Expression, Activation, and Biochemical Properties of a Novel Arabidopsis Protein Kinase[1]

Deming Gong, Zhizhong Gong, Yan Guo, and Jian-Kang Zhu*

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

An Arabidopsis *SOS2* (*salt overly sensitive 2*)-like protein kinase gene, *PKS6*, was expressed in leaves, stems, and siliques, but not detectable in roots of adult plants; its expression in young seedlings was up-regulated by abscisic acid. To determine the biochemical properties of the PKS6 protein, we expressed the PKS6 coding sequence as a glutathione S-transferase fusion protein in *Escherichia coli*. The bacterially expressed glutathione S-transferase-PKS6 fusion protein was inactive in substrate phosphorylation. We have constructed constitutively active forms of PKS6 by either a deletion of its putative auto-inhibitory FISL motif (i.e. PKS6ΔF) or a substitution of threonine-178 with aspartic acid within the putative activation loop. We found that PKS6ΔF exhibited a strong preference for Mn^{2+} over Mg^{2+} as a divalent cation cofactor for kinase activity. PKS6ΔF displayed substrate specificity against three different peptide substrates and had an optimal pH of approximately 7.5 and temperature optimum of 30°C. The apparent K_m values for ATP and the preferred peptide substrate p3 of PKS6ΔF were determined to be 1.7 and 28.5 μM, respectively. These results provide significant insights into the regulation and biochemical properties of the protein kinase PKS6. In addition, the constitutively active, gain-of-function kinase mutants will be invaluable for future determination of the in planta function of PKS6.

Perception and transduction of environmental cues is critical for the immotile plants. Protein kinases are involved in cellular signaling and metabolic regulation in plants. The Arabidopsis genome encodes more than 1,000 typical protein kinases (Arabidopsis Genome Initiative, 2000). Many developmental, environmental, and hormonal signals induce a transient increase in cytoplasmic calcium levels in plant cells. In most cases, the calcium signal is then transduced via a combination of protein phosphorylation and dephosphorylation cascades. It has been thought that the majority of calcium-stimulated protein phosphorylation is carried out by calcium-dependent protein kinases or calmodulin-like domain protein kinases (CDPKs) in plants (Trewavas and Malho, 1997; Sanders et al., 1999). This family of protein kinases, currently known only in plants and protozoa, contains a kinase catalytic domain fused with a calmodulin-like regulatory domain, which enables activation directly through calcium binding (Roberts and Harmon, 1992; Roberts, 1993). In animals, two major types of protein kinases, calcium/calmodulin-dependent kinases (CaMKs) and protein kinase C (PKC), are known to decode calcium signals.

We have recently isolated and characterized the Arabidopsis *SOS2* gene that encodes a Ser/Thr protein kinase with an N-terminal kinase catalytic domain similar to SNF1/AMPK (Hardie, 1999) and a novel C-terminal regulatory domain (Liu et al., 2000). SOS2 interacts with and is activated by a myristoylated EF-hand calcium-binding protein SOS3 (salt overly sensitive 3; Liu and Zhu, 1998; Halfter et al., 2000; Ishitani et al., 2000). The FISL motif, a 21-amino acid sequence in the C-terminal regulatory domain of SOS2, has been recently shown to be necessary and sufficient to bind SOS3 (Guo et al., 2001). Arabidopsis genome contains 23 *SOS2*-like protein kinase genes (*PKSes*). Recent studies suggest that the family of PKSes in plants may be functionally analogous to CaMKs in animals (Guo et al., 2001).

To elucidate the biochemical properties and physiological functions of the PKS protein kinases, we have recently cloned several of these *PKSes* and expressed them in *Escherichia coli*. However, none exhibited substrate phosphorylation activity in the absence of specific SOS3-like calcium-binding proteins (SCaBPs). It, thus, became necessary to create constitutively active forms of the PKS proteins. These constitutively active PKSes are critical for investigation of the in planta functions of these novel protein kinases as well as for the determination of their enzymatic properties.

In this paper, we report the gene cloning and expression, and activation and enzymatic properties of an Arabidopsis SOS2 homolog (PKS6). *PKS6* was differentially expressed in different organs of adult plants, and its expression was up-regulated by abscisic acid (ABA). Constitutively active forms of PKS6 were constructed by a deletion of the FISL motif in the C-terminal regulatory domain or a substitution of Thr-178 with Asp within the putative activation loop (Johnson et al., 1996) in the N-terminal kinase catalytic domain. These results indi-

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* Corresponding author; e-mail jkzhu@ag.arizona.edu; fax 520–621–7186.

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cate that PKS6 kinase activity is controlled by both auto-inhibition and phosphorylation-dependent activation in the activation loop. We have also determined the cofactor preference, substrate specificity, pH and temperature dependence, and kinetic properties of PKS6. Our results provide significant insights into the regulation and biochemical properties of this novel protein kinase.

RESULTS

Tissue-Specific Expression and ABA Regulation of PKS6 Transcript

We obtained the open reading frame of *PKS6* cDNA by reverse transcriptase-PCR. *PKS6* contains an open reading frame of 1,344 bp, and the deduced protein consists of 448 amino acid residues with a calculated molecular mass of 50.5 kD. As the first step toward elucidating the physiological functions of PKS6 in plants, the steady-state levels of *PKS6* transcript in different tissues and under various stresses were investigated by northern-blot analysis. Total RNA was extracted from various organs of adult Arabidopsis plants or from stress-treated young seedlings. RNA blots were hybridized to a radiolabeled *PKS6*-specific DNA probe. *PKS6* was expressed in leaves, stems, flowers, and siliques. Interestingly, *PKS6* was not detectable in Arabidopsis roots (Fig. 1A). The transcript abundance of *PKS6* in young seedlings (Fig. 1B) was lower than those in most organs (except roots) of mature plants, suggesting developmental regulation of *PKS6* gene expression. To investigate potential regulation of *PKS6* expression by environmental and hormonal factors, we analyzed the transcript levels of *PKS6* in young Arabidopsis seedlings treated with salt, cold, drought, or ABA. *PKS6* expression was significantly increased by ABA treatment and slightly increased by salt and drought treatments but was not affected by cold treatment (Fig. 1B).

