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Characterization of a PDK1 homologue from the moss Physcomitrella patens

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ABSTRACT

The Ser/Thr protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a highly conserved eukaryotic kinase that is a central regulator of many AGC kinase subfamily members. Through its regulation of AGC kinases, PDK1 controls many basic cellular processes from translation to cell survival. While many of these PDK1-regulated processes are conserved across kingdoms, it is not well understood how PDK1 may have evolved within kingdoms. In order to better understand PDK1 evolution within plants, we have isolated and characterized the PDK1 gene from the moss Physcomitrella patens (PpPDK1), a non-vascular representative of early land plants. PpPDK1 is similar to other plant PDK1s in that it can functionally complement a yeast PDK1 knockout line. However, unlike PDK1 from other plants, the P. patens PDK1 protein does not bind phospholipids due to a lack of the lipid-binding pleckstrin homology domain, which is used for lipid-mediated regulation of PDK1 activity. Sequence analysis of several PDK1 proteins suggests that lipid regulation of PDK1 may not commonly occur in algae and non-vascular land plants. PpPDK1 can phosphorylate AGC kinase substrates from tomato and P. patens at the predicted PDK1 phosphorylation site, indicating that the PpPDK1 substrate phosphorylation site is conserved with higher plants. We have also identified residues within the PpPDK1 kinase domain that affect kinase activity and show that a mutant with highly reduced kinase activity can still confer cell viability in both yeast and P. patens. These studies lay the foundation for further analysis on the evolution of PDK1 within plants.
The kinase subfamily known as the AGC kinases is responsible for regulating many basic cellular functions and are found in diverse species including yeast, mammals, and plants (Manning et al., 2002; Bögre et al., 2003; Pearce et al., 2010). This group of kinases is named for three of its representatives, cAMP-dependent protein kinase 1 (PKA), cGMP-dependent protein kinase (PKG), and protein kinase C (PKC). Given that the AGC kinase subfamily has been maintained across vast evolutionary distances and divergent life strategies it appears that it is of ancient origin.

Protein kinases within the AGC group are regulated by two main conserved phosphorylation sites. The first site is a Ser or Thr located in a hydrophobic motif typically found at the C-terminus of the protein (Frödin et al., 2002). The second phosphorylation site is a Ser or Thr located in a region of variable length referred to as the activation loop or T-loop (Bögre et al., 2003). In many AGC kinases from both mammals (Bayascas, 2008; Bayascas, 2010) and plants (Bögre et al., 2003; Anthony et al., 2004; Anthony et al., 2006; Devarenne et al., 2006; Zegzouti et al., 2006; Zegzouti et al., 2006), this activation loop site is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1). Activation loop phosphorylation by PDK1 typically occurs after docking of the phosphorylated hydrophobic motif of the AGC kinase substrate within a pocket located in the small lobe of the PDK1 kinase domain (Biondi et al., 2002). Accordingly, the hydrophobic motif of AGC kinases is also known as the PDK1-interacting fragment (PIF) and the pocket with which it interacts is called the PIF-binding pocket. Many substrate AGC kinases also contain a PIF-binding pocket for binding their own PIF, which stabilizes the kinase-active conformation generated from PDK1 phosphorylation (Frödin et al., 2002; Biondi, 2004).

PDK1 is itself an AGC kinase and was initially identified by chromatographic fractionation of muscle and brain protein extracts as the upstream kinase responsible for activation loop phosphorylation of protein kinase B (PKB; aka Akt) (Alessi et al., 1997; Stokoe et al., 1997). In the years since its identification, PDK1 has been demonstrated to phosphorylate at least 23 of the 60 human and 12 of the 39 Arabidopsis AGC kinases (Zegzouti et al., 2006; Pearce et al., 2010), thereby coordinating numerous and diverse cellular processes, including cell survival and apoptosis, cell growth and division, hormone responses, and protein synthesis (Pearce et al., 2010). Evidently, the ability of PDK1 to activate a large array of AGC kinases is a crucial regulatory mechanism for many important signaling pathways.
The phosphorylation of PKB by PDK1 requires production of the signaling lipid phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$), which directs colocalization of PDK1 and PKB at the plasma membrane to promote their interaction (Borgatti et al., 2003; Calleja et al., 2007). Subsequently, several other AGC kinases have been shown to be phosphorylated by PDK1 in response to PtdIns(3,4,5)P$_3$ production (Bayascas, 2010). A domain within PDK1 known as the pleckstrin homology (PH) domain directly binds PtdIns(3,4,5)P$_3$ (Bögre et al., 2003). Even though plants do not produce PtdIns(3,4,5)P$_3$ (Bögre et al., 2003), the PH domain has been shown to bind several phosphoinositides (Deak et al., 1999), but phosphatidic acid appears to be the phospholipid that induces PDK1 activity toward substrates (Anthony et al., 2004; Anthony et al., 2006).

Though not present in prokaryotes, PDK1-like sequences have been identified in almost all eukaryotes studied so far including yeast, multicellular fungi, nematodes, insects, gastropods, mammals, and plants (Silber et al., 2004). As might be expected because of its role as a key regulator of AGC kinase signaling, loss of PDK1 is lethal in yeast (Casamayor et al., 1999; Niederberger and Schweingruber, 1999), D. melanogaster (Rintelen et al., 2001), mice (Lawlor et al., 2002), and tomato (Devarenne et al., 2006). Interestingly, in contrast to the other studied PDK1 genes, Arabidopsis PDK1 (AtPDK1) appears to be non-essential for plant survival (Camehl et al., 2011). PDK1s from both human and Arabidopsis have been shown to complement loss of the S. cerevisiae PDK1 homologues PKH1 and PKH2 (Casamayor et al., 1999; Deak et al., 1999), suggesting that while some aspects of PDK1 function are common to yeast, mammals, and plants, additional organism-specific AGC kinase-mediated cellular regimes await further exploration.

In recent years the moss Physcomitrella patens has emerged as an exciting new model organism due to several attractive advantages over other plant models including a dominant haploid gametophyte stage (Cove, 2005) and highly efficient homologous recombination (Schaefer, 2002). This makes targeted gene disruption and replacement possible for the first time in a plant. Additionally, as a member of the bryophytes P. patens is an ideal model for plant evolutionary studies. Bryophytes, consisting of mosses, liverworts, and hornworts, are the closest extant relatives of early land plants, which first appear in the fossil record approximately 440-460 million years ago (Wellman et al., 2003; Wellman, 2010). Almost all other plant models are angiosperms, a much more recent lineage that emerged approximately 140-180
million years ago (Soltis et al., 2008). Therefore, studies performed in *P. patens* can be used to uniquely illuminate the evolutionary changes that have occurred since the ancestors of modern plants first emerged from aquatic habitats to colonize the land.

In this study we identify and characterize a homologue of PDK1 from *P. patens* (*PpPDK1*) and analyze *PDK1* sequences from divergent plant species to examine the protein features of *PpPDK1* in an evolutionary context. These experiments present a starting point from which additional aspects of PDK1 function and evolution may be investigated.

**RESULTS**

**Identification of a Putative PDK1 from *P. patens***

The *P. patens* genome database at Phytozome (www.phytozome.net) was searched using the *AtPDK1* sequence (GenBank accession AF132742.1) (Deak et al., 1999). The two top hits, *Pp1s217_11V6.2* and *Pp1s217_11V6.1* (Supplemental Fig. S1A), are alternate transcripts produced from the same genomic locus, *Pp1s217_11V6*, which spans 3,941 nt of genomic DNA and contains a 2,803 nt coding region of nine exons/eight introns (Fig. 1A, Supplemental Fig. S2A). *Pp1s217_11V6.2* is listed as the primary transcript, and thus will be the main focus of this study. Its coding sequence comprises 1,044 nt, which produces a protein of 347 a.a. (Fig. 1A, B and Supplemental Fig. S2B, C). *Pp1s217_11V6.1* is a splice variant with a 1,041 nt coding sequence generated by including the final 3 nt of exon 5 with intron 5. This produces a protein lacking a single glutamate that would originate from the junction between exons 5 and 6 (data not shown). *Pp1s217_11V6* locus transcripts are annotated as PDK1 by Kyoto Encyclopedia of Genes and Genomes (KEGG). The third and fourth hits (*Pp1s118_230V6* and *Pp1s4_386V6.1*) are both annotated as putative homologues of ribosomal S6 kinase (S6K) by Eukaryotic Orthologous Groups (KOG) and have coding sequences of 1,488 nt and 1,518 nt, respectively (Supplemental Fig. S1A). *Pp1s217_11V6.2, Pp1s118_230V6*, and *Pp1s4_386V6.1* were all amplified from total moss mRNA, cloned into *E. coli* and yeast expression vectors, and sequenced. Hereafter the *Pp1s217_11V6.2, Pp1s118_230V6*, and *Pp1s4_386V6.1* genes are referred to as *PpPDK1, Pp188412*, and *Pp174181*, respectively (Supplemental Table S1).
Based on ClustalW protein sequence alignment with \textit{AtPDK1} and tomato PDK1 (\textit{Solanum lycopersicum}, \textit{SlPDK1}), \textit{PpPDK1} appears to possess characteristics of a typical PDK1: a pocket for binding the PIF motif of substrate AGC kinases, a kinase domain containing the conserved Lys residue (Lys67) required for ATP coordination by all protein kinases, and an activation loop within the kinase domain (Fig. 1B). However, \textit{PpPDK1} lacks a C-terminal lipid-binding PH domain, and thus shares only 34.4\% amino acid identity with \textit{AtPDK1}, whereas \textit{SlPDK1} and \textit{AtPDK1} are 79.9\% identical (Fig. 1B). Like \textit{PpPDK1}, yeast Phk1 and Phk2 also lack PH domains, but both proteins are much larger than \textit{PpPDK1} (Phk1, 766 a.a.; Phk2, 1,061 a.a.) and their overall sequence identity to other PDK1s is low (Casamayor et al., 1999).

