# AtPIP5K1, an *Arabidopsis thaliana* phosphatidylinositol phosphate kinase, synthesizes PtdIns(3,4)$P_2$ and PtdIns(4,5)$P_2$ *in vitro* and is inhibited by phosphorylation

Tomas WESTERGREN*, Stephen K. DOVE†, Marianne SOMMARIN* and Christophe PICAL*[1]

*Department of Plant Biochemistry, Lund University, PO Box 124, SE-221 00 Lund, Sweden, and †Centre for Clinical Research in Immunology and Signalling, University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, U.K.

PtdIns phosphate kinases (PIPkins), which generate PtdIns$P_2$ isomers, have been classified into three subfamilies that differ in their substrate specificities. We demonstrate here that the previously identified *AtPIP5K1* gene from *Arabidopsis thaliana* encodes a PIPkin with dual substrate specificity *in vitro*, capable of phosphorylating PtdIns3*P* and PtdIns4*P* to PtdIns(3,4)$P_2$ and PtdIns(4,5)$P_2$ respectively. We also show that recombinant AtPIP5K1 is phosphorylated by protein kinase A and a soluble protein kinase from *A. thaliana*. Phosphorylation of AtPIP5K1 by protein kinase A is accompanied by a 40% inhibition of its catalytic activity. Full activity is recovered by treating phosphorylated AtPIP5K1 with alkaline phosphatase.

Key words: lipid kinase, phosphoinositide, plant, regulation.

## INTRODUCTION

PtdIns and its phosphorylated derivatives are involved in many cellular processes in animal cells. Seven phosphorylated derivatives of PtdIns have been identified in animal cells. Hydrolysis of PtdIns(4,5)$P_2$ by phosphoinositide-specific phospholipase C results in the generation of two second messengers, Ins(1,4,5)$P_3$ and diacylglycerol, that respectively release $Ca^{2+}$ from internal stores and stimulate protein kinase C [1]. PtdIns(4,5)$P_2$ and other phosphoinositides serve many additional critical functions in animal cells, by regulating the actin cytoskeleton, vesicular trafficking, protein activities, nuclear functions and endocytosis [2–6].

Although plants have been shown to contain all the phosphoinositides identified in animal cells, except for PtdIns5*P* and PtdIns(3,4,5)$P_3$, comparatively little information exists on their metabolism and functions in plant cells. However, microinjected 'caged' Ins(1,4,5)$P_3$ can release $Ca^{2+}$ from internal stores [7] and can trigger stomatal closure [8,9]. There is also evidence that phosphoinositides might participate in the regulation of cytoskeletal structures in plant cells [10,11], in polar pollen tube growth [12] and in nuclear function [13]. We and others have recently shown that salt stress stimulates the synthesis of two types of PtdIns$P_2$, PtdIns(4,5)$P_2$ in suspension-cultured *Arabidopsis thaliana* cells [14,15], and PtdIns(3,5)$P_2$ in *Chlamydomonas* and other plant cells [16].

The synthesis of PtdIns(3,4)$P_2$, PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$ from PtdIns*P* isomers is catalysed by PtdIns*P* kinases (PIPkins). These enzymes form a family of lipid kinases distinct from PtdIns 3-kinases and PtdIns 4-kinases, and are classified into three subfamilies (types I–III), depending on their substrate specificity. Type I enzymes are PtdIns4*P* 5-kinases, type II enzymes are PtdIns5*P* 4-kinases [17] and type III enzymes are PtdIns3*P* 5-kinases; these last are represented by the *Saccharomyces cerevisiae FAB1* gene product and its homologues from other species [18]. *In vitro*, type I and II PIPkins can also phosphorylate PtdIns3*P* and PtdIns [19,20]. It is important to note that in animal cells PtdIns(3,4)$P_2$ can be generated via phosphorylation of PtdIns4*P* on the 3-OH position by heterodimeric inositol lipid 3-kinases of the p110 family [21].

