A calcium-independent activation of the *Arabidopsis* PKS24 kinase by its interacting SCaBP1 calcium sensor

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Running title: Regulation of PKS activity in *Arabidopsis*

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Abstract

The salt stress-induced SALT-OVERLY-SENSITIVE (SOS) pathway in *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 (PKS) 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 threonine²¹¹ and threonine²¹², 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 (PM) H⁺-ATPase. Together, these results suggest the existence of a novel SCaBP-PKS regulatory mechanism in plants.
Introduction

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-SENSITIVE3 (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 mutant (Liu et al., 1998).

SCaBP/CBL proteins interact with the SOS2-LIKE PROTEIN KINASES (PKs)/CBL-INTERACTING PROTEIN KINASES (CIPKs) kinases (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 Na⁺/H⁺ antiporter by directly phosphorylating the SOS1C-terminus (Shi et al., 2000; Qiu et al., 2002; Quintero et al., 2002; Yu et al., 2010; Quintero et al., 2011).

In addition to the calcium-dependent activation of PKS kinases by SCaBP calcium sensors, two other regulatory mechanisms have been identified for these protein families. First, PKS kinases 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 kinase regulatory domain interacts with its kinase domain via the FISL motif to repress PKS kinase 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 PKS kinases phosphorylate a serine 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 threonine residues in the SCaBP1 C-terminal tail.

Results

The activation of PKS24 by SCaBP1 is calcium independent
We have previously shown 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 kinase activity, we found that, in comparison to what was seen for other SCaBP-PKS pairs (Shi et al, 1999; Halfter et al, 2000; Guo et al., 2001; Quan et al, 2009), activation of PKS24 by SCaBP1 (auto-phosphorylation) increased with increasing amounts of PKS24 protein and was independent of calcium (Figure 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 (Figure 1A). Consistent with previous results, PKS24 phosphorylated SCaBP1. These results indicate that SCaBP1 plays a critical role in regulating PKS24 activity.

To determine if calcium is involved in this regulation, we first assayed whether calcium has a direct effect on PKS24 activity using increasing concentrations of calcium (from 0 to 20 mM) and the calcium chelator EGTA. No difference in PKS24 auto- or SCaBP1 phosphorylation was seen in the presence of EGTA or at concentrations of calcium ranging from 0.25 to 5 mM (Figure 1B and 1C). 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 (Figure 1B and 1C). These results indicate that the activation of PKS24 by SCaBP1 is calcium-independent in vitro. Maximum PKS24 autophosphorylation was
observed when 10 mM Mg\(^{2+}\) was included in the assay; in comparison, only 0.5 mM Mn\(^{2+}\) was required to produce the maximum level of PKS24 activity, indicating a preference for Mn\(^{2+}\) over Mg\(^{2+}\) (Supplemental Figure 1).

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 (Figure 2A) and the ability of PKS24 to trans-phosphorylate SCaBP1 and Myelin Basic Protein (MBP, 200 ng) was monitored (Figure 2B). The results show that increasing SCaBP1 increased both the auto- and trans-phosphorylation 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 Figure 2).

**The FISL motif does not inhibit PKS24 activity**

SOS2 exhibits weak auto- and trans-phosphorylation 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 Threonine at position 168 (T\(^{168}\)) in the kinase activation loop to Aspartic Acid (D) or by deleting the FISL motif in the regulatory domain and both of these features (a Threonine in the kinase activation loop and the FISL motif) are conserved throughout the PKS family (Guo et al., 2001). To determine if the conserved Threonine and FISL motif in PKS24 affect its kinase activity, we generated three PKS24 mutants: one with the T\(^{178}\)/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 vitro* kinase assays. In contrast to the results for SOS2, deleting the FISL motif did not alter the auto- or trans-phosphorylation activity of PKS24 when MBP was used as the substrate; however, the T\(^{178}\)/D mutation strongly increased PKS24 activity and the kinase
activity of PKS24T/DDF was similar to that of the T^{178}/D mutant (Figure 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).