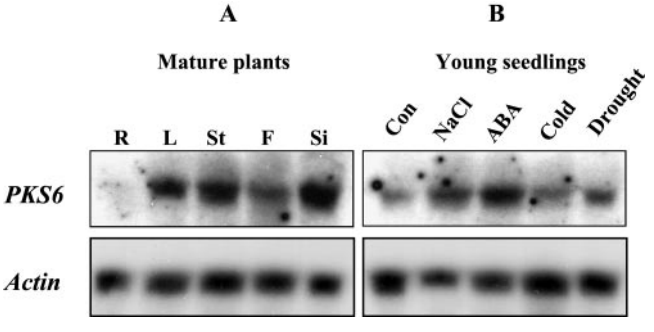

Figure 1. Expression of *PKS6* in different tissues and in response to various stresses analyzed by northern blot. A, Expression of *PKS6* in different tissues of mature Arabidopsis plants. R, Roots; L, leaves; St, stems; F, flowers; and Si, siliques. B, Expression of *PKS6* under different stress conditions. Con, Control; NaCl, 300 mM NaCl for 5 h; ABA, 100 μM ABA for 3 h; Cold, 0°C for 24 h; and Drought, dehydration for 30 min. Young seedlings that showed low *PKS6* transcript level were subjected to the above treatments so that an induction of *PKS6* expression could be determined. Twenty micrograms of total RNA was analyzed by RNA gel blotting. The blot was hybridized with the gene-specific DNA probe for *PKS6*. The northern blot was exposed to x-ray film for 7 d. Actin is shown as a loading control (exposed to x-ray film for 1 d).

Deletion of the FISL Motif or Substitution of Thr-178 with Asp Activates PKS6

To further characterize the enzymatic properties of PKS6, we constructed a PKS6 fusion protein in which glutathione S-transferase (GST) was fused in frame to the N-terminal end of PKS6. After expression in bacteria, the GST-fusion protein was affinity-purified by glutathione Sepharose. Kinase assays indicated that the purified GST-PKS6 fusion protein was unable to phosphorylate a number of commonly used protein or peptide substrates (data not shown). Therefore, we wanted to generate activated forms of PKS6.

We have recently defined the auto-inhibitory and activation domains of SOS2 and created constitutively active SOS2 mutant proteins (Guo et al., 2001). Like SOS2, PKS6 has a FISL motif in the C-terminal regulatory domain (Fig. 2, A and B). The FISL motif in SOS2 is necessary and sufficient to bind SOS3 and to keep SOS2 inactive in substrate phosphorylation (Guo et al., 2001). We hypothesized that the FISL motif in PKS6 may also be auto-inhibitory to the kinase activity. To test this, we constructed a PKS6 mutant, designated PKS6ΔF, by deleting the FISL motif between Met-319 and Arg-339 (Fig. 2B) through site-directed mutagenesis. PKS6 also contains a putative "activation loop" in the N-terminal kinase catalytic domain, located between the conserved Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) sequences (Fig. 2, A and B). This indicates that Thr-178 in the putative activation loop could be the critical target site for phosphorylation by an upstream activating kinase(s). To investigate whether or not a substitution of Thr-178 with Asp that partially mimics this phosphorylation is sufficient to activate PKS6, we constructed an activation loop mutant, designated PKS6T/D, by changing Thr-178 to Asp (Fig. 2B) through site-directed mutagenesis.

The wild type (designated PKS6WT) and PKS6 mutants were expressed in *E. coli* as GST-fusion proteins, and purified by affinity chromatography on glutathione Sepharose (Fig. 3A). An apparent molecular mass of approximately 80 kD, the expected size from the deduced amino acid sequence of PKS6, was observed for these purified GST-fusion proteins. The PKS family of proteins tested thus far does not show any kinase activity against commonly used protein substrates, such as myelin basic protein, histone H1, and casein. However, a Ser-containing synthetic peptide p3 (ALARAASAAALARRR), derived from the recognition sequences of PKC or SNF1/AMPK, is known to be phosphorylated by SOS2 (Halfter et al., 2000).

