A Calcium-Independent Activation of the Arabidopsis SOS2-Like Protein Kinase24 by Its Interacting SOS3-Like Calcium Binding Protein1

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The salt stress-induced SALT-OVERLY-SENSITIVE (SOS) pathway in Arabidopsis (Arabidopsis thaliana) involves the perception of a calcium signal by the SOS3 and SOS3-like CALCIUM-BINDING PROTEIN8 (SCaBP8) calcium sensors, which then interact with and activate the SOS2 protein kinase, forming a complex at the plasma membrane that activates the SOS1 Na+/H+ exchanger. It has recently been reported that phosphorylation of SCaBP proteins by SOS2-like protein kinases (PKSs) stabilizes the interaction between the two proteins as part of a regulatory mechanism that was thought to be common to all SCaBP and PKS proteins. Here, we report the calcium-independent activation of PKS24 by SCaBP1 and show that activation is dependent on interaction of PKS24 with the C-terminal tail of SCaBP1. However, unlike what has been found for other PKS-SCaBP pairs, multiple amino acids in SCaBP1 are phosphorylated by PKS24, and this phosphorylation is dependent on the interaction of the proteins through the PKS24 FISL motif and on the efficient activation of PKS24 by the C-terminal tail of SCaBP1. In addition, we show that Thr-211 and Thr-212, which are not common phosphorylation sites in the conserved PFPF motif found in most SCaBP proteins, are important for this activation. Finally, we also found that SCaBP1-regulated PKS24 kinase activity is important for inactivating the Arabidopsis plasma membrane proton-translocating adenosine triphosphatase. Together, these results suggest the existence of a novel SCaBP-PKS regulatory mechanism in plants.

Calcium is a ubiquitous second messenger that plays an important role in the regulation of plant growth and development. Many different types of calcium-binding proteins have been identified in plants (Harper et al., 2004), including the SALT-OVERLY-SENSITIVE (SOS3)-LIKE CALCIUM BINDING PROTEINS (SCaBPs; Liu and Zhu, 1998; Gong et al., 2004). Because the calcium-binding domain of these proteins shares sequence similarity with the yeast calcineurin B subunit, they have also been called CALCINEURIN B-LIKE PROTEINS (CBLs; Kudla et al., 1999; Luan et al., 2002). The founding member of this gene family, SOS3, was identified in a genetic screen from a salt-sensitive Arabidopsis (Arabidopsis thaliana) mutant (Liu and Zhu, 1998). SCaBP/CBL proteins interact with the SOS2-LIKE PROTEIN KINASES (PKSs)/CBL-INTERACTING PROTEIN KINASES (CIPKs; Shi et al., 1999; Halfter et al., 2000; Guo et al., 2001). The genetic linkage between these two families was established after identification of SOS2 from a genetic screen similar to the one that identified the sos3 mutant (Liu et al., 2000). SOS3 interacts with SOS2 in vivo and in vitro and activates SOS2 in a calcium-dependent manner in vitro (Halfter et al., 2000). The SOS3-SOS2 complex further activates SOS1, a plasma membrane (PM) Na+/H+ antiporter, by directly phosphorylating the SOS1 C terminus (Shi et al., 2000; Qiu et al., 2002; Quintero et al., 2002, 2011; Yu et al., 2010).

In addition to the calcium-dependent activation of PKSs by SCaBP calcium sensors, two other regulatory mechanisms have been identified for these protein families. First, PKSs have a conserved 21-amino acid peptide (FISL motif) in their regulatory domain that is necessary for efficient interaction with the SCaBP calcium sensors (Guo et al., 2001; Albrecht et al., 2001; Gong et al., 2004). The PKS regulatory domain interacts with its kinase domain via the FISL motif to repress PKS activity; interaction of SCaBP with the PKS FISL motif releases the kinase domain inhibition allowing for kinase activity (Guo et al., 2001; Gong et al., 2004). Second, the PKSs phosphorylate a Ser residue in the conserved C-terminal PFPF motif of the SCaBP proteins. This phosphorylation enhances the interaction between the two proteins and...
fully activates the complex (Lin et al., 2009; Du et al., 2011; Hashimoto et al., 2012).