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 if 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 (Figure 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, kinase assays were performed using versions of PKS24, PKS24DF, PKS24T/D, and PKS24T/DDF fused to GST. The presence of SCaBP1 in the reactions increased both the auto- and trans-phosphorylation activities of PKS24 and PKS24T/D. In contrast, neither PKS24DF nor PKS24T/DDF was able to phosphorylate SCaBP1 or be activated by SCaBP1 (Figure 4), although they could phosphorylate MBP. These results demonstrate that an interaction between the two proteins at a site in addition to the active site is required for both the phosphorylation of SCaBP1 by PKS24 and the activation of PKS24 by SCaBP1.

To determine if other SCaBP proteins also activate PKS24, SCaBP8 and SCaBP6, which share 59.8% and 90.4% amino acid sequence similarity with SCaBP1, respectively, were fused to the Glutathione S-Transferase (GST) and the constructs were expressed in *Escherichia coli*. Histidine (His)-PKS5, GST-PKS24, GST-SOS2, GST-SCaBP1, GST-SCaBP6, and GST-SCaBP8 fusion proteins were then purified and used in kinase assays (Figure 5). SCaBP1 and SCaBP6 but not SCaBP8 were phosphorylated by PKS24 and activated PKS24 (Figure 5A). In vitro pull-down
assays demonstrated that GST-PKS24 pulled down SCaBP1 and SCaBP6, but not
SCaBP8 (Supplemental Figure 3). Both SOS2 and PKS5 phosphorylated SCaBP1,
SCaBP6 and SCaBP8. However, their auto-phosphorylation activities were not
enhanced by the phosphorylated SCaBP proteins (Figure 5). These results suggest that
the activation of PKS24 by SCaBP1 and SCaBP6 is a specific regulatory process in
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-
day-old seedlings were harvested and the proteins were extracted. Anti-Myc beads
were used to immunoprecipitate Myc-PKS24 or Myc-PKS24DF (Figure 6A) and
MBP was used as the substrate for the kinase assays (Figure 6B). The results show
that PKS24 from transgenic plants harboring Flag-SCaBP1 had the highest activity
(Figure 6C). MBP was very weakly phosphorylated by PKS24DF, whether the
transgenic plants contained Flag-SCaBP1 or not (Figure 6C). However, the
phosphorylation of MBP by Myc-PKS24 from transgenic plants that did not contain
Flag-SCaBP1 was stronger than phosphorylation by PKS24DF (Figure 6C). These
results suggest that endogenous SCaBP1 in 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.

Previously, we have shown that PKS24 phosphorylates Serine\(^{216}\) of SCaBP1 (Du
et al, 2011). Polyclonal phosphospecific antibodies (anti-S216P) detected this
phosphorylation in vitro (Supplemental Figure 4). 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. (Figure 6A).
Flag-SCaBP1 was only detected in the Myc-PKS24 but not Myc-PKS24DF co-
immunoprecipitated products (Figure 6D). A stronger signal was detected with anti-S216P in transgenic plants harboring both Myc-PKS24 and Flag-SCaBP1 (Figure 6E). A weaker signal was also detected in plants expressing only Myc-PKS24, suggesting that endogenous SCaBP1 was pulled down and phosphorylated by PKS24 (Figure 6E); however, no signal was detected in the PKS24DF co-immunoprecipitated products (Figure 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.