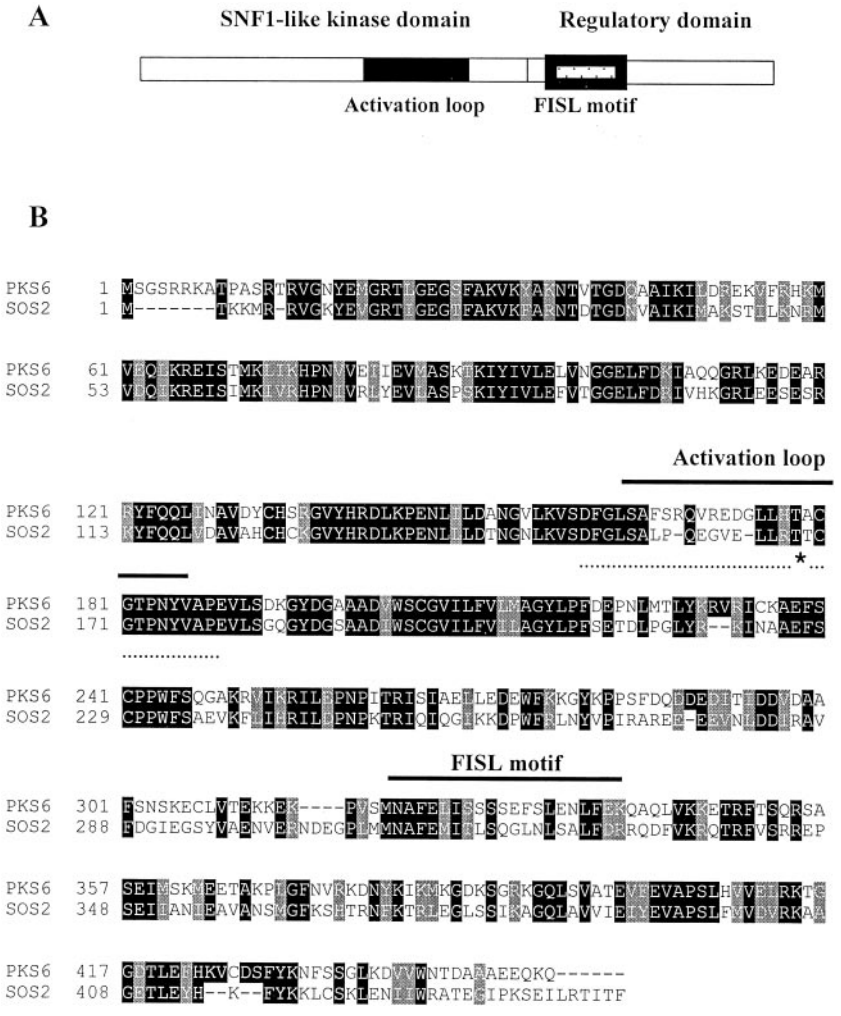

Figure 2. Alignment of the deduced amino acid sequences of PKS6 and SOS2. A, Schematic diagram of the domain structure of SOS2. B, Alignment of PKS6 and SOS2 was generated using multiple sequence alignment analysis and processed using "boxshade" at http://www.ch.embnet.org/. PKS6 is identical to gene product with GenBank accession number AAF26468. The open reading frame of *PKS6* was amplified by reverse transcriptase-PCR, cloned, and completely sequenced (data not shown). Amino acids are numbered on the left. Identical residues and conservative replacements are shown with black and gray shading, respectively. The N-terminal kinase catalytic domain is highly conserved. The C-terminal non-catalytic regulatory domain contains the conserved FISL motif (marked). Also marked is the putative activation loop between the conserved DFG and APE motifs (dots) and the conserved Thr residue (asterisk) that may be phosphorylated by an upstream protein kinase(s). Dashed lines represent spaces that were introduced to maximize alignment.

We, thus, measured phosphorylation activity with the peptide p3 as a substrate and autophosphorylation activity for the mutant and wild-type kinases in the presence of 5 mM Mg^{2+}. The activation loop mutant PKS6T/D exhibited a 36-fold higher activity in p3 phosphorylation than PKS6WT (Fig. 3B). The FISL motif deletion mutant PKS6ΔF was found to be even more active than PKS6WT, with an 80-fold increase in substrate phosphorylation activity compared with PKS6WT (Fig. 3B). Both PKS6 mutants also had higher autophosphorylation activity than PKS6WT (data not shown). Because of its high kinase activity, we chose PKS6ΔF for further biochemical analysis.

Biochemical Properties of PKS6ΔF

Little is known about the enzymatic properties of the Arabidopsis PKS proteins. The constitutively active forms of PKS6 provide us with invaluable materials to study the biochemical properties of these novel protein kinases. To investigate the cofactor requirement of PKS6ΔF, we measured substrate phosphorylation activity in the presence of various concentrations of Mg^{2+} or Mn^{2+}. As shown in Figure 4, p3 phosphorylation by PKS6ΔF was dependent on either divalent cation. PKS6ΔF exhibited no p3 phosphorylation activity in the absence of Mg^{2+} or Mn^{2+}. Substrate phosphorylation activity of PKS6ΔF increased as the concentration of Mn^{2+} or Mg^{2+} increased from 0 to 2.5 or 5.0 mM. Mg^{2+} at concentrations of less than 1 mM did not activate PKS6ΔF substrate phosphorylation activity. Optimal activation was achieved at 5 mM Mg^{2+} (Fig. 4). In contrast, as low as 0.25 mM Mn^{2+} could activate substrate phosphorylation of PKS6ΔF. Optimal activation was observed at 2.5 mM of Mn^{2+}, and higher concentrations ($>$ 5 mM Mn^{2+}) became inhibitory (Fig. 4). These results demonstrate that PKS6ΔF prefers Mn^{2+} over Mg^{2+} as a cofactor for substrate phosphorylation.

To determine the cofactor requirement of PKS6ΔF for autophosphorylation activity, we measured autophosphorylation activity in the presence of various concentrations of Mg^{2+} or Mn^{2+} (Fig. 5). Autophosphorylation activity of PKS6ΔF was also dependent on either Mg^{2+} or Mn^{2+}. PKS6ΔF exhibited no autophosphorylation activity in the absence of Mg^{2+} or Mn^{2+}. Mg^{2+} only weakly activated the autophos-

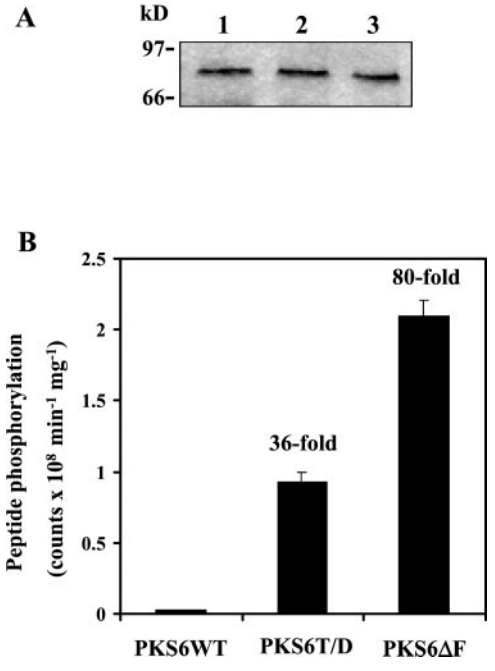

Figure 3. Activation of PKS6 by FISL motif deletion or substitution of Thr-178 with Asp within the putative activation loop. A, SDS-PAGE analysis of relevant wild-type and mutant PKS6 proteins. PKS6 wild type (PKS6WT), T to D change mutant (PKS6T/D), and FISL motif deletion mutant (PKS6ΔF) were expressed as GST-tagged fusion proteins and purified by glutathione Sepharose affinity chromatography. Purified proteins were analyzed by SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue R-250. Lanes 1 through 3, PKS6WT, PKS6T/D, and PKS6ΔF, respectively. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left. The protein standards are rabbit phosphorylase b (97 kD) and bovine serum albumin (66 kD). B, Activation of PKS6 by FISL motif deletion and substitution of Thr-178 with Asp within the putative activation loop. Peptide phosphorylation activities of PKS6WT, PKS6T/D, and PKS6ΔF were assayed using 150 μM p3 as a substrate, 10 μM ATP, and 5 mM $MgCl_2$ as described in "Materials and Methods." The number on top of each bar is -fold increase over the wild-type control. Results represent the means ± SD from three experiments.

