THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 31, Issue of August 2, pp. 28340–28350, 2002

Printed in U.S.A.

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

Received for publication, August 13, 2001, and in revised form, May 23, 2002
Published, JBC Papers in Press, May 23, 2002, DOI 10.1074/jbc.M107719200

Deming Gong, Zhizhong Gong, Yan Guo, Xiuyin Chen, and Jian-Kang Zhu‡

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

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 tyrosine 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 Mn^{2+} over Mg^{2+} 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)[1]-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). *SOS3* 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,[2] 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 de-

* This work was supported by National Institutes of Health Grant R01GM59138 (to J.-K. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Plant Sciences, University of Arizona, Tucson, AZ 85721. Tel.: 520-626-2229; Fax: 520-621-7186; E-mail: jkzhu@ag.arizona.edu.

[1] The abbreviations used are: SOS2, Salt Overly Sensitive 2; ABA, abscisic acid; AMPK, AMP-activated protein kinase; BisTris, [bis(2-hydroxyethy)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; PMSF, phenylmethanesulfonyl fluoride; SNF1, sucrose-non-fermenting protein kinase; SCaBP, SOS3-like calcium-binding proteins; PKSes, SOS2-like protein kinases.

[2] D. Gong, Z. Gong, Y. Guo, X. Chen, and J.-K. Zhu, unpublished observations.

pendence. 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 2-week-old wild-type *Arabidopsis* (Columbia ecotype) plants. *PKS11*-specific primer pairs containing *Kpn*I and *Eco*RI sites at the termini are as follows: 5′-GCGGTACCATGGTGGTAAGGAAGGTGGGCATATG-3′ (forward) and 5′-CGGAATTCAACGTCTTTTACTCTTGGCCTTGGTGAC-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 3-week-old-seedlings, 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 *Bam*HI site at the 5′ end and an *Sma*I site at the 3′ end to facilitate cloning: 5′-CGGGATCCATTATTTAGGAGAC-3′ (*Bam*HI site underlined) and 5′-TCCCGGGCATTTCTTCAAGTCTAG-3′ (*Sma*I site underlined). The fragment was cloned into *Bam*HI- and *Sma*I-digested pBI101 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 ferricyanide, 5 mM potassium ferrocyanide, and 0.03% (v/v) Triton X-100.

Site-directed Mutagenesis—Both the T/S/Y to D change within the activation loop and the FISL motif deletion mutation of the PKS11 were introduced using oligonucleotide-directed *in vitro* mutagenesis. The mutagenic primers for T/S/Y to D changed mutation are as follows: pPKS11T161D-forward, 5′-CAAGGAGTTACCATCCTAAAGGACACATGTGGAACTCCC-3′; pPKS11T161D-reverse, 5′-AATTGGGAGTTCCACATGTGTCCTTTAGGATGGTAACTC-3′; pPKS11S154D-forward, 5′-ATATCTGATTTTGCCTCGACGCATTACCTGAACAAGGAG-3′; pPKS11S154D-reverse, 5′-TCCTTGTTCAGGTAATGCGTCGAGGCCAAAATCAGATATC-3′; pPKS11Y173D-forward, 5′-ACATGTGGAACTCCCAATGACGTTGCTCCTGAGGTTCTCAG-3′; and pPKS11Y173D-reverse, 5′-GAGAACCTCAGGAGCAACGTCATTGGGAGTTCCACATGTTG-3′. The mutagenic primers for deletion mutation are as follows: pPKS11Δ F-forward, 5′-TCCACTAACTGGAAAGGACTCCATGAAGCACCAGACAAGG-3′; and pPKS11ΔF-reverse, 5′-AGTCCTTTCCAGTTAGTGGACCTGTGTCTCTTGTTCCATC-3′.

Mutagenesis reactions were carried out on the 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) with the following PCR cycle: 95 °C for 30 s, followed by 16 cycles of 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 7 min. The PCR products were gel-purified and treated with *Dpn*I to digest the parental supercoiled double-stranded DNA. The digested PCR products were transformed into DH5α-competent cells. The sequences of mutation as well as the fidelity of the rest of the DNA in all constructs were confirmed by direct DNA sequencing.

Expression and Purification of Fusion Proteins—Glutathione *S*-transferase (GST)-PKS11 was obtained as described previously (8). The PKS11 open reading frame was cloned into pGEX-2T-CMS vector and expressed in bacteria as a C-terminal fusion protein with the bacterial GST under control of the isopropyl β-D-thiogalactopyranoside-inducible *tac* promoter. All GST fusion constructs were transformed into *Escherichia coli* BL21 codon plus cells (Stratagene). A 10-ml overnight Luria-Bertani (LB) culture was transferred to a fresh 1000 ml of LB and further cultured at 37 °C until the A_{600} reached about 0.8. Recombinant protein expression was induced by 0.6 mM isopropyl β-D-thiogalactopyranoside for 4 h. The cells were harvested by centrifugation, and the pellets were resuspended in ice-cold lysis buffer, pH 7.5, containing 140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10% (v/v) glycerol, 5 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM phenylmethanesulfonyl fluoride. Lysozyme (1 mg/ml) and Triton X-100 (1%, v/v) were added to the suspension and incubated on ice with gentle shaking for 1 h before sonication. The sonicate was then clarified by centrifugation, and the recombinant proteins were affinity-purified by glutathione-Sepharose 4B (Amersham Biosciences). SDS-PAGE (10%, w/v) analysis was used to evaluate the protein composition of each preparation. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