\textit{PpPDK1 is a Functional Homologue of \textit{S. cerevisiae} Phk1/2}

\textit{AtPDK1} was initially identified as a functional homologue of PDK1 by its ability to complement a deletion strain of the \textit{S. cerevisiae PDK1} homologues \textit{PKH1} and \textit{PKH2} (Deak et al., 1999). The previously characterized diploid yeast strain AC306 is heterozygous for deletion of both \textit{PKH1} and \textit{PKH2} (Casamayor et al., 1999). After sporulation of this strain, haploid cells lacking both \textit{PKH1} and \textit{PKH2} (\textit{pkh1}Δ::HIS3/pkh2Δ::TRP1) are inviable, but expression of either human \textit{PDK1} or \textit{AtPDK1} in these cells rescues the lethal phenotype (Casamayor et al., 1999; Deak et al., 1999). A similar complementation experiment was performed to test whether \textit{PpPDK1} is a functional homologue of Phk1/2. AC306 yeast transformed with empty vector, \textit{MBP-AtPDK1-6His}, \textit{MBP-SlPDK1-6His}, or \textit{MBP-PpPDK1-6His} were sporulated and haploid spores grown on nonselective YPD medium to enable growth of all viable spores. Analysis of at least thirty tetrads from each yeast culture recovered no viable \textit{pkh1}Δ::HIS3/pkh2Δ::TRP1 spores from untransformed yeast or from yeast transformed with empty vector (Fig. 2A). However, after transformation with \textit{MBP-AtPDK1-6His}, \textit{MBP-SlPDK1-6His}, or \textit{MBP-PpPDK1-6His} viable \textit{pkh1}Δ::HIS3/pkh2Δ::TRP1 spores were recovered, indicating that each \textit{PDK1} tested was able to rescue the lethal phenotype of the \textit{PKH1/2} deletion (Fig. 2A). Western blotting with α-MBP confirmed expression of the PDK1 proteins (Fig. 2B). The ability of each \textit{PDK1} to confer \textit{pkh1}Δ::HIS3/pkh2Δ::TRP1 spore viability was confirmed by growing spores on medium with 5-fluoroorotic acid (5-FOA), which induces loss of the URA3-marked plasmids containing the \textit{PDK1} constructs. No viable \textit{pkh1}Δ::HIS3/pkh2Δ::TRP1 spores were recovered from 5-FOA.
plates (Fig. 2C), suggesting that like AtPDK1 and SlPDK1, PpPDK1 is a functional homologue of Pkh1/2.

The Pp174181 and Pp188412 kinase genes identified in our search of P. patens for PDK1-like sequences appear to be more closely related to the AGC kinase S6K rather than PDK1 based on protein BLAST analysis (Supplemental Fig. S1A). To verify that P. patens possesses a single functional homologue of PDK1, a second complementation experiment was performed with PpPDK1, Pp174181, and Pp188412. The previously characterized haploid S. cerevisiae strain INA106-3B lacks PKH2 and possesses a point mutation in PKH1 (D398G; pkh2Δ::LEU2/pkh1D398G) that confers a temperature sensitive phenotype; INA106-3B yeast is able to grow at the permissive temperature of 25°C but not at the restrictive temperature of 35°C (Inagaki et al., 1999). INA106-3B yeast were transformed with empty vector, MBP-PpPDK1-6His, MBP-Pp174181, or MBP-Pp188412. Transformed yeast cultures were grown in liquid medium lacking uracil at 25°C and spotted onto plates which were incubated at either 25°C or 35°C. Only yeast transformed with MBP-PpPDK1-6His were able to complement pkh2Δ::LEU2/pkh1D398G, as assessed by growth at 35°C (Supplemental Fig. S1B). Western blotting with α-MBP confirmed expression of all proteins (Supplemental Fig. S1B). Taken together, these results suggest that like rice (Matsui et al., 2010), and in contrast to Arabidopsis (Bögre et al., 2003; Camehl et al., 2011) and tomato (Devarenne et al., 2006), the P. patens genome contains a single PDK1 gene.

Lipid-binding ability of PpPDK1

Because of its lack of a PH domain (Fig. 1A), the ability of PpPDK1 to bind lipids was analyzed. PpPDK1 did not appear to strongly bind phospholipids, though very weak binding to several phospholipids was detected using protein-lipid overlay assays (Supplemental Fig. S3A). This result is in contrast to AtPDK1 and SlPDK1, which both strongly interact with a number of phospholipids including multiple phosphorylated phosphatidylinositols and phosphatidic acid (PA; Supplemental Fig. S3A) (Deak et al., 1999). PpPDK1 also appeared not to bind sphingolipids (Supplemental Fig. S3B), contrary to human PDK1 which is activated by sphingosine (King et al., 2000), and Pkh1/2 which are activated by sphingoid bases (Friant et al., 2001). These results raise the possibility that, unlike the other plant PDK1s analyzed so far...
Characterization of PpPDK1 Activity

The kinase activity (auto- and trans-phosphorylation) of PpPDK1 was tested by in vitro kinase assays. MBP-PpPDK1-6His used either Mg\(^{2+}\) or Mn\(^{2+}\) as a divalent cation for autophosphorylation and phosphorylation of the artificial kinase substrate myelin basic protein (Fig. 3A). As expected, since Lys67 coordinates ATP, mutation of Lys67 to Gln (K67Q) abolished both PpPDK1 autophosphorylation and phosphorylation of myelin basic protein (Fig. 3A). To test whether PpPDK1 can activate AGC kinases, PpPDK1 was incubated with either a known PDK1 AGC kinase substrate, Adi3 from tomato (Devarenne et al., 2006), or a novel putative AGC kinase isolated from P. patens, Pp2484, which was identified by searching the P. patens genome database at Phytozome using the Adi3 sequence (accession #AY849914). Based on ClustalW protein sequence alignment with Adi3, Pp2484 appears to possess characteristics of a typical AGC kinase: a kinase domain containing the invariant ATP coordinating Lys (Lys341), an activation loop within the kinase domain, and a C-terminal PIF motif for interaction with PDK1 (Supplemental Fig. S4A). Pp2484 was further verified as a functional kinase using in vitro kinase assays to confirm the contribution of conserved residues to its activity. As expected, mutation of the conserved ATP-coordinating Lys341 to Gln (K341Q) completely abolished MBP-Pp2484 autophosphorylation and phosphorylation of myelin basic protein (Supplemental Fig. S4B). Mutation of the conserved activation loop Ser to the phosphomimetic amino acid Asp (S577D) increased MBP-Pp2484 kinase activity compared to wild-type protein (Supplemental Fig. S4B). Next, the ability of PpPDK1 to phosphorylate these AGC kinases was tested. MBP-PpPDK1-6His phosphorylated kinase-inactive MBP-Adi3\(^{K337Q}\) and MBP-Pp2484\(^{K341Q}\) (Fig. 3B). As has been seen for SlPDK1 phosphorylation of Adi3 (Devarenne et al., 2006), mutation of the conserved PDK1 phosphorylation site in the activation loop of MBP-Adi3\(^{K337Q}\) and MBP-Pp2484\(^{K341Q}\) (Ser539 in Adi3 and Ser577 in Pp2484) to Ala decreased total phosphorylation of both proteins by approximately 60 percent (Fig. 3B). These results provide evidence that, like other PDK1s, PpPDK1 is able to phosphorylate AGC kinases in vitro, including a P. patens
AGC kinase that is a potential substrate in vivo. Taken with the yeast complementation results, it seems likely that PpPDK1 functions as the only PDK1 used by P. patens to regulate the activity of its AGC kinases.