In plants, the relative abundance of the different PtdIns$P_2$ isomers is very variable, depending on the cell types and/or growth conditions. For example, in suspension-cultured cells of *A. thaliana*, the only PtdIns$P_2$ isomer detectable is PtdIns(4,5)$P_2$ [14], whereas PtdIns(3,4)$P_2$ is the predominant PtdIns$P_2$ form in *Commelina communis* stomatal guard cells [22]. In some plant cells the production of PtdIns(3,5)$P_2$ is stimulated on salt stress [16,23]. A plant enzyme activity capable of phosphorylating PtdIns4*P* to a PtdIns$P_2$ isomer has long been known to be present in the plasma membrane [24]. The identity of the PtdIns$P_2$ isomer was not determined [24]. Both PtdIns(3,4)$P_2$ and PtdIns(3,5)$P_2$ are generated from endogenous phosphoinositides when plant plasma membranes are incubated with ATP (S. K. Dove and B. K. Drøbak, unpublished work). The only PtdIns 3-kinases identified so far in plants [25,26] are homologues of the yeast Vps34p protein, which only phosphorylates PtdIns, but not its phosphorylated derivatives, on the 3-OH group. Furthermore, the genome of *A. thaliana* does not contain any genes that encode proteins similar to the heterodimeric inositol lipid 3-kinases from animals that are able to phosphorylate PtdIns, PtdIns4*P* and PtdIns(4,5)$P_2$. This suggests that a distinct enzyme is responsible for the synthesis of PtdIns(3,4)$P_2$ in plant cells. A gene encoding a functional plant PIPkin has been identified [27]. This gene from *A. thaliana*, *AtPIP5K1*, encodes a protein with sequence similarity to type I and II PIPkins. The authors tested only PtdIns4*P* as a substrate, and the structure of the lipid product formed was not determined [27]. We have now analysed the properties of AtPIP5K1 more closely, and demonstrate that *in vitro* it generates PtdIns(4,5)$P_2$ and PtdIns(3,4)$P_2$ from PtdIns4*P* and PtdIns3*P* respectively. This substrate specificity is identical with that of the Mss4p PIPkin from *S. cerevisiae*. Sequence comparison also demonstrates that AtPIP5K1 and Mss4p are more closely related to each other than to PIPkins from other species. In addition, we show that AtPIP5K1 can be phosphorylated by protein kinase A (PKA) and a protein kinase activity present in a cytosol fraction prepared from *A. thaliana* plants. The PKA-catalysed

Abbreviations used: GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; PIPkin, PtdIns*P* kinase; PKA, protein kinase A.

[1] To whom correspondence should be addressed (e-mail christophe.pical@plantbio.lu.se).

phosphorylation of AtPIP5K1 had an inhibitory effect on the catalytic activity of the plant enzyme.

## MATERIALS AND METHODS

### Materials

[$^3$H]Inositol was from Amersham Pharmacia Biotech. [$\gamma$-$^{32}$P]ATP was synthesized as described [28]. PtdIns3*P* and PtdIns(3,4)$P_2$ were synthesized as described [29]. PtdIns and PtdIns4*P* were from Sigma; PtdIns5*P* was from Echelon Research Laboratories. Silica gel 60-A TLC plates were from Merck. PKA and alkaline phosphatase were from Boehringer Mannheim.

### Expression of recombinant AtPIP5K1

Sequencing of the cDNA for AtPIP5K1 provided by Kazuo Shinozaki (Riken, Ibaraki, Japan) (GenBank® accession number AB005902) [27] revealed one mistake in the nucleotide sequence provided: the thymidine base at position 562 is not present in the cDNA, resulting in a frameshift and a wrong and shorter N-terminus for the deduced amino acid sequence of AtPIP5K1 given by Mikami et al. [27] (see Figure 1). A different cDNA for the same gene, with a correct sequence, has been reported (accession number AF019380) [30]. The entire coding sequence of *AtPIP5K1* was amplified by PCR from the cDNA clone provided by K. Shinozaki with *Pfu* polymerase (Stratagene) using primers that inserted *Sal*I and *Not*I restriction sites at the 5′ and 3′ ends respectively. The amplified fragment was digested and ligated into the expression vector pGEX-4T-2 (Amersham Pharmacia Biotech) digested with *Sal*I and *Not*I. The resulting construct was named pGPIP5K1. The sequence of the amplified fragment was confirmed by automated DNA sequencing.