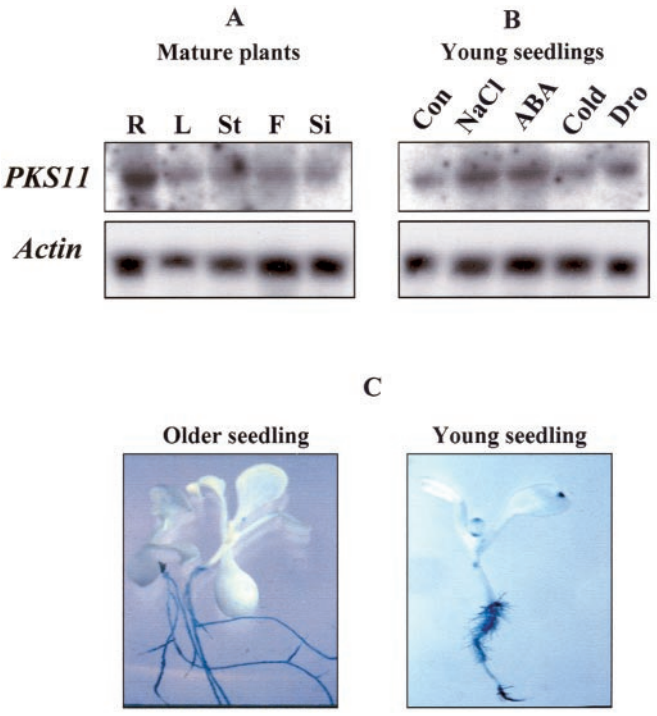

FIG. 1. **Expression of *PKS11* gene in different tissues and in response to various stresses.** *A*, expression of *PKS11* in different tissues analyzed by Northern blot. *R*, root; *L*, leaf; *St*, stem; *F*, flower; *Si*, silique. *B*, expression of *PKS11* under different stress conditions. *Con*, control (MS salt only); *NaCl*, 300 mM NaCl for 5 h; *ABA*, 100 μM abscisic acid for 3 h; *Cold*, 0 °C for 24 h; *Dro*, dehydration for 30 min. Thirty micrograms of total RNA was analyzed by RNA gel blotting. The blot was hybridized with a gene-specific DNA probe for *PKS11*. The Northern blot was exposed to x-ray film for 7 days. *Actin* is shown as a loading control (exposed to x-ray film for 12 h). *C*, localization of *PKS11* promoter-glucuronidase activity in seedlings of *Arabidopsis* transgenic plants.

Phosphorylation Assays—In vitro phosphorylation assays using a synthetic peptide p3 (ALARAASAAALARRR, Research Genetics, Huntsville, AL) as substrate were performed as described previously (8) with modification. Peptide phosphorylation was measured as the incorporation of radioactivity from [γ-^{32}P]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 $MnCl_2$ 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 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-

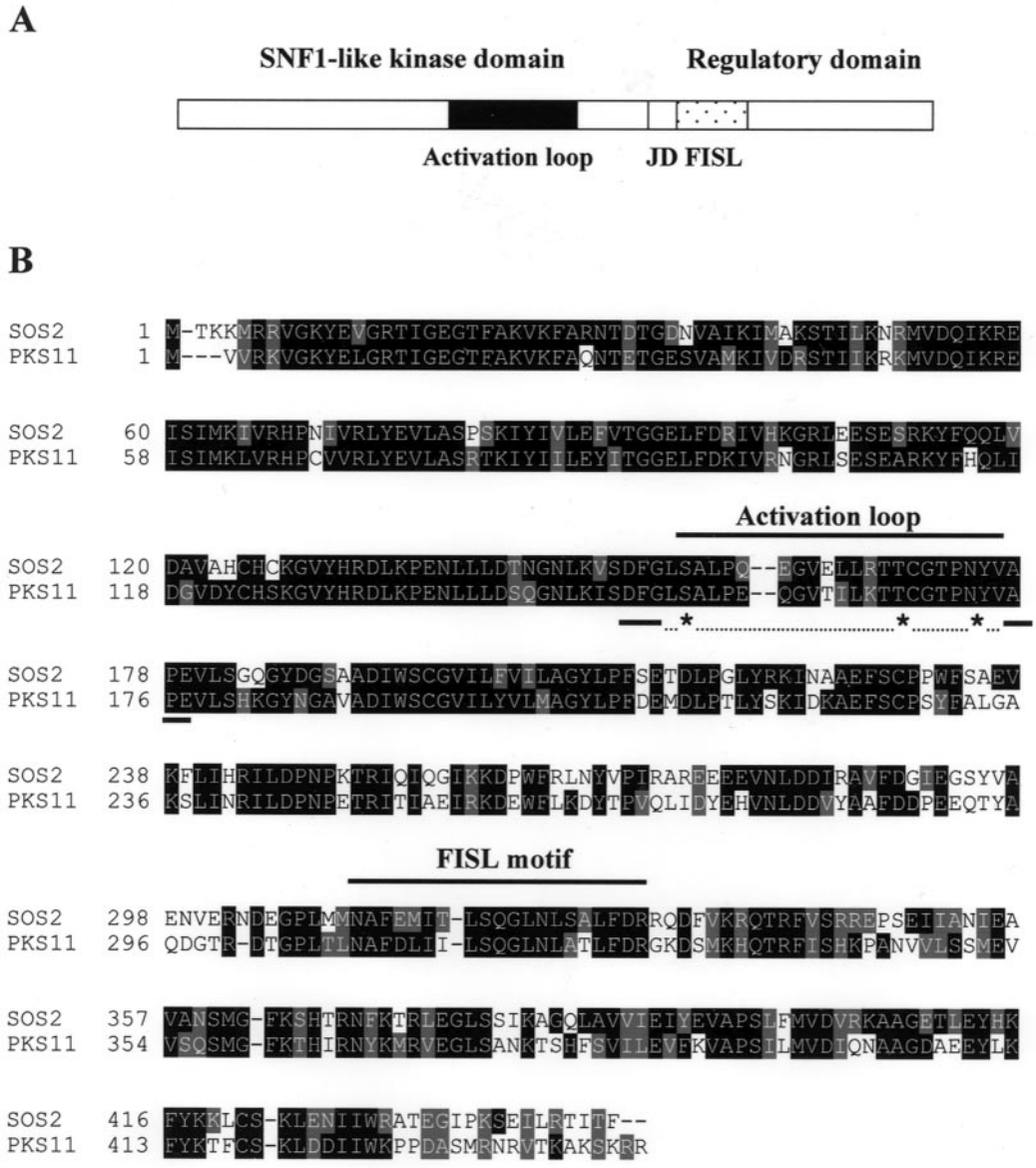

FIG. 2. **Alignment of the deduced amino acid sequences of PKS11 and SOS2.** *A*, shown is a schematic diagram of the domain structure for SOS2. *JD*, junction domain; *FISL*, FISL motif. *B*, an alignment of PKS11 and SOS2 was generated using multiple sequence alignment analysis and was processed using "boxshade" at www.ch.embnet.org/. PKS11 is identical to gene product with the GenBank™ accession number T09903. The cDNA for *PKS11* was amplified by RT-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 regulatory domain contains the conserved FISL (marked). Also marked is the putative activation loop between the conserved DFG and APE motifs (*dots*), and the conserved threonine, serine or tyrosine residue (*asterisk*) that may be phosphorylated by an upstream protein kinase(s). *Dashed lines* represent spaces that were introduced to maximize alignment.

nated 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) 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 $MnCl_2$ or $MgCl_2$, 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 $MnCl_2$ 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 *Xba*I/*Sst*I containing primers (5′-GCTCTAGAATGGTGGTAAGGAAGGTGGGCAAGTG-3′, forward, *Xba*I site underlined, and 5′-CGAGCTCTCAACGTCTTTTACTCTTGGCCTTGGTG-3′, reverse, *Sst*I site underlined) on the *PKS11T161D* cDNA template. The PCR products were purified from agarose gel, digested, and cloned into the

