Biochemical and Functional Characterization of PKS11, a Novel Arabidopsis Protein Kinase*

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The Arabidopsis SOS2 (Salt Overly Sensitive 2)-like protein kinases (PKS) are novel protein kinases that contain an SNF1-like catalytic domain with a putative activation loop and a regulatory domain with an FISL motif that binds calcium sensors. Very little biochemical and functional information is currently available on this family of kinases. Here we report on the expression of the PKS11 gene, activation and characterization of the gene product, and transgenic evaluation of its function in plants. PKS11 transcript was preferentially expressed in roots of Arabidopsis plants. Recombinant glutathione-S-transferase fusion protein of PKS11 was inactive in substrate phosphorylation. However, the kinase can be highly activated by a threonine 161 to aspartate substitution (designated PKS11T161D) in the putative activation loop. Interestingly, PKS11 can also be activated by substitution of either a serine or threonine with aspartate within the activation loop. Deletion of the FISL motif also resulted in a slight activation of PKS11. PKS11T161D displayed an uncommon preference for Mn2+ over Mg2+ for substrate phosphorylation and autophosphorylation. The optimal pH and temperature values of PKS11T161D were determined to be 7.5 and 30 °C, respectively. The activated kinase showed substrate specificity, high affinity, and catalytic efficiency for a peptide substrate p3 and for ATP, AMP or ADP at concentrations from 10 μM to 1 mM did not activate PKS11T161D. Transgenic Arabidopsis plants expressing PKS11T161D were more resistant to high concentrations of glucose, suggesting the involvement of this protein kinase in sugar signaling in plants. These results provide insights into the function as well as regulation and biochemical properties of the PKS protein kinase.

Protein phosphorylation plays crucial roles in cellular functions, including cell division, metabolism, and response to hormonal, developmental, and environmental signals. The Arabidopsis genome encodes a large number of protein kinases (1). The calcium-dependent protein kinase or calmodulin-like domain protein kinase family is responsive to calcium, because they contain a kinase catalytic domain fused with a calmodulin-like regulatory domain (2). Recent studies suggest that the family of Salt Overly Sensitive 2 (SOS2)-like protein kinases (i.e. PKSes) in plants is also responsive to calcium through interaction with the SOS3 (Salt Overly Sensitive 3) family of calcium-binding proteins, and thus may be functionally analogous to animal calcium/calmodulin-dependent protein kinases (3).

The Arabidopsis SOS2 and SOS3 genes are required for sodium and potassium ion homeostasis and salt tolerance (4, 5). SOS2 encodes a myristoylated EF-hand calcium-binding protein (5, 6) that may sense the calcium signal elicited by salt stress. SOS2 encodes a serine/threonine protein kinase with an N-terminal kinase catalytic domain similar to SNF1/AMPK (7) and a novel C-terminal regulatory domain (4). SOS3 physically interacts with SOS2 in the yeast two-hybrid system as well as in vitro (8). A 21-amino acid sequence in the regulatory domain of SOS2, the FISL motif, has been determined to be necessary and sufficient to bind SOS3 (3). In the presence of calcium, SOS3 activates the substrate phosphorylation of SOS2 (8). Salt stress up-regulation of the SOS1 (Salt Overly Sensitive 1) gene encoding a putative Na+/H+ antiporter is partially under control of the SOS3-SOS2 regulatory pathway (9).

Arabidopsis contains 23 PKS genes, several of which have been cloned and their transcript expression analyzed (3). However, neither the biochemical properties nor the physiological functions of the PKS gene products are known. By analogy to SOS2, these PKSes do not seem to have substrate phosphorylation activity in the absence of specific interacting proteins, i.e. the SOS3-like calcium-binding proteins, and thus further characterization is difficult to carry out. Therefore, it is of crucial importance to make these inactive PKSes active in order to characterize their biochemical properties. In addition to being excellent materials for biochemical characterization, active forms of PKSes may be expressed in plants to probe their in vivo functions.