PIF-Binding Pocket Mutations That Affect PpPDK1 Activity

Interaction between many AGC kinase substrates and PDK1 is mediated by hydrophobic interactions between the PIF motif at the C-terminus of the substrate and the PIF-binding pocket of PDK1 (Bögre et al., 2003). Mutating any of several important residues in the PIF-binding pocket decreases PDK1 interaction with substrates (Frödin et al., 2002). In human PDK1, the PIF-binding pocket residues that interact with the PIF motif of PKA include Lys115, Ile119, Gln150, or Leu155, and each residue is required for efficient PDK1-PIF interaction (Biondi et al., 2000). These residues correspond to Lys341, Ala345, Glu376, and Leu383 of Adi3, which also has a PIF-binding pocket that interacts with its own PIF motif, and mutation of Lys341, Gln376, or Leu383 reduces Adi3 autophosphorylation (Devarenne et al., 2006). Based on ClustalW protein sequence alignment with human PDK1 and Adi3, the corresponding PIF-binding pocket residues of PpPDK1 are Lys71, Ile75, Gln106, and Leu111 (Supplemental Fig. S5A). Each of these residues were individually mutated to test the effects on PpPDK1 activity and interaction with Pp2484.

First, the autophosphorylation activity of PpPDK1 PIF-binding pocket mutants was tested by in vitro kinase assays. As has been seen in Fig. 3A, autophosphorylation was completely abolished in the kinase-inactive MBP-PpPDK1K67Q-6His (Fig. 4A). The K71A, K71Q, and L111A mutations all drastically reduced MBP-PpPDK1-6His autophosphorylation compared to wild-type PpPDK1 (Fig. 4A). In contrast, the I75A and Q106A mutations produced similar autophosphorylation levels compared to wild-type PpPDK1 (Fig. 4A). These results are somewhat unexpected because PDK1 proteins lack a PIF motif, and autophosphorylation is not known to require PIF-binding pocket residues. It is possible that in PpPDK1, Lys71 and Leu111 are required for the general maintenance of PpPDK1 in a kinase-active state, possibly by ensuring proper conformation of the small lobe of the kinase domain where the PIF-binding pocket is found.
Next, the *Pp*PDK1 PIF-binding pocket mutants were incubated with kinase-inactive *Pp*2484K341Q in *in vitro* kinase assays to test the activity of the *Pp*PDK1 mutants on a potential substrate. MBP-*Pp*PDK1K67Q-6His displayed no detectable activity on MBP-*Pp*2484K341Q (Fig.4B). The activity of the K71A and K71Q MBP-*Pp*PDK1-6His mutants toward MBP-*Pp*2484K341Q was drastically reduced compared to wild-type *Pp*PDK1 (Fig. 4B). The activity of MBP-*Pp*PDK1L111A-6His on MBP-*Pp*2484K341Q was also reduced compared to wild-type *Pp*PDK1 (Fig. 4B), but was not as dramatic as the decrease in autophosphorylation shown in Fig. 4A. Interestingly, though the autophosphorylation of MBP-*Pp*PDK1I75A-6His and MBP-*Pp*PDK1Q106A-6His was comparable to wild-type *Pp*PDK1 (Fig. 4A), these mutants displayed reduced activity on MBP-*Pp*2484K341Q compared to wild-type *Pp*PDK1 (Fig. 4B). In kinase assays containing the *Pp*PDK1 PIF-binding pocket mutants and kinase-inactive Adi3K337Q, similar results were obtained for the K67Q, K71A, K71Q, and L111A mutants (Supplemental Fig. S5B). However, in contrast to the kinase assays with *Pp*2484, activity of MBP-*Pp*PDK1-6HisI75A and MBP-*Pp*PDK1Q106A-6His on MBP-Adi3K337Q was comparable to wild-type *Pp*PDK1 (Supplemental Fig. S5B).

To further investigate the contributions of Lys71, I75, Gln106, and Leu111 to substrate binding, *in vitro* pulldown experiments were performed between *Pp*PDK1 and *Pp*2484. Wild-type and PIF-binding pocket mutants of MBP-*Pp*PDK1-6His were pre-incubated with Ni²⁺ resin, then either MBP or MBP-*Pp*2484 was added, and the interactions assessed by western blotting with α-MBP. No individual point mutation was able to abolish interaction between *Pp*PDK1 and *Pp*2484, though several *Pp*PDK1 mutants (K67Q, K71A, K71Q, and L111A) were able to pull down less *Pp*2484 than wild-type *Pp*PDK1 (Fig. 4C). Taken together with the kinase assays, these data may indicate that the analyzed *Pp*PDK1 PIF-binding pocket residues are required for proper conformation of an active kinase as well as participation in substrate binding.

Finally, the ability of *Pp*PDK1 PIF-binding pocket mutants to complement Pkh1/2 was tested with the same INA106-3B yeast used to test *Pp*174181 and *Pp*188412 complementation (Supplemental Fig. S1B). INA106-3B yeast were transformed with either empty vector, wild-type MBP-*Pp*PDK1-6His, or a MBP-*Pp*PDK1-6His PIF-binding pocket mutant and analyzed as in Supplemental Fig. S1B. Unexpectedly, only yeast transformed with MBP-*Pp*PDK1K67Q-6His, which completely lacks kinase activity (Fig. 4A and B), was unable to fully complement *PKH1/2*, as assessed by growth at 35°C (Fig. 4D). Yeast transformed with MBP-*Pp*PDK1K71A-
6His, which is capable of minimal autophosphorylation (Fig. 4A) and trans-phosphorylation (Fig. 4B), displayed comparable growth at 35°C to yeast transformed with wild-type MBP-PpPDK1-6His (Fig. 4D). Western blotting with α-MBP confirmed expression of all MBP-PpPDK1-6His proteins (Fig. 4D). These results suggest that, while kinase-active PpPDK1 is required to perform tasks related to cell survival and growth, very low levels of activity are sufficient to enable PpPDK1 to fulfill these vital functions.

Analysis of pdk1 knockout and PpPDK1-6His and PpPDK1K71A-6His Transformed Moss

In every organism studied so far except Arabidopsis (Camehl et al., 2011), deletion of PDK1 is lethal (Casamayor et al., 1999; Niederberger and Schweingruber, 1999; Rintelen et al., 2001; Lawlor et al., 2002; Devarenne et al., 2006). To test whether deletion of PpPDK1 is lethal, we attempted to knockout PpPDK1 via homologous recombination using a construct containing the hygromycin resistance marker between 5’ and 3’ PpPDK1 targeting fragments (Fig. 5A). At the same time we took an alternate approach in case the pdk1 knockout line was lethal. Because PpPDK1K71A had the largest reduction in kinase activity (Fig. 4A and B) but still enabled yeast viability (Fig. 4D), we used gene targeting to replace the endogenous PpPDK1 with either PpPDK1-6His or PpPDK1K71A-6His (Fig. 5B). After transformation and two rounds of antibiotic selection, the surviving moss colonies were PCR genotyped using primers 1 and 2 shown in Fig. 5A and B.

Initial genotyping showed that the majority of the pdk1 knockout transformants did not lack the endogenous PpPDK1 gene, whereas the majority of gene replacement transformants yielded genotyping products for both the endogenous PpPDK1 (532 nt) and PpPDK1-6His (2,356 nt; data not shown), suggesting that these colonies might have been unstably transformed. Further analysis identified one pdk1 knockout line, one PpPDK1-6His transformant, and one PpPDK1K71A-6His transformant. A representative genotyping PCR is shown in Fig. 5C indicating the pdk1 knockout line lacked the endogenous PpPDK1 gene and the gene replacement lines lacked the endogenous copy of PpPDK1 and were most likely stable transformants.

Additional genotyping was used to verify 5’ and 3’ integration of all constructs into the endogenous PpPDK1 locus of the P. patens genome. Using the primer combinations 3 + 4 and
5 + 6 shown in Fig. 5A and B, moss colonies with PCR products of the correct size were amplified from genomic DNA extracted from the pdk1 knockout line and the gene replacement lines, but not from wild-type P. patens (Fig. 5D). This indicated that all transformants had integrated the exogenous DNA into the PpPDK1 locus. These PCR products were cloned and sequenced to confirm that proper 5’ and 3’ integration had occurred.