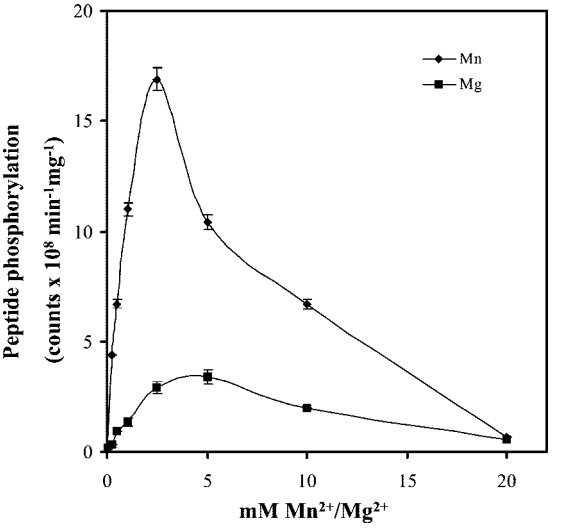

Figure 4. Dependence of substrate phosphorylation activity of PKS6ΔF on Mn^{2+} or Mg^{2+}. Phosphorylation of p3 by the kinase was determined in the presence of 0, 0.25, 0.5, 1, 2.5, 5, 10, and 20 mM of Mn^{2+} (as $MnCl_2$) or Mg^{2+} (as $MgCl_2$). Initial rates were measured and plotted against the Mn^{2+} or Mg^{2+} concentrations. Three independent experiments were performed, and the average was shown here. Error bars indicate ±SD (n = 3).

phorylation activity, and the activation required millimolar concentrations of Mg^{2+}. In striking contrast, Mn^{2+} strongly activated PKS6ΔF autophosphorylation even in the micromolar range. These results suggest that PKS6ΔF also prefers Mn^{2+} over Mg^{2+} for autophosphorylation activity. We compared the substrate phosphorylation and autophosphorylation activities of PKS6T/D and PKS6ΔF in the presence of 2.5 mM Mn^{2+}. PKS6ΔF and PKS6T/D displayed even higher peptide phosphorylation activity (186- and 76-fold higher than PKS6WT for PKS6ΔF and PKS6T/D, respectively) and autophosphorylation activity under the condition (Fig. 6, A and B).

We have previously shown that SOS2 could phosphorylate two synthetic peptide substrates p1 (LRRASLG) and p2 (VRKRTLRRL), derived from the recognition sequences of PKC or SNF1/AMPK, and p3 (Halfter et al., 2000). These three peptides were, thus, chosen to analyze the substrate specificity of PKS6ΔF in this study. To test the substrate specificity of PKS6ΔF, we compared the substrate phosphorylation activities of PKS6ΔF on two Ser-containing peptide substrates (p1 and p3) and a Thr-containing peptide substrate (p2). As shown in Figure 7, PKS6ΔF only weakly phosphorylated p2 but strongly phosphorylated both p1 and p3, with p3 giving higher

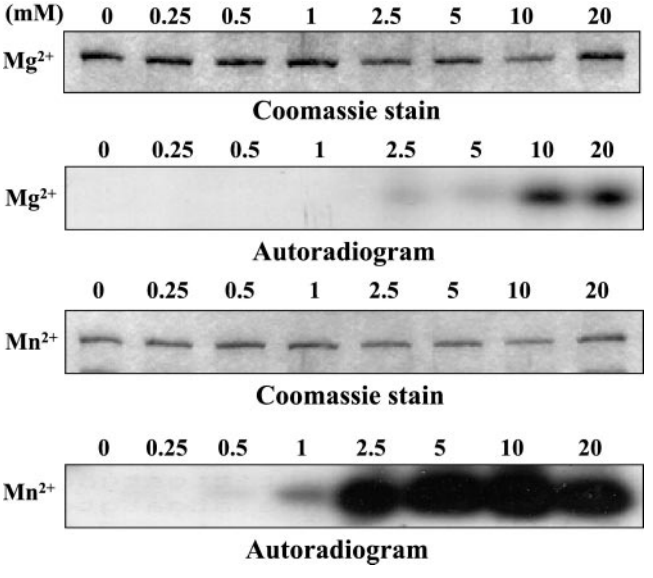

Figure 5. Dependence of autophosphorylation activity of PKS6ΔF on Mg^{2+} or Mn^{2+}. Autophosphorylation activity of PKS6ΔF in the presence of various concentrations of Mg^{2+} (as $MgCl_2$) or Mn^{2+} (as $MnCl_2$) as shown on the top of each panel was presented as the density of autoradiographic bands. Three independent experiments were performed, and a typical result is shown here.

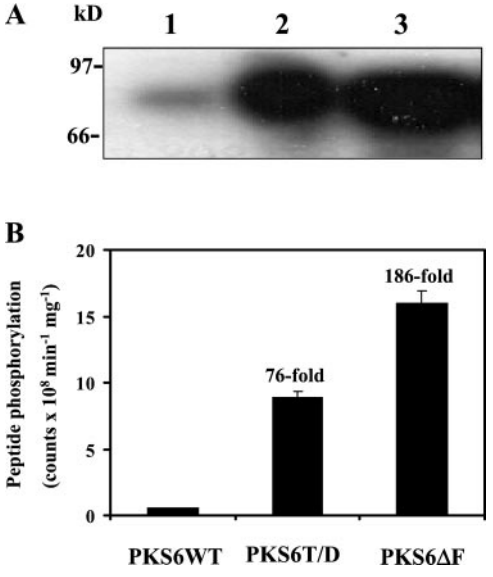

Figure 6. Autophosphorylation and peptide phosphorylation activities of PKS6WT, PKS6T/D, and PKS6ΔF in the presence of Mn^{2+}. A, Autophosphorylation of PKS6WT, PKS6T/D, and PKS6ΔF. Lanes 1 through 3, PKS6WT, PKS6T/D, and PKS6ΔF, respectively. The autoradiogram shown is representative of three independent experiments with similar results. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left. B, Peptide phosphorylation activity of PKS6WT, PKS6T/D, and PKS6ΔF. Peptide phosphorylation and autophosphorylation activities were assayed in the presence of 2.5 mM $MnCl_2$, 150 μM p3, and 10 μM ATP in the kinase buffer as described in "Materials and Methods." Error bars indicate ±SD (n = 3).