In this current work, we cloned the cDNA and analyzed the tissue-specific expression of a PKS gene, PKS11. We found that PKS11 was preferentially expressed in roots of Arabidopsis plants. A highly active PKS11 mutant form was constructed by substituting a threonine residue with aspartate (designated PKS11T161D) within the putative activation loop (10). This observation strongly suggests that activation loop phosphorylation may be an important determinant of the kinase activity in vivo. We then further characterized the activated PKS11 in terms of cofactor preference, substrate specificity, kinetic properties, effect of ADP and AMP, and pH and temperature dependencies.

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\* The abbreviations used are: SOS2, Salt Overly Sensitive 2; ABA, abscisic acid; AMPK, AMP-activated protein kinase; BisTris, (bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; CDPK, calcium-dependent/calmodulin-like domain protein kinase; GST, glutathione S-transferase; MS, Murashige and Skoog; PKC, protein kinase C; PKD, protein kinase D; PMSP, phenylmethanesulfonyl fluoride; SNF1, sucrose-non-fermenting protein kinase; ScnBP, SOS3-like calcium-binding proteins; PKSes, SOS2-like protein kinases.

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We expressed the constitutively active PKS11 kinase mutant in transgenic Arabidopsis, and we found that the transgenic plants were more resistant to high levels of glucose. Our results provide the first detailed biochemical characterization of the PKS and suggest that PKS11 is involved in sugar signaling in plants.

**EXPERIMENTAL PROCEDURES**

**Reverse Transcriptase-PCR and Northern Blot Analysis**—A cDNA containing the complete open reading frame of PKS11 was obtained by reverse transcriptase (Invitrogen)-PCR. Template mRNA was isolated from 3-week-old, wild-type Arabidopsis thaliana ecotype Columbia plants. PKS11-specific primer pairs containing KmI and EcoRI sites at the termini are as follows: 5'-GGGTTAGCTTGAAGGAGGGA-3' (forward) and 5'-CCCATGACGTTGCTCCTGAGGTTCTCAG-3' (reverse) (MWG Biotec, High Point, NC). The PCR products were gel-purified, digested, and cloned into a modified pGEX-2T-CMS vector and completely sequenced. Arabidopsis wild-type seedlings were grown on Murashige and Skoog (MS) nutrient agar plates under continuous light (11), and 10-day-old seedlings were treated with NaCl, abscisic acid (ABA), cold, and drought as described previously (9, 12). For the collection of different tissues, wild-type plants were grown in Turface soil to facilitate root harvesting. Roots and leaves were collected from different tissues, and stems, flowers, and siliques were harvested from mature plants. Total RNA isolation and Northern blot analysis were performed as described previously (13). For analysis of transgene expression, total RNA was isolated from 10-day-old seedlings grown on MS agar plates containing 3% glucose. Thirty micrograms of total RNA was loaded in each lane, size-fractionated by electrophoresis, and blotted onto a nylon membrane. The blot was hybridized with a gene-specific DNA probe for PKS11.

**Promoter-Glucuronidase Analysis**—A 1207-bp promoter region of the PKS11 gene was amplified by PCR from genomic DNA with the following primer pair introducing a BamHI site at the 5' end and an SmaI site at the 3' end to facilitate cloning: 5'-CAGGATCCCCCTATATGTTCTCTCTCAAGTGTCG-3' (BamHI site underlined) and 5'-ACCAGACAAGG-3' (SmaI site underlined). The fragment was cloned into BamHI- and SmaI-digested pBluescript SHI vector to obtain a transcriptional fusion of the PKS11 promoter and the β-glucuronidase coding sequence. Transgenic plants harboring this construct were generated as described previously (12). For β-glucuronidase assay, materials were stained at 37 °C overnight in 100 mM sodium phosphate buffer, pH 7.0, containing 1 mg/ml 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.03% (v/v) Triton X-100.