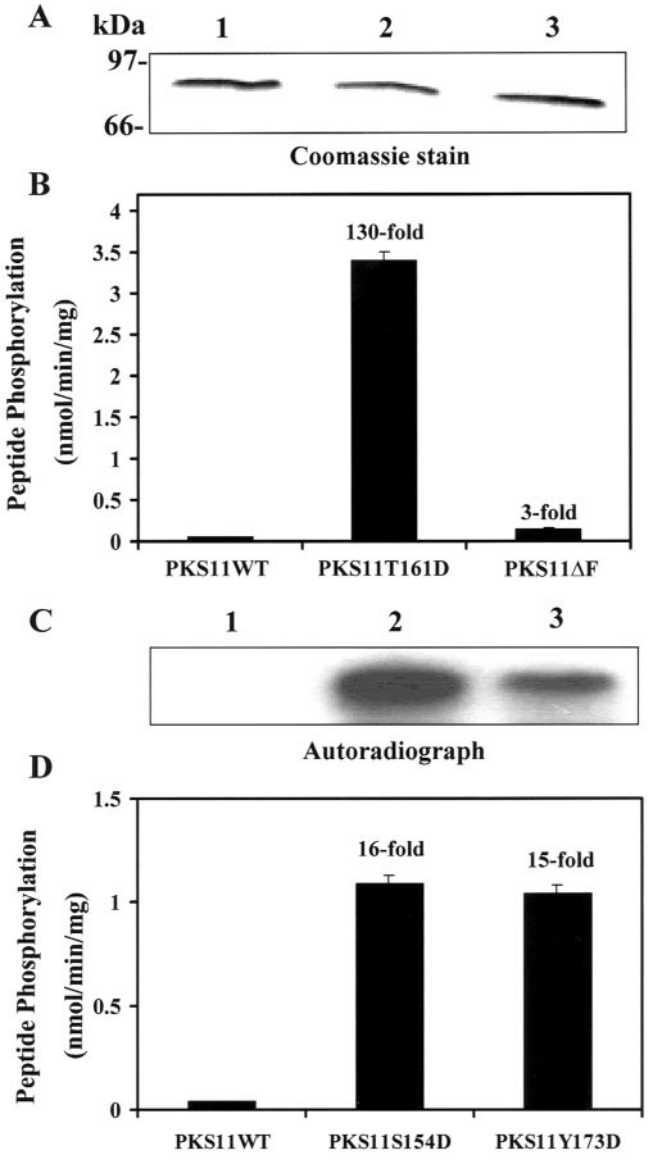

FIG. 3. **Activation of PKS11 by FISL motif deletion and by a substitution of Thr, Ser, or Tyr with Asp within the putative activation loop.** PKS11 wild-type (PKS11WT) and mutant cDNAs (PKS11T161D and PKS11ΔF) were expressed as GST-tagged fusion proteins in *E. coli* BL21 (codon plus), and purified by glutathione-Sepharose affinity chromatography. *A*, expressed recombinant proteins were analyzed by SDS-PAGE and the gels stained with Coomassie Brilliant Blue R-250. *Lanes 1–3*, purified PKS11WT, PKS11T161D, and PKS11ΔF, respectively. The protein standards were rabbit phosphorylase *b* (97 kDa) and bovine serum albumin (66 kDa). *B*, peptide phosphorylation activities of wild-type (*WT*), FISL motif deletion (Δ*F*), and Thr to Asp (T161D) changed PKS11. Peptide phosphorylation was assayed using 150 μM p3 as a substrate, 10 μM ATP, and 5 mM $MgCl_2$ as described under "Experimental Procedures." *C*, autophosphorylation activities of PKS11WT, PKS11T161D, and PKS11ΔF. The autoradiogram shown is representative of three independent experiments with similar results. *D*, peptide phosphorylation activities of wild-type (*WT*), serine (S154D), or tyrosine (Y173D) to aspartate changed mutants of PKS11. The number on *top of each bar* is fold increase over the wild-type control. Results represent the mean ± S.D. from three experiments.

binary vector, pBIB, under control of the super promoter (14). This promoter is located upstream of the *PKS11T161D* coding region and causes expression in all tissues constitutively. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and *Arabidopsis* transformation was performed according to the method described previously (15). The wild-type *PKS11* coding sequence was similarly overexpressed in *Arabidopsis* under control of the super promoter. After harvesting, the seeds were planted on MS agar 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_2 and T_3 generation transgenic plants expressing *PKS11T161D* 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 control lines were germinated and 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.

Statistical Analysis and Kinetic Parameter Calculations—The values of apparent K_m and maximal velocity V_{max} 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.

RESULTS

cDNA Cloning and Expression Patterns of PKS11 in Different Tissues and in Response to Environmental Stresses—In order to determine experimentally the open reading frame of *PKS11* gene, the cDNA was cloned by reverse transcription-PCR. The deduced amino acid sequence of PKS11 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 *PKS11* 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 *PKS11*. *PKS11* 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. 1*A*). 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. 1*B*). No significant induction or repression of *PKS11* was observed under any of the treatments.

A promoter-glucuronidase reporter fusion was used to investigate further the tissue distribution of *PKS11* 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. 1*C*).

Sequence Alignment and Analysis—Like SOS2, the founding member of the PKS family, PKS11 also contains an N-terminal SNF1-like kinase catalytic domain and a C-terminal regulatory domain (Fig. 2*A*). An alignment of the deduced amino acid sequence of PKS11 with SOS2 showed that these kinases are highly conserved throughout the entire length (Fig. 2*B*). In the superfamily of protein kinases, the PKSes belong to SNF1/AMPK family (16). Like many other protein kinases including SOS2, PKS11 contains a putative "activation loop" or "activation segment" in the kinase catalytic domain, located between the conserved DFG and APE sequences (Fig. 2*B*). The kinase also contains a conserved FISL motif, a stretch of 21 amino acid residues, located near the kinase domain (Fig. 2*B*). The FISL motif in SOS2 has been identified recently as the SOS3-interacting sequence and is autoinhibitory to substrate phosphorylation (3). PKS11 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. *PKS11* is located on chromosome 4, based on information in the *Arabidopsis* genomic sequence database (www.arabidopsis.org).