**Threonine 211 and 212 in the C-terminus of SCaBP1 are important for activation of PKS24**

Previous results have demonstrated that PKS24 phosphorylates SCaBP1 at Serine$^{216}$ 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 (Figure 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, SCaBP1$^{S216A}$, SCaBP1N210 and SCaBP1ND30 in kinase assays (Figure 7A). When compared with wild-type SCaBP1, SCaBP1N210 was only weakly phosphorylated by PKS24 and did not activate PKS24. SCaBP1ND30 activated the auto-phosphorylation activity of PKS24 to a level similar to that of SCaBP1, but the level of SCaBP1 phosphorylation by PKS24 was dramatically reduced. SCaBP1$^{S216A}$ was phosphorylated by PKS24 and activated PKS24 at a level similar to that of wild-type SCaBP1 (Figure 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 (Figure 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 Figure 5) suggest that, while the C-terminus of SCaBP1 is not needed for binding to PKS24 via the FISL domain, it possesses the ability to activate PKS24. As 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, T213) to determine if they are important for activation of PKS24. The three Threonine residues were replaced with Alanine and the mutant proteins were used in kinase assays. SCaBP1T211A did not activate PKS24 and showed little evidence of phosphorylation by PKS24 while SCaBP1T212A activated PKS24 and was weakly phosphorylated by it. The SCaBP1T211A T212A double mutant, like SCaBP1T211A, it did not activate PKS24. In comparison, SCaBP1T213A, like wild-type SCaBP1, activated PKS24 and was phosphorylated by it (Figure 7B). These results demonstrate that both SCaBP1T211 and SCaBP1T212 are important for the activation of PKS24.

PKS24 regulates PM H⁺-ATPase activity

To analyze the biological function of PKS24, we obtained two T-DNA insertion lines for PKS24, (SALK_147899 and SALK_009699, referred to pks24-1 and pks24-2, respectively) and confirmed their status as gene knockouts (Supplemental Figure 7). The phenotype of each mutant was monitored following exposure to glucose, abiotic stresses and plant hormones including ABA, 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 H⁺-ATPase 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 (Fuglsang et al., 2007) and to activate PKS24 in Arabidopsis (Figure 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; Qiu et al., 2004; Guo et al., 2004). To determine if 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 SCAβP1 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 SCAβP1 and PKS24 in combination (Figure 8A and F). H⁺-transport activity also decreased with the addition of active PKS24 kinase, either PKS24T/D or PKS24T/DDF (Figure 8B, C, and F); the level of reduction was less than that seen in the presence of SCAβP1 in combination with PKS24 (Figure 8F). SCAβP1 in combination with PKS24T/D was more effective than the SCAβP1 and PKS24 in reducing H⁺-transport activity while SCAβP1 in combination with PKS24T/DDF had a similar effect on H⁺-transport activity as PKS24T/DDF (Figure 8C and F). When added as a control, denatured (boiled) protein did not alter H⁺-transport in wild-type vesicles (Figure 8D 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 (Figure 8E and H). These data suggest that the PKS24 kinase plays a role in negatively regulating PM H⁺-ATPase activity.

Discussion

Previous data has shown that SCAβPs are calcium sensors that physically interact with and activate PKS protein kinases in a calcium-dependent manner (Luan et al, 2002; Gong et al, 2004). In this study, we found that activation of PKS24 by SCAβP1 is calcium-independent but requires the C-terminus of SCAβP1 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 PKS kinases is essential for its interaction with the SCaBP calcium sensors. This is supported by analysis of the complex structures of SOS3-SOS2 (Sanchez-Barrena et al, 2007) and CBL2/SCaBP1-CIPK14/PKS24 (Akaboshi et al, 2008). The PKS kinases 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 molecular weight form suggesting that calcium changes the conformation of the SOS2-SOS3 complex and stabilizes the interaction (Sanchez-Barrena et al, 2007). In contrast to 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 PKS kinases in the conserved SCaBP PFPF motif and phosphorylation increases the interaction between the two proteins (Du et al, 2011). SCaBP8\(^{S237}\) is the only amino acid phosphorylated by SOS2 (Lin et al, 2009); however, SCaBP1\(^{S216A}\) can be phosphorylated by PKS24 and activated PKS24 \textit{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 phosphorylation sites in this region (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 Threonine 211 and 212 are required for PKS24 activation. In contrast, Threonine 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
Threonine 211 and 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 multi-step 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 also 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 PKS24 kinases 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 is required to activate the PKS5 kinase in vivo and to further deactivate the PM H⁺-ATPase.

