stress tolerance. The SOS pathway consists of three proteins, SOS1, SOS2, and SOS3. SOS1 is a plasma membrane Na^+/H^+ antiporter (Shi et al., 2000). SOS2 is a member of the SNF1-related protein kinase 3 (SnRK3) family (Liu et al., 2000; Hrabak et al., 2003). SOS3 is a myristoylated calcium-binding protein (Liu and Zhu, 1998; Ishitani et al., 2000). SOS3 forms a complex with SOS2, activates the substrate phosphorylation activity of SOS2 in a calcium-dependent manner (Halfter et al., 2000; Liu et al., 2000), and also recruits SOS2 to the plasma membrane. The SOS3–SOS2 protein kinase complex phosphorylates SOS1 to stimulate its Na^+/H^+ antiport activity (Qiu et al., 2002; Quintero et al., 2002). Thus, the SOS pathway regulates Na^+ homeostasis under salt stress, and SOS2 is one of the pivotal kinases under salt stress. Loss-of-function mutations in SOS1, SOS2, or SOS3 cause hypersensitivity to NaCl (Zhu et al., 1998). On the other hand, overexpression of *SOS1* (Shi et al., 2003) or ectopic expression of an activated form of SOS2 (Guo et al., 2004) increases salt tolerance in transgenic plants.

Because SOS2 protein is not constitutively active in substrate phosphorylation *in vitro* (Gong et al., 2002), its activation is a key signaling event under salt stress. In SOS2, the catalytic domain is located in the N-terminal region, and the FISL motif is located in the C-terminal regulatory region and serves as an autoinhibitory domain. Removing the FISL motif or the entire C-terminal regulatory region makes SOS2 constitutively active (Guo et al., 2001; Qiu et al., 2002). The FISL motif also interacts with SOS3 (Halfter et al., 2000; Liu et al., 2000), and evidence suggests that the binding of Ca–SOS3 relieves the autoinhibitory function of the FISL motif. Recently, another binding partner of SOS2 has been reported (Quan et al., 2007). SOS3-like

[1] To whom correspondence should be addressed. E-mail jian-kang.zhu@ucr.edu, fax 951-827-7115, tel. 951-827-7117.

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calcium-binding protein 8 (SCaBP8, also referred to as Calcineurin B-like10) also interacts with and regulates SOS2 through the FISL motif. SOS3 functions mainly in the root whereas SCaBP8 functions mainly in the shoot (Quan et al., 2007).

Another activation mechanism of SOS2 is phosphorylation in the activation loop. The activation loop is a highly conserved region among various kinases, and many protein kinases are activated by phosphorylation of one or more residues within their activation loop (Hanks and Hunter, 1995; Johnson et al., 1996). In the SnRK3 subfamily, the activation loop is located between the conserved DFG and APE residues in the kinase catalytic domain (Guo et al., 2001). Ser 156, Thr 168, and Tyr 175 residues are completely conserved among all members of this subfamily. When one of these residues was changed to Asp to mimic phosphorylation, the mutant proteins were constitutively active *in vitro* (Gong et al., 2002). The activation loop mutation and the autoinhibitory domain deletions have a synergistic effect on the kinase activity of SOS2 (Guo et al., 2001; Qiu et al., 2002). Furthermore, transgenic plants expressing the T168D (Thr168 is mutated to Asp) form of SOS2 in both wild-type and *sos3* background had improved salt tolerance (Guo et al., 2004).

Besides the mechanisms described above (interaction with SOS3 and phosphorylation in the activation loop), autophosphorylation might regulate SOS2 activity because the GST (glutathione S transferase)-fused constitutively active form of SOS2 shows intramolecular autophosphorylation *in vitro* (Gong et al., 2002). Several kinases have been reported to regulate its function through intramolecular autophosphorylation (Schulze-Muth et al., 1996; Yang et al., 2005; Jansen et al., 2006). In this study, we analyzed the autophosphorylation of SOS2. We identified an autophosphorylation site and examined the importance of this site using transgenic plants expressing SOS2 with mutations at the site.