Activation of PKS11 by Amino Acid Substitutions in the

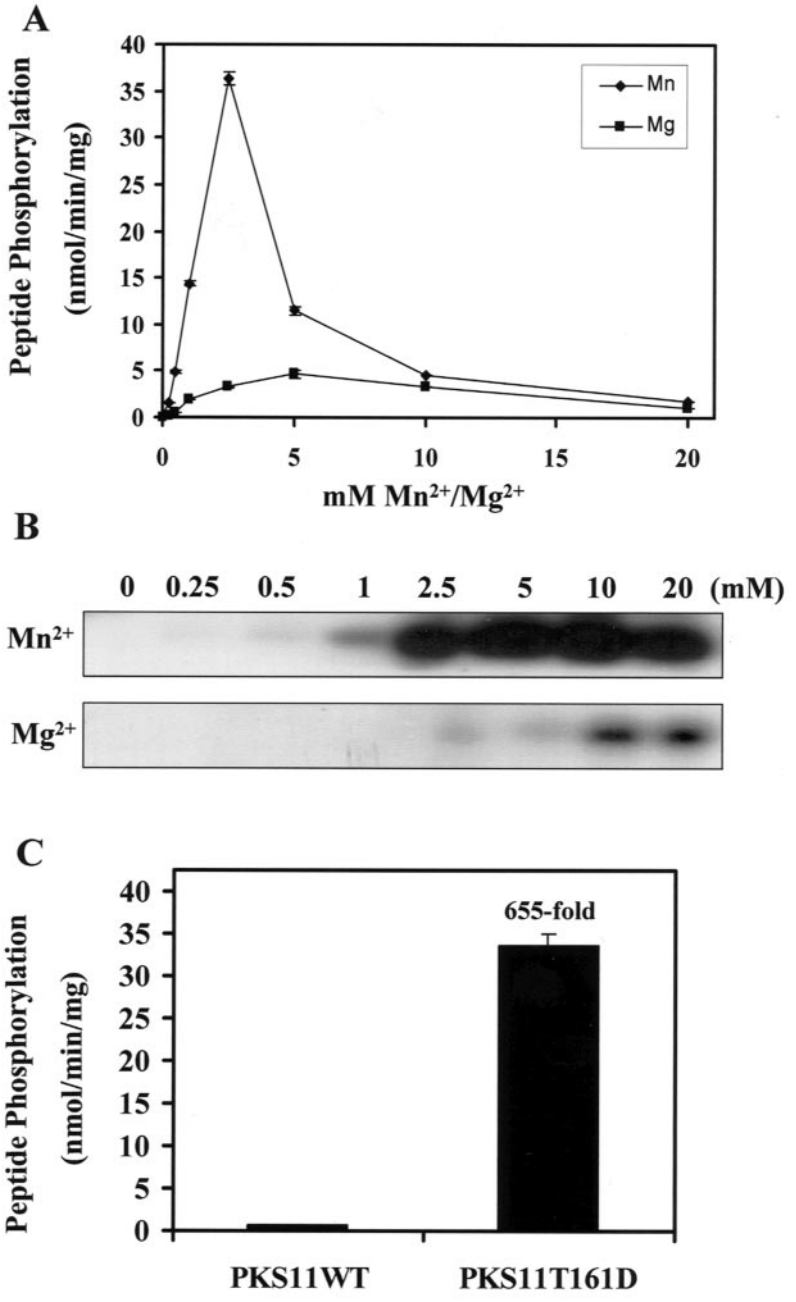

FIG. 4. **Dependence of substrate phosphorylation and autophosphorylation of PKS11T161D on divalent metal cations Mn^{2+} and Mg^{2+}.** *A*, peptide phosphorylation. Phosphorylation of p3 by the kinase was determined at various concentrations of Mn^{2+} (as $MnCl_2$) or Mg^{2+} (as $MgCl_2$) as indicated. Initial rates were measured and plotted against the Mn^{2+} or Mg^{2+} concentrations. Three independent experiments were performed, and the average is shown here. *Error bars* indicate ± S.D. ($n = 3$). *B*, autophosphorylation. Autophosphorylation of PKS11T161D in the presence of various concentrations of Mn^{2+} (as $MnCl_2$) or Mg^{2+} (as $MgCl_2$), as indicated, was presented as the density of autoradiographic bands. Three independent experiments were performed, and a typical result is shown here. *C*, comparison of p3 phosphorylation of PKS11T161D with PKS11WT in the presence of 2.5 mM Mn^{2+}. The number on *top of the bar* is fold increase over the wild-type control.

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 Thr^{168} 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. 2*B*). This suggests that the threonine residue in PKS11, Thr^{161}, 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 Thr^{161} to Asp (Fig. 2*B*). 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 Lys^{304} and Leu^{325} (Fig. 2*B*) 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. 3*A*). 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 5 mM Mg^{2+} as a cofactor. The FISL motif deletion mutant, PKS11ΔF, displayed a 3-fold increase in substrate phosphorylation compared with the wild-type kinase (designated PKS11WT) (Fig. 3*B*). In contrast, the activation loop mutant, PKS11T161D, was extremely

