Phosphorylation of SOS3-like Calcium Binding Proteins by their interacting SOS2-like Protein Kinases is a common regulatory mechanism in Arabidopsis

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Running title: Regulation of PKS kinases in Arabidopsis

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Abstract

Arabidopsis genome encodes 9 SOS3-like Calcium Binding Proteins (SCaBP, also named Calcineurin B-Like Protein {CBL}) and 24 SOS2-like Protein Kinases (PKS, also named as CBL-Interacting Protein Kinase {CIPK}). A general regulatory mechanism between these two families is that SCaBP calcium sensors activate PKS kinases through interacting with their FISL motif. In this study, we demonstrated that phosphorylation of SCaBPs by their functional interacting PKSes is another common regulatory mechanism. The phosphorylation site serine216 at the C-terminal of SCaBP1 by PKS24 was identified by LC/MS/MS analysis. This serine residue is conserved within the PFPF motif at C-terminus of SCaBP proteins. Phosphorylation of this site of SCaBP8 by SOS2 has been determined previously. We further showed that CIPK23/PKS17 phosphorylated CBL1/SCaBP5 and CBL9/SCaBP7 and PKS5 phosphorylated SCaBP1 at the same site in vitro and in vivo. Furthermore, the phosphorylation stabilized the interaction between SCaBP and PKS proteins. This tight interaction neutralized the inhibitory effect of PKS5 on plasma membrane H+-ATPase activity. These data indicate that SCaBP phosphorylation by their interacting PKS kinases is a critical component of SCaBP-PKS regulatory pathway in Arabidopsis.
Introduction

Calcium is a uniform second messenger involved in many plant responses to environmental stimuli. There are many different types of calcium binding proteins identified in plants (Luan et al., 2002; Harper et al., 2004). One of them shares significant sequence similarity with yeast calcineurine B subunit (Luan et al., 2002; Gong et al., 2004). The first gene cloned from this family is SOS3 (Salt Overly Sensitive3) by genetic screening of Arabidopsis salt sensitive mutants and map-based cloning (Liu and Zhu, 1998). SOS3 physically interacts with and activates SOS2 (Salt Overly Sensitive2), a protein kinase (Halfter et al., 2000), and this complex in turn activates SOS1 (Salt Overly Sensitive1), a plasma membrane Na⁺/H⁺ antiporter (Shi et al., 2000; Qiu et al., 2002). SOS3 interacts with the FISL motif of SOS2 at its C-terminal regulatory domain, a SOS2 kinase-inhibiting domain (Guo et al., 2001). Recently a SOS3 homologous, SCaBP8 (SOS3 like calcium binding protein8), is identified that interacts with and activates SOS2 to protect Arabidopsis shoots from salt stress, while SOS3 primarily protects roots (Quan et al., 2007). SOS3 has a N-terminal myristoylation signal peptide and it is required for SOS3 function in plant salt tolerance (Ishitani et al., 2000). SCaBP8 shares most of the biochemical features of SOS3 in regulating SOS2 kinase activity and recruiting SOS2 to plasma membrane, however it lacks such myristoylation signal sequence, suggesting that multiple regulatory processes exist in SCaBP family (Quan et al., 2007). Although capable of performing similar functions in biochemical and cellular tests, SCaBP8 and SOS3 must fulfill distinct regulatory functions in the salt stress response, as they could not replace each other in genetic complementation experiments. SOS2 phosphorylates SCaBP8 at its C terminus serine\(^{337}\) but does not phosphorylate SOS3. This phosphorylation is induced by salt stress, occurs at the plasma membrane, stabilizes the SCaBP8-SOS2 interaction, and enhances plasma membrane Na⁺/H⁺ exchange activity (Lin et al., 2009).
In Arabidopsis, there are 9 SOS3 like calcium binding proteins (SCaBPs/CBLs), and 24 SOS2 like protein kinases (PKSes/CIPKs) (Luan et al., 2002; Gong et al., 2004). The regulation of PKS kinase activity by SCaBP calcium sensors is essential for their function in vivo (Gong et al., 2002; Guo et al., 2004). It is a general understanding that PKS kinase domain interacts with the C-terminal regulatory domain and this intramolecular interaction blocks the substrates accessing into the kinase domain (Guo et al., 2001; Gong et al., 2004). Activation of PKS kinases requires both the phosphorylation of their kinase activation loop by upstream kinase (Guo et al., 2001; Gong et al., 2002) and interaction with SCaBPs that releases the kinase domain for the target access (Gong et al., 2004).

Similar as SOS regulatory process, other two pathways have been identified related to these two families. CBL1- and CBL9-CIPK23 complex phosphorylates and regulates AKT1 to protect plants from low potassium stress (Xu et al., 2006). SCaBP1-action is required for PKS5 in order to phosphorylate and negatively regulate AHA2 to mediate the cytoplasmic pH homeostasis (Fuglsang et al., 2007; Yang et al., 2010). In addition to regulate ion transporter activity, PKS kinases have been found to interact with protein phosphatases (Guo et al., 2002; Ohta et al., 2003; Lee et al., 2006), however the regulatory mechanism is not well understood.