**Site-directed Mutagenesis**—Both the T507Y to D change within the activation loop and the F513L motif deletion mutation of the PKS11 were introduced using oligonucleotide-directed in vitro mutagenesis. The promotor mutants for T507Y to D changed as are: pPKS11T516D-forward, 5'-ACAGAGTTTACCTCTCTTAAAGGACATCATGGAACTCC-3'; pPKS11T516D-reverse, 5'-AATGGGAGCTCTCCGAGATTTGTCTTGGTTC-3'; pPKS11T516D-forward, 5'-ACATCTGATTTTGGCCCTGCAGGATTACCTACCAAGAGGAG-3'; pPKS11S154D-forward, 5'-TCTTGTTCAGGAAATGTCGAGACACAAAATCAATACTAC-3'; pPKS11S154D-reverse, 5'-TCTTGTTCAGGAAATGTCGAGACACAAAATCAATACTAC-3'. The mutagenic primers for deletion mutagenesis are as follows: pPKS11A11F-forward, 5'-TCTCTACTACGTGAAAAACCTCAATGAACACGCGAGGAG-3'; and pPKS11A11F-reverse, 5'-AGCTCCCTTCTTCTTTGAAGCAGGCCAAGGG-3'.

**Phosphorylation Assays**—In vitro phosphorylation assays using a synthetic peptide p3 (ALARAASAAALARRR) were performed as described previously (8) with modification. Peptide phosphorylation was measured as the incorporation of [γ-32P]ATP (PerkinElmer Life Sciences) into the peptide substrate. Reactions without the peptide p3 or kinase proteins were used as controls. The kinase assay buffer contained 20 mM Tris-HCl, pH 7.2, 2.5 mM MgCl2, or 5 mM MnCl2, 0.1 mM ATP, and 2 mM dithiothreitol. Reactions in the absence of phosphorylation were started by adding 100 μM ATP and 5 μCi of [γ-32P]ATP (specific activity of 600 cpm/pmol), and reaction mixtures were immediately transferred to 30 °C for 30 min. All reactions contained 400–500 ng of purified proteins. Protein concentration was determined by the Bradford method using a dye binding assay (Bio-Rad) with bovine serum albumin as a standard. The stained bands on SDS-PAGE gels were also compared with a bovine serum albumin dilution series to adjust for the potential presence of other minor proteins that may copurify with the kinases. Enzyme activities were linear with respect to incubation time and amount of enzyme assayed. Reactions were termi-
nated by adding 1 μl of 0.5 mM 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) for peptide phosphorylation analysis. The P-81 paper was then washed 4 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 (Amersham Biosciences) with the ImageQuant software. To the remaining 25 μl of reaction mixture, 5 μl of 6× SDS-PAGE sample buffer was added and denatured by boiling for 3 min, the samples were then separated by a 10% SDS-PAGE gel. The gel was dried and exposed to x-ray film (Eastman Kodak) to detect kinase autophosphorylation.

For the analysis of a cofactor requirement, peptide phosphorylation and autophosphorylation assays were performed in the kinase assay buffer with 0–20 mM MnCl2 or MgCl2, whereas the concentrations of p3 (150 μM) and ATP (10 μM) were fixed. For substrate specificity assays, peptides p1 (LRRASLG) and p2 (VRKRTLRRL) (Sigma) were used in addition to the p3. Individual kinetic parameters were determined by varying the concentrations of p3 (0–300 μM) while holding ATP constant (10 μM). Alternatively, ATP concentrations were varied (0–20 μM) while keeping p3 constant (150 μM). The amount of recombinant proteins added to individual assays and the time of incubation were varied to maintain substrate conversion within a linear range. Optimal concentration of MnCl2 was used in the activity assays for the determination of kinetic parameters. Kinase assay buffers containing 10 μM to 1 mM ADP or AMP were used to test the effect of ADP or AMP on substrate phosphorylation. For the determination of optimal temperature of substrate phosphorylation, reaction mixtures were incubated at 15–42 °C instead of 30 °C. The effect of pH on substrate phosphorylation activity was determined using 20 mM BisTris titrated to the desired pH with either HCl or KOH in place of 20 mM Tris-HCl buffer.