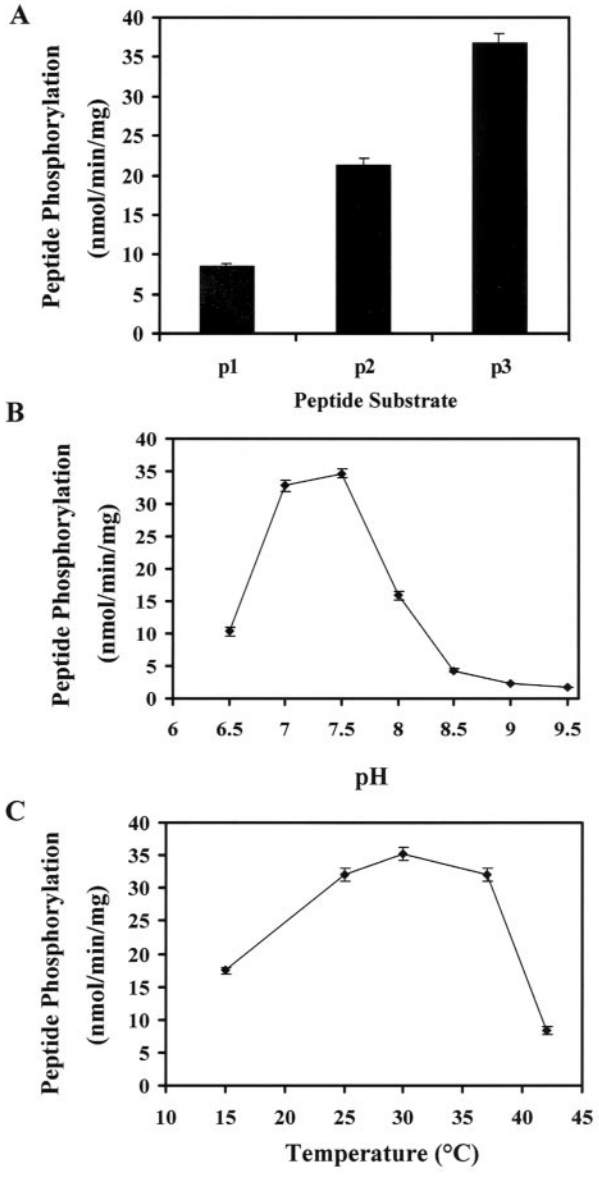

FIG. 5. **Substrate specificity and dependence on pH and temperature of PKS11T161D.** *A*, substrate specificity. PKS11T161D protein was incubated in the kinase assay buffer containing 150 μM of each peptide substrate at 30 °C for 30 min as described under "Experimental Procedures." *B*, pH dependence. Enzyme assays at each pH value were buffered by 20 mM BisTris. *C*, temperature dependence. Kinase assays were performed at each temperature indicated as described under "Experimental Procedures." Each result is the mean ± S.D. from three experiments.

active, with 130-fold higher activity in p3 phosphorylation than PKS11WT (Fig. 3*B*). Both PKS11 mutants also had higher autophosphorylation activity than PKS11WT (Fig. 3*C*).

In addition to the threonine residue, a serine and a tyrosine residue within the activation loop are also completely conserved in all PKSes (Fig. 2*B* and data not shown). We wanted to investigate whether changing the conserved serine or tyrosine to aspartate could also make the kinase constitutively active. We thus constructed PKS11S154D and PKS11Y173D, respectively, by mutating Ser^{154} to Asp and Tyr^{173} to Asp, respectively, via site-directed mutagenesis. Kinase assays showed that PKS11S154D and PKS11Y173D exhibited 16- and 15-fold higher activity, respectively, in p3 phosphorylation than the wild-type kinase (Fig. 3*D*). The most active mutant, PKS11T161D, was therefore used for subsequent biochemical and functional analysis.

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. 4*A*). 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. 4*A*). 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. 4*B*). 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. 4*C*) 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. 5*A*). 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. 5*B*). 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. 5*C*). 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-

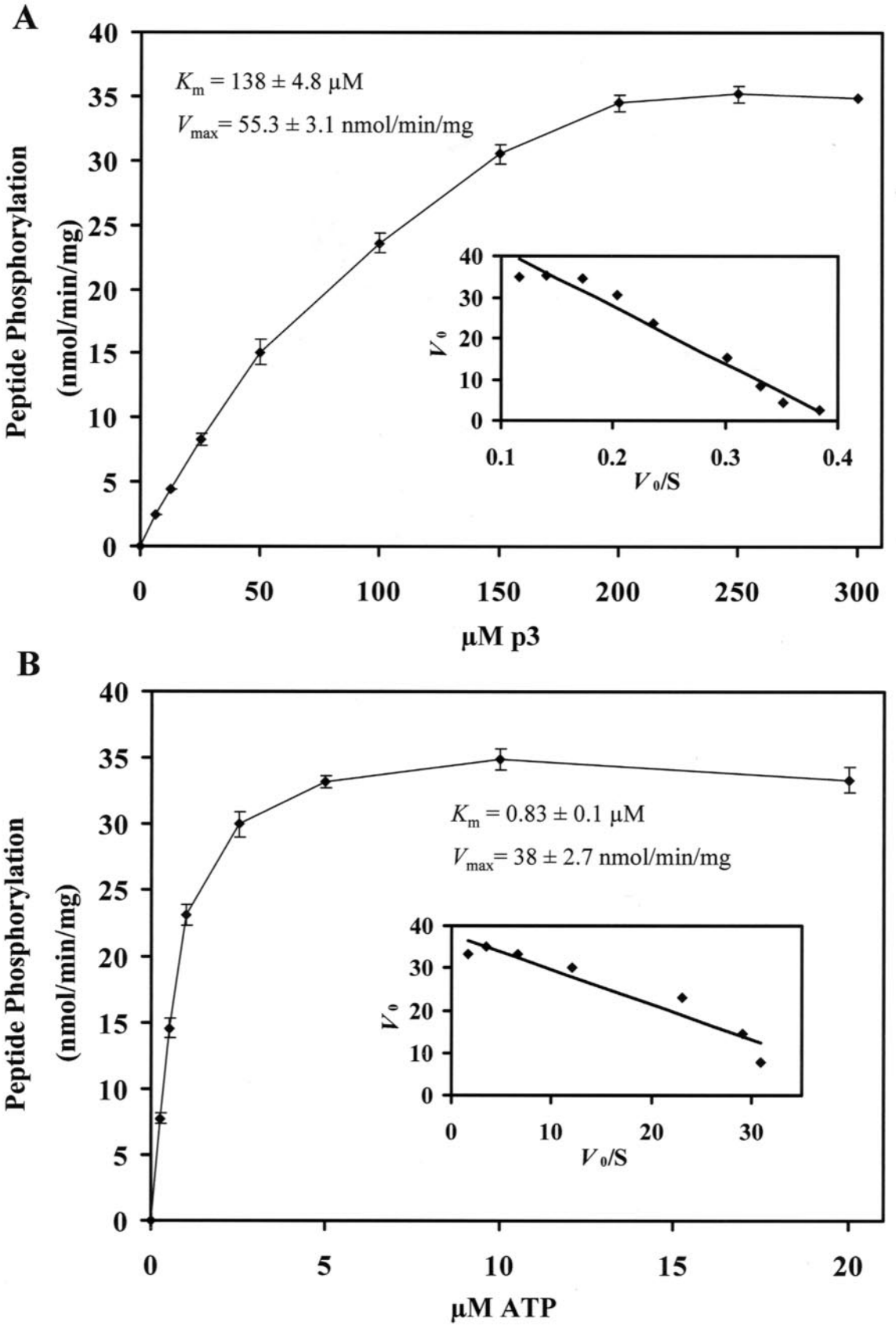

FIG. 6. **Dependence of substrate phosphorylation of PKS11T161D on peptide substrate p3 or ATP.** *A*, dependence of substrate phosphorylation on peptide substrate p3. Phosphorylation of p3 by PKS11T161D was assayed at 30 °C in the presence of 2.5 mM $MnCl_2$ as described under "Experimental Procedures." 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-Hosftee plots of the average values for each data set. *Error bars* indicate ± S.D. (n = 3).