In this study, we have demonstrated that phosphorylation of SCaBP calcium sensors by their functional interaction PKS kinases enhances the complex stability.

**Results**

**Phosphorylation of SCaBP calcium sensors by their interaction PKS protein kinases**

Previously we showed that SCaBP8 is specifically phosphorylated by SOS2 under salt stress in Arabidopsis (Lin et al., 2009). To determine if this phosphorylation regulation occurs in other functional interaction SCaBP-PKS pairs, in vitro kinase assays were
performed between SCaBP1 and PKS5 (Fuglsang et al., 2007; Yang et al., 2010), CBL1/SCaBP5 or CBL9/SCaBP7 and CIPK23/PKS17 (Xu et al., 2006), and SCaBP1 and PKS24 (Akaboshi et al., 2008). PKS24, CIPK23, CBL1 and CBL9 cDNA was fused to a GST gene. The GST fusion proteins were purified from E. coli and then subjected to a kinase assay. As a positive control, we first conformed that SOS2 phosphorylated SCaBP8 (Figure 1A). When we tested the other pairs, we found that both PKS5 and PKS24 phosphorylated SCaBP1 and CIPK23 phosphorylated both CBL1 and CBL9 (Figure 1B-1E). Our results indicate that the phosphorylation of SCaBP proteins by their functional interaction PKS kinases may exist in plants.

Mapping of SCaBP1 phosphorylation site

To map the phosphorylation site(s) in SCaBP1 by PKS24, we incubated GST-PKS24 that was on the GST-beads and SCaBP1 in kinase buffer with cold ATP and digested the SCaBP1 protein with trypsin and enriched the phosphopeptides through the IMAC (Immobilized Metal Affinity Chromatography) technology. The phosphorylation site(s) was identified by LC/MS/MS analysis subsequently. Figure 2 shows a MS spectrum of a triply charged phosphopeptide after IMAC enrichment. The measured mass of this peptide corresponds to the theoretical mass of peptide DITTTFPSFVFHSQVEDT plus one phosphate group. The CID (Collision-induced Dissociation) spectrum of this phosphopeptide identified as DITTTFPpSFVFHSQVEDT. This phosphorylation site is serine 216 at the C-terminal.

Arabidopsis genome encodes ten SCaBP calcium sensors. All of them contain four EF-hand calcium-binding motifs (Akaboshi et al., 2008) and a conserved 23-amino acid motif at the C-terminus (Figure 3). It seems that this motif is found only in the SCaBP subfamily of calcium sensors. Interestingly, both SCaBP8S237, phosphorylated by SOS2, and SCaBP1S216, phosphorylated by PKS24, are located and conserved in this motif.
Sequence analyses of these putative motifs indicate that residues P, M, L, F, P, and F are conserved absolutely. Hence we name this motif as PFPF motif. The putative phosphorylation serine residue is conserved in the PFPF motif of eight out of ten SCaBP proteins and surrounded by FP and F residues. SCaBP4 contains a threonine residue at this position. However SCaBP2 is the only one without this putative phosphorylated residue in the PFPF motif.

**PKS kinases phosphorylate SCaBP calcium sensors at the conserved serine residue of the PFPF motif**

To determine if SCaBP calcium sensors are phosphorylated by PKS kinases in planta, phosphorylation site-specific antibodies were generated by immunizing rabbits with the chemically synthesized phosphorylated peptide Cys-TFPpSFVFH-NH₂ (phosphorylated form) for SCaBP1, CBL1 and CBL9. To select the phospho-specific antibodies, the serum was first incubated with the phosphopeptides that had been coupled to SulfoLink resin. After washing the column, the phospho-specific antibodies were eluted at pH 2.7 and immediately neutralized. The antibodies were then run over a column containing the unphosphorylated polypeptide to remove antibodies not specific for the phosphoserine. The flow-through was collected and characterized (Lin et al., 2009). To evaluate the specificity of the antibodies for the serine phosphorylation site in the PFPF motif, we mutated the serine residue in the PFPF to alanine. SCaBP1, CBL1, CBL9 and their mutant SCaBP1\(^{S216A}\), CBL1\(^{S201A}\), CBL9\(^{S201A}\) proteins were incubated with their functional interaction PKS proteins in kinase buffer in the presence or absence of ATP. The proteins were then separated using SDS-PAGE and detected by immunoblotting. A strong signal was detected only when SCaBP proteins were incubated with both PKS kinases and ATP (Supplemental Figure 1A-1D). In the absence of ATP or when the serine was changed to alanine, only a very weak signal appeared (Supplemental Figure 1A-1D).
These results suggest that the antibodies specifically recognize the phosphorylated serine in PFPF motif of SCaBP calcium sensors and the mutant SCaBP proteins were no longer phosphorylated by the PKS kinases. Consistent with our previous finding, SOS2 phosphorylated SCaBP8 at serine$^{237}$ (Supplemental Figure 1E, Lin et al., 2009).