activity than p1. The results suggest that PKS6ΔF prefers p3 over p1 and p2 as a peptide substrate. To determine the pH optimum of PKS6ΔF for substrate phosphorylation activity, we monitored p3 phosphorylation across the pH range of 6.5 to 9.5 (Fig. 8A). PKS6ΔF exhibited a narrow pH-activity profile with optimal pH values between 7.0 and 7.5. Very low substrate phosphorylation activity was observed

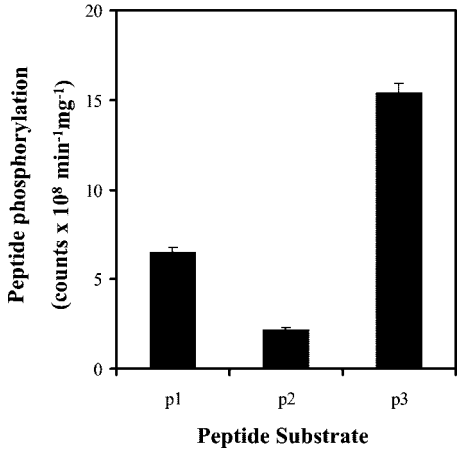

Figure 7. Substrate specificity of PKS6ΔF. PKS6ΔF was incubated with the kinase buffer containing 150 μM of peptide substrates p1, p2, or p3 at 30°C for 30 min as described in "Materials and Methods." Each result is the mean ± SD from three experiments.

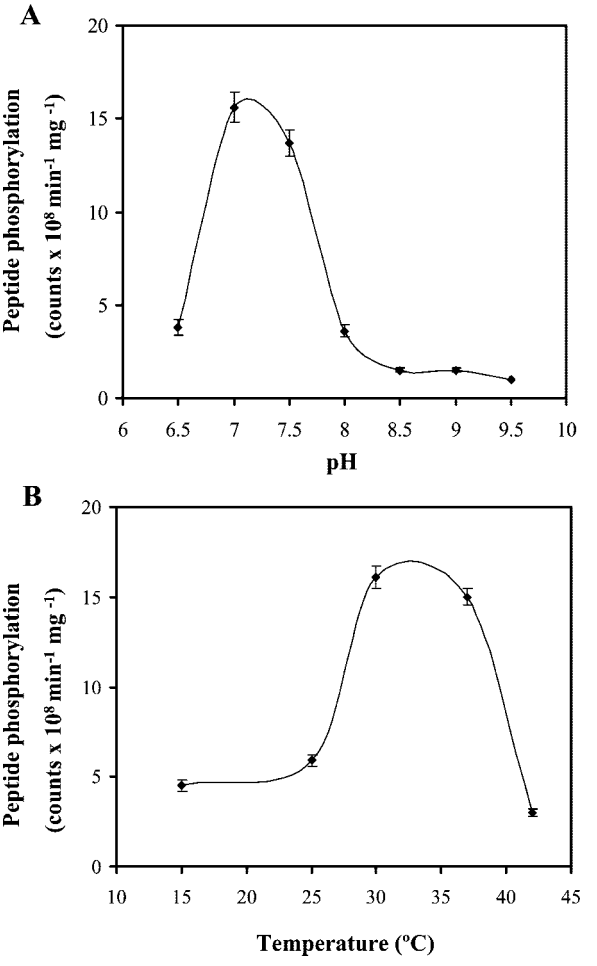

Figure 8. Dependence of substrate phosphorylation activity of PKS6ΔF on assay pH and temperature. A, pH dependence. Enzyme assays at each pH value were buffered by 20 mM BIS-TRIS propane. B, Temperature dependence. Enzyme assays were performed at each temperature indicated as described in "Materials and Methods." Error bars indicate ±SD (n = 3).

at pH values higher than 8.0 (Fig. 8A). The effect of temperatures from 15°C to 42°C on p3 phosphorylation by the kinase was also determined (Fig. 8B). The temperature optimum for substrate phosphorylation activity of PKS6ΔF was found to be approximately 30°C. The PKS6ΔF protein exhibited Michaelis-Menten kinetics with respect to ATP and the preferred peptide substrate p3. Data from three independent experiments are shown as saturation curves with specific activity (counts per minute per milligram of protein) plotted versus concentrations of the substrate p3 or ATP (Fig. 9, A and B). The K_m values of PKS6ΔF for p3 and ATP, determined from Eadie-Hofstee plots of V versus V/[S] (Fig. 9, inset), were 28.5 and 1.69 μM, respectively, indicating that PKS6ΔF has high affinity for p3 and ATP. The ratio of V_{max} to K_m was determined to be approximately 6 and 98 for p3 and ATP, respectively. In addition, ATP at concentrations of 25 μM or higher was found to