**Generation of Transgenic Plants Expressing PKS11T161D**—To generate the expression construct of PKS11T161D, PCR was carried out using two restriction sites XbaI/SstI containing primers (5′-GCTCTAGAATGGTGTAAGGAAGGTGGGCAAGTG-3′, forward, XbaI site underlined, and 5′-CGAGCTCAGACGTCTTTTACTCTTGCCGCTTG-3′, reverse, SstI site underlined) on the PKS11T161D cDNA template. The PCR products were purified from agarose gel, digested, and cloned into the...
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medium containing 40 mg/liter hygromycin and 500 mg/liter vancomycin, and the transgenic lines were selected out. The transformed seedlings were transferred into soil to set seed under routine conditions. Seeds of wild-type and transgenic plants were surface-sterilized in 100% bleach for 10 min, followed by 5 washes in sterile distilled water. The seeds were embedded on MS agar plates and germinated and grown on the vertical plates at 22 °C, 300 PAR, 16-h light and 8-h dark photoperiod. Seed germination and seedling growth of the wild-type control plants, T<sub>0</sub> and T<sub>1</sub> generation transgenic plants expressing PKSI1T161D or PKS11 were tested for responses to various concentrations of ABA, salt, mannitol, and glucose treatments. To observe the effect of glucose on seed germination and seedling growth, transgenic and wild-type lines were grown on MS plates containing 1–5% glucose, 0.1 to 0.5 mM 2-deoxyglucose, or 1–5% 3-O-methylglucose. Seedlings were grown in the dark for 6 days, and hypocotyl length was measured from 20 seedlings at each glucose concentration in the control and transgenic plants.

Results

C DNA Cloning and Expression Patterns of PKSI1 in Different Tissues and in Response to Environmental Stresses—In order to determine experimentally the open reading frame of PKSI1 gene, the cDNA was cloned by reverse transcription-PCR. The deduced amino acid sequence of PKSI1 was found to be identical to that in the database, which was obtained from computer-based annotation. As a first step toward functional analysis, the steady-state transcript level of PKSI1 gene in different tissues of mature plants as well as under various stresses was determined. Blots of total RNA from different tissues or from stress-treated young seedlings were hybridized to a specific DNA probe for PKSI1. PKSI1 was expressed in all tissues examined, but the expression level in roots was substantially higher than that in leaves, stems, flowers, or siliques of mature Arabidopsis plants (Fig. 1A). Because of our interest in plant stress responses, potential regulation of the PKS gene by salt, cold, drought, and ABA was examined in young Arabidopsis seedlings (Fig. 1B). No significant induction or repression of PKSI1 was observed under any of the treatments.

A promoter–glucuronidase reporter fusion was used to investigate further the tissue distribution of PKSI1 expression. In Arabidopsis seedlings, promoter–glucuronidase staining was readily detected in roots, but the staining in other tissues was very weak or below the detection limit (Fig. 1C).

Sequence Alignment and Analysis—Like SOS2, the founding member of the PKS family, PKSI1 also contains an N-terminal SNF1-like kinase catalytic domain and a C-terminal regulatory domain (Fig. 2A). An alignment of the deduced amino acid sequence of PKSI1 with SOS2 showed that these kinases are highly conserved throughout the entire length (Fig. 2B). In the superfamily of protein kinases, the PKSes belong to SNF1/AMPK family (16). Like many other protein kinases including SOS2, PKSI1 contains a putative “activation loop” or “activation segment” in the kinase catalytic domain, located between the conserved DFG and APE sequences (Fig. 2B). The kinase also contains a conserved FISL motif, a stretch of 21 amino acid residues, located near the kinase domain (Fig. 2B). The FISL motif in SOS2 has been identified recently as the SOS3-interacting sequence and is autoinhibitory to substrate phosphorylation (3). PKSI1 contains an open reading frame of 1338 bp and is predicted to encode a protein of 446 amino acid residues with an estimated molecular mass of 50.4 kDa. PKSI1 is located on chromosome 4, based on information in the Arabidopsis genomic sequence database (www.arabidopsis.org).