In this study, we identified a novel PKS activation mechanism involving the calcium-independent activation of PKS24 by SCaBP1 and show that it requires binding of SCaBP1 to the FISL motif of PKS24 and the involvement of two Thr residues in the SCaBP1 C-terminal tail.

RESULTS
The Activation of PKS24 by SCaBP1 Is Calcium Independent

We previously showed that phosphorylation of SCaBP1 by PKS24 increases the interaction between the two proteins (Du et al., 2011). While testing the effect of SCaBP1 on PKS24 activity, we found that compared with what was seen for other SCaBP-PKS pairs (Shi et al., 1999; Halfter et al., 2000; Guo et al., 2001; Quan et al., 2007), activation of PKS24 by SCaBP1 (autophosphorylation) increased with increasing amounts of PKS24 protein and was independent of calcium (Fig. 1). Low concentrations of PKS24 (6.67 ng/μl) displayed much higher kinase activity than high concentrations (133.3 ng/μl) in the absence of SCaBP1, and there was a dramatic increase in kinase activity detected with increasing concentrations of PKS24 (Fig. 1A). Consistent with previous results, PKS24 phosphorylated SCaBP1. These results indicate that SCaBP1 plays a critical role in regulating PKS24 activity.

To determine whether calcium is involved in this regulation, we first assayed whether calcium has a direct effect on SCaBP1 phosphorylation. We found that calcium had no direct effect on SCaBP1 phosphorylation (Fig. 1B). However, when calcium was added to the reaction buffer without calcium ions, SCaBP1 was phosphorylated, indicating that calcium is not required for SCaBP1 phosphorylation. These results suggest that calcium is not involved in the regulation of PKS24 activation by SCaBP1.
effect on PKS24 activity using increasing concentrations of calcium (from 0 to 20 mM) and the calcium chelator EGTA. No difference in PKS24 autophosphorylation or SCaBP1 phosphorylation was seen in the presence of EGTA or at concentrations of calcium ranging from 0.25 to 5 mM (Fig. 1, B and C). Similar to what has been shown for kinase assays involving SOS2 (Guo et al., 2001), decreased levels of these PKS24 activities were observed at concentrations of calcium above 10 mM (Fig. 1, B and C). These results indicate that the activation of PKS24 by SCaBP1 is calcium independent in vitro. Maximum PKS24 autophosphorylation was observed when 10 mM Mg2+ was included in the assay; in comparison, only 0.5 mM Mn2+ was required to produce the maximum level of PKS24 activity, indicating a preference for Mn2+ over Mg2+ (Supplemental Fig. S1).

To further characterize the interaction between SCaBP1 and the kinase activity of PKS24, a fixed amount of PKS24 (1 μg) was incubated with increasing concentrations of SCaBP1 (Fig. 2A) and the ability of PKS24 to transphosphorylate SCaBP1 and myelin basic protein (MBP, 200 ng) was monitored (Fig. 2B). The results show that increasing SCaBP1 increased both the autophosphorylation and transphosphorylation activities of PKS24. We then investigated the stoichiometry of MBP phosphorylation by PKS24 in the absence or presence of SCaBP1. In the presence of 2.5 μM MBP and 10 μM ATP, MBP phosphorylation by PKS24 (0.7 μM) reached saturation within 15 min with or without 2.0 μM SCaBP1. Maximum phosphate incorporation was 0.15 ± 0.02 mol/mol of MBP without SCaBP1 but increased dramatically to 1.2 ± 0.2 mol/mol with addition of SCaBP1 (Supplemental Fig. S2).