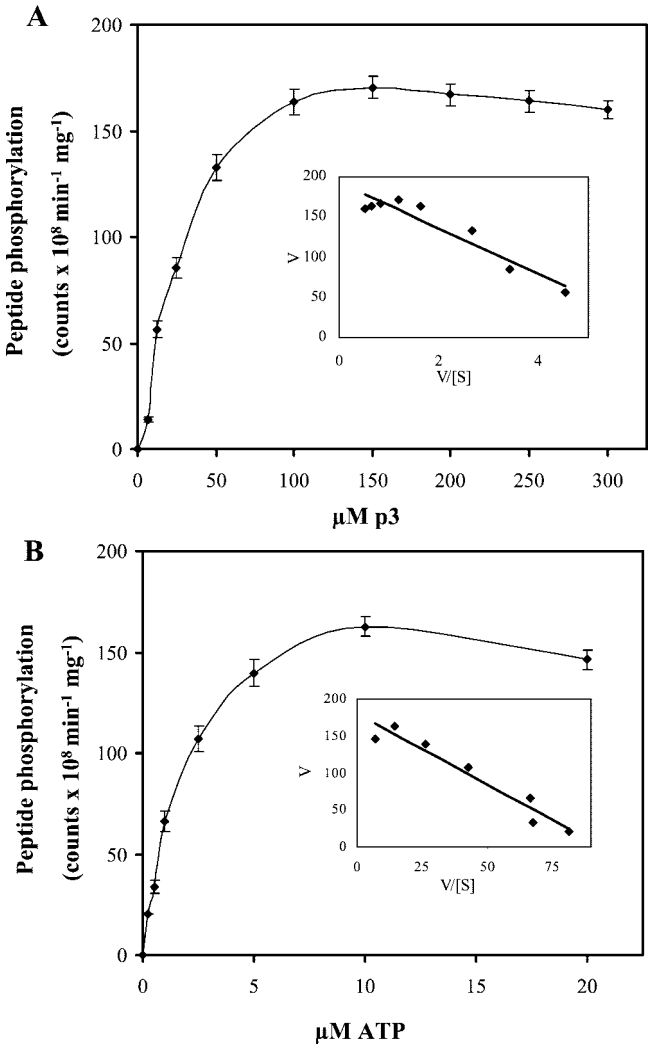

Figure 9. Dependence of substrate phosphorylation activity of PKS6ΔF on peptide substrate p3 and ATP. A, Dependence of substrate phosphorylation of PKS6ΔF on peptide substrate p3. Phosphorylation of p3 by PKS6ΔF was assayed at 30°C in the presence of 2.5 mM $MnCl_2$ as described in "Materials and Methods." Results shown are the averages of three independent assays presented as saturation curves with specific activity versus p3 concentration as indicated. ATP concentration in the assay buffer was set constant at 10 μM. B, Dependence of substrate phosphorylation of PKS6ΔF on ATP. Phosphorylation of p3 by PKS6ΔF was assayed at 30°C in the presence of 2.5 mM $MnCl_2$ as described in "Materials and Methods." Results shown are the averages of three independent assays presented as saturation curves with specific activity versus ATP concentration as indicated. P3 concentration in the assay buffer was set constant at 150 μM. The insets are Eadie-Hofstee plots of the average values for each data set. Error bars indicate ±SD (n = 3).

inhibit PKS6ΔF substrate phosphorylation activity (data not shown).

DISCUSSION

The Arabidopsis *PKS6* gene encodes a protein kinase that shares high sequence homology with SOS2, and can be classified as a member of the SnRK3 family of SNF1-related kinases (Hardie, 2000). In this study, we have shown that *PKS6* transcript was expressed in leaves, stems, and siliques, but not in roots (Fig. 1A). Another highly related gene, *PKS11*, has been recently shown to be expressed exclusively in Arabidopsis roots (J.-K. Zhu, unpublished data). These results indicate that closely related PKS isoforms have tissue-specific expression patterns and, thus, may not have redundant in planta functions. The shoot-specific expression of *PKS6* suggests that it mainly functions in photosynthetic tissues. In addition, the low transcript abundance of *PKS6* in young seedlings compared with that in mature plants strongly suggests that *PKS6* expression is developmentally regulated. *PKS6* expression was substantially up-regulated by ABA (Fig. 1B), implying that it may play a role in ABA-dependent signaling in plants. A member of the SnRK2 family of protein kinases, PKABA1 from wheat (*Triticum aestivum*), was the first protein kinase shown to be up-regulated by ABA at the transcript level (Anderberg and Walker-Simmons, 1992). PKABA1 has recently been shown to mediate ABA repression of α-amylase expression in barley (*Hordeum vulgare*) seeds (Gomez-Cadenas et al., 1999). This is the first report, to our knowledge, of transcript up-regulation of an Arabidopsis PKS/SnRK3 family kinase in response to ABA.

PKS6 contains a FISL motif in the C-terminal regulatory domain and a putative activation loop in the N-terminal kinase catalytic domain (Fig. 2B). In this study, strong activation of PKS6 was achieved by a deletion of the FISL motif. The kinase activity of SOS2 was dependent on both its interacting protein SOS3 and calcium (Halfter et al., 2000). We have recently found that SOS2 interaction with SOS3 was mediated via the FISL motif. The FISL motif is necessary and sufficient to keep SOS2 inactive and serves as an auto-inhibitory domain (Guo et al., 2001). Our result with the FISL motif deletion mutant of PKS6 shows that the FISL motif is also auto-inhibitory in PKS6. In our preliminary experiments of this study, we found that SOS3 did not activate PKS6. A yeast two-hybrid assay also showed that PKS6 interacted very weakly with SOS3 (Guo et al., 2001). PKS6 did display preferential interaction with SCaBP1, SCaBP5, and SCaBP6. Whether these proteins could modulate auto-inhibition of PKS6 remains to be determined.

In this study, activation of PKS6 was also achieved by a substitution of the conserved Thr residue with Asp within the putative activation loop. The result suggests that PKS6 may be activated in vivo through activation loop phosphorylation at the Thr residue by an upstream kinase(s). Further studies are required to identify an upstream kinase(s) that phosphorylates the Thr residue in the PKS catalytic segment and to fully elucidate the molecular mechanisms of PKS activity regulation. Thr residues in the activation

loop of catalytic subdomain VIII of an Arabidopsis somatic embryogenesis receptor kinase AtSERK1 have recently been suggested to be potential targets for phosphorylation (Shah et al., 2001). In addition, Ser-164 in the putative activation loop of PKS6 and all other PKSes is absolutely conserved (Fig. 2B; data not shown). Phosphorylation at Ser-744 and Ser-748 within the activation loop of protein kinase D (PKD) has been observed recently during PKC-mediated PKD activation (Waldron et al., 2001). A PKD mutant with both Ser residues substituted with Glu residues was constitutively active (Iglesias et al., 1998). It remains to be seen whether the activation of PKS6 may also be obtained by a Ser-164 to Glu mutation.