SOS2 phosphorylated SCaBP8 serine$^{237}$ under salt stress (Figure 4A), which is consistent with our previous finding (Lin et al., 2009). To examine SCaBP proteins are phosphorylated by their interaction PKS kinases in vivo, we generated 35S:3flag-SCaBP1, 35S:3flag-SCaBP1$^{S216A}$, 35S:3flag-CBL1, 35S:3flag-CBL1$^{S201A}$, 35S:3flag-CBL9, 35S:3flag-CBL9$^{S201A}$, 35S:6myc-PKS5, 35S:6myc-PKS24 and 35S:6myc-CIPK23 plasmids. Each pair of SCaBP-PKS plasmids was co-transformed into Arabidopsis protoplasts. Total proteins were extracted from the transformed protoplasts and subjected to immunoblots. Anti-myc antibody readily detected myc-labeled PKS kinases and anti-flag antibody detected flag-labeled SCaBP proteins in the transfected protoplasts. The SCaBP proteins were immunoprecipitated by anti-flag and the phosphorylation was detected by the anti-phosphoserine antibodies. The phosphoserine$^{216}$ signal of SCaBP1 was detected only in protein extracts from plants co-expressing flag-SCaBP1 and myc-PKS5 or flag-SCaBP1 and myc-PKS24 (Figure 4B and 4C). The phosphoserine$^{201}$ signal of CBL1 and CBL9 was detected in protein extracts from plants co-expressing myc-CIPK23 and flag-CBL1 or myc-CIPK23 and flag-CBL9 (Figure 4D). No phosphoserine signal was seen in plants expressing flag-SCaBP$^{S/A}$ mutants and their interaction myc-PKS (Figure 4B-4D). As a control, SOS2 did not phosphorylated SCaBP$^{S216}$ when flag-SCaBP1 and myc-SOS2 were co-expressed in Arabidopsis protoplasts (Figure 4E). Nearly equal amounts of flag-SCaBP or myc-PKS proteins were present in the assays as demonstrated by immunoblots with anti-myc or anti-flag antibodies (Figure 4). Our data demonstrate that PKS kinases specifically phosphorylate their functional interaction SCaBP calcium sensors in planta at the serine residue in the PFPF motif.
Phosphorylation of SCaBP proteins enhance their interaction with PKS kinases

SCaBP8 phosphorylation enhances its interaction with SOS2 (Lin et al., 2009). To determine if PKS phosphorylation of SCaBP has a similar biological function as SOS2 and SCaBP8, we performed yeast two-hybrid assays. As shown in Figure 5, mutation of serine\textsuperscript{216} to alanine in SCaBP1 significantly decreased the interaction between SCaBP1 and PKS5 (Figure 5A) or PKS24 (Figure 5B), and mutation of serine\textsuperscript{201} to alanine in CBL1 or CBL9 significantly decreased the interaction between CBL1 or CBL9 and CIPK23 (Figure 5C, 5D). In contrast, when the conserved serine was substituted to an aspartic acid to mimic a phosphorylated serine residue, the SCaBP\textsuperscript{S/D} mutation enhanced the interaction with the PKS kinases (Figure 5A-5D). To further examine the quantitative binding affinities between phosphorylated and nonphosphorylated SCaBP forms with PKS kinases, a β-galactosidase activity assays in yeast for LacZ reporter expression was performed (Figure 5E-5H). These interaction differences were not due to differences in levels of SCaBP, SCaBP\textsuperscript{S/A} or SCaBP\textsuperscript{S/D} expression (Supplemental Figure 2). Our results indicate that the interaction between PKS and phosphorylated SCaBP is stronger than that between PKS and nonphosphorylated SCaBP.