Materials and Methods

Plasmid construction

*PKS24* and *SCaBP1* cDNA was obtained by RT-PCR from wild-type Arabidopsis (*Arabidopsis thaliana*; Columbia ecotype) RNA. The amplified products were gel-purified, digested and cloned into the pCAMBIA2307-6×Myc and pCAMBIA1307-3×Flag vectors, respectively. Site-directed mutagenesis was used to construct the T to
D substitutions and/or a FISL motif deletion mutant of PKS24 (PKS24T/D, PKS24DF and PKS24T/DDF). The gel-purified amplified products were digested with BamHI and SalI, cloned into the pGEX-6P-1 vector and PKS24T/D and PKS24T/DDF were subcloned into the pCAMBIA2307-6×Myc vector. To construct the GST-SCaBP1 fusion protein and its mutant forms, SCaBP1 was extracted from pCAMBIA1307-3×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 Serine/Threonine to Alanine substitutions in SCaBP1 (SCaBP1N210, SCaBP1S216A, SCaBP1T211A, SCaBP1T212A, SCaBP1T211A T212A and SCaBP1T213A). The resulting products were then cloned into pGEX-6P-1. To construct the GST-SCaBP6 fusion plasmid, SCaBP6 cDNA was obtained by RT-PCR from wild-type Arabidopsis RNA. The gel-purified amplified product was digested with BamHI and SalI, cloned 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-PKS5 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 Table1.

**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 μg/mL) until OD600 = 1.0 was reached. Recombinant protein expression was induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside at 16°C overnight. The recombinant proteins were affinity purified according to the manufacturer’s protocol (GE Healthcare Life Science) and analyzed by SDS-PAGE.

Kinase assays were performed as described previously (Lin et al., 2009). Kinase buffer included 20 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 1 mM CaCl2, 10 μM ATP, and 1 mM dithiothreitol. The kinase reaction was performed in a total volume of 15 μL and was started by the addition of 0.1 μL of γ-32P-ATP (1μCi), and the mixtures were incubated at 30°C for 0.5 h. Reactions were terminated by adding 6× 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 in Gong et al. (2002). Stoichiometry was measured and calculated as described in Halbrügge 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-PKS24 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 μg of SCaBPs were incubated with 1 μg (or 5μg) each of GST-PKS24 or its variations for 30 min at room temperature in 100 μL 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% glutathione-bead slurry was added into the binding system, and incubated for 30 min at room temperature. After centrifuge at 500×g for 2 min, the supernatant was discarded. The glutathione beads were washed with 500 μL kinase buffer for five times. The pull-down products were resuspended in 100 μL of SDS-PAGE loading buffer, and 20 μL examined by 12% SDS-PAGE gel, with visualization by staining with Commassie Brilliant Blue R 250.

**Co-immunoprecipitation assays**

Twelve-day-old seedlings were harvested and homogenized on ice in extraction buffer containing 10 mM Tris-HCl (pH7.6) 150 mM NaCl, 1 mM EDTA, 0.5% NP-40. Ten microliters of anti-cMyc conjugated agarose (Sigma) was incubated with the extract supernatant for 1 h at 4°C. The beads were washed 5 times with extraction buffer. The co-IP products were detected via immunoblots using anti-cMyc (Sigma), anti-Flag (Sigma) and anti-S216P antibodies.