The FISL Motif Does Not Inhibit PKS24 Activity

SOS2 exhibits weak autophosphorylation and transphosphorylation activity when p3 peptide is used as the substrate (Halfter et al., 2000; Quan et al., 2007). Active SOS2 can be created by changing the Thr at position 168 (T168) in the kinase activation loop to Asp (D) or by deleting the FISL motif in the regulatory domain and both of these features (a Thr in the kinase activation loop and the FISL motif) are conserved throughout the PKS family (Guo et al., 2001). To determine whether the conserved Thr and FISL motif in PKS24 affect its kinase activity, we generated three PKS24 mutants: one with the T178/D substitution (PKS24T/D), one with the FISL motif deleted (PKS24DF), and one with both mutations (PKS24T/DDF). The three mutant proteins and wild-type PKS24 were then used in in vitro kinase assays. In contrast with the results for SOS2, deleting the FISL motif did not alter the autophosphorylation or transphosphorylation activity of PKS24 when MBP was used as the substrate; however, the T178/D mutation strongly increased PKS24 activity and the kinase activity of PKS24T/DDF was similar to that of the T178/D mutant (Fig. 3A). These results suggest that the FISL motif in PKS24 does not serve as a kinase-inhibitory domain as it does in SOS2 (Guo et al., 2001).

Figure 2. PKS24 phosphorylates SCaBP1 and SCaBP1 activates PKS24. A, Phosphorylation of SCaBP1 by PKS24. The activity of PKS24 was enhanced as the concentration of SCaBP1 was increased. Coomassie Blue-stained polyacrylamide gel (top); PKS24 autophosphorylation and SCaBP1 transphosphorylation activity (bottom). B, Phosphorylation of SCaBP1 and MBP by PKS24. Coomassie Blue-stained polyacrylamide gel (top); PKS24 autophosphorylation and SCaBP1/MBP transphosphorylation activity (bottom). The concentration of SCaBP1 in lanes 1 to 16 were as follows (in ng/μl): 0, 0.67, 3.33, 6.67, 13.4, 33.3, 66.7, 133, 266.7, 0, 6.67, 13.4, 33.3, 66.7, 133, and 266.7. PKS24 and SCaBP1 were purified as GST-fusion proteins; the GST tag was removed from SCaBP1 by PreScission protease. SC1, SCaBP1.
SOS3 and SCaBP8 interact with the SOS2 FISL motif in a SOS2 kinase activity- and calcium-independent manner (Halfter et al., 2000; Guo et al., 2001; Quan et al., 2007). To test whether the phosphorylation of SCaBP1 by PKS24 requires an interaction between the two proteins at a site in addition to the active site, assays were performed to determine whether SCaBP1 interacts with PKS24 and PKS24T/D in the presence or absence of calcium and ATP. Neither PKS24DF nor PKS24T/DDF was able to interact with SCaBP1 (Fig. 3B), indicating that the interaction between SCaBP1 and PKS24 requires the FISL motif but is independent of calcium and PKS24 activity.

The Activation of PKS24 by SCaBP1 Is Interaction Dependent

To test whether the phosphorylation of SCaBP1 by PKS24 requires an interaction between the two proteins,
assays (Fig. 5). SCaBP1 and SCaBP6 but not SCaBP8 were phosphorylated by PKS24 and activated PKS24 (Fig. 5). In vitro pull-down assays demonstrated that GST-PKS24 pulled down SCaBP1 and SCaBP6, but not SCaBP8 (Supplemental Fig. S3). Both SOS2 and PKS5 phosphorylated SCaBP1, SCaBP6, and SCaBP8. However, their autophosphorylation activities were not enhanced by the phosphorylated SCaBP proteins (Fig. 5). These results suggest that the activation of PKS24 by SCaBP1 and SCaBP6 is a specific regulatory process in the SCaBP-PKS pathway.

**Myc-SCaBP1 Activates Flag-PKS24 in Planta**

To determine the activation of PKS24 by SCaBP1 in vivo, Myc-PKS24, Myc-PKS24DF, and Flag-SCaBP1 were transformed into wild-type plants. Transgenic plants containing Myc-PKS24 and Flag-SCaBP1 or Myc-PKS24DF and Flag-SCaBP1 were generated by crossing transgenic plants harboring single transgenes. Twelve-d-old seedlings were harvested and the proteins were extracted. Anti-Myc beads were used to immunoprecipitate Myc-PKS24 or Myc-PKS24DF (Fig. 6A) and MBP was used as the substrate for the kinase assays (Fig. 6B). The results show that PKS24 from transgenic plants harboring Flag-SCaBP1 had the highest activity (Fig. 6C). MBP was very weakly phosphorylated by PKS24DF, whether the transgenic plants contained Flag-SCaBP1 or not (Fig. 6C). However, the phosphorylation of MBP by Myc-PKS24 from transgenic plants that did not contain Flag-SCaBP1 was stronger than phosphorylation by PKS24DF (Fig. 6C). These results suggest that endogenous SCaBP1 in the wild type plays a role in activating the activity of PKS24. Consistent with this, PKS24DF, which does not interact with SCaBP1, was not activated by either endogenous or overexpressed SCaBP1.