To examine this interaction in Arabidopsis cell, we generated 35S:3\text{flag-SCaBP}\textsuperscript{1S216D}, 35S:3\text{flag-CBL1}\textsuperscript{S201D}, and 35S:3\text{flag-CBL9}\textsuperscript{S201D} plasmids and transformed the combinations of 35S:6\text{myc-PKS5} with 35S:3\text{flag-SCaBP1}, 35S:3\text{flag-SCaBP1}\textsuperscript{S216A}, or 35S:3\text{flag-SCaBP1}\textsuperscript{S216D}, the combinations of 35S:6\text{myc-PKS24} with 35S:3\text{flag-SCaBP1}, 35S:3\text{flag-SCaBP1}\textsuperscript{S216A}, or 35S:3\text{flag-SCaBP1}\textsuperscript{S216D}, the combinations of 35S:6\text{myc-CIPK23} with 35S:3\text{flag-CBL1}, 35S:3\text{flag-CBL1}\textsuperscript{S201A}, or 35S:3\text{flag-CBL1}\textsuperscript{S201D}, and the combinations of 35S:6\text{myc-CIPK23} with 35S:3\text{flag-CBL9}, 35S:3\text{flag-CBL9}\textsuperscript{S201A}, or 35S:3\text{flag-CBL9}\textsuperscript{S201D} into Arabidopsis protoplasts. Myc-PKS (Figure 6A and 6B) or flag-SCaBP (Figure 6C and 6D) was immunoprecipitated and flag-SCaBP or myc-PKS
was detected via immunoblot analysis. In comparison to SCaBP and SCaBP^{S/D} mutant proteins, SCaBP^{S/A} mutation significantly reduced the interaction with PKSes (Figure 6A-6D). Our results suggest that the phosphorylation of SCaBP calcium sensors by their interaction PKS kinases shares the similar biological function that enhances the interaction between PKS and SCaBP proteins.

**Phosphorylation of SCaBP1 by PKS5 is required for AHA2 regulation in yeast**

Previously we demonstrated that PKS5 and SCaBP1 can inactivate PM H^{+}-ATPase AHA2 in yeast and plant (Fuglsang et al., 2007; Yang et al., 2010). In order to test the effect of the phosphorylation SCaBP1^{S216} by PKS5 on the regulation of AHA2 activity, we reconstituted SCaBP1-PKS5-AHA2 regulatory pathway in the yeast strain RS-72. The detailed information for the reconstitution was described by Fuglsang et al (2007). When we expressed AHA2 in RS-72, the function of the endogenous H^{+}-pump was complemented and the yeast was able to grow on glucose medium (Figure 7). Consistent with our previous finding, when PKS5 and SCaBP1 were co-expressed with AHA2, the growth of the yeast was significantly inhibited compared to expression AHA2 alone. However, when PKS5 and SCaBP1^{S216D} were co-expressed with AHA2, the inhibition of AHA2 activity was abolished compared with co-expression of PKS5 and SCaBP1 (Figure 7). PKS5 and SCaBP1^{S216A} had a similar effect on AHA2 as PKS5 and SCaBP1^{S216D} (Figure 7). These results demonstrate that residue S216 in SCaBP1 is essential for PKS5 mediated inactivation of AHA2 in the yeast system.

**Discussion**

Outside stimuli increase cytoplasmic calcium concentration and this in turn triggers plant responses. SCaBP family proteins are a group of calcium sensors that physically interact
with and activate PKS kinases. It is likely that ion transporters are the preferential targets of the SCaBP-PKS complex (Quintero et al., 2002; Qiu et al., 2002; Gong et al., 2002; Xu et al., 2006; Fuglsang et al., 2007; Quan et al., 2007; Batelli et al., 2007). In this study, we determined that phosphorylation of SCaBP proteins by PKS kinases is a common regulatory mechanism in Arabidopsis. The phosphoserine is located in the conserved PFPP motif at the C-terminus of SCaBP proteins and this phosphorylation enhances the interaction between PKS kinases and SCaBP calcium sensors.

Previous genetic studies suggested that SCaBP proteins functions upstream of PKS kinases (Halfter et al., 2000; Xu et al., 2006; Fuglsang et al., 2007; Quan et al., 2007). The SOS pathway is described, as SOS3 and SCaBP8 perceives calcium signal evoked by salt stress, interacts with and activates SOS2, recruits SOS2 to plasmas membrane, and this protein complex further activates SOS1. It is believed that is general regulatory mechanism for other SCaBP-PKS pathways. Upon the perception of a calcium signal, SCaBP calcium sensors interact with and activate PKS kinases and that in turn triggers downstream responses (Halfter et al., 2000; Xu et al., 2006; Fuglsang et al., 2007).

Phosphorylation modification is critical for protein precise function in vivo. Our finding of phosphorylation of SCaBP8 by SOS2 led to the discovery that the phosphorylation is important for SCaBP8 function in salt tolerance by stabilizing the plasma membrane localization of the SCaBP8-SOS2 complex (Lin et al., 2009). In this study, we further determined that the phosphorylation of SCaBP proteins by PKS kinases exists in other functional interaction SCaBP-PKS pairs. These results provide important insights into the regulation of activities of the PKS protein kinases by SCaBP calcium sensors.

SCaBP calcium sensors interact with PKS protein kinases through the FISL motif that is conserved in all PKS proteins (Guo et al., 2001; Gong et al., 2004). In this study, we found that the phosphorylation of SCaBP proteins by PKS kinases locates in the PFPP motif, a 23 amino-acid fragment, which is found in all SCaBP proteins and located at
their C-terminus. It is known that various parts of SOS3 participate in its binding to SOS2 and no single motif was found to be sufficient for SOS2 binding (Ishitani et al., 2000). Analysis of SOS3-SOS2 (Sanchez-Barrena et al., 2007) and CBL2/SCaBP1-CIPK14/PKS24 (Akaboshi et al., 2008) complex structures suggest that the recognition process of PKS kinases by their interacting calcium sensor SCaBPs is similar: the FISL motif inserts into a hydrophobic cleft generated by SCaBP/CBL, however, the specificity may be conferred by calcium-binding and protein-interaction activity.