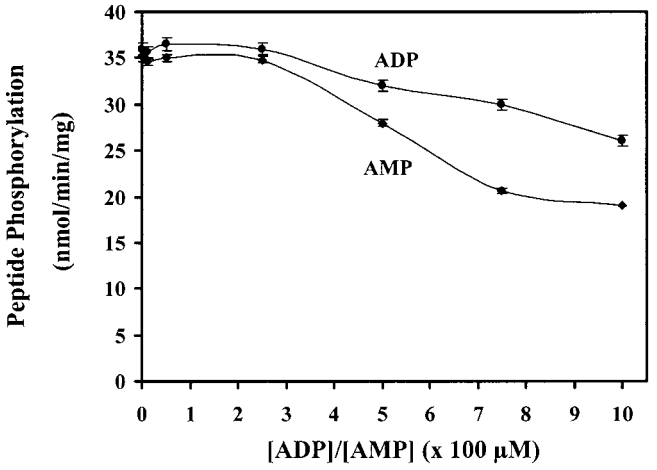

FIG. 7. **Effect of ADP and AMP on substrate phosphorylation of PKS11T161D.** p3 phosphorylation of PKS11T161D was assayed in different concentrations of either ADP or AMP as indicated. *Error bars* indicate ± S.D. ($n = 3$).

tein) plotted *versus* concentrations of the substrate p3 or ATP (Fig. 6, *A* and *B*). Apparent kinetic parameters were determined from the Eadie-Hofstee plots (shown in the *inset*). The K_m values toward p3 and ATP were ~138 and 0.83 μM, respectively; the V_{max} values were ~55 and 38 nmol/min/mg for p3 and ATP, respectively.

AMP was found to activate mammalian AMPK by severalfold (18–19) but did not activate AMPK homologs from cauliflower, carrot, rapeseed, and spinach and with modest inhibition at 200 μM AMP (20–21). We tested the effect of ADP or AMP on p3 phosphorylation by the active kinase. Neither ADP nor AMP at concentrations from 10 to 250 μM activated PKS11T161D. Instead, there was a modest inhibition by these chemicals at concentrations higher than 500 μM (Fig. 7).

Phenotypes of Transgenic Arabidopsis Plants Expressing PKS11T161D—We have reported that SOS2 kinase functions in plant salt tolerance (4). To test whether this novel SOS2-like protein kinase, PKS11, has distinct functions *in vivo*, we introduced *PKS11T161D* under control of the super promoter into *Arabidopsis.* As a control, *PKS11WT* was also introduced into transgenic *Arabidopsis* plants under control of the promoter. Over 20 transgenic lines were generated for each construct by using an *Agrobacterium*-mediated transformation method (15). The presence of the transgene was determined by hygromycin resistance and DNA gel blot analyses (data not shown). Seed germination and seedling growth in response to ABA, salt, mannitol, or glucose treatment were tested for 12 of the transgenic lines in T_2 and T_3 generations. We found that the *PKS11T161D* transgenic lines exhibited an altered response to glucose but not to any of the other treatments.

High concentrations of glucose exhibit an inhibitory effect on seed germination and seedling growth (22). Compared with untransformed control plants, the seeds of *PKS11T161D* transgenic plants germinated earlier (data not shown), and seedlings grew better on MS media supplemented with 2 or 4% glucose, but there was no difference in germination (data not shown) and seedling growth without glucose (Fig. 8*A*). Quantitative measurements of root length showed that 4% glucose significantly inhibited root growth of the untransformed control and of transgenic plants expressing *PKS11WT* (Fig. 8*B*). In contrast, transgenic plants expressing the constitutively active *PKS11T161D* were much less inhibited by the glucose treatment. To rule out the possibility that the difference in glucose response between the transgenic and control plants is due to an osmotic effect, we tested the plant response to the same concentration of mannitol. No difference was observed between the control and transgenic plants when they were planted on 4% mannitol (Fig. 8*A*). Interestingly, we also did not find any difference between the control and transgenic plants in their responses to the glucose analog 2-deoxyglucose or 3-*O*-methylglucose (data not shown).

Because hypocotyls elongate extensively in the dark, the effect of glucose on hypocotyl elongation could be quantified easily in the dark. Seeds were germinated and seedlings were grown on plates containing 4% glucose for 6 days in the dark to determine the effect of glucose on hypocotyl elongation (22). As shown in Fig. 8*C*, hypocotyl length of either control plants or transgenic plants expressing *PKS11WT* on 4% glucose was only ~30% that on 0% glucose. In contrast, the hypocotyl length on 4% glucose in transgenic plants expressing *PKS11T161D* was ~70% that on 0% glucose.

To determine whether the altered glucose response in the *PKS11T161D* transgenic plants correlated with the level of transgene expression, we conducted Northern blot analysis using a *PKS11* gene-specific probe. The results (Fig. 9*A*) showed that all the transgenic lines tested overexpressed the transgene at various levels. Furthermore, the transgene expression level in the different transgenic lines correlated well with their root growth (Fig. 9*B*) and hypocotyl elongation (data not shown) on 4% glucose.

DISCUSSION

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. 3*D*). 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-