We previously showed that PKS24 phosphorylates Ser-216 of SCaBP1 (Du et al., 2011). Polyclonal phosphospecific antibodies (anti-S216P) detected this phosphorylation in vitro (Supplemental Fig. S4). Anti-S216P was used to detect the phosphorylation status of FLAG-SCaBP1 immunoprecipitated with anti-FLAG from the transgenic plants described above. The presence of Myc-PKS24 or Myc-PKS24DF in these immunoprecipitates was detected with anti-MYC (Fig. 6A). Flag-SCaBP1 was only detected in the Myc-PKS24 but not Myc-PKS24DF coimmunoprecipitated products (Fig. 6D). A stronger signal was detected with anti-S216P in transgenic plants harboring both Myc-PKS24 and Flag-SCaBP1 (Fig. 6E). A weaker signal was also detected in plants expressing only Myc-PKS24, suggesting that endogenous SCaBP1 was pulled down and phosphorylated by PKS24 (Fig. 6E); however, no signal was detected in the PKS24DF coimmunoprecipitated products.
products (Fig. 6E). These results suggest that SCaBP1 is phosphorylated by PKS24 in vivo, and that phosphorylation is dependent on the interaction of SCaBP1 with the FISL domain of PKS24.

Thr-211 and Thr-212 in the C Terminus of SCaBP1 Are Important for Activation of PKS24

Previous results have demonstrated that PKS24 phosphorylates SCaBP1 at Ser-216 and that this phosphorylation enhances the interaction between the two proteins (Du et al., 2011). Interaction between PKS24 and SCaBP1 is also required for the activation of PKS24 (Fig. 4). To determine the region of SCaBP1 that is required for the activation of PKS24, we made two additional SCaBP1 mutant proteins by removing the last 16 amino acids (from 211 to 226, SCaBP1N210) or the first 30 amino acids from SCaBP1 (SCaBP1ND30) and used GST-fused SCaBP1, SCaBP1S216A, SCaBP1N210, and SCaBP1ND30 in kinase assays (Fig. 7A). Compared with wild-type SCaBP1, SCaBP1N210 was only weakly phosphorylated by PKS24 and did not activate PKS24. SCaBP1ND30 activated the autophosphorylation activity of PKS24 to a level similar to that of SCaBP1, but the level of SCaBP1 phosphorylation by PKS24 was dramatically reduced. SCaBP1S216A was phosphorylated by PKS24 and activated PKS24 at a level similar to that of wild-type SCaBP1 (Fig. 7A). These results indicate that the C terminus of SCaBP1 is required for the activation of PKS24 and multiple sites in the N terminus of SCaBP1 are phosphorylated by PKS24. Because Ser-216 is one of multiple phosphorylation sites in SCaBP1, additional reductions in the presence of the Ser-216 mutation were not seen.

Interaction between PKS24 and SCaBP1 is essential for both activation of PKS24 and phosphorylation of SCaBP1 (Fig. 4). We tested whether SCaBP1N210 interacts with PKS24 and PKS24DF. The results showing that SCaBP1N210 was pulled down by PKS24 but not by PKS24DF (Supplemental Fig. S5) suggest that although the C terminus of SCaBP1 is not needed for binding to PKS24 via the FISL domain, it possesses the ability to activate PKS24. Because the phosphorylation of SCaBP1 is often coupled with the activation of PKS24, we then tested three putative phosphorylation sites within the last 16 amino acids of SCaBP1 (T211, T212, and T213) to determine whether they are important for activation of PKS24. The three Thr residues were replaced with Ala and the mutant proteins were used in kinase assays. SCaBP1T211A did not activate PKS24 and showed little evidence of phosphorylation by PKS24, whereas SCaBP1T212A activated PKS24 and was weakly phosphorylated by it. The SCaBP1T211AT212A double mutant, like SCaBP1T211A, did not activate PKS24. In comparison, SCaBP1T213A, like wild-type SCaBP1, activated PKS24 and was phosphorylated by it (Fig. 7B). These results demonstrate that both SCaBP1T211 and SCaBP1T212 are important for the activation of PKS24. In vitro pull-down assays provided evidence that these three point mutations did not change the ability of the proteins to bind to PKS24 (Supplemental Fig. S6).