Crystal structure analysis of the SOS3-SOS2 C-terminal domain revealed that calcium stabilizes the SOS3-SOS2 complex and that the C-terminal EF4 calcium-binding site of SOS3 may be responsible for activating SOS2 (Sanchez-Barrena et al., 2007). These findings suggest that calcium is involved in both the interaction of SOS3 with SOS2 and the activation of SOS2 by SOS3. The manners of SCaBPs/CBLs binding to calcium may provide the structural basis and specificity for the interaction between SCaBPs and PKSes (Nagae et al., 2003; Sanchez-Barrena et al., 2005; Sanchez-Barrena et al., 2007; Akaboshi et al., 2008). Phosphorylation of SCaBP8 by SOS2 enhances their interaction. However, this is independent of calcium (Lin et al., 2009). These results suggest that calcium signal and phosphorylation modification may function in separate steps in regulating the PKS-SCaBP interaction. This is consistent with that calcium has a less effect on the interactions between SCaBP1/CBL2 and the regulatory domain of PKS24/CIPK14 (Akaboshi et al., 2008). The structural differences between SCaBP1 and SOS3 are mainly in their N- and C-terminal domains. The crystal structure of the C-terminus of SCaBP1 has been examined in detail (Nagae et al., 2003), whereas that of SOS3 was found to be disordered (Sanchez-Barrena et al., 2005). This may imply a reason that SCaBP1 is phosphorylated by both PKS5 and PKS24 and SOS3 is not phosphorylated by SOS2. These results suggest the PFPF motif of SCaBP proteins may confer specificity and strength to the regulation of their target protein kinases.
SCaBP1 is phosphorylated by PKS5 and PKS24 at its C-terminal serine\(^{216}\) and only phosphorylation at serine\(^{216}\) was detected by LC/MS/MS. The SCaBP1\(^{S216D}\) mutation enhances SCaBP1 interaction with PKS5, while the SCaBP1\(^{S216A}\) mutation reduces this interaction. However, both of the mutations abolish the inhibition of AHA2 activity in yeast system, suggesting that formation of a tight complex might change the effect of SCaBP1 and PKS5 on downstream targets. The serine\(^{S/A}\) mutation in the PFPF motif reduces the interaction between SCaBP and PKS proteins, but does not block the interaction and affect the PKS kinase activity (Lin et al., 2009), suggesting the phosphorylation mechanism may be involved in fine-tuning the regulatory activity of SCaBP calcium sensors.

Materials and Methods

Construction of Plasmids

PKS and SCaBP cDNAs were obtained by reverse transcription PCR (RT-PCR) from wild-type Arabidopsis (Columbia ecotype). PKS5, PKS24, CIPK23, SCaBP1, SCaBP1\(^{S216A}\), SCaBP1\(^{S216D}\), CBL1, CBL1\(^{S201A}\), CBL1\(^{S201D}\), CBL9, CBL9\(^{S201A}\) and CBL9\(^{S201D}\) CDS were cloned into the BamHI-SalI or SpeI-KpnI sites in pCAMBIA1307-6myc or pCAMBIA1307-3Flag binary vector, respectively. To produce PKS5, PKS24, SCaBP1, CBL1, CBL9 and the mutant GST fusion protein, the DNA fragments were excised from corresponding pCAMBIA1307-6myc or pCAMBIA1307-3Flag plasmid and subcloned into the BamHI-SalI sites in pGEX-6p-1 vector. CIPK23 was amplified using the 6myc-CIPK23 as templates and cloned into BamHI-NotI sites in the pGEX-6p-1 or pET-28a–c vector. All primers and plasmid constructs used in these studies are listed in supplemental Table 1 online.

Protein Purification 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 (LB) medium with ampicillin (100µg/ml) or kanamycin (100µg/ml) until the OD$_{600}$ reached 1.0. Recombinant protein expression was induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside for 12 hr at 20°C. The recombinant proteins were affinity-purified according to the manufacturer's protocol, and analyzed by SDS-PAGE.

The kinase assays were performed as described as previously (Lin et al., 2009). The kinase buffer included 20 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 10 µM ATP, and 1 mM DTT. The kinase reaction was in a total volume of 20 µl and was started by adding 0.5 µl of $\gamma$-32P-ATP (5 µCi), and the mixtures were immediately incubated at 30°C for 0.5 hr. Reactions were terminated by adding 6×SDS loading buffer and incubating at 95°C for 5 min. The reaction mixtures were separated by 12% (w/v) SDS-PAGE and stained with Commassie Brilliant Blue R-250. Then the gels were exposed to a phosphor screen (Amersham Biosciences). After 12 hr of exposure, signals were captured with a Typhoon 9410 phosphor imager (Amersham Biosciences).