In this study, we found that PKS6 preferred Mn^{2+} over Mg^{2+} for substrate phosphorylation as well as autophosphorylation activity. An Arabidopsis Ser/Thr kinase, AtCIPK1/PKS13 (Shi et al., 1999); a tobacco (*Nicotiana tabacum*) SNF1 homolog, NPK5 (Muranaka et al., 1994); and an Arabidopsis receptor-like protein kinase, RLK5 (Horn and Walker, 1994), have been shown to prefer Mn^{2+} for autophosphorylation activity. Some Ser/Thr protein kinases from animal and yeast systems and receptor Tyr kinases from animals also prefer Mn^{2+} as a cofactor, such as *Xenopus laevis* XEEK1 (Sturany et al., 1996), human (*Homo sapiens*) STE20-like protein kinase (Schinkmann and Blenis, 1997), and Brewer's yeast (*Saccharomyces cerevisiae*) YGR262c (Stocchetto et al., 1997). The preference of Mn^{2+} for enzyme activity in some kinases was suggested to reflect involvement of the kinase in a complex for full activation (Sturany et al., 1996). Micromolar amounts of Mn^{2+}, the physiological concentrations in plant cells, were sufficient to activate PKS6ΔF (Fig. 4). These results indicate a physiological role of Mn^{2+} in modulating the protein kinase activity of PKS6. Mn^{2+} at concentrations between 0.25 and 2.5 mM activated PKS6ΔF in the presence of 10 μM ATP, whereas Mn^{2+} at concentrations above 5 mM became inhibitory (Fig. 4). It is estimated that 97% to 98% of ATP would be in the form of MnATP under the concentrations of 0.5 mM Mn^{2+} and 50 μM ATP, and MnATP does not increase as the concentration of Mn^{2+} increases (White et al., 1984). Therefore, kinase activation by increasing Mn^{2+} concentration may be due to free Mn^{2+} binding to a distinct site on the PKS6 protein. The roles that Mn^{2+} plays in the catalytic mechanism of PKS6 and what amino acid residue(s) binds Mn^{2+} need further investigation. Mg^{2+} is a preferred cofactor for phosphofructo-1-kinase from *Entamoeba histolytica* (Chi et al., 2001) and pyruvate kinase from cyanobacterium *Synechococcus* sp. (Knowles et al., 2001). Autophosphorylation of AtSERK1 was dependent on Mg^{2+} but was inhibited by Mn^{2+} (Shah et al., 2001). The kinase activity of SOS2 required Mg^{2+} as a cofactor (Halfter et al., 2000). The wheat WPK4, which is very similar to PKS6, cannot use Mn^{2+} in place of Mg^{2+} for either autophosphorylation or phosphorylation of MBP (Ikeda et al., 1999). It seems that the activities of protein kinases, particularly the PKS family of kinases, may be differentially regulated by divalent cations. In addition, high concentrations (> 5 mM) of either Mn^{2+} or Mg^{2+} were found to be inhibitory to substrate phosphorylation of PKS6ΔF (Fig. 4). This is in contrast to a human Ser/Thr protein kinase, protein kinase D2 (Sturany et al., 1996), and a cyanobacterial pyruvate kinase (Knowles et al., 2001). The human and cyanobacterial kinases require 30 mM Mg^{2+} for maximal kinase activity. Optimal autophosphorylation activity was also observed at 10 mM Mg^{2+} for AtSERK1 (Shah et al., 2001).

PKS6ΔF preferably phosphorylated two Ser-containing peptides (p1 and p3) compared with a Thr-containing peptide substrate (p2). We have recently found that SOS2 equally phosphorylated the three peptide substrates, although it did not show any kinase activity against commonly used protein substrates (Halfter et al., 2000). AtCIPK1/PKS13 also did not phosphorylate commonly used protein substrates including casein, MBP, histone H_1, and histone IIIS (Shi et al., 1999). These results suggest that different PKS isoforms may have distinct substrate preferences. The apparent K_m values of PKS6ΔF are within the range of the reported values for SNF1 and AMPK from yeast, mammals, and higher plants (Ruzzene and Pinna, 1999).

MATERIALS AND METHODS

Plant Materials

Arabidopsis (Columbia ecotype) wild-type seedlings were grown on Murashige and Skoog nutrient agar plates under continuous light (Wu et al., 1996), and 10-d-old seedlings were treated with NaCl, ABA, cold, and drought as described previously (Shi et al., 2002). For the collection of different tissues, plants were grown in Turface soil to facilitate root harvesting. Roots and leaves were collected from 3-week-old seedlings, and stems, flowers, and siliques were harvested from adult plants.

Reverse Transcriptase-PCR and Northern-Blot Analysis

The complete open reading frame of *PKS6* was obtained by reverse transcriptase-PCR. For reverse transcriptase-PCR, the single-strand cDNA was synthesize by reverse transcriptase (Invitrogen, Carlsbad, CA) from mRNA and was then used as the template for PCR reaction. Template mRNA was isolated from 2-week-old wild-type Arabidopsis plants. *PKS6*-specific primer pair containing *Bam*HI and *Xba*I sites at the termini are as follows: 5′-CGGGATCCATGAGTG-GAAGCAGAAGGAAGGCGACG-3′ (forward, the *Bam*HI site is underlined) and 5′-GCTCTAGATTATTGCTTTTGT-TCTTCAGCG GCTGCA-3′ (reverse, the *Xba*I site is underlined; MWG-Biotech, High Point, NC). The PCR products were gel-purified, digested, cloned into a modified pGEX-2TK vector, and completely sequenced. RNA isolation and northern-blot analysis were performed as described previ-

ously (Zhu et al., 1998). Twenty micrograms of total RNA was loaded in each lane, size-fractionated by electrophoresis, and blotted onto a nylon membrane. A ^{32}P-labeled probe was a gene-specific fragment from the 3′ end of the gene including a 3′-untranslated sequence.