PKS24 Regulates PM Proton-Translocating Adenosine Triphosphatase Activity

To analyze the biological function of PKS24, we obtained two transfer DNA insertion lines for PKS24 (SALK_147899 and SALK_009699, referred to as pks24-1 and pks24-2, respectively) and confirmed their status as gene knockouts (Supplemental Fig. S7). The phenotype of each mutant was monitored after exposure to Glc, abiotic stresses, and plant hormones including abscisic acid, ethylene, and auxin (data not shown). No significant differences were observed between wild-type and mutant plants with any of these treatments. PKS5 negatively regulates the PM proton-translocating adenosine triphosphatase activity (Fuglsang et al., 2007) and PKS24 shares the highest sequence similarity with PKS5 among the 25 PKS family members. SCaBP1
has been shown to activate PKS5 when expressed in yeast (*Saccharomyces cerevisiae*; Fuglsang et al., 2007) and to activate PKS24 in Arabidopsis (Fig. 6), suggesting that PKS24 may also play a role in regulating PM H⁺-ATPase activity. It has been shown that addition of activated SOS2 kinase (T/DSOS2DF) can directly stimulate the activities of the PM and tonoplast Na⁺/H⁺ antiporters (Qiu et al., 2002, 2004; Guo et al., 2004). To determine whether PKS24 plays a role in the regulation of PM H⁺-ATPase activity, we purified PM vesicles from wild-type plants and measured the H⁺-transport activity of the H⁺-ATPase in the presence of different combinations of PKS24 and SCaBP1 proteins. Addition of PKS24 protein had no significant effect on the H⁺-transport activity in wild-type vesicles; however, this activity was significantly reduced by adding SCaBP1 and PKS24 in combination (Fig. 8, A and F). H⁺-transport activity also decreased with the addition of active PKS24, either PKS24T/D or PKS24T/DDF (Fig. 8, B, C, and F); the level of reduction was less than that seen in the presence of SCaBP1 in combination with PKS24 (Fig. 8F). SCaBP1 in combination with PKS24T/DDF was more effective than the SCaBP1 and PKS24 in reducing H⁺-transport activity, whereas SCaBP1 in combination with PKS24T/DDF had a similar effect on H⁺-transport activity as PKS24T/DDF (Fig. 8, C and F). When added as a control, denatured (boiled) protein did not alter H⁺-transport in wild-type vesicles (Fig. 8, D and G).

To further investigate the role of PKS24 on the regulation of PM H⁺-ATPase activity, we purified PM vesicles from wild-type plants, the PKS24 knockout mutants and the pks24-1 mutant harboring PKS24 or PKS24T/D and measured H⁺-transport activity. The two mutants showed the highest activity, the transgenic plants harboring PKS24 showed activity that was the same as in wild-type and transgenic plants harboring PKS24T/D possessed the lowest activity (Fig. 8, E and H). These data suggest that PKS24 plays a role in negatively regulating PM H⁺-ATPase activity.

**DISCUSSION**

Previous data have shown that SCaBPs are calcium sensors that physically interact with and activate PKSs in a calcium-dependent manner (Luan et al., 2002; Gong et al., 2004). In this study, we found that activation of PKS24 by SCaBP1 is calcium independent but requires the C terminus of SCaBP1 and the FISL motif of PKS24, and that the phosphorylation of the SCaBP1 C terminus by PKS24 may also play an important role in PKS24 activation.

The FISL motif in the PKSs is essential for its interaction with the SCaBP calcium sensors. This is supported by analysis of the complex structures of SOS3-SOS2 (Sánchez-Barrena et al., 2007) and CBL2/...
SCaBP1-PKS24 (Akaboshi et al., 2008). The PKSs recognize their interacting calcium sensor SCaBPs by the interaction of the FISL motif with a hydrophobic cleft generated by the four calcium-binding domains (EF hands) of SCaBP.