**Phosphopeptide enrichment and mass spectrometry analysis**

The recombinant SCaBP1 protein was digested with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH$_4$HCO$_3$ (pH 7.8) at 37 °C overnight. The resulting peptide mixture was desalted through a C18 reversed phased cartridge and then vacuum dried in a CentriVap concentrator (Labconco, Kansas City, MO, USA). The dry peptides were esterified in dry methanol/thionyl chloride (25:1 vol/vol) for 1 hr at room temperature. The mixture was vacuum-dried again and the resulting peptide methyl esters were reconstituted in acetonitril/methanol/0.01% acetic acid (1:1:1 vol/vol/vol). The phosphorylated peptide methyl esters were enriched through a home-made IMAC (Immobilized Metal Affinity Chromatography) column and submitted to a QSTAR XL mass spectrometer (MDS SCIEX, Toronto, Canada) for LC/MS/MS analysis.
Preparation of anti-phosphoserine SCaBP polyclonal antibodies

Anti-phosphoserine\textsuperscript{216} (SCaBP1) polyclonal antibodies were made by AbMart (www.ab-mart.com.cn). Two 8-amino acid peptides (corresponding to amino acids 213-220 of SCaBP1 and 198-205 of CBL1/9) with N-terminal cysteines, Cys-TFPpSFVFH-NH\textsubscript{2} (phosphorylated form) and Cys-TFPSFVFH-NH\textsubscript{2} (non-phosphorylated form), were also synthesized by AbMart. The serine phosphospecific peptide (Cys-TFPpSFVFH-NH\textsubscript{2}) was used to produce polyclonal phosphospecific antibodies and the non-phosphorylated peptide (Cys-TFPSFVFH-NH\textsubscript{2}) was used for screening and purification.

Co-immunoprecipitation assays and SCaBP proteins phosphorylation in planta

Transgenic plant seedlings expressing 35S:6myc-SCaBP\textsubscript{8} and 35S:6myc-SCaBP\textsubscript{8S237A} was treated with 100mM NaCl for 12 hours. Plant protein was extracted using 2× cold extraction buffer containing 10 mM Tris-Cl, 150 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP-40, 2× protease inhibitor (Roche) and 2× phosphatase inhibitor (Roche). The resulting samples were then analyzed by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore). The blots were probed with primary anti-SCaBP\textsubscript{8}-phosphoserine\textsuperscript{237} or anti-myc (Sigma-Aldrich) antibodies. Plasmids of 35S:6myc-PKS5 or 35S:6myc-PKS24 were co-transformed with 35S:3flag-SCaBP\textsubscript{1}, or 35S:3flag-SCaBP\textsubscript{1S216A}, respectively; and 35S:6myc-CIPK23 was co-transformed with 35S:3flag-CBL\textsubscript{1}, 35S:3flag-CBL\textsubscript{1S201A}, 35S:3flag-CBL\textsubscript{9}, or 35S:3flag-CBL\textsubscript{9S201A} into Arabidopsis protoplasts. The protoplasts were lysed with the extraction buffer, and immunoprecipitated with anti-Flag antibody-conjugated agarose (Sigma-Aldrich). The immunoprecipitates were analyzed by western blot with the anti-phosphoserine antibodies.

For co-immunoprecipitation assays, the protoplasts were incubated for 12h after
transformed, and lysed, sonicated and centrifuged. Ten µL of anti-myc or anti-Flag conjugated agarose (Sigma) was incubated with the extract supernatant overnight at 4°C. The co-immunoprecipitation products were detected via immunoblot analysis using anti-myc or anti-flag antibodies.

**Yeast two-hybrid assays**

PKS5, PKS24 and CIPK23 were amplified using the myc-tag plasmids as templates and cloned into the pGBKT7 vector. SCaBP1, SCaBP1S216A, SCaBP1S216D, CBL1, CBL1S201A, CBL1S201D, CBL9, CBL9S201A and CBL9S201D were amplified using the flag-tag plasmids as templates and cloned into the pGADT7 vector. All primers were used for these constructs are listed in Supplemental Table 1 online.

Yeast strain AH109 was used in the yeast two-hybrid assay. Yeast transformation, growth assays and β-galactosidase activity assays were performed as described in the Yeast Protocols Handbook (Clontech).

**Yeast complementation Assays**

SCaBP1S216A and SCaBP1S216D were amplified and cloned into NotI sites in the pMP1645 vector using the primer pairs SCaBP1 NotIF and SCaBP1 NotIR (see Supplemental Table 1). PKS5 and AHA2 were co-transformed with SCaBP1, SCaBP1S216A or SCaBP1S216D, respectively, into Saccharomyces cerevisiae strain RS-72 (Mat a; adel-100 his4-519 leu2-3,312 pPMA1::pGAL1) for complementation tests. Growth of the transformed yeast on solid and liquid medium was performed as described previously (Fuglsang et al., 2007).