*Escherichia coli* strain BL21(DE3)pLys (BL21) was used for the expression of a recombinant fusion protein of glutathione S-transferase (GST) with AtPIP5K1. An overnight culture of BL21, transformed with pGPIP5K1, was diluted 1:100 with fresh culture medium and grown at 30 °C with shaking at 200 rev./min until a $D_{600}$ of 0.6–0.8 was reached, at which point isopropyl $\beta$-D-thiogalactoside (IPTG) was added to a final concentration of 30 $\mu$M. Cells were incubated for an additional 6 h at 16 °C and 200 rev./min; they were then collected by centrifugation and frozen until the recombinant protein was to be purified. Frozen cells were thawed and resuspended in TBS buffer [10 mM Tris/HCl (pH 8.0)/140 mM NaCl] containing 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM dithiothreitol and 1 mM PMSF (extraction buffer). The crude extract was sonicated and centrifuged for 15 min at 10000 ***g*** and 4 °C. The supernatant was incubated with glutathione–Sepharose resin (Amersham Pharmacia Biotech), pre-equilibrated with extraction buffer, for 1 h at 4 °C. The resin was then washed with extraction buffer supplemented with 0.3 M NaCl, followed by extraction buffer. Proteins bound to the resin were stored in aliquots at −80 °C until use.

### Lipid kinase activity assay

Inositol lipid phosphorylation was performed as described [31]. In brief, phosphoinositides, in chloroform/methanol (2:1, v/v), were dried under a stream of nitrogen. They were resuspended in 2 × kinase assay buffer [50 mM Hepes/KOH (pH 7.4)/3 mM $MgCl_2$/10 mM 2-glycerophosphate/2 mM dithiothreitol/240 mM NaCl] supplemented with 2% (w/v) sodium cholate. Glutathione–Sepharose with recombinant protein was washed once with 1 × kinase assay buffer before activity measurement. Each reaction consisted of 5 $\mu$g of lipid substrate, resin-bound GST–AtPIP5K1, and was started by the addition of 50 $\mu$M [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci per assay) in a final volume of 50 $\mu$l. The reaction was performed for 5 min at room temperature and stopped by the addition of 1 ml of chloroform/methanol (1:1, v/v) and 20 $\mu$l of brain extract (Folch Fraction I; Sigma) in chloroform (10 mg/ml) was added to provide carrier lipids. The mixture was vortex-mixed, and 0.5 ml of 1.2 M HCl was added to separate the phases. After centrifugation, the lower phase was washed once with 0.5 ml of methanol and 0.5 ml of 0.1 M HCl. The washed lower phase was transferred to a new tube and dried under a stream of nitrogen. The lipid products were resuspended in 30 $\mu$l of chloroform and separated by TLC with a solvent mixture composed of chloroform/methanol/water/$NH_4OH$ (45:45:9:5, by vol.). Radiolabelled lipids were detected with a PhosphorImager (Molecular Dynamics).

### Identification of lipid products

After lipid kinase assay, lipids were dried with nitrogen and deacylated with a methylamine solution prepared as described by Dove and Michell [32]. A total phospholipid deacylate was prepared from a *S. cerevisiae* strain radiolabelled with [$^3$H]inositol and treated with 1 M NaCl to stimulate the synthesis of PtdIns(3,5)$P_2$ [23]. An aliquot of this total $^3$H-labelled deacylate was added to the deacylation products obtained from the PtdIns4*P* phosphorylation reactions before they were separated on a Partisphere 46 mm × 250 mm SAX HPLC column (5 $\mu$m pore size; Whatman) with an $(NH_4)_2HPO_4$ gradient (gradient B in [32]). For the identification of the product generated by AtPIP5K1 from PtdIns3*P*, an aliquot of a total deacylate obtained from *S. cerevisiae* overexpressing a farnesylated p110$\gamma$ subunit of mouse inositol lipid 3-kinase and radiolabelled with [$^3$H]inositol was used. This deacylate contained substantial amounts of [$^3$H]Gro*P*Ins(3,4)$P_2$ and [$^3$H]Gro*P*Ins(3,4,5)$P_3$ as well as the inositol lipids normally found in yeast cells. Fractions (1 ml) were collected, and the radioactivity in each fraction was determined with a scintillation counter.