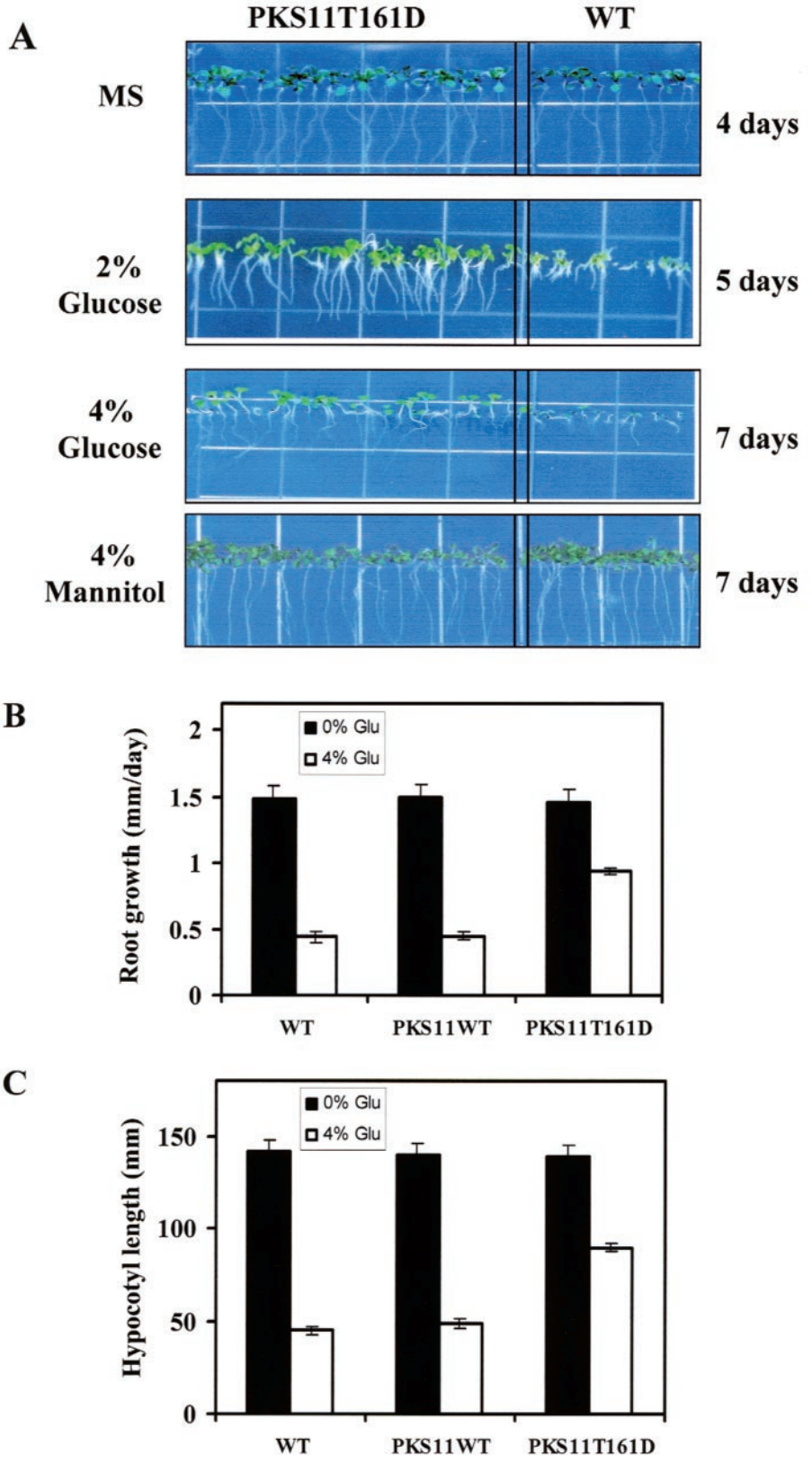

FIG. 8. **Transgenic *Arabidopsis* plants expressing *PKS11T161D* were more resistant to glucose.** *A*, seeds of homozygous *PKS11T161D* transgenic *Arabidopsis* plants (8th line) and untransformed control plants (*WT*) were germinated and grown in MS agar plates containing 0 (MS only), 2, and 4% glucose 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.

ton's tyrosine kinase or Src family tyrosine kinases (29, 30). 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 T*X*Y 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-

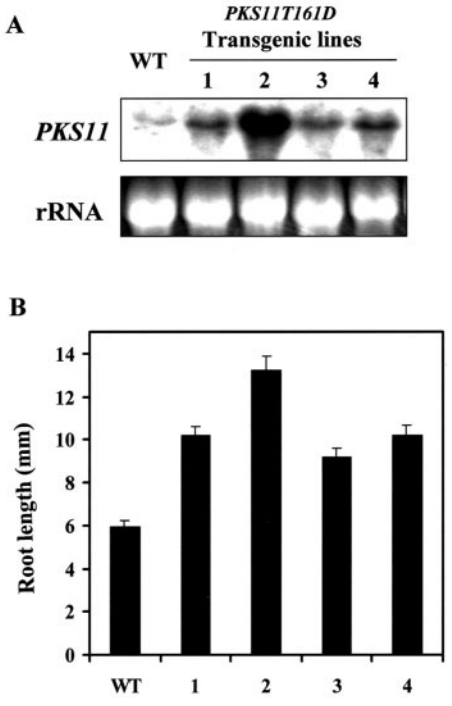

FIG. 9. **Correlation between transgene expression level and root growth on 4% glucose.** *A*, Northern blot analysis of the transgene expression. *WT*, untransformed wild-type control plants; *lanes 1–4* represent *PKS11T161D* transgenic lines 1, 8, 10, and 11, respectively. Thirty micrograms of total RNA samples from 10-day-old seedlings grown on 3% glucose plates were used for RNA gel blot analysis. A *PKS11* gene-specific probe was used to detect *PKS11* transcripts in the control untransformed (*WT*) plants and different transgenic lines. The Northern blot was exposed to an x-ray film for 20 h. Ethidium bromide-stained rRNA is shown as a loading control for equal loading. *B*, root growth of different transgenic lines. *WT*, untransformed control plants; *lanes 1–4* represent *PKS11T161D* transgenic lines 1, 8, 10 and 11, respectively. Seeds of the transgenic *Arabidopsis* plants and control plants were germinated and grown in MS agar plates containing 4% glucose under constant light. Root length was measured 7 days after seed imbibition. *Error bars* represent S.D. from 10 to 20 seedlings.

ation of residues in the activation segment belong to RD kinases (10) in which the aspartate residue in the activation loop has an adjacent arginine. The arginine residue has been hypothesized to form an ionic bridge with the phosphorylated serine or threonine residue, and this ionic bridge can stabilize the catalytically active conformation (10). *Arabidopsis* PKSes are non-RD protein kinases because they lack such a structural feature within the putative activation loop (Fig. 2*B* and data not shown). PKD was the first example of a non-RD kinase that is activated by phosphorylation of Ser^{744} and Ser^{748} (26). In this regard, PKS11 provides another interesting example of non-RD kinases being activated through possible activation loop phosphorylation. Further studies are needed to identify the upstream kinase(s) that phosphorylate PKS11 in its catalytic segment and to fully elucidate the molecular mechanism of activity regulation of PKS11 and other PKS proteins.

In this study, slight activation of PKS11 was achieved by deleting the FISL motif in the C-terminal regulatory domain. Substrate phosphorylation activity of SOS2 was dependent on both its interacting protein SOS3 and calcium (8). We have recently found that SOS2 interaction with SOS3 was mediated via the FISL motif. The FISL motif is sufficient to keep SOS2 inactive and serves as an autoinhibitory domain (3). Our result with the FISL motif deletion mutant of PKS11 shows that the FISL motif is also autoinhibitory in the PKS. Activation of PKS11 by the activation loop T to D change (Fig. 3, *B* and *C*) suggests that activation segment phosphorylation in the kinase domain is capable of at least partially releasing the inhibitory effect of the FISL motif.

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. 4*A*). 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. 4*A*). 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 monovalent and divalent metal cation cofactors (42–45).

The apparent K_m and V_{max} values toward ATP or the peptide substrate p3 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 (HMRSAMSGLHLVKRR), 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 animal 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

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. 3*B*). 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.