**Plasma membrane Na⁺/H⁺ antiport assays**
Na+/H+ antiport activity was measured as a Na\(^{+}\)-induced dissipation of the pH gradient (\(\Delta \text{pH}\); 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 pre-incubated for 10 min at room temperature with plasma membrane vesicles isolated from plants. An inside-acid \(\Delta \text{pH}\) was formed in the vesicles by the activity of the H\(^{+}\)-ATPase and measured as a decrease (quench) in the fluorescence of quinacrine (a pH-sensitive fluorescent probe). Assays (2 mL) contained 5 \(\mu\)M quinacrine, 3 mM MgSO\(_4\), 100 mM KCl, 25 mM 1, 3-bis [Tris (hydroxymethyl) methylamino] propane-HEPES, pH 6.5, 250 mM mannitol, and 50 \(\mu\)g/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 \(\Delta \text{pH}\) was measured at excitation and emission wavelengths of 430 and 500 nm, respectively. When the maximum \(\Delta \text{pH}\) was formed (reached steady state), NaCl was added to initiate Na\(^{+}\) transport. At the end of each reaction, 10 \(\mu\)M (final concentration) of the protonophore m-chlorophenylhydrazone (CCCP) was added to dissipate any remaining \(\Delta \text{pH}\). Specific activity was calculated by dividing the initial rate by the mass of plasma membrane protein in the reaction (\(\% \text{F/min per mg of protein}\)). Unless indicated, all data represent means ± SE of at least three replicate experiments.

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REFERENCES


PROTEIN8 by SOS2 protein kinase stabilizes their protein complex and regulates salt tolerance in Arabidopsis. Plant Cell **21**, 1607–1619


FIGURE LEGENDS

Figure 1. Activation of PKS24 by SCaBP1 is calcium-independent.

(A) Effect of SCaBP1 on PKS24 activity. Increasing concentrations of PKS24 were incubated without or with a fixed amount of SCaBP1. The concentrations of PKS24 in lane 1-6 in ng/µl were: 6.7, 13.3, 33.5, 67, 100 and 133; Lane 7, 133 ng/µl PKS24 without SCaBP1; Lane 8, (negative control) SCaBP1 (33.3 ng/µl) was incubated in reaction buffer without PKS24. Coomassie blue staining (upper panel) and kinase activity (lower panel).

(B-C) Coomassie blue-stained polyacrylamide gel (upper panel), auto-phosphorylation activity of PKS24 (B) and trans-phosphorylation of SCaBP1 (C) in the presence of increasing concentrations of calcium (lower panel). SC1, SCaBP1; E, 5 mM EGTA was added into the reaction buffer without calcium ions. PKS24 and SCaBP1 were purified as GST-fusion proteins; the GST tag was removed from SCaBP1 by PreScission protease.

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 (upper panel); PKS24 auto- and SCaBP1 trans-phosphorylation activity (lower panel).

(B) Phosphorylation of SCaBP1 and Myelin Basic Protein (MBP) by PKS24. Coomassie blue-stained polyacrylamide gel (upper panel); PKS24 auto- and SCaBP1/MBP trans-phosphorylation activity (lower panel). The concentration of SCaBP1 in lane 1-16 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, 266.7. PKS24 and SCaBP1 were purified as GST-fusion proteins; the GST tag was removed from SCaBP1 by PreScission protease.

Figure 3. In vitro pull-down and phosphorylation of SCaBP1 by wild-type and
mutant PKS24.

(A) Auto- and trans-phosphorylation activity of PKS24, PKS24T/D, PKS24DF and PKS24T/DDF. Coomassie blue staining (upper panel) and phosphorylation activity (lower panel).

(B) The interaction of PKS24 with SCaBP1 requires the FISL motif but not calcium or ATP. The pull-down experiments were performed in kinase assay buffer. Lane 1, input containing 200 ng of PKS24 and 1 μg of SCaBP1 as markers. Lane 2, the pull-down products from lane 1 after five washes. Lanes 3-9, SCaBP1 pull-down by PKS24, PKS24DF, PKS24T/D and PKS24T/DDF in the presence or absence of calcium. Upper panel, reactions in kinase buffer lacking ATP; lower panel, reactions in kinase buffer containing ATP. SC1, SCaBP1.