### *A. thaliana* subcellular fractionation

*A. thaliana* plants were grown in soil in a greenhouse with supplementary light (23 W/m², 350–800 nm; Philips G/86/2 HPLR 400W) and with full nutrient supply.

Plants (4 weeks old; everything above the soil) were harvested and homogenized in 50 mM Mops/KOH (pH 7.8)/330 mM sucrose/0.2% (w/v) casein (boiled hydrolysate)/5 mM EDTA/5 mM ascorbic acid/5 mM dithiothreitol/0.6% (w/v) polyvinylpolypyrrolidone/1 mM PMSF. The homogenate was filtered and centrifuged at 10000 ***g*** for 15 min. The resulting supernatant was centrifuged at 30000 ***g*** for 1 h, giving a supernatant, termed the cytosol, and a pellet, termed the microsomal fraction.

### Protein phosphorylation

Resin with bound GST–AtPIP5K1 was washed with PKA buffer [100 mM Tris/HCl (pH 7.5)/0.5 M NaCl/100 mM $MgCl_2$/1 mM EGTA]. GST–AtPIP5K1 bound to glutathione–Sepharose was phosphorylated with PKA in accordance with the manufacturer's instructions (Boehringer Mannheim). In brief, 20 $\mu$l of PKA buffer was mixed with 20 $\mu$l of washed resin and 40 m-units of PKA; the volume was adjusted to 90 $\mu$l with water. The reaction was started with 10 $\mu$l of 50 $\mu$M ATP (0.5 $\mu$Ci/$\mu$l) and incubated at 20 ± 2 °C. The reaction was terminated after 30 min with Laemmli sample buffer [33]. In some cases, phosphorylation by PKA was followed by treatment with alkaline phosphatase. Phosphorylated GST–AtPIP5K1 was washed three times (5 min each) with alkaline phosphatase buffer [50 mM Tris/HCl

(pH 8.5)/0.1 mM EDTA] followed by incubation with 10 units of alkaline phosphatase for 30–60 min at 20 ± 2 °C. Control samples were exposed to the same treatment except that no PKA or alkaline phosphatase was present.

Phosphorylation of GST–AtPIP5K1 bound to the resin with the cytosol and microsome fraction from *A. thaliana* plants was performed exactly as with PKA, except that incubation time was prolonged to 2 h. The effect of $Ca^{2+}$ was also tested, by replacing 1 mM EGTA in the incubation with 0.1–1 mM $CaCl_2$.

### Protein sequence analysis

Alignment of amino acid sequences was performed with the ClustalW program available from the ExPASY Molecular Biology Server (http://www.expasy.ch).

### SDS/PAGE

Proteins were separated on 10 % (w/v) polyacrylamide gels as described previously [33].

### Protein determination

Protein concentration was determined essentially as described by Bearden [34], with BSA as standard protein.

## RESULTS

### Sequence comparison

The *A. thaliana* gene *AtPIP5K1* encodes a protein sharing sequence similarity with PIPkins and possesses PtdIns*P* kinase activity [27]. Sequence comparison clearly shows that AtPIP5K1 is significantly more similar to Mss4p than to any other PIPkin (Figure 1). The amino acid sequences of AtPIP5K1 and Mss4p are 25 % identical (195 residues), with an additional 127 residues conserved between them. Only 15 % of the amino acids are identical when comparing AtPIP5K1 with animal PIPkins. This reflects the fact that AtPIP5K1 and Mss4p both have an N-terminal extension, which contains a number of identical or conserved residues (Figure 1, upper panel). This extension is absent from the type I and type II PIPkins and is not conserved in type III PIPkins (Figure 1, lower panel). Even when comparing the putative catalytic domains, AtPIP5K1 and Mss4p are more similar to each other than to type I, II and III PIPkins (Figure 1, lower panel). In addition, its calculated molecular mass (85.8 kDa) is similar to that of Mss4p (89.3 kDa), whereas type I and II PIPkins from animals are smaller (60–72 and 47 kDa respectively) [17] and FAB1p and its homologues are much bigger (200 kDa or more) [18].