REFERENCES

1. *Arabidopsis* Genome Initiative (2000) *Nature* **408,** 796–815
2. Roberts, D. M. (1993) *Curr. Opin. Cell Biol.* **5,** 242–246
3. Guo, Y., Halfer, U., Ishitani, M., and Zhu, J.-K. (2001) *Plant Cell* **13,** 1383–1399
4. Liu, J., Ishitani, M., Halfter, U., Kim, C.-S., and Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3730–3734
5. Liu, J., and Zhu, J.-K. (1998) *Science* **280,** 1943–1945
6. Ishitani, M., Liu, J., Halfter, U., Kim, C.-S., Wei, M., and Zhu, J.-K. (2000) *Plant Cell* **12,** 1667–1677
7. Hardie, D. G. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 97–131
8. Halfter, U., Ishitani, M., and Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3735–3740
9. Shi, H., Ishitani, M., Kim, C.-S., and Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6896–6901
10. Johnson, L. N., Noble, M. E. M., and Owen, D. J. (1996) *Cell* **85,** 149–158
11. Wu, S., Ding, L., and Zhu, J.-K. (1996) *Plant Cell* **8,** 617–627
12. Shi, H., Xiong, L., Stevenson, B., Lu, T., and Zhu, J.-K. (2002) *Plant Cell* **14,** 465–477
13. Zhu, J.-K., Liu, J., and Xiong, L. (1998) *Plant Cell* **8,** 1181–1191
14. Narasimhulu, S. B., Deng, X., Sarria, R., and Gelvin, S. B. (1996) *Plant Cell* **8,** 873–886
15. Clough, S. J., and Bent, A. F. (1998) *Plant J.* **16,** 735–743
16. Hanks, S. K., and Hunter, T. (1995) *The Protein Kinase Facts Book*, pp. 7–47, Academic Press, London
17. White, M. F., and Kahn, C. R. (1986) *Enzymes* **17,** 248–361
18. Ferrer, A., Caelles, C., Massot, N., and Hegardt, F. G. (1985) *Biochem. Biophys. Res. Commun.* **132,** 497–504
19. Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) *Eur. J. Biochem.* **186,** 129–136
20. Mackintosh, R. W., Davies, S. P., Clarke, P. R., Weekes, J., Gillespie, J. G., Gibb, B. J., and Hardie, D. G. (1992) *Eur. J. Biochem.* **209,** 923–931
21. Toroser, D., Plaut, Z., and Huber, S. C. (2000) *Plant Physiol.* **123,** 403–411
22. Jang, J.-C., Leon, P., Zhou, L., and Sheen, J. (1997) *Plant Cell* **9,** 5–19
23. McCartney, R. R., and Schmidt, M. C. (2001) *J. Biol. Chem.* **276,** 36460–36466
24. Dajani, R., Fraser, E., Roe, S. M., Yong, N., Good, V., Dale, T. C., and Pearl, L. H. (2001) *Cell* **105,** 721–732
25. Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) *J. Biol. Chem.* **276,** 32606–32615
26. Iglesias, T., Waldron, R. T., and Rozengurt, E. (1998) *J. Biol. Chem.* **273,** 27662–27667
27. Ali, N., Halfter, U., and Chua, N.-H. (1994) *J. Biol. Chem.* **269,** 31626–31629
28. Shah, K., Vervoort, J., and Vries, S. C. (2001) *J. Biol. Chem.* **276,** 41263–41269
29. Kawakami, Y., Kitaura, J., Hartman, S. E., Lowell, C. A., Siraganian, R. P., and Kawakami, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7423–7428
30. Liu, Y., Witte, S., Liu, Y.-C., Doyle, M., Elly, C., and Altman, A. (2000) *J. Biol. Chem.* **275,** 3603–3609
31. Crosby, D., and Poole, A. W. (2002) *J. Biol. Chem.* **277,** 9958–9965
32. Zhou, B., and Zhang, Z.-Y. (2002) *J. Biol. Chem.* **277,** 13889–13899
33. Muranaka, T., Banno, H., and Machida, Y. (1994) *Mol. Cell. Biol.* **14,** 2958–2965
34. Horn, M. A., and Walker, J. C. (1994) *Biochim. Biophys. Acta* **1208,** 65–74
35. Su, J. Y., Eriksob, E., and Maller, J. L. (1996) *J. Biol. Chem.* **271,** 14430–14437
36. Schinkmann, K., and Blenis, J. (1997) *J. Biol. Chem.* **272,** 28695–28703
37. Stocchetto, S., Marin, O., Carignani, G., and Pinna, L. A. (1997) *FEBS Lett.* **414,** 171–175
38. White, M., Haring, H.-U., Kasuga, M., and Kahn, C. R. (1984) *J. Biol. Chem.* **259,** 255–264
39. Chi, A. S., Deng, Z., Albach, R. A., and Kemp, R. G. (2001) *J. Biol. Chem.* **276,** 19974–19981
40. Knowles, V. L., Smith, C. S., Smith, C. R., and Plaxton, W. C. (2001) *J. Biol. Chem.* **276,** 20966–20972
41. Sturany, S., Lint, J. V., Muller, F., Wilda, M., Hameister, H., Hocker, M., Brey, A., Gern, U., Vandnhede, J., Gress, T., Adler, G., and Seufferlein, T. (2001) *J. Biol. Chem.* **276,** 3310–3318
42. Ireland, R. J., DeLuca, V., and Dennis, D. T. (1980) *Plant Physiol.* **65,** 1188–1193
43. Lin, M., Turpin, D. H., and Plaxton, W. C. (1989) *Arch. Biochem. Biophys.* **269,** 219–227
44. Lin, M., Turpin, D. H., and Plaxton, W. C. (1989) *Arch. Biochem. Biophys.* **269,** 228–238
45. Smith, C. R., Knowles, V. L., and Plaxton, W. C. (2000) *Eur. J. Biochem.* **267,** 4477–4485
46. Barker, J. H., Slocombe, S. P., Ball, K. L., Hardie, D. G., Shewry, P. R., and Halford, N. G. (1996) *Plant Physiol.* **112,** 1141–1149
47. Sheen, J., Zhou, L., and Jang, J.-C. (1999) *Curr. Opin. Plant Biol.* **2,** 410–418
48. Zhang, Y., Shewry, P. R., Jones, H., Barcelo, P., Lazzeri, P. A., and Halford, N. G. (2001) *Plant J.* **28,** 431–441
49. Halford, N. G., and Hardie, D. G. (1998) *Plant Mol. Biol.* **37,** 735–748
50. Furuichi, T., and Muto, S. (2001) *The 12th International Workshop, Plant Membrane Biology,* p. 63, Madison, WI