Site-Directed Mutagenesis

FISL motif deletion and substitution of Thr with Asp mutation within the activation loop of PKS6 were introduced using oligonucleotide-directed in vitro mutagenesis. The sequences of mutagenic primers for deletion mutation were 5′-CAAGCGCAACTTGTGAAGAAAGAAAC-3′ (forward) and 5′-GGATACAGGTTTCTCCTTCTTCT-CAG-3′ (reverse). The sequences of mutagenic primers for Thr to Asp change mutation were 5′-GAAGATGGTTTGCT-TCATGACGCTTGTGGAACGCCAAAC-3′ (forward) and 5′-GTTTGGCGTTCCACAAGCGTCATGAAGCAAACCA-TCTTC-3′ (reverse). PCR reactions were carried out on double-stranded plasmid DNA using an enzyme mix of *LA Tag* (TaKaRa Shuzo, Ltd., Kyoto) and *Pfu* Turbo DNA polymerase (1:1; Stratagene, La Jolla, CA). The PCR amplification consisted of 16 cycles with 30 s at 95°C, 1.0 min at 58°C, and 7 min at 72°C. The amplified products were isolated from agarose gel, purified, and then treated with *Dpn*I to digest the parental supercoiled dsDNA. For the FISL motif deletion mutation, the digested PCR product was phosphorylated by T4 polynucleotide kinase (Invitrogen) and then ligated by T4 DNA ligase (Invitrogen) before transformation into DH5α competent cells. For the T to D mutation, the digested PCR product was transformed into the same competent cells. The sequence of mutations as well as the fidelity of the rest of the DNA in both constructs were confirmed by direct DNA sequencing.

Expression of PKS6 Fusion Proteins in *Escherichia coli* and Purification

Expression of GST-PKS6 fusion proteins was performed as described previously (Guo et al., 2001). All PKS6 mutant and wild-type fusion constructs were transformed into *E. coli* BL21 codon plus (DE3) cells (Stratagene) for protein expression. A 10-mL overnight culture was transferred into a fresh 1,000 mL of Luria-Bertani medium, and the cells were grown at 37°C to an A_{600} of 0.8 and then induced with 0.6 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The cells were collected by centrifugation and resuspended in ice-cold phosphate-buffered saline lysis solution (pH 7.5) containing 10% (v/v) glycerol, 5 mM dithiothreitol, 2 μg mL^{-1} aprotinin, 2 μg mL^{-1} leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Lysozyme (1 mg mL^{-1}) and Triton X-100 (1%, v/v) were added to the suspension, sonicated, and clarified by centrifugation (15,000*g*, 30 min, 4°C). The GST-fusion proteins were affinity-purified from the bacterial lysates with glutathione Sepharose 4B (Amersham-Pharmacia Biotech, Uppsala) chromatography according to the manufacturer's instructions. The purity of the purified protein of each preparation was evaluated by 10% (w/v) SDS-PAGE analysis. The gels were stained with Coomassie Brilliant Blue.

Phosphorylation Assays

Peptide phosphorylation was measured as the incorporation of radioactivity from [γ-^{32}P]ATP (PerkinElmer Life Sciences, Foster City, CA) into the peptide substrate p3 (ALARAASAAALARRR, Research Genetics, Huntsville, AL) according to that described previously (Halfter et al., 2000) with modification. Control reactions were either without a peptide substrate or without kinase proteins. The kinase buffer contained 20 mM Tris-HCl, pH 7.2, 2.5 mM $MnCl_2$ or 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 μM ATP, and 2 mM dithiothreitol. Kinase reactions (in a total volume of 40 μL) were started by adding 150 μM p3 and 5 μCi of [γ-^{32}P]ATP (specific radioactivity of 600 cpm pmol^{-1}), and reaction mixtures were incubated for 30 min at 30°C. All reactions contained approximately 300 to 400 ng of purified proteins. The protein concentrations were estimated by comparison with serial dilutions of bovine serum albumin on the same gel. Enzyme activities were linear with respect to incubation time and amount of enzyme assayed. Reactions were terminated by adding 1 μL of 0.5 M EDTA, and the GST fusion proteins bound to glutathione Sepharose beads were pelleted. Fifteen microliters of the supernatant was spotted onto P-81 phosphocellulose paper (Whatman, Clifton, NJ) for peptide phosphorylation analysis. The P-81 paper was then washed four times in cold 1% (v/v) phosphoric acid (10 min per wash) and dried, and the phosphorylated peptide was quantified by phosphorimaging using a STORM 860 PhosphorImager from Molecular Dynamics (Sunnyvale, CA) with the ImageQuant software. The remaining 25-μL reaction mixture was used to determine autophosphorylation activity of the fusion proteins. Five microliters of 6× Laemmli SDS-PAGE sample buffer was added to the 25-μL reaction mixture, denatured by boiling for 4 min, and then separated by 10% (w/v) SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to verify equal loading and dried. The dried gel was autoradiographed with x-ray film (Eastman Kodak, Rochester, NY).

To determine cofactor requirements, we assayed peptide phosphorylation and autophosphorylation activity in the kinase buffer with 0 to 20 mM of $MnCl_2$ or $MgCl_2$. To analyze the effect of pH on substrate phosphorylation activity, we used 20 mM BisTris propane titrated to the desired pH with either HCl or KOH in place of 20 mM Tris-HCl buffer. To test the dependence of substrate phosphorylation on temperature, reaction mixtures were incubated at 15°C to 42°C instead of 30°C. For substrate specificity analysis, two peptide substrates p1 (LRRASLG; Kemptide, Sigma, St. Louis) and p2 (VRKRTLRRL; Sigma, St. Louis) were used in addition to p3. Kinetic parameters were determined by varying the concentration of p3 (0–300 μM) in the presence of 2.5 mM $MnCl_2$ while holding ATP constant (10 μM). As an alternative, ATP concentrations were varied (0–30 μM) while keeping p3 constant (150 μM). The values of apparent K_m, maximal velocity V_{max}, and V_{max}/K_m for p3 and ATP were determined by at least

triplicate measurements of initial velocity for different concentrations of p3 and ATP. Eadie-Hofstee regression was used to fit the data in a defined concentration range to a straight line, and K_m and V_{max} values were determined from the regression equation.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the materials. Obtaining any permissions will be the responsibility of the requestor.

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