### AtPIP5K1 has dual substrate specificity *in vitro*

Mikami et al. [27] showed that the putative catalytic domain of AtPIP5K1 phosphorylated commercial PtdIns4*P* to a PtdIns$P_2$ isomer but the identity was not determined. No other PtdIns*P* isomers were tested as substrates. To determine the substrate specificity of the protein encoded by *AtPIP5K1*, we expressed full-length AtPIP5K1 as a GST fusion protein (Figure 2). Induction of the expression of *GST–AtPIP5K1* in *E. coli* with IPTG resulted in the accumulation of a protein of approx. 110 kDa (lane + in Figure 2), which corresponds well to the calculated size (115 kDa) for the GST–AtPIP5K1 fusion protein. Non-induced cells did not contain any significant amount of this protein (lane − in Figure 2). Affinity purification on glutathione–Sepharose beads produced a clear enrichment of the 110 kDa protein (lane P in Figure 2). We then tested the activity

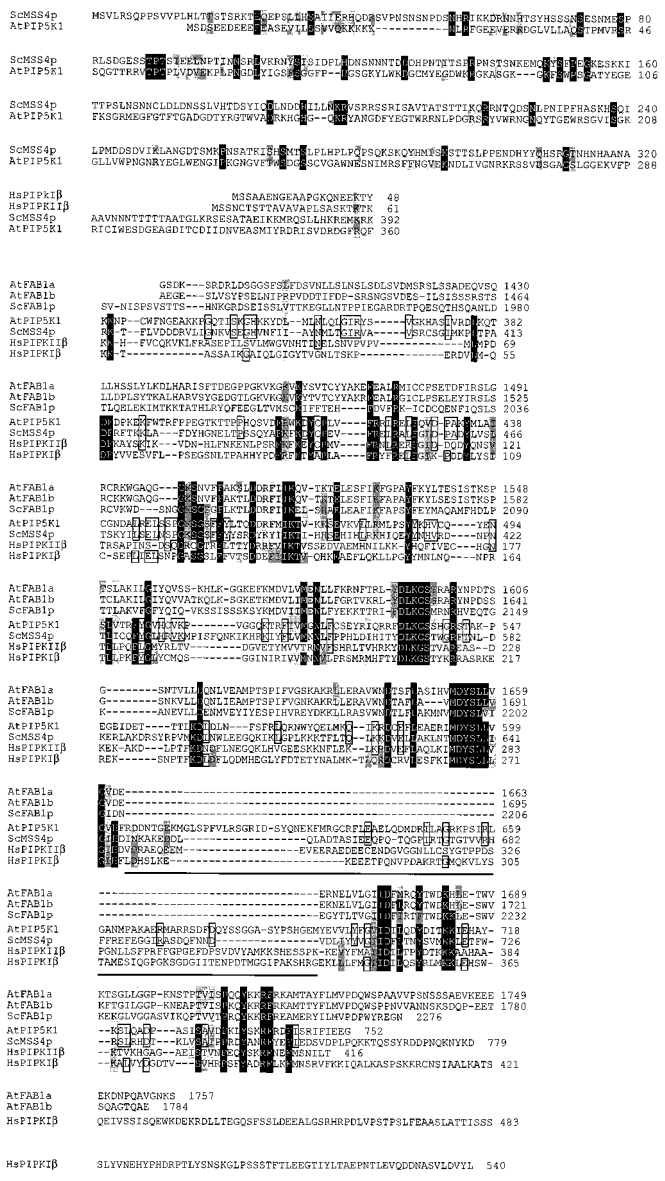

**Figure 1 Multiple sequence alignment of AtPIP5K1 with Mss4p, HsPIPKI$\beta$, HsPIPKII$\beta$ and FAB1 proteins**

The sequences of AtPIP5K1 (accession no. AF019380), Mss4p (D13716) HsPIPKI$\beta$ (X92493), HsPIPKII$\beta$ (U85245), predicted AtFAB1a (AL035525), predicted AtFAB1b (AB022220) and ScFAB1p (U01017) were aligned by using the ClustalW sequence alignment program and adjusted by hand. Residues identical in at least the four first sequences are shown in white on a black background, while those conserved or semi-conserved are shaded in grey. Additional residues identical in at least AtPIP5K1 and Mss4p are boxed. The insertion in the catalytic domain of PtdIns*P* 4/5-kinases is indicated by a line below the aligned sequences. Numbers at the right indicate the position of the last amino acid in each lane for the respective proteins. Upper panel: alignment of the N-terminal regions of AtPIP5K1, ScMss4p, HsPIPKI$\beta$ and HsPIPKII$\beta$. Lower panel: alignment of the catalytic domains.