Calcium also plays a role in regulation of PKS and SCaBP interaction. When SOS3 is not in a complex with SOS2, all four EF hands are in a Ca\(^{2+}\)-bound form; however, when SOS3 interacts with SOS2, only EF1 and EF4 contain Ca\(^{2+}\). In a Ca\(^{2+}\)-free system, the SOS3-SOS2 complex aggregates to a high M\(_2\) form, suggesting that calcium changes the conformation of the SOS2-SOS3 complex and stabilizes the interaction (Sánchez-Barrena et al., 2007). In contrast with the SOS2-SOS3 complex, Ca\(^{2+}\) does not affect the SCaBP1/CBL1-PKS24/CIPK14 interaction and complex stability. In both Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms, this complex exists as a monomer in solution (Akaboshi et al., 2008). However, it is not understood how specificity in interaction is achieved for each SCaBP-PKS pair.

SCaBP proteins are phosphorylated by their interacting PKSs in the conserved SCaBP PPF motif and phosphorylation increases the interaction between the two proteins (Du et al., 2011). SCaBP8\(^{237}\) is the only amino acid phosphorylated by SOS2 (Lin et al., 2009); however, SCaBP1\(^{216A}\) can be phosphorylated by PKS24 and activated PKS24 in vitro, indicating that there are multiple PKS24-phosphorylation sites in SCaBP1. When we removed 16 amino acids from the C terminus of SCaBP1 or mutated three putative sites (SCaBP1\(^{T211A}\), SCaBP1\(^{T212A}\), and SCaBP1\(^{T213A}\)), the mutated proteins still interacted with PKS24, but SCaBP1\(^{T211A}\) and SCaBP1\(^{T212A}\) did not activate the kinase, indicating that the Thr-211 and Thr-212 are required for PKS24 activation. By contrast, Thr-213 is not required for the activation of PKS24. Our results indicate that both the interaction between PKS24 and SCaBP1 through the FISL motif and Thr-211 and Thr-212 in the C terminus of SCaBP1 are essential for the activation of PKS24.

Although we do not know whether the phosphorylation of the C terminus of SCaBP1 is required for this activation, such a multistep phosphorylation mechanism could be involved in fine-tuning the regulatory activity of SCaBP1, and the phosphorylation of these alternate sites could alter the structure of the PKS24-SCaBP1 complex and further activate PKS24. Both PKS24 and PKS24DF are able to phosphorylate MBP; however, the phosphorylation of SCaBP1 by PKS24 requires the FISL motif, suggesting that structure-based recognition between these two proteins is important for the phosphorylation of SCaBP1 by PKS24 as well as their interaction. Deletion of the FISL motif abolishes the interaction between SOS2 and SCaBP8; however, SOS2DF still phosphorylates SCaBP8, although it is weaker than SOS2 phosphorylation (Lin et al., 2009). These results suggest that the activation of PKS24 by SCaBP1 and the activation of SOS2 by SOS3/SCaBP8 are regulated by different mechanisms.

Similar to PKS5, PKS24 is required for inactivating PM H\(^{+}\) transport. Activated PKS24s reduced PM H\(^{+}\)-ATPase activity and the reduction in the level of the activity correlated with the activity of the kinases. These results suggest that the kinase activities of PKS5 and PKS24 play a central role in regulating H\(^{+}\)-transport activity. Because SCaBP1 does not activate PKS5 in vitro, it is possible that other cofactors, additional proteins, or posttranslational modification of PKS5 are required to activate PKS5 in vivo and to further deactivate the PM H\(^{+}\)-ATPase.