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REFERENCES


FIGURE LEGENDS

Figure 1. SCaBP proteins were phosphorylated by their functional interacting PKS kinases.

(A) Phosphorylation of SCaBP8 by SOS2. Coomassie blue-stained polyacrylamide gel (left panel); SOS2 auto- and SCaBP8 trans-phosphorylation activity (right panel).

(B) Phosphorylation of SCaBP1 by PKS5. Coomassie blue-stained polyacrylamide gel (left panel); PKS5 auto- and SCaBP1 trans-phosphorylation activity (right panel).

(C) Phosphorylation of SCaBP1 by PKS24. Coomassie blue-stained polyacrylamide gel (left panel); PKS24 auto- and SCaBP1 trans-phosphorylation activity (right panel).

(D) Phosphorylation of CBL1 by CIPK23. Coomassie blue-stained polyacrylamide gel (left panel); CIPK23 auto- and CBL1 trans-phosphorylation activity (right panel).

(E) Phosphorylation of CBL9 by CIPK23. Coomassie blue-stained polyacrylamide gel (left panel); CIPK23 auto- and CBL9 trans-phosphorylation activity (right panel).

SC, SCaBP.

Figure 2. The phosphorylation site in the SCaBP1 is mapped to the PFPF motif at the serine^216 by LC/MS/MS.

(A) Parent ion of the triply-charged peptide DITTTFPSFVFHSQVEDT with one phosphate group.

(B) CID spectrum of DITTTFPpSFVFHSQVEDT. The major y- and b-type product ions are indicated.

Figure 3. Amino acids sequence alignment of the Arabidopsis SCaBP proteins.
The phosphoserine residue is conserved in the PFPF motif at C-terminus of SCaBP proteins. The alignment is generated with DNAMAN software. Conserved and identical amino acids sequences are shown on a black background. The EF hand and the PFPF motif were underlined in black line. Arrows indicate the conserved serine/threonine
residue and stars indicate the conserved amino acids in the PFPP motif. SC, SCaBP.

Figure 4. Phosphorylation of the conserved serine in the PFPP motif by PKS kinases.

(A) Phosphorylation of SCaBP8$^{S237}$ by SOS2 is induced by salt stress. SCaBP8 proteins were immunoprecipitated from the transgenic plants expressing 6myc-SCaBP8 and 6myc-SCaBP8$^{S237A}$ and detected with anti-myc antibody (top panel) or anti-phosphoserine$^{237}$ antibodies (bottom panel). Seedlings were treated with water or 100 mM NaCl for 12h.

(B) Phosphorylation of SCaBP1$^{S216}$ by PKS5 in vivo. Myc-PKS5 was co-transformed with flag-SCaBP1 or flag-SCaBP1$^{S216A}$ into Arabidopsis protoplasts. PKS5 was detected by myc-antibody (top panel) and SCaBP1 was detected by anti-flag antibody (middle panel). Flag-SCaBP1 and flag-SCaBP1$^{S216A}$ were immunoprecipitated and phosphorylation of serine$^{216}$ was detected by anti-phosphoserine$^{216}$ antibodies (bottom panel).

(C) Phosphorylation of SCaBP1$^{S216}$ by PKS24 in vivo. Myc-PKS24 was transformed or co-transformed with flag-SCaBP1 or flag-SCaBP1$^{S216A}$ into Arabidopsis protoplasts. SCaBP1 was detected by anti-flag antibody (top panel) and PKS24 was detected by myc-antibody (middle panel). Flag-SCaBP1 and flag-SCaBP1$^{S216A}$ were immunoprecipitated and phosphorylation of serine$^{216}$ was detected by anti-phosphoserine$^{216}$ antibodies (bottom panel).

(D) Phosphorylation of CBL1$^{S201}$ and CBL9$^{S201}$ by CIPK23 in vivo. Myc-CIPK23 was co-transformed with flag-CBL1, flag-CBL1$^{S201A}$, flag-CBL9, or flag-CBL9$^{S201A}$ into Arabidopsis protoplasts. CIPK23 was detected by myc-antibody (top panel) and CBL1/9 was detected by anti-flag antibody (middle panel). Flag-CBL1, flag-CBL1$^{S201A}$, flag-CBL9, and flag-CBL9$^{S201A}$ were immunoprecipitated and phosphorylations of serine$^{201}$ were detected by anti-phosphoserine$^{201}$ antibodies.

(E) SCaBP1 was not phosphorylated by SOS2 in vivo. Flag-SCaBP1 was co-transformed
with \textit{myc-SOS2} or \textit{myc-PSK5} into \textit{Arabidopsis} protoplast. PKS5 and SOS2 were detected by myc-antibody (top panel) and SCaBP1 was detected by anti-flag antibody (middle panel). Flag-SCaBP1 was immunoprecipitated and phosphorylation of serine\textsuperscript{216} was detected by anti-phosphoserine\textsuperscript{216} antibodies (bottom panel).