SCaBP1, PKS24 and its variations were purified as GST-fusion proteins; the GST tag was removed from SCaBP1 by PreScission protease for the pull-down assay.

Figure 4. PKS24 activation by SCaBP1 requires the interaction of PKS24 with SCaBP1.

Kinase assays were performed using combinations of PKS24, PKS24DF, PKS24T/D or PKS24T/DDF and SCaBP1, MBP or SCaBP1 plus MBP as indicated. Coomassie blue-stained polyacrylamide gel (upper panel); phosphorylation activity (lower panel). SC1, SCaBP1. PKS24 and SCaBP1 were purified as GST-fusion proteins; the GST tag was removed from SCaBP1 by PreScission protease.

Figure 5. SCaBP1 and SCaBP6 specifically activate PKS24.

Kinase assays were performed using combinations of GST-PKS24, GST-SOS2 or His-PKS5 and GST-SCaBP1, GST-SCaBP6 or GST-SCaBP8 as indicated. Coomassie blue-stained polyacrylamide gel (upper panel); phosphorylation activity (lower panel). SC1, SCaBP1; SC6, SCaBP6; SC8, SCaBP8. PKS24, SOS2, SCaBP1, SCaBP6, and SCaBP8
were fused to GST tag; PKS5 was fused to His tag.

Figure 6. SCaBP1 activates PKS24 in vivo.
Co-immunoprecipitation was performed with transgenic plants as indicated, and the products were used in kinase assays or immunoblots. The result of immunoblots with anti-cMyc antibody (panel A); Coomassie blue-stained polyacrylamide gel of the substrate MBP for the kinase assay (panel B); autoradiography of MBP phosphorylation (panel C); the result of immunoblots with anti-flag antibody (panel D) and anti-S216P antibody (panel E). WB, Western Blot. SCaBP1 was fused to Flag tag; PKS24 and PKS24DF were fused to Myc tag.

Figure 7. Threonine 211 and 212 in the C-terminus of SCaBP1 are important for activation of PKS24.
(A) Activation of PKS24 by SCaBP1, SCaBP1S216A and SCaBP1DN30 but not by SCaBP1N210. Coomassie blue staining (upper panel) and kinase activity (lower panel). (B) Activation of PKS24 by SCaBP1, SCaBP1T212A or SCaBP1T213A. Coomassie blue staining (upper panel) and kinase activity (lower panel). SC1, SCaBP1. PKS24, SCaBP1 and its variations were fused to GST tag.

Figure 8. PKS24 negatively regulates plasma membrane H+-ATPase activity.
Plasma membrane vesicles were isolated from wild-type plants, pks24 mutants and transgenic plants treated with 250 mM NaCl for 3 d. Intravesicular acidification was initiated by addition of 3 mM ATP and the pH gradient was collapsed by addition of 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP). The H+ transport activity (ΔpH formation) was measured in wild-type vesicles (A-G), and in the presence of PKS24 (A, F), SCaBP1 and PKS24 (A, F), PKS24T/D (B, F), SCaBP1 and PKS24T/D (B, F), PKS24T/DDF (C, F), SCaBP1 and PKS24T/DDF (C, F), denatured PKS24, SCaBP1 and
PKS24, PKS24T/D, SCaBP1 and PKS24T/D, PKS24T/DDF, SCaBP1 and PKS24T/DDF (D, G). The H+ transport activity (ΔpH formation) was measured in vesicles isolated from wild-type plants (A-D, F, G) or the pks24 mutants and transgenic plants (E, H). Units of H+ transport activity are ΔF/min per mg protein. All data represent means ± standard errors of at least three replicate experiments. Each replicate experiment was performed using independent membrane preparations. One representative experiment of three replicates is shown in A, B, C, D and E. Student’s test was used for determining the statistical significance. Significant differences (P≤0.05) are indicated by different lowercase letters. SC1, SCaBP1, TD, PKS24T/D; TDDF, PKS24T/DDF. SCaBP1, PKS24 and its variations were fused to GST tag.