RESULTS

Ser228 Is Autophosphorylated *In Vitro*

A constitutively active form of SOS2 (SOS2CA), in which Thr168 is changed to Asp and the C-terminal 139 amino acids are deleted, shows strong autophosphorylation activity *in vitro* (Gong et al., 2002). To determine the autophosphorylation site(s), phosphorylated GST-fused SOS2CA was analyzed by mass spectrometry. Recombinant GST–SOS2CA was purified from *E. coli* and phosphorylated by incubating with non-radioactive ATP. After separation with SDS–PAGE, GST–SOS2CA was digested in-gel with trypsin and analyzed by liquid chromatography and tandem mass spectrometry (LC/MS/MS). A phosphorylated peptide, INAAEF pS CPPWFSAEVK, corresponding to residues 222–238 of SOS2, was found (Figure 1).

After the mutation Ser228 to Ala (SOS2CA S228A) or Ser228 to Asp (SOS2CA S228D) was introduced to the constitutively active SOS2 (SOS2CA), autophosphorylation of the mutated proteins was examined. GST-fused proteins purified from *E.*

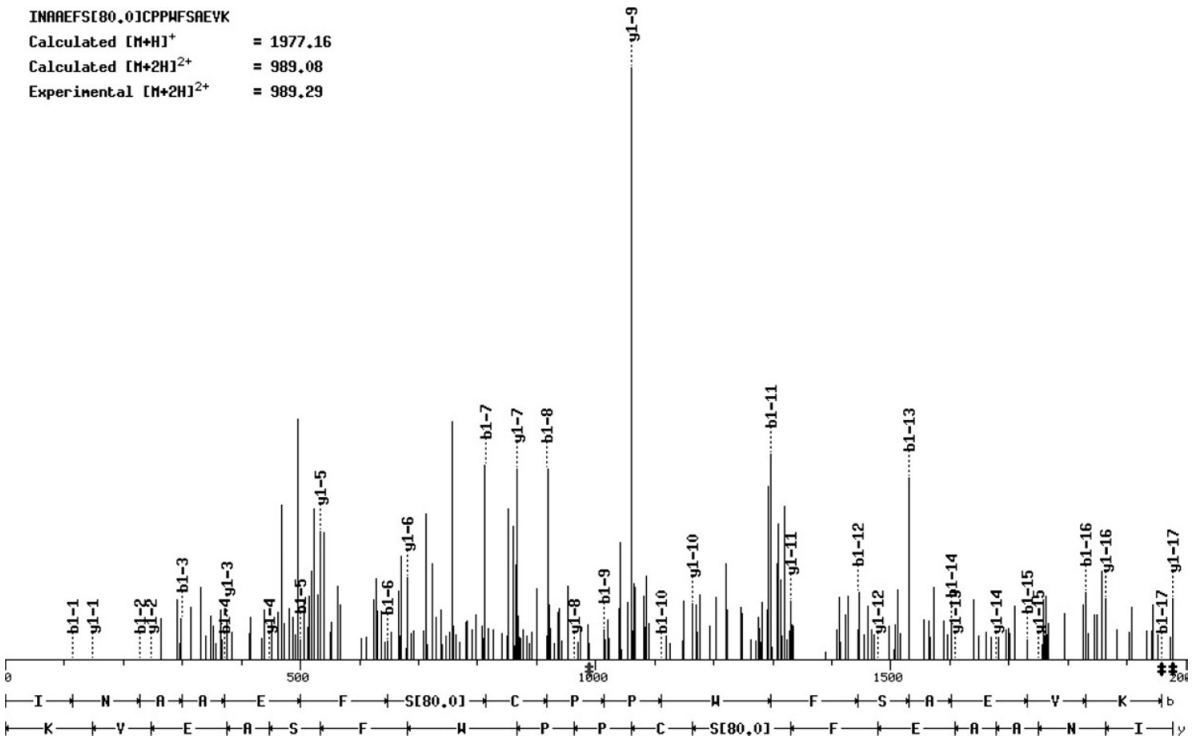

Figure 1. The Presence of a Phosphate Group on Ser228 in MS/MS Peptide Sequence Data. The N- and C-terminal peptide fragment ions are indicated as b-ion and y-ion series, respectively.