SC, SCaBP.

Figure 5. Phosphorylation of SCaBP proteins by PKS kinases enhances their interaction in yeast two-hybrid assays. PKS CDS in the pGBKKT7 vector were co-transformed with their interaction SCaBP, SCaBP\textsuperscript{S/D} or SCaBP\textsuperscript{S/A} in the pGADT7 vector into yeast strain AH109. A, PKS5-SCaBP1; B, PKS24-SCaBP1; C, CIPK23-CBL1; and D, CIPK23-CBL9. Growth of the transformed yeast was assayed on media minus Trp and Leu (left panel), minus Trp, Leu and His with 2.5 mM 3-AT (middle panel) or minus Trp, Leu and His with 5 mM 3-AT (bottom panel). 3-AT, 3-amino-1,2,4-triazole.

(E) to (H), Quantitative \(\beta\)-galactosidase assays were performed. Yeast containing various PKS and SCaBP proteins were tested for \textit{LacZ} activity using ONPG as substrate. E, PKS5-SCaBP1; F, PKS24-SCaBP1; G, CIPK23-CBL1; and H, CIPK23-CBL9. All data represent means±SEM (n=3). SC, SCaBP.

Figure 6. Phosphorylation of SCaBP proteins by PKS kinases enhances their interaction in Arabidopsis. Myc-PKS plasmids were co-transformed with \textit{flag-SCaBP}, \textit{flag-SCaBP\textsuperscript{S/D}} or \textit{flag-SCaBP\textsuperscript{S/A}} plasmids into \textit{Arabidopsis} protoplasts, respectively. A, transformation of PKS5 and SCaBP1; B, transformation of PKS24 and SCaBP1; C, transformation of CIPK23 and CBL1; and D, transformation of CIPK23 and CBL9. Total protein was analyzed using anti-myc and anti-flag antibodies (Input). After immunoprecipitation with anti-flag or anti-myc conjugated agarose as labeled, the PKS or SCaBP proteins in the
pellet were detected via immunoblot analysis using anti-myc or anti-flag antibody (Output). SC, SCaBP.

Figure 7. The phosphorylation of SCaBP1\textsuperscript{S216} is required for regulating AHA2 activity. SCaBP1, SCaBP1\textsuperscript{S216A} or SCaBP1\textsuperscript{S216D} was expressed in the yeast RS-72 strain harboring two plasmids AHA2 and PKS5. A suspension of each transformed strain was diluted in sterile water at three concentrations (OD\textsubscript{600}=0.1, 0.01 and 0.001), and spotted on solid media either containing galactose (left panel) or glucose (right panel) pH 6.5. The growth of the cells was monitored 4 days after transfer. All data represent means of three replicate experiments.
Supplemental Figure 1. Phosphorylation of the conserved serine in the PFPF motif by PKS kinases in vitro.

(A) and (B) phosphorylation of SCaBP1$^{S216}$ by PKS5 (A) and PKS24 (B) in vitro. SDS-PAGE with Comassie blue-stained SCaBP1 and SCaBP1$^{S216\alpha}$ proteins (top panel); SCaBP1$^{S216}$ phosphorylation was detected by western blot using anti-phosphoserine$^{216}$ (bottom panel).

(C) and (D) Phosphorylation of CBL1$^{S201}$ and CBL9$^{S201}$ by CIPK23 in vitro. SDS-PAGE with Comassie blue-stained CBL1, CBL1$^{S210\alpha}$ (C), or CBL9 and CBL9$^{S201\alpha}$ proteins (D) (top panel); CBL1$^{S201}$ and CBL9$^{S201}$ phosphorylation was detected by western blot using anti-phosphoserine$^{201}$ (bottom panel).

(E) phosphorylation of SCaBP8$^{S237}$ by SOS2 in vitro. SDS-PAGE with Comassie blue-stained SCaBP8 and SCaBP8$^{S237\alpha}$ proteins (top panel); SCaBP8$^{S237}$ phosphorylation was detected by western blot using anti-phosphoserine$^{237}$ (bottom panel).

Supplemental Figure 2. Expression of PKS and SCaBP proteins in yeast.
Proteins were extracted from the yeast expressing c-Myc-PKS and HA-SCaBP (Figure 5): A, PKS5-SCaBP1; B, PKS24-SCaBP1; C, CIPK23-CBL1 and D, CIPK23-CBL9.
Expression level of PKS (top panel) and SCaBP and their mutants (middle panel) was detected by western blotting with anti-cMyc and anti-HA antibodies, respectively.
SDS-PAGE with Coomassie blue-stained total yeast proteins (bottom panel).
Supplemental Table 1

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