Finally, Southern blot hybridization was performed to verify that PpPDK1-6His and PpPDK1K71A-6His were present in the correct location in the P. patens genome. Southern blot analysis was not carried out on the pdk1 knockout line due to a lack of large amounts of viable tissue (see below). A probe based in the 35S promoter of the G418 resistance cassette (Supplemental Fig. S6A) hybridized to a predicted DNA fragment of approximately 5 kb in NdeI/Smal/XbaI digested genomic DNA from PpPDK1-6His and PpPDK1K71A-6His strains, but not from wild-type P. patens (Supplemental Fig. S6B), confirming that the PpPDK1-6His constructs had integrated into the endogenous PpPDK1 locus. The PpPDK1-6His transformant produced four additional hybridizing DNA fragments, whereas the PpPDK1K71A-6His transformant produced one additional hybridizing fragment, suggesting that both strains probably contain multiple insertions of the PpPDK1-6His constructs into the PpPDK1 locus, which is a common occurrence in transformed lines of P. patens (Schaefer and Zryd, 1997; Schaefer, 2002). Overall, these results indicate that the transformed moss strains lack the endogenous PpPDK1 gene and contain at least one copy of PpPDK1-6His or PpPDK1K71A-6His DNA that have been integrated into the PpPDK1 locus by homologous recombination.

In order to confirm loss of PpPDK1 expression in the pdk1 knockout line and expression of the PpPDK1-6His and PpPDK1K71A-6His constructs, RT-PCR expression analysis was performed on all transformants as described previously using ubiquitin as a control (Harries et al., 2005). PpPDK1 mRNA was absent from the pdk1 knockout line and was present at similar levels in wild-type and PpPDK1K71A-6His moss, whereas PpPDK1-6His moss contained higher levels of PpPDK1 mRNA (Fig. 5E). These results indicate that the pdk1 knockout line is lacking the endogenous PpPDK1 and the gene replacement lines are expressing either PpPDK1-6His or PpPDK1K71A-6His. Attempts to pull down PpPDK1-6His and PpPDK1K71A-6His proteins from moss using Ni²⁺ resin were unsuccessful (data not shown), so future efforts to purify PpPDK1 from moss may require the use of additional tags, such as a tandem affinity purification (TAP) tag, or robust overexpression of PpPDK1.
To assess macroscopic effects from the loss of PDK1 and the K71A mutation on *P. patens* growth and development, wild-type, *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1K71A-6His* strains were grown and allowed to develop protonema and leafy gametophore tissue over a 12 week time course. The *pdk1* knockout line and wild-type moss grew similarly for the first 2 weeks (Fig. 6A). However, at later time points the *pdk1* knockout line produced fewer protonema, some of which began to turn brown and die (Supplemental Fig. S7). By 6 weeks, significant browning of the *pdk1* knockout protonema was seen and at 12 weeks much of the protonemal tissue was brown, though some green filaments were still visible (Fig. 6A, B). A small amount of apparently healthy gametophore tissue formed in the center of the *pdk1* knockout colony (Fig. 6A, B), but the majority of the tissue appeared to be dead. For the gene replacement lines, the colony viability and sizes of both transformed strains appeared to be similar to wild-type moss for the first 4 weeks, after which expansion of the transformed moss colonies through protonemal growth was reduced compared to wild-type moss (Fig. 6A). At the same time, production of gametophore tissue in the gene replacement lines appeared to form earlier than the wild-type moss, but was not apparently different in morphology from the wild-type moss (Fig. 6A, B). This result is in agreement with yeast complementation results (Fig. 4D) and suggests that the minimally active *PpPDK1K71A* is able to carry out essential functions related to moss growth and viability. It should be noted that the reduced colony size of the gene replacement moss lines could be due to improper RNA processing since the vector used for gene replacement lacks a terminator sequence for the introduced *PpPDK1* gene. However, this vector has been used previously for gene replacement without noticeable differences from wild-type (Shakirov et al., 2010; Spinner et al., 2010). Additionally, from the *pdk1* knockout line it appears that loss of *PpPDK1* is not completely lethal, but does not produce healthy moss tissue. In comparison, this would suggest that the *PpPDK1-6His* and *PpPDK1K71A-6His* constructs in the gene replacement lines are functional since this moss tissue was fully viable and did not show browning like the *pdk1* knockout line.

As an additional test of the functionality of the *PpPDK1-6His* and *PpPDK1K71A-6His* transformants and the impaired growth of the *pdk1* knockout line, we tested whether these moss were hypersensitive to heat (30°C) and osmotic stress (0.9 M mannitol). For heat stress, incubation at 30°C for 14 days gave surviving tissue in all moss lines except for the *pdk1* knockout line, which appeared to be completely dead (Supplemental Fig. S8A). After 14 days of
recovery at 25°C all moss lines except the \textit{pdk1} knockout line were able to recover and grow healthy, viable tissue that resembled the unstressed control (Supplemental Fig. S8A). Similarly, the \textit{pdk1} knockout line was most severely affected by 30 days of growth on 0.9 M mannitol and 14 days of recovery without mannitol restored growth of all lines except the \textit{pdk1} knockout line (Supplemental Fig. S8B). These data suggest that the \textit{PpPDK1-6His} and \textit{PpPDK1}^{K71A-6His} constructs in the gene replacement lines are able to function comparably to the wild-type \textit{PpPDK1}, but that the \textit{pdk1} knockout line is compromised in both normal growth and in response to heat and osmotic stresses.

Features of PDK1s from Algae, Primitive Land Plants, and Angiosperms

Given the position of \textit{P. patens} as a representative of early land plants, we sought to compare the \textit{PpPDK1} sequence to those of other plants to gain insights into the evolution of plant PDK1 protein domain composition and functions. With the genome sequences from many different higher and lower plants currently available it is now possible to do such an analysis based on a comparison of PDK1 protein domain features. We obtained 23 PDK1 sequences from ten higher plants (nine angiosperms, one lycophyte) and seven lower plants (one bryophyte, five green algae, one red alga, one brown alga) for a comparative analysis (Fig. 7A). Interestingly, a search of the genomes of two diatoms (\textit{Thalassiosira pseudonana}, http://genome.jgi-psf.org/Thaps3/Thaps3.home.html; \textit{Phaeodactylum tricornutum}, http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) and one additional green alga (\textit{Micromonas pusilla}, http://genome.jgi-psf.org/MicpuC2/MicpuC2.home.html) did not reveal obvious PDK1 sequence homologues (Fig. 7A), raising the possibility that these organisms either lack \textit{PDK1} altogether or possess \textit{PDK1} genes that are very different from known sequences, which could make them difficult to identify by sequence homology. It is also possible that their genome sequences require further annotation to identify \textit{PDK1} sequences.

Next, we analyzed all 23 identified PDK1 protein sequences for conserved features, including kinase domain, PIF-binding pocket, activation loop, and PH domain. Multiple sequence alignment revealed that the kinase domain of every PDK1 analyzed possesses conserved PIF-binding pocket residues, but that algal and \textit{P. patens} PDK1s differ from vascular plant PDK1s in the lack of a PH domain (Supplemental Fig. S9; Fig. 7B). As might be expected,
a maximum-likelihood tree constructed from all 23 plant PDK1 sequences closely resembled the known phylogenetic placement of these plants, with higher plant PDK1s much more closely related to each other than to primitive land plant or algal PDK1s (Fig. 7B). Our sequence analysis suggests that, while many residues in the PDK1 catalytic domain and PIF-binding pocket have been maintained in highly divergent plant taxa throughout hundreds of millions of years of evolution, the lipid-binding PH domain may only be required in higher plant PDK1s, and is not a characteristic feature of PDK1s from non-vascular land plants and algae.

DISCUSSION

Regulation of many basic processes in eukaryotic cells occurs through phosphorylation of several members of the AGC kinase subfamily by PDK1 (Bögre et al., 2003; Bayascas, 2010). Thus, PDK1 function appears to be highly conserved among many different organisms suggesting PDK1 is an evolutionarily ancient gene. While several PDK1 genes have been reported from higher plants (Deak et al., 1999; Devarenne et al., 2006; Matsui et al., 2010), PDK1 from organisms representative of more ancient plant systems, such as lower, non-vascular plants, has not been reported. Here were identified and characterized such a PDK1 gene from the moss P. patens and these studies offer insights into deciphering how the PDK1 gene may have evolved within plants.

Is PpPDK1 a True PDK1?

From our studies it may be questioned whether or not PpPDK1 is truly a PDK1 since it lacks a PH domain. However, in the 14 years since the human PDK1 was identified, the definition of what constitutes a PDK1 protein has emerged. Containing a PH domain does not appear to be a requirement to catalog a protein as a PDK1 since a PH domain it is not required for function of yeast Pkh1/2 (Casamayor et al., 1999). These proteins were identified as PDK1s by similarity of the kinase domains to known PDK1s, the ability of Pkh1/2 to phosphorylate known AGC kinase substrates, and by the ability of human PDK1 to rescue yeast lacking Pkh1/2 (Casamayor et al., 1999). Thus, the conserved characteristics that define a PDK1 protein include the presence of a PIF-binding pocket and the ability to phosphorylate AGC kinase substrates at
the conserved activation loop phosphorylation site (Bögre et al., 2003). Some PDK1 proteins have also been shown to phosphorylate the activation loop site on AGC kinases from a different species. For example, AtPDK1 can phosphorylate and activate endogenous AGC kinases (Anthony et al., 2004; Anthony et al., 2006; Zegzouti et al., 2006; Zegzouti et al., 2006) as well as the mammalian AGC kinase PKB (Deak et al., 1999). PDK1 proteins form other species have also been identified by the ability of the associated gene to complement the lethality of the yeast Pkh1/2 knockout (Deak et al., 1999).