MATERIALS AND METHODS

Plasmid Construction

PKS24 and SCaBP1 complementary DNA was obtained by reverse transcription PCR from wild-type Arabidopsis (Arabidopsis thaliana Columbia ecotype) RNA. The amplified products were gel-purified, digested, and cloned into the pCAMBIA2307-6\(^{x}\)/Myc and pCAMBIA1307-3\(^{x}\)/Flag vectors, respectively. Site-directed mutagenesis was used to construct the T to D substitutions and/or a FISL motif deletion mutant of PKS24 (PKS24\(^{T/D}\), PKS24DF, and PKS24\(^{T/DDF}\). The gel-purified amplified products were digested with BamHI and SalI, cloned into the pGEX-6P-1 vector, and PKS24\(^{T/D}\) and PKS24\(^{T/DDF}\) were subcloned into the pCAMBIA2307-6\(^{x}\)/Myc vector. To construct the GST-SCaBP1 fusion protein and its mutant forms, SCaBP1 was extracted from pCAMBIA1307-3\(^{x}\)/Flag-SCaBP1 vector using BamHI and SalI and subcloned into pGEX-6P-1. Site-directed mutagenesis was used to construct the C-terminal deletion mutant and Ser/Thr to Ala substitutions in SCaBP1 (SCaBP1\(^{N210}\), SCaBP1\(^{S216A}\), SCaBP1\(^{T211A}\), SCaBP1\(^{T212A}\), SCaBP1\(^{T213A}\), and SCaBP1\(^{T211A\;T212A\;T213A}\)). The resulting products were then cloned into pGEX-6P-1. To construct the GST-SCaBP6 fusion protein, SCaBP6 complementary DNA was obtained by reverse transcription PCR from wild-type Arabidopsis RNA. The gel-purified amplified product was digested with BamHI and SalI and subcloned into the pGEX-6P-1 vector. The pGEX-2TK-SOS2 and the pGEX-6P-1-SCaBP8 plasmids were described by Lin et al. (2009). The pQE-30-PK55 plasmid was described by Yang et al. (2010). The entire insert in each of the constructs was sequenced. All primers in this study are listed in Supplemental Table 1.

Fusion Protein Expression and Purification, Pull-Down, and Kinase Assays

All GST or His fusion constructs were transformed into Escherichia coli BL21 (DE3). The transformed cells were grown at 37°C in Luria-Bertani medium with ampicillin (100 \(\mu\)g/mL) until Optical Density 600 = 1.0 was reached. Recombinant protein expression was induced by 1.0 mM isopropyl-\(\beta\)-D-thiogalactopyranoside at 16°C overnight. The resulting recombinants were affinity purified according to the manufacturer’s protocol (GE Healthcare Life Science) and analyzed by SDS-PAGE.

Kinase assays were performed as previously described (Lin et al., 2009). Kinase buffer included 20 mM Tris-HCl (pH 8.0), 5 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 \(\mu\)M ATP, and 1 mM dithiothreitol. The kinase reaction was performed in a total volume of 15 \(\mu\)L and was started by the addition of 0.1 \(\mu\)g of \(\gamma\)-\(\text{32P}\)-ATP (L\(_{\text{c}}\)C), and the mixures were incubated at 30°C for 0.5 h. Reactions were terminated by adding 6X SDS loading buffer followed by incubation at 95°C for 5 min. Proteins were separated by 12% (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue R-250 followed by exposure to a phosphor screen (Amersham Biosciences). After a 12-h exposure, signals were captured with a Typhoon 9410 phosphor imager (Amersham Biosciences). Enzyme activity was measured and quantified as described by Gong et al. (2002). Stoichiometry was measured and calculated as described by Halbrittge et al. (1990).

For the pull-down assay, SCaBP6, SCaBP8, SCaBP1, and its mutated versions were cleaved by PreScission protease from the GST tag; GST-PK55 and its mutated versions were eluted from glutathione beads according to the manufacturer’s protocol (GE Healthcare Life Science). The glutathione was removed using Amicon Ultra-10 centrifugal filters (Millipore). Five \(\mu\)g of SCaBPs were incubated with 1 \(\mu\)g (or 5 \(\mu\)g) each of GST-PK55 or its variants for 30 min at room temperature in 100 \(\mu\)L of kinase buffer (20 mM
Tris-HCl, pH 8.0, 5 mM MgCl₂, and 1 mM dithiothreitol; calcium or ATP were added to the buffer when testing their effects. Then 20 μl of the 50% (v/v) glutathione-bead slurry was added into the binding system and incubated for 30 min at room temperature. After centrifugation at 9000 rpm for 2 min, the supernatant was discarded. The glutathione beads were washed with 500 μl kinase buffer five times. The pull-down products were resuspended in 100 μl of SDS-PAGE loading buffer, and 20 μl was examined by 12% (w/v) SDS-PAGE gel, with visualization by staining with Coomassie Brilliant Blue R 250.