Activation of PKSI1 by Amino Acid Substitutions in the
Activation Loop—We expressed PKS11 and a number of other PKS proteins in bacteria, and we found that none had any kinase activity against commonly used protein or peptide substrates (data not shown). In order to biochemically characterize the enzyme, we attempted to construct active forms of the kinase. Previously, we have found that aspartate substitution of Thr168 in the putative activation loop of SOS2 could activate the kinase (3). A comparison of the putative activation loop of PKS11 and a number of other PKSes (data not shown) with that of SOS2 showed that the threonine residue is conserved (Fig. 2B). This suggests that the threonine residue in PKS11, Thr161, could be a target site for phosphorylation by a putative upstream activating kinase(s). To produce active PKS11 protein, we substituted the threonine residue with aspartate to partially mimic phosphorylation by an upstream kinase(s) using site-directed mutagenesis on the PKS11 cDNA. The resulting mutant, designated PKS11T161D, was produced by changing Thr161 to Asp (Fig. 2B). In addition, the FISL motif in PKS11 may be autoinhibitory to the kinase activity. Therefore, a FISL motif deletion mutant, designated PKS11ΔF, was constructed by deleting the FISL motif between Lys104 and Leu125 (Fig. 2B) using site-directed mutagenesis.

PKS11 wild-type and mutant proteins were expressed in E. coli BL21 cells as GST fusion proteins and purified by affinity chromatography on glutathione-Sepharose (Fig. 3A). The expression level of the recombinant PKS11 mutant proteins was similar to that of the wild-type counterpart, as shown by SDS-PAGE analysis. These purified GST-PKS11 fusion proteins showed the expected apparent molecular mass of about 80 kDa, with GST-PKS11ΔF slightly smaller. We have shown previously (8) that SOS2 can phosphorylate a peptide p3 in the presence of SOS3. We measured substrate phosphorylation of the peptide and autophosphorylation in vitro for the mutant and wild-type kinases in the presence of 2.5 mM Mn2+ or Mg2+. (Fig. 3B). In contrast, the activation loop mutant, PKS11T161D, was extremely
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Cofactor Preference of PKS11T161D—To determine the cofactor preference for divalent cations in vitro of PKS11T161D, we measured substrate phosphorylation activity in the presence of various concentrations of two divalent cations, Mg$^{2+}$ and Mn$^{2+}$. Divalent cations were absolutely required for substrate phosphorylation of p3 as well as autophosphorylation of the kinase, as shown by the lack of activity in the absence of the cations (Fig. 4A). Substrate phosphorylation increased as the concentrations of Mn$^{2+}$ or Mg$^{2+}$ in the range of 0–2.5 mM (Mn$^{2+}$) or 0–5.0 mM (Mg$^{2+}$) increased. Interestingly, Mn$^{2+}$ appeared to be a much more effective cofactor than Mg$^{2+}$ for PKS11T161D. As low as 0.25 mM Mn$^{2+}$ could activate substrate phosphorylation of PKS11T161D. Optimal activation was observed at around 2.5 mM Mn$^{2+}$, and higher concentrations (>5 mM Mn$^{2+}$) became inhibitory. In contrast, Mg$^{2+}$ did not activate PKS11T161D at concentrations of less than 1 mM. Optimal activation was achieved at 5 mM or higher concentrations of Mg$^{2+}$ (Fig. 4A). The intracellular concentration of Mn$^{2+}$ is in the micromolar range, whereas that of Mg$^{2+}$ is in the millimolar range (17). Nevertheless, these results suggest that Mn$^{2+}$ could play a role in activity regulation of the PKS under physiological conditions.