The PpPDK1 identified here meets all of these definitions of PDK1s and thus should be considered a true PDK1. PpPDK1 contains a PIF-binding pocket that mediates interaction with substrates (Fig. 4D), phosphorylates P. patens and tomato AGC kinases at the conserved activation loop site (Fig. 3B), and can complement the yeast Pkh1/2 knockout (Fig. 2, 4D). These complementation assays indicate that PpPDK1 can regulate some of the known PDK1 functions in a heterologous system and suggests that PpPDK1 may carry out the same functions within P. patens. These studies would also suggest that the mechanism for activation of substrates by PpPDK1 is conserved with what has been discovered for other PDK1s.

The PpPDK1 Knockout is Developmentally Compromised

Unlike other organisms, deletion of PpPDK1 was not lethal even though much of the pdkl knockout moss colony appeared dead after 12 weeks of growth. Subsequent replating can recover growth of green protonema, but the same browning, non-viable phenotype appears as the moss ages. This would suggest that the pdkl knockout line has severe developmental abnormalities. The only other report of a non-lethal PDK1 knockout is for Arabidopsis (Camehl et al., 2011). However, plants lacking both AtPDK1-1 and AtPDK1-2 are stunted and less fertile than wild type (Camehl et al., 2011) suggesting that a loss of PDK1 in plants leads to growth retardation. The only other plant that was analyzed for the loss of PDK1 was tomato, where loss of PDK1 by virus induced gene silencing was lethal (Devarenne et al., 2006). Confirmation of lethality in tomato would require the production of a PDK1 knockout, which is currently not possible in tomato. These contradictory results regarding the effects of PDK1 loss in photosynthetic organisms suggests that divergent roles for PDK1 may have evolved within plants. Further analysis on PDK1 from additional plants will be required to clarify this situation.
It is also interesting to note that the minimally kinase active $Pp$PDK1$^{K71A}$ protein could confer viability and does not produce the browning, non-viable phenotype seen with the $pdk1$ knockout line (Fig. 6). The $Pp$PDK1$^{K71A}$ protein was also capable of supporting viability in yeast (Fig. 4D). There are several possible explanations for why $Pp$PDK1 proteins with drastically reduced kinase activity are still able to confer cell viability. First, the $Pp$PDK1 mutations did not completely eliminate interaction with a $P. patens$ AGC kinase (Fig. 4C) and thus, any phosphorylation taking place could be sufficient for cell viability. Second, some functions of PDK1 may be independent of interaction with AGC kinases. For example, several non-AGC kinase proteins have been identified in mammals that are required for PDK1 function (Nakamura et al., 2008; Sephton et al., 2009). Finally, interaction of PDK1 with some substrates is independent of PIF binding (Collins et al., 2003). For example, in mammalian cells PKB, which also has a PH domain for lipid binding, appears to associate with PDK1 through the lipid interaction of their PH domains (Collins et al., 2003). This would suggest lipid binding is sufficient to activate some PDK1 substrates. However, $Pp$PDK1 does not have a PH domain (Fig. 1B) and does not strongly bind lipids (Supplemental Fig. S3). Thus, it will be of interest to determine if novel mechanisms exist in $P. patens$ for PDK1 interaction with substrates. Additionally, more studies with the $P. patens$ PDK1$^{K71A}$ line generated here will be needed to determine if there is altered interaction with and activation of endogenous AGC kinase substrates.

**Comparison of $Pp$PDK1 to other PDK1 genes**

Our phylogenetic analysis shows that there is a clear difference between higher (vascular) and lower (non-vascular) plants; the PDK1 proteins from these classes of plants can be distinguished by the presence or absence of the PH domain, respectively (Fig. 7B). Thus, it is possible that the PH domain for lipid binding is an aspect of PDK1 function that developed at the time of vascular system formation. Additionally, the sequences for the green algae $O. lucimarinus$ and $O. tauri$, the red alga $C. merolae$, and the brown alga $E. siliculosus$ contain extensive amino acid sequences on the N-terminal and C-terminal sides of the kinase domain (Fig. 7B). The significance of these extra sequences towards function remains to be determined since activity of these proteins has not been reported.
The possible lack of \textit{PDK1} sequences from diatoms raises some interesting questions. Diatoms can be considered a more recently evolved algae in relation to green and red algae (Sims et al., 2006; Moustafa et al., 2009). Thus, the diatom eukaryotic non-photosynthetic ancestor may not have contained a \textit{PDK1}-like sequence(s), they may have lost their \textit{PDK1} gene(s) since the main endosymbiotic event, or they may have \textit{PDK1}-like sequences divergent enough from conserved \textit{PDK1}s that they are not recognizable in typical comparison searches. Further analysis of diatom genomes for \textit{PDK1}-like sequences will help to clarify this situation.

Our analysis of plant PDK1 proteins is limited by the number of \textit{PDK1} sequences from lower plants and non-flowering vascular plants. With the addition of genome sequences from additional lower plants such as the bryophyte liverwort \textit{Marchantia polymorpha} (currently, the \textit{M. polymorpha} EST sequences contain 4,024 contigs but not an obvious \textit{PDK1} sequence homologue, http://www.genome.jp/kegg-bin/show_organism?org=empm), more green, red, and brown algae, and vascular plants such as ferns, this comparison will become more robust and allow for a more concrete assessment on the domain evolution of PDK1 proteins.

**MATERIALS AND METHODS**

**Cloning and Site Directed Mutagenesis**

\textit{PpPDK1} (Pp1s217\_11V6.2 at Phytozome, GenBank accession JN049607), \textit{Pp188412} (Pp1s118\_230V6 at Phytozome, GenBank accession JN049610), and \textit{Pp174181} (Pp1s4\_386V6.1 at Phytozome, GenBank accession JN049609) coding sequences were identified by searching Phytozome using the \textit{AtPDK1} sequence (GenBank accession AF132742.1) (Deak et al., 1999). \textit{Pp2484} (Pp1s224\_73V6.1 at Phytozome, GenBank accession JN049608) was identified by searching Phytozome using the \textit{Adi3} sequence (GenBank accession AY849914) (Devarenne et al., 2006).

Total RNA was extracted from \textit{P. patens} and \textit{A. thaliana} tissue using TRIzol Reagent (Invitrogen) and reverse transcription was performed using SuperScriptIII reverse transcriptase (Invitrogen). Full length cDNAs for the following genes were amplified from 1\textsuperscript{st} strand cDNA using forward primers beginning at the ATG start codon and reverse primers beginning at the stop codon or last amino acid; \textit{PpPDK1}, forward primer 5’-ATGGCCATGGATGGGACC-3’,
reverse primer 5'-TACGTCGTATACAAATGCATCCAAACC-3'; \textit{Pp188412}, forward primer 5'-ATGGCGGCAACAAATCGCATTAAC-3', reverse primer 5'-CTATGATGAGCCCAGCCACG-3'; \textit{Pp174181}, forward primer 5'-ATGACACTTGCTACCACCTCTCAGG-3', reverse primer 5'-TTATGAGGATTCCCCACTCTTGAG-3'; \textit{AtPDK1}, forward primer 5'-ATGTTGGCAATGGAG-3', reverse primer 5'-GCGGTTGTGAAGAGTC-3'. The annotated full-length 2,328 nt \textit{Pp2484} cDNA could not be amplified from 1st strand cDNA. Instead, a 1,560 nt cDNA for \textit{Pp2484} was amplified from 1st strand cDNA using forward primer 5'-ATGAGTGGAAAGTTGAGCATGAG-3', reverse primer 5'-TCAAAAAAAGTCAAAAATCCACGTAGCTGAC-3'. The amplified 1,560 nt \textit{Pp2484} cDNA lacks the first 768 nt of exon 1 but the encoded protein contains all conserved AGC kinase elements.

Cloning of \textit{SlPDK1} and \textit{Adi3} was previously reported (Devarenne et al., 2006). \textit{PpPDK1}, \textit{AtPDK1}, and \textit{SlPDK1} lacking the endogenous stop codon were cloned into pET22-b(+) (Novagen) to incorporate a C-terminal 6His tag. \textit{PpPDK1-6His}, \textit{AtPDK1-6His}, and \textit{SlPDK1-6His} were amplified from pET22-b(+) and cloned into pMAL-c2x (New England Biolabs) for N-terminal MBP translational fusions. \textit{Pp188412}, \textit{Pp147181}, and \textit{Pp2484} without a 6His tag were also cloned into pMAL-c2x.