Coimmunoprecipitation Assays

Twelve-d-old seedlings were harvested and homogenized on ice in extraction buffer containing 10 μl Tris-HCl (pH 7.6) 150 mM sodium chloride, 1 mM EDTA, 0.5% (v/v) Nonidet P-40. Ten μl of anti-cMyc conjugated agarose (Sigma) was incubated with the extract supernatant for 1 h at 4°C. The beads were washed five times with extraction buffer. The coimmunoprecipitation products were detected via immunoblots using anti-cMyc (Sigma), anti-Flag (Sigma), and anti-S216P antibodies.

PM Na⁺/H⁺ Antiport Assays

Na⁺/H⁺ antiport activity was measured as a Na⁺-induced dissipation of the pH gradient (change in pH [ΔpH]; i.e. a Na⁺-induced increase in quinacrine fluorescence) as described by Lin et al. (2009). Recombinant SCaBP1, PKS24, PKS24T/D, or PKS24T/DDF protein (250 ng/ml) was preincubated for 10 min at room temperature with plasma membrane vesicles isolated from plants. An inside-out ΔpH was formed in the vesicles by the activity of the H⁺-ATPase and was measured as a decrease (quench) in the fluorescence of quinacrine (a pH-sensitive fluorescent probe). Assays (2 mL) contained 5 μM quinacrine, 3 mM MgSO₄, 100 mM KCl, 25 mM 1,3-bis(tris(hydroxymethyl)methyl) methylamino)propane-HEPES, pH 6.5, 250 μM mannitol, and 50 μg/mL of plasma membrane protein. Reactions were mixed by inversion several times and then placed in a dark chamber in a fluorescence spectrophotometer (Hitachi F-4500). Reactions were equilibrated in the dark with stirring for 5 min before beginning fluorescence readings. The assay was initiated by the addition of ATP to a final concentration of 3 mM, and formation of ΔpH was measured at excitation and emission wavelengths of 430 and 500 nm, respectively. When the maximum ΔpH formed was reached (steady state), sodium chloride was added to initiate Na⁺ transport. At the end of each reaction, 10 μM (final concentration) of the protonophore m-Chlorophenylhydrazone was added to dissipate any remaining ΔpH. Specific activity was calculated by dividing the initial rate by the mass of plasma membrane protein in the reaction (ΔpH/min per mg of protein). Unless indicated, all data represent the mean ± s.e. of at least three replicate experiments.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ACT2, At1g38700; PKS24, AT5G01820; PKS5, AT2G30560; SCaBP1, AT5G53990; SCaBP6, AT4G26570; SCaBP8, At4g33000; and SOS2, At5g35410.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Kinase activity of wild-type and mutant PKS24 and the effect of Mn²⁺ and Mg²⁺.

Supplemental Figure S2. Time course of MBP phosphorylation by PKS24 without (A) or with SCaBP1 (B).

Supplemental Figure S3. PKS24 interacts with SCaBP1 and SCaBP6, but not SCaBP8 in vitro.

Supplemental Figure S4. Anti-S216P antibody can be used to detect the phosphorylation of SCaBP1 by PKS24 in vitro.

Supplemental Figure S5. SCaBP1N210 interacts with PKS24, but not PKS24DF in vitro.

Supplemental Figure S6. PKS24 interacts with SCaBP1, SCaBP1S216, SCaBP1T217, SCaBP1S216T217, and SCaBP1T217 in vitro.

Supplemental Figure S7. Transfer DNA insertion position in pks24-1 (SALK_147899) and pks24-2 (SALK_09699) mutant lines and PKS24 expression analysis.

Supplemental Table S1. Primers used in this study.

Regulation of SOS2-Like Protein Kinase Activity in Arabidopsis

ACKNOWLEDGMENTS

We thank Drs. José M. Pardo and Jianmin Zhou for critical reading of the manuscript and stimulating discussions.

Received November 7, 2013; accepted February 11, 2014; published February 12, 2014.

LITERATURE CITED