We tested whether PKS11T161D also preferred Mn$^{2+}$ over Mg$^{2+}$ as a cofactor for autophosphorylation. Autophosphorylation was assayed in the presence of various concentrations of the two divalent cations. Mn$^{2+}$ also strongly activated autophosphorylation of PKS11T161D even in the micromolar range (Fig. 4B). In contrast, Mg$^{2+}$ only weakly activated the autophosphorylation, and the activation required millimolar concentrations of Mg$^{2+}$. These results suggest that PKS11 is a novel protein kinase with an uncommon cofactor preference. With 2.5 mM Mn$^{2+}$ as a cofactor in the kinase assay, PKS11T161D displayed even higher peptide phosphorylation (Fig. 4C) as well as autophosphorylation activity (data not shown).

Substrate Specificities and Kinetic Parameters—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, three synthetic peptide substrates (p1, p2, and p3), derived from the recognition sequences of protein kinase C or SNF1/AMPK, are known to be phosphorylated by SOS2 (8). These peptides were thus chosen to analyze the substrate specificity of PKS11T161D in the present study. The above results show that PKS11T161D can phosphorylate the peptide substrate p3. To determine the substrate specificity of PKS11T161D, we compared two serine-containing peptide substrates p1 and p3 and a threonine-containing peptide substrate p2. PKS11T161D phosphorylated both p1 and p3, with p3 giving higher activity than p1 (Fig. 5A). PKS11T161D also phosphorylated p2. These results demonstrate that p3 is a preferred peptide substrate for PKS11T161D.

P3 phosphorylation by the kinase was determined over the pH range of 6.5 to 9.5. PKS11T161D exhibited a narrow pH activity profile with optimal pH values between 7.0 and 7.5 (Fig. 5B). The effect of temperature from 15 to 42 °C on p3 phosphorylation by the kinase was also determined. The substrate phosphorylation activity of PKS11T161D increased as the temperature was raised. The temperature optimum was found to be 30 °C (Fig. 5C). Higher temperatures decreased the activity of the kinase. At 25 °C, PKS11T161D displayed ~90% of the maximal activity.

To test the affinity and catalytic efficiency toward p3 and ATP of PKS11T161D, we determined the apparent kinetic parameters. Data from three independent experiments are shown as saturation curve with specific activity (nmol/min/mg pro-
FIG. 6. Dependence of substrate phosphorylation of PKS11T161D on peptide substrate p3 or ATP. A, dependence of substrate phosphorylation on peptide substrate p3. Phosphorylation of p3 by PKS11T161D was assayed at 30 °C in the presence of 2.5 mM MnCl₂ as described under “Experimental Procedures.” Result shown is the average of three independent assays presented as saturation curve with specific activity versus p3 concentration as indicated. ATP concentration in the kinase assay buffer was set constant at 10 μM. B, dependence of substrate phosphorylation of PKS11T161D on ATP. Result shown is the average of three independent assays presented as saturation curve with specific activity versus ATP concentration as indicated. p3 concentration in the kinase 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 ± S.D. (n = 3).
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The mechanism of activity regulation of the PKS family of protein kinases is not well understood. Like SOS2, PKS11 contains a very conserved SNF1/AMPK-like kinase catalytic domain with a putative activation loop and a regulatory domain with an FISL motif. In this study, activation of PKS11 was achieved by changing a conserved threonine residue within the activation loop to aspartate to mimic phosphorylation by an upstream kinase(s). Activation of the yeast SNF1 kinase also required the phosphorylation of a conserved threonine residue in the activation loop of the catalytic subunit (23). These results suggest that PKS11 may be activated in vivo through activation loop phosphorylation at the threonine residue by an upstream kinase(s). Two requirements have been thought to be critical for the catalytic activity of protein kinases. One is the correct juxtaposition of catalytic groups contributing to the transfer of the γ-phosphate group from ATP to a serine, threonine, or tyrosine side chain of the substrate (24). The other is the accessibility and correct positioning of the substrate-binding site(s) (10). Some protein kinases can achieve a catalytically active conformation in the absence of activation segment phosphorylation (24). However, many other protein kinases possess an activation loop that contains amino acid residues that are themselves subject to phosphorylation. The mechanism of PKS activation by phosphorylation, therefore, could be that phosphorylation promotes a conformation of the activation loop in which the catalytic and substrate-binding sites are correctly formed, resulting in a significant increase in kinase activity (24).