Site-directed mutagenesis was performed on genes cloned into pMAL-c2x using Pfu Turbo (Stratagene) according to the manufacturer’s instructions. Finally, \textit{MBP-PpPDK1-6His}, \textit{MBP-AtPDK1-6His}, \textit{MBP-SlPDK1-6His}, \textit{MBP-Pp188412}, \textit{MBP-Pp174181}, and \textit{MBP-PpPDK1-6His} point mutants were cloned into the plasmid p416GPD (Mumberg et al., 1995) for yeast complementation studies. p416GPD contains a URA3 marker and a constitutive GPD promoter to drive expression of the gene of interest.

**Yeast Strains and Complementation of \textit{PKH1} and \textit{PKH2}**

Two previously described strains of \textit{S. cerevisiae} were used in complementation experiments: strain AC306 (Casamayor et al., 1999) was used for tetrad analysis of \textit{PKH1/2} complementation and strain INA106-3B (Inagaki et al., 1999) was used for temperature sensitivity \textit{PKH1/2} complementation. AC306 is a diploid strain that is heterozygous for \textit{PKH1} and \textit{PKH2} deletion (genotype: MATa/MATa \textit{PKH1/pkh1Δ::HIS3 PKH2/pkh2Δ::TRP1 ade2-1
INA106-3B is a haploid strain that lacks *PKH2* and contains a point mutation in *PKH1* (genotype: MATa *pkh1<sup>D398G</sup> pkh2<sup>Δ::LEU2</sup> ade1 his3-2 trp1 ura3*). Both strains were transformed with the *PDK1* constructs in p416GPD.

After transformation, AC306 yeast transformed with the desired plasmid was grown at 30°C on plates lacking uracil. Sporulation was induced by incubating transformed yeast in 1% potassium acetate at 25°C for 7 days. After sporulation, tetrad dissections were performed on at least 30 tetrads of each sample using standard techniques. After dissection, spore genotype and viability were analyzed by replica plating on minimal medium and on medium lacking histidine, tryptophan, or uracil. After spore analysis, representative cultures of each haploid spore were grown at 30°C in liquid YPD medium and spotted on both YPD plates and plates containing 5-FOA (Research Products International), which selects for loss of the URA3-marked p416GPD plasmid.

For temperature sensitive *PKH1/2* complementation, liquid cultures of INA106-3B yeast transformed with the desired plasmid were grown at 25°C in liquid medium lacking uracil and spotted on two identical plates lacking uracil. One plate was incubated at 25°C for 4 days and the other plate was incubated at 35°C for 2 days, then plates were viewed and photographs taken.

To analyze the expression of all genes cloned into p416GPD, total protein was extracted as previously described (Yaffe and Schatz, 1984) from yeast grown at 30°C in liquid YPD medium (AC306 yeast) or 25°C in liquid medium lacking uracil (INA106-3B yeast). MBP fusion proteins were then detected by α-MBP (1:10,000; New England Biolabs) western blot.

**In vitro Kinase Assays**

*PpPDK1-6His, Pp2411, AtPDK1-6His, SlPDK1-6His, Pp2411,* and Adi3 were expressed as MBP fusion proteins using pMAL-c2 in *E. coli* BL21(DE3) and purified with amylose resin (New England Biolabs) according to the manufacturer’s instructions. *In vitro* kinase assays were performed by combining the purified proteins in a 30 μl final volume of kinase buffer containing 10 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub> or 10 mM MnCl<sub>2</sub>, and 1 mM DTT. The reactions were started by the addition of 1 μCi γ-[<sup>32</sup>P]ATP and nonradiolabeled ATP to a final concentration of 20 μM followed by incubation at room temperature for 15 min. Reactions were stopped by the addition of 4x SDS-PAGE sample buffer. Protein phosphorylation was visualized by
phosphorimager (Bio-Rad Molecular Imager) after separation by SDS-PAGE and signals were quantified using Quantity One software (Bio-Rad). All kinase assays were performed a minimum of three times, with representative images shown in figures.

**In vitro Pulldown Assays**

MBP, MBP-\textit{Pp}2411, and MBP-\textit{PpPDK1-6His} were purified with amylose resin as described above. Five $\mu$g of purified MBP-\textit{PpPDK1-6His} was added to 10 $\mu$l of Ni$^{2+}$ resin (Novagen) in 200 $\mu$l of binding buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton-X-100) and mixed at 4°C for 1 hr to allow MBP-\textit{PpPDK1-6His} to bind the resin. Next, 5 $\mu$g of purified MBP or MBP-\textit{Pp2411} was added to the sample and mixed at 4°C for 1 hr to allow the proteins to interact with MBP-\textit{PpPDK1-6His}. After the incubation period samples were centrifuged at 400 x g for 1 min, the supernatant removed, and the samples were washed 3 times with 1 ml binding buffer followed by the addition of 100 $\mu$l of 4x SDS-PAGE sample buffer. Ten $\mu$l of each sample was then separated by SDS-PAGE and MBP fusion proteins detected by $\alpha$-MBP western blot. Protein inputs were analyzed by performing SDS-PAGE on 5 $\mu$g of purified MBP, MBP-\textit{Pp2411}, and MBP-\textit{PpPDK1-6His} and staining with coomassie.

**Protein-Lipid Overlays**

Binding of MBP, MBP-\textit{AtPDK1-6His}, MBP-\textit{SpPDK1-6His}, and MBP-\textit{PpPDK1-6His} to several common phospholipids and sphingolipids was tested by protein-lipid overlay using PIP Strips and Sphingo Strips (Echelon Bioscience) according to the manufacturer’s instructions and as previously described (Zegzouti et al., 2006). Briefly, lipid strips were blocked with 3% fatty acid-free BSA in 1x PBS for 16 hr, 5 $\mu$g of each purified protein was incubated with a lipid strip for 1.5 hr at room temperature, the lipid strips were washed 3 times with 1x PBST, and proteins bound to lipids were detected using $\alpha$-MBP as with typical western blots.

**Tissue Culture of \textit{P. patens} and Generation of \textit{pdk1} knockout and \textit{PpPDK1-6His} Constructs**
Wild type *P. patens* Gransden (Ashton and Cove, 1977) was routinely grown on BCD plates overlaid with cellophane disks (AA Packaging) at 25°C in continuous light following standard protocols (Roberts et al., 2011). Seven days before transformation, wild type *P. patens* was passaged to BCD plates containing 5 mM di-ammonium tartrate.

For each moss stress treatment, all 4 moss lines (wild-type, *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1K71A-6His*) were grown on the same plate to ensure results were not due to variation between plates. For heat stress, moss lines were moved to BCD plates and incubated at 30°C for 14 days, then allowed to recover on the same plate at 25°C for 14 days. For osmotic stress, moss lines were moved to BCD plates containing 0.9 M mannitol for 14 days, then moved to new BCD plates lacking mannitol and allowed to recover for 14 days.

For both knockout and gene replacement generation, the pBHRF and pBNRF vectors were used, respectively. In short, pBNRF and pBHRF contain 35S:NptII (G418/kanamycin resistance) or 35S:Hph (hygromycin resistance) EcoRI fragments from pH23b or pGL2, respectively, cloned into the EcoRI site of pBilox, a derivative of pMCS5 (MoBiTec), with two direct repeats of the loxP sites cloned into the *XhoI*-KpnI and BglII/SpeI sites (Schaefer et al., 2010). The construct used to generate the *pdk1* knockout line was made by amplifying a 1,091 nt 5’ targeting fragment from genomic DNA with primers 5’-GACAAGCTTTCTCAGAAAGTGCAAAAGGCTTTTCATTC-3’ (*HindIII* restriction site in bold) and 5’-GACCTCTCAGTTCTTGATGTTGGTGGCC-3’ (*XhoI* restriction site in bold) and cloned into the *HindIII* and *XhoI* sites of the vector pBHRF (Schaefer et al., 2010) upstream of *Hph*. Similarly, a 1,151 nt 3’ targeting fragment was amplified from genomic DNA with primers 5’-GACGCAGCGCCATTATCTAGACTCACATG-3’ (*NotI* restriction site in bold) and 5’-GACAGATCTGTATGTAACCAACCTAG-3’ (*BglII* restriction site in bold) and cloned into the *NotI* and *BglII* sites of pBNRF, 3’ to *Hph*. A diagram of the complete construct can be found in Fig. 5A.