Supplemental Figure 1. Kinase activity of wild-type and mutant PKS24 and the effect of Mn2+ and Mg2+.

(A-B) Auto-phosphorylation activity of GST-PKS24 using Mg2+ (A) or Mn2+ (B) as the co-factor. Coomassie blue staining (upper panel) and auto-phosphorylation activity (lower panel).

(C) Auto-phosphorylation activity in (A) and (B) measured using a Typhoon 9410 PhosphorImager.

Supplemental Figure 2. Time course of MBP phosphorylation by PKS24 without (A) or with SCaBP1 (B).

MBP (1 μg, 2.5 μM) was phosphorylated by GST-PKS24 (0.7 μM) without (A) or with GST-SCaBP1 (B) for the times indicated and separated by SDS-PAGE. 32P incorporation was quantified by cutting out and counting the area defined by the MBP band. All data represent means ± standard errors of at least three replicate experiments. One representative experiment of three replicates is shown.
Supplemental Figure 3. PKS24 interacts with SCaBP1 and SCaBP6, but not SCaBP8 in vitro.
Pull-down experiments were performed in kinase assay buffer. Lane 1, 1 μg of PKS24 was added as a marker; Lanes 2, 4 and 6, input of 1 μg of PKS24 and 1 μg of SCaBP1, SCaBP6 or SCaBP8; Lanes 3, 5 and 7, pull-down products from lane 2, 4 and 6 after five washes. SCaBP1, SCaBP6, SCaBP8, and PKS24 were purified as GST-fusion proteins; the GST tag of SCaBP1, SCaBP6, and SCaBP8 was removed by PreScission protease for the pull-down assay.

Supplemental Figure 4. Anti-S216P antibody can be used to detect the phosphorylation of SCaBP1 by PKS24 in vitro.
Panels A and B, Coomassie blue-stained SCaBP1 proteins (upper panels) and SCaBP1 phosphorylation detected using SCaBP1S216P antibodies (lower panels). SCaBP1, PKS24 and its variations were fused to GST tag.

Supplemental Figure 5. SCaBP1N210 interacts with PKS24, but not PKS24DF in vitro.
Pull-down experiments were performed in kinase assay buffer. Lanes 1 and 3, 1 μg of SCaBP1N210 and 1 μg of PKS24 and PKS24DF; Lanes 2 and 4, pull-down products from lane 1 and 3, after five washes. SCaBP1N210, PKS24 and its variations were purified as GST-fusion proteins; the GST tag of SCaBP1N210 was removed by PreScission protease for the pull-down assay.

Supplemental Figure 6. PKS24 interacts with SCaBP1, SCaBP1S216, SCaBP1T211, SCaBP1T212 and SCaBP1T213 in vitro.
Pull-down experiments were performed in kinase assay buffer. Lane 1, 1μg of PKS24
was added as a marker; Lanes 2, 4, 6, 8, 10 and 12, 1μg of PKS24 and 1 μg of SCaBP1, SCaBP8, SCaBP1S216, SCaBP1T211, SCaBP1T212 and SCaBP1T213; Lanes 3, 5, 7, 9, 11 and 13, pull-down products from lanes 2, 4, 6, 8, 10 and 12 after five washes. PKS24, SCaBP1 and its variations were purified as GST-fusion proteins; the GST tag of SCaBP1 and its variations was removed by PreScission protease for the pull-down assay.

Supplemental Figure 7. T-DNA insertion position in pks24-1 (salk_147899) and pks24-2 (salk_09699) mutant lines and PKS24 expression analysis.

(A) T-DNA insertion sites in the pks24-1 and pks24-2 mutants. (B) RT-PCR analysis of PKS24 expression in homozygous pks24-1 and pks24-2 plants. ACTIN2 was amplified as an internal control.