In this study, activation of PKS11 was also achieved by changing the conserved serine or tyrosine residue to aspartate (Fig. 3D). In protein kinase D (PKD), activation loop phosphorylation at Ser^744 and Ser^748 has been found during protein kinase C (PKC)-mediated activation (25). A PKD mutant with both the serine residues substituted with glutamic acid to mimic phosphorylation became very active (26). An Arabidopsis dual specificity kinase phosphorylated tyrosine as well as serine and threonine residues (27). Another Arabidopsis dual specificity receptor kinase phosphorylated myelin basic protein predominantly on tyrosine residue (28). Isoforms of PKC subfamily can be activated by tyrosine phosphorylation by Bru-
Tyrosine phosphorylation of a member of the PKC subfamily was dependent on the activity of a Bruton’s tyrosine kinase that may directly phosphorylate the PKC (31). Full activation of a mitogen-activated protein kinase, ERK2, required dual phosphorylation of the Thr and Tyr residues in the TXY motif of the activation loop by a mitogen-activated protein kinase kinase (32). Our results strongly suggest that PKS11 may be activated in vivo through activation loop phosphorylation on the conserved threonine, serine, and/or tyrosine residue. PKS11 activation by tyrosine phosphorylation within the activation loop is of interest because it suggests possible involvement of PKS11 in tyrosine kinase-mediated signaling pathways. Most protein kinases that are activated by phosphoryl-

Fig. 8. Transgenic Arabidopsis plants expressing PKS11T161D were more resistant to glucose. A, seeds of homozygous PKS11T161D transgenic Arabidopsis plants (8th line) and untransformed control plants (WT) were germinated and grown in MS agar plates containing 0 (MS only), 2, and 4% glucose or mannitol. Arabidopsis seedlings were grown under constant light for 4–7 days after seed imbibition. Wild-type control plants (right) are shown for comparison. The pictures were taken 4, 5, or 7 days after seed imbibition. B and C, quantitation of root growth (of light grown seedlings) and hypocotyl length (of dark grown seedlings) in 0 and 4% glucose. Error bars represent S.D. from 10 to 20 samples.
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PKS11T161D showed a strong preference for Mn$^{2+}$ over Mg$^{2+}$. The cofactor preference is similar to that described for autophosphorylation of a tobacco serine/threonine kinase NPK5 (33) and an Arabidopsis receptor-like kinase RLK5 (34). Some serine/threonine protein kinases from animal and yeast systems and receptor tyrosine kinases from animals also preferred Mn$^{2+}$ as a cofactor (35–37). 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 (35). Micromolar amounts of Mn$^{2+}$, the physiological concentrations in plant cells, were found to be sufficient for activation of PKS11 (Fig. 4, A and B). These results indicate a potential physiological role of Mn$^{2+}$ in activity regulation of PKS11. In this study, Mn$^{2+}$ at concentrations from 0.25 to 2.5 mM activated PKS11T161D in the presence of 10 μM ATP (Fig. 4A). It is estimated that 97–98% of ATP would be in the form of MnATP under the concentrations of 0.5 mM Mn$^{2+}$ and 50 μM ATP, and MnATP did not increase as the concentration of Mn$^{2+}$ increased (38). Therefore, kinase activation by increasing Mn$^{2+}$ concentration may be due to free Mn$^{2+}$ binding to a distinct site on the PKS protein. The role that Mn$^{2+}$ plays in the catalytic mechanism of PKS11 and what amino acid residue(s) bind Mn$^{2+}$ need further investigation. Phosphofructo-1-kinase preferred Mg$^{2+}$ to Mn$^{2+}$, and substituting Mn$^{2+}$ in the assay resulted in 16% of the observed activity with Mg$^{2+}$ (39). Mg$^{2+}$ was also the preferred cofactor for pyruvate kinase (40). In addition, high concentrations (>5 mM) of either Mn$^{2+}$ or Mg$^{2+}$ were found to be inhibitory to substrate phosphorylation of PKS11T161D (Fig. 4A). This is in contrast to a serine/threonine protein kinase D2 (41) and pyruvate kinase (40). These kinases required 30 mM Mg$^{2+}$ for maximal kinase activity in vitro. Some kinases required both mono- and divalent metal cation cofactors (42–45).