The construct used to replace the endogenous *PpPDK1* locus with *PpPDK1-6His* or *PpPDK1K71A-6His* was made by amplifying a 1,091 nt 5’ targeting fragment plus the entire *PpPDK1* coding region from genomic DNA with primers 5’-AAGTGCAAAAGGCT TTCAATCTC-3’ and 5’-TACGTCGTATACAAATGCATCC-3’ and cloning into pCR2.1-TOPO (Invitrogen). A C-terminal 6His tag, a 5’ *XbaI* site, and a 3’ *XhoI* site were added to this *PpPDK1* by PCR and cloned into the *XbaI* and *XhoI* sites of pBNRF upstream of *NptII*. Similarly, a 1,151 nt 3’
targeting fragment was amplified from genomic DNA with primers 5’-GACGCGGCCCAGTATGACTACATG-3’ (NotI restriction site in bold) and 5’-GACAGATCTTACACCCACCATG-3’ (BglII restriction site in bold) and cloned into the NotI and BglII sites of pBNRF, 3’ to NptII. A diagram of the complete construct can be found in Fig. 5B. The K71A mutation was then introduced by site-directed mutagenesis using Pfu Turbo according to the manufacturer’s instructions.

To transform *P. patens*, 50 μg of each construct was linearized by simultaneous digestion with 80 units of HindIII and SpeI for pdk1 knockout generation or XbaI and SpeI for the *PpPDK1* gene replacement. Linearized DNA was introduced into wild type *P. patens* Gransden protoplasts using a previously described PEG transformation protocol (Roberts et al., 2011). Transformants were initially selected by 7 days of growth on plates containing 20 μg/ml hygromycin or 25 μg/ml G418 followed by 10 days of growth on nonselective plates and an additional 7 days of growth on hygromycin or G418 plates. Surviving colonies were analyzed by genotyping PCR and Southern blotting (see below) for integration of the *PpPDK1-6His* constructs into the correct location in the genome.

Genomic DNA Extraction

Genomic DNA was extracted from each moss colony surviving two rounds of hygromycin or G418 selection by grinding the tissue (approximately 10-25 mm² per colony) in a microcentrifuge tube with 200 μl extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl) followed by incubation at 65°C for 30 min. An equal volume (200 μl) of phenol/chloroform was added and the mixture was vortexed vigorously for 15 sec followed by centrifugation at 15,000 x g for 15 min. The upper aqueous phase was added to a new microcentrifuge tube containing 170 μl isopropanol and 10 μl of 3 M sodium acetate, pH 5.2. Samples were incubated at -20°C for 1 hr followed by centrifugation at 15,000 x g for 20 min. The supernatant was aspirated and pellets washed with 200 μl of 70% ethanol followed by centrifugation at 15,000 x g for 20 min. Pellets were dried for 1 hr at room temperature and resuspended at 37°C in 20 μl of autoclaved dH₂O containing 10 μg RNase A.
Genotyping

Genomic DNA extracted as described above was subjected to genotyping PCR. Initial genotyping was performed with primers that amplify a 532 nt product if the endogenous PpPDK1 is present, a 2,356 nt product if a PpPDK1-6His construct is present, and no product if the endogenous PpPDK1 is not present; primer 1, 5’-GAGAGACTGGGAGTTCAAGGCTATG-3’ and primer 2, 5’-CTCCAACTGGTAATTAATGCTGCAATGG-3’. A diagram of the locations of these genotyping primers can be found in Fig. 5A and B.

Additional genotyping PCR was used to test for proper 5’ and 3’ integration of the pdk1 knockout and PpPDK1-6His constructs. The first set of primers tests for 5’ integration. These primers are based outside the 5’ targeting fragment (primer 3) and in the 35S promoter (primer 4) and amplify the entire 5’ targeting fragment for the pdk1 knockout construct and the entire 5’ targeting fragment plus the PpPDK1-6His coding sequence for the gene replacement constructs (see Fig. 5A and B for primer location); primer 3, 5’-GGTAGGTGGTATTTCTAACACTCAATGATG-3’ and primer 4, 5’-CGTGCTCCACCATGTTGACGAAG-3’. The second set of primers tests for 3’ integration. These primers are based in the NptII gene (primer 5) and outside the 3’ targeting fragment (primer 6) and amplify the entire 3’ targeting fragment for all constructs (see Fig. 5A and B for primer location); primer 5, 5’-GCTGAAATCACCAGTCTCTCTCTCTAC-3’ and primer 6, 5’-GGCAATGGTTCAAAAACCTCTTTATAAGTCC-3’.

Southern Blot

Southern blot analysis was performed essentially as previously described (Nelson et al., 2011) to verify that PpPDK1-6His or PpPDK1K71A-6His was integrated into the correct location in the P. patens genome and to assess the number of integration events. Forty μg of genomic DNA extracted from wild-type, PpPDK1-6His, or PpPDK1K71A-6His moss was simultaneously digested with 120 units each of NdeI, SalI, and XbaI and separated on a 1% agarose gel, which was then transferred to a nylon membrane (GE Healthcare). The membrane was hybridized at 65°C with a High Prime (Roche) internally labeled DNA probe comprised of 234 nt of the 35S promoter used to drive NptII expression in pBNRF. This probe was amplified from the pBNRF
vector using primers 5’-CGTCAACATGGTGAGCACG-3’ and 5’-GCAGAGGCATCTTCAACGATGG-3’. A diagram of the location of the probe can be found in Supplemental Fig. S6A.

Expression Analysis of *PpPDK1* in Wild-Type, *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1*<sup>K71A</sup>-6His Moss

Total RNA was extracted from *P. patens* tissue using TRIzol Reagent (Invitrogen) and reverse transcription was performed using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer’s instructions. RT-PCR was performed as previously described (Harries et al., 2005). As an expression control, ubiquitin was amplified with forward primer 5’-ACTACCCCTGAAGTTGTATAGTTCGG-3’ and reverse primer 5’-CAAGTCACATTACTCGCTGTCTAG-3’. *PpPDK1* was amplified with forward primer 5’-TTCAAGCTGCGACAGAATATTTGAC-3’ and reverse primer 5’-CTTGCCATTTTTTCTTCTCATCCAAAC-3’. Each gene was amplified by 30 cycles of PCR, utilizing a 30 sec denaturing step at 94°C, a 30 sec annealing step at 57°C, and a 30 sec extension step at 72°C.

Phylogenetic Analysis of PDK1 Proteins

The following PDK1 protein sequences were obtained from NCBI, Phytozome, and individual species genome databases using a BLAST search with either the *O. tauri* PDK1 sequence (accession XP_003078129.1 at GenBank) or the *A. thaliana* PDK1-1 sequence (accessions AED90755.1 at GenBank and AT5G04510 at Phytozome): *A. thaliana* PDK1-2 (accessions NP_187665.2 at GenBank and AT3G10540 at Phytozome), *C. merolae* PDK1 (CDS #2387, locus CMO090C from genome website: http://merolae.biol.s.u-tokyo.ac.jp/), *C. reinhardtii* PDK1 (accessions XP_001701378.1 at GenBank and Cre18.g749950 at Phytozome), *Chlorella variabilis* NC64A PDK1 (protein id #26267 from genome website: http://genome.jgi-psf.org/ChlNC64A_1/ChlNC64A_1.home.html), *E. siliculosus* PDK1 (accession CBJ33478 at GenBank and Esi0491_0016 at http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Ectsi), *G. max* PDK1-1 (accessions
XP_002262670.1 at GenBank and Glyma10g34430 at Phytozome), G. max PDK1-2 (accession Glyma20g33140 at Phytozome), O. lucimarinus PDK1 (protein id #14571 from genome website: http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html), O. sativa PDK1 (accessions BAF06862 at GenBank and LOC_Os01g65230 at Phytozome), P. patens PDK1 (accessions JN049607 at GenBank and Pp1s217_11V6 at Phytozome), P. trichocarpa PDK1-1 (accessions XP_002315349.1 at GenBank and POPTR_0010s23910.1 at Phytozome), P. trichocarpa PDK1-2 (accession POPTR_0008s02890.1 at Phytozome), R. communis PDK1 (accessions XP_002533941.1 at GenBank and 27428.t000007 at Phytozome), S. bicolor PDK1 (accessions XP_002458841.1 at GenBank and Sb03g041290 at Phytozome), S. lycopersicum PDK1-1 (accession AAW38936 at GenBank), S. lycopersicum PDK1-2 (gene model SL1.00sc03032_9.1.1 at genome website: http://solgenomics.net), S. moellendorfii PDK1 (accessions XP_002960408.1 at GenBank and 164063 at Phytozome), V. carteri PDK1 (accessions XP_002957069.1 at GenBank and 83913 at Phytozome), V. vinifera PDK1 (accessions CBI40191.3 at GenBank and GSVIVG01034673001 at Phytozome), Z. mays PDK1-1 (accession ABB71956.1 at GenBank), and Z. mays PDK1-2 (accessions ACG46841.1 at GenBank and GRMZM2G097821 at Phytozome).