The apparent $K_m$ and $V_{max}$ values toward ATP or the peptide substrate p$\varepsilon$ for PKS11T161D are similar to the reported values for SNF1 or SNF1-related kinases from yeast, mammals, and higher plants (7). For example, the $K_m$ and $V_{max}$ values of a partially purified barley SnRK1 kinase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase kinase, for a synthetic peptide SAMS (HMRSAMSGLHILKVRK), were determined to be 47 μM and 25 nmol/min/mg, respectively (46). The apparent $K_m$ for ATP of a spinach SnRK1 was ~6 μM (21). ADP or AMP has no effect on the substrate phosphorylation activity of PKS11T161D (Fig. 7). AMP did not activate SnRK1 protein kinases from cauliflower, carrot, and rapeseed (20). These observations are in contrast to the AMPK that is activated by AMP (18–19). Our results suggest that PKS11 may not have an AMP-binding site. Alternatively, it is conceivable that additional effectors are required for AMP regulation of PKS11 in plants.

The protein kinase SOS2 is required for plant salt tolerance (4). Little is known about the physiological function of the other PKSes in plants. In this study, we investigated PKS11 function by expressing its activated form in plants. Expression of the activated, dominant PKS11 kinase in plants may avoid problems caused by genetic redundancy that is often associated with large gene families. Expression of the activated kinase in transgenic plants may also reveal whether the kinase activity is functionally sufficient for the respective physiological processes. The phenotype of the PKS11T161D transgenic plants suggests that PKS11 is involved in sugar signaling in plants (Fig. 8). Our experiments with glucose analogs indicate that PKS11 functions in sugar signaling may be independent of the hexokinase pathway (47). The molecular mechanisms by which
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plant cells sense sugars and transduce the signals are not well understood. Plant SnRK1 protein kinases are orthologs of yeast SNF1 and have been implicated to regulate carbon metabolism through both gene expression and direct control of enzyme activation state (48). The involvement of a PKS protein in sugar signaling is unexpected as the PKS family of kinases is known to interact with the SOS3 family of calcium-binding proteins, and as such are involved in calcium signaling (3). In contrast, yeast SNF1 or SNF1 orthologs in plants that function in sugar signaling have different interacting proteins and are regulated by AMP and not calcium (49). Recently, it was reported that high levels of sugars could trigger a strong and transient increase in cytosolic calcium in Arabidopsis seedlings (50). Although the calcium sensor(s) that interact with PKS11 have yet to be identified, the presence of a functional FISL motif in PKS11 suggests that this kinase binds to one or more of the calcium sensors in the SOS3 family (3). Therefore, we propose that PKS11 functions in mediating calcium signaling in response to sugar signals. Future identification of PKS11-interacting partners and substrate proteins will help to clarify the precise role of this kinase in sugar responses. In addition, PKS11 wild-type kinase is inactive by itself in substrate phosphorylation (Fig. 3B). Overexpression of the wild-type kinase does not seem to have any significant phenotypes. This may be because we did not co-overexpress any specific interacting partner (i.e. SOS3-like regulatory protein) of the kinase, and thus the overexpressed wild-type kinase may not be as active as the overexpressed PKS11T161D mutant. These observations strongly suggest that kinase activity is required for the PKS11 function in plants, and the constitutively active kinase may efficiently phosphorylate and regulate its downstream substrate(s) in a regulatory protein-independent manner.

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