A multiple alignment of all 23 PDK1 protein sequences was created using MUSCLE (MUltiple Sequence Comparison by Log-Expectation). A maximum-likelihood phylogenetic tree was then created from aligned PDK1 sequences using MEGA version 5 (Tamura et al., 2011) and the resulting phylogram labeled in Adobe Illustrator. The cladogram showing phylogenetic relationships between plants whose PDK1 sequences were analyzed (Fig. 7A) was created in Adobe Illustrator based on published literature (Bhattacharya and Medlin, 1998; Merchant et al., 2007; Cock et al., 2010; Banks et al., 2011).

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

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FIGURE LEGENDS

Figure 1. Features of *P. patens* PDK1 (*PpPDK1*) and comparison with PDK1 from tomato (*SlPDK1*) and *A. thaliana* (*AtPDK1*). A, Diagram of locus *Pp1s217_11V6*, which is located on *P. patens* genomic scaffold 217. The *Pp1s217_11V6* locus spans 3941 nt of genomic DNA consisting of a 901 nt 5’ UTR, a 2803 nt coding region of nine exons/eight introns, and a 237 nt 3’ UTR. Expression of this gene produces a transcript with 1044 nt of coding sequence. Numbers below the dashed lines indicate the start and end locations of the *PpPDK1* coding sequence on genomic scaffold 217. B, Features of *PpPDK1* protein compared with *SlPDK1-1* and *AtPDK1-1*, as determined by ClustalW multiple alignment of protein sequences. The conserved lysine that is required for Mg2+ coordination in all kinases is K67 in *PpPDK1*, K77 in *SlPDK1*, and K73 in *AtPDK1*. The percent identity of each protein to *AtPDK1* was determined using the EMBOSS Needle pairwise sequence alignment program.

Figure 2. *PpPDK1* rescues lethality caused by deletion of both *S. cerevisiae* PDK1 homologues, *PKH1* and *PKH2*. Yeast strain AC306 was transformed with p416GPD containing the indicated *PDK1* cDNAs; NT, untransformed AC306; EV, empty vector transformed AC306. Sporulation was induced and the four haploid cells produced by each diploid cell were grown on rich medium (YPD) and medium containing 5-FOA. Spore phenotypes are indicated on left and were determined by replica plating on medium lacking histidine, tryptophan, or uracil. A, On YPD plates *pkh1Δ::HIS3/pkh2Δ::TRP1* spores are only viable when *AtPDK1*, *SlPDK1*, or *PpPDK1* is expressed. B, α-MBP western blot showing protein expression. C, On 5-FOA plates *pkh1Δ::HIS3/pkh2Δ::TRP1* spores are not viable due to loss the URA3-marked p416GPD plasmid.

Figure 3. Characterization of *PpPDK1* kinase activity on myelin basic protein and AGC kinases from tomato and *P. patens*. A, Wild-type (wt) MBP-*PpPDK1-6His* autophosphorylates and phosphorylates myelin basic protein in the presence of either Mg2+ or Mn2+, but the K67Q mutation abolishes all kinase activity. Top two panels, *PpPDK1* autophosphorylation; bottom two panels, *PpPDK1* phosphorylation of myelin basic protein. B, *PpPDK1* phosphorylates AGC kinases Adi3 (tomato) and *Pp2484* (*P. patens*) at the conserved PDK1 phosphorylation site in the
activation loop of both kinases. Mutation of the conserved activation loop serine to alanine (S539A in Adi3 and S577A in \(Pp2484\)) results in reduced phosphorylation of Adi3 and \(Pp2484\) by \(PpPDK1\).

**Figure 4.** Functional analysis of conserved \(PpPDK1\) PIF-binding pocket residues. A, Mutation of \(PpPDK1\) PIF-binding pocket residues reduces autophosphorylation. Values are reported as the percentage of wild-type (wt) \(PpPDK1\) autophosphorylation and are the mean of three independent experiments. Error bars indicate standard error. B, Mutation of \(PpPDK1\) PIF-binding pocket residues reduces phosphorylation of \(Pp2484\). Values are reported as the percentage of wt \(PpPDK1\) phosphorylation of \(Pp2484\) and are the mean of four independent experiments. Error bars indicate standard error. C, Mutation of \(PpPDK1\) PIF-binding pocket residues reduces, but does not abolish, interaction with \(Pp2484\). The left panel shows protein inputs analyzed by SDS-PAGE and staining with coomassie, and the right panel shows Ni\(^{2+}\) resin pulldowns of MBP-\(PpPDK1\)-6His analyzed by \(\alpha\)-MBP western blot. D, All \(PpPDK1\) PIF-binding pocket mutants that possess kinase activity are able to fully complement a temperature sensitive allele of \(S.\ cerevisiae\ PKH1\). Haploid strain pkh2\(\Delta/pkh1^{D398G}\) temperature-sensitive yeast was transformed with p416GPD containing the indicated \(PpPDK1\) constructs. Transformed yeast were grown in liquid medium lacking uracil, spotted on plates lacking uracil, and grown at the indicated temperatures and times. Total protein was extracted from cultures grown in liquid medium at 25°C and analyzed by \(\alpha\)-MBP western blot to verify expression of each \(PpPDK1\).

**Figure 5.** Production of a \(pdk1\) knockout line and replacement of endogenous \(PpPDK1\) with \(PpPDK1\)-6His. A and B, The constructs used to replace the endogenous \(PpPDK1\) locus with a hygromycin marker to create a \(pdk1\) knockout line (A) and to replace the endogenous \(PpPDK1\) locus with \(PpPDK1\)-6His or \(PpPDK1^{K71A}\)-6His (B). The 6His tag (maroon box) is not drawn to scale for ease of visibility. Dashed lines indicate regions of homologous recombination. The locations of genotyping primers used in (C) and (D) are depicted with arrows above the diagram. C, PCR-based genotyping with primers 1 \(+\) 2 shows that the indicated \(P.\ patens\) transformants do not contain a wild-type \(PpPDK1\) gene. D, PCR-based genotyping of the indicated \(P.\ patens\) transformants showing proper integration of constructs into the endogenous \(PpPDK1\) locus.
Wild-type *P. patens* genomic DNA was used as a negative control for integration of the constructs. E, RT-PCR analysis of gene expression for the indicated genes. Analysis of ubiquitin gene expression was used as an internal control.

**Figure 6.** *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1K71A-6His* moss macroscopic phenotype compared to wild-type *P. patens*. A, The indicated moss were plated and photographed over a 12 week period. B, Images of protonema and gametophore tissue for the indicated moss lines taken at the 12 week period.

**Figure 7.** Phylogenetic relationship and protein domains of PDK1 proteins from plants. A, Cladogram showing evolutionary relationship of plants used in B. B, Comparison of PDK1 protein sequences. On the left side, the indicated proteins were aligned and a maximum-likelihood phylogenetic tree produced using MEGA5. On the right side, the indicated domains were identified in each protein and aligned based on the activation loop domain. Horizontal dashed line indicates split between vascular (above line) and non-vascular (below line) plants.
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Figure 4. Functional analysis of conserved PpPDK1 PHF-binding pocket residues. Based on ClustalW multiple alignment, K71, I75, Q106, and L111 of PpPDK1 may participate in binding substrate C-terminal PHFs. A, Mutation of PpPDK1 PHF-binding pocket residues reduces autophosphorylation. Values are reported as the percentage of wt PpPDK1 autophosphorylation and are the mean of three independent experiments. Error bars indicate standard error. B, Mutation of PpPDK1 PHF-binding pocket residues reduces phosphorylation of Pp2484. Values are reported as the percentage of wt PpPDK1 phosphorylation of Pp2484 and are the mean of four independent experiments. Error bars indicate standard error. C, Mutation of PpPDK1 PHF-binding pocket residues reduces, but does not abolish, interaction with Pp2484. The left panel shows protein inputs analyzed by SDS-PAGE and staining with coomassie, and the right panel shows Ni²⁺ resin pulldowns of MBP-PpPDK1-6His pulled down by α-MBP western blot. D, All PpPDK1 PHF-binding pocket mutants were transformed in vivo into a Pp2484 deletion strain lacking a temperature sensitive allele of X. cernuus PK1.1. Haploid strain cells were grown to late log phase in liquid medium containing p416GPD containing the indicated PpPDK1 constructs under control of the constitutive GPD promoter. Transformed yeast were grown in liquid medium lacking uracil, spotted on plates lacking uracil, and grown at the indicated temperatures and times. Total protein was extracted from cultures grown in liquid medium at 25°C and analyzed by α-MBP western blot to verify expression of each PpPDK1.
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