of recombinant GST–AtPIP5K1 bound to the glutathione resin with PtdIns, PtdIns3*P*, PtdIns4*P*, PtdIns5*P* and PtdIns(3,4)$P_2$ as substrates. Strong lipid kinase activity was detected when PtdIns4*P* was the substrate, slightly weaker activity with PtdIns3*P* and only very weak activity with PtdIns5*P* (Figure 3A). No lipid kinase activity was detected with PtdIns or PtdIns(3,4)$P_2$ as substrate (Figure 3A). As expected, GST alone did not phosphorylate PtdIns4*P*. The major lipid products

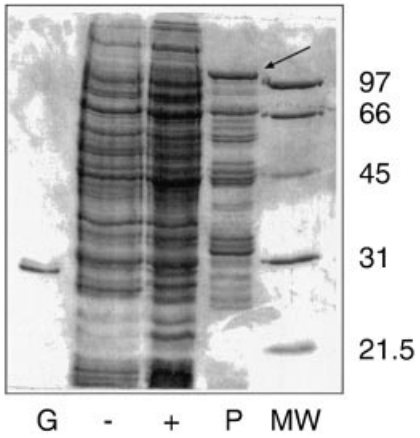


**Figure 2 Expression of recombinant proteins**

BL21 cells harbouring the pGPIP5K1 construct were grown overnight at 30 °C. A sample was withdrawn before induction with IPTG. Recombinant GST–AtPIP5K1 was purified from the induced cells. Recombinant GST was affinity-purified from BL21 cells harbouring the pGEX-4T-2 plasmid. Recombinant GST (lane G) and GST–AtPIP5K1 (lane P), as well as supernatants obtained after the sonication of cell extracts from non-induced (−) and induced (+) cells, were separated by SDS/PAGE and stained with Coomassie Brilliant Blue G-250. The arrow indicates GST–AtPIP5K1. Molecular mass standards (lane MW) are indicated in kDa at the right.

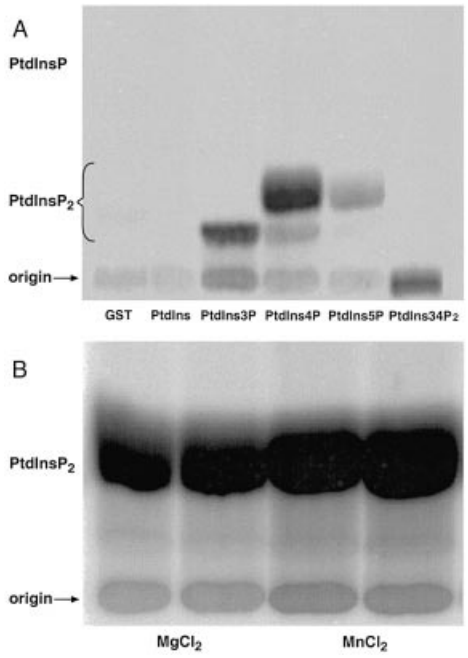


**Figure 3 Lipid kinase activity of GST–AtPIP5K1 *in vitro***

The activity of recombinant GST–AtPIP5K1 bound to glutathione–Sepharose beads was assayed with five different lipids as substrate [PtdIns, PtdIns3*P*, PtdIns4*P*, PtdIns5*P* and PtdIns(3,4)$P_2$] in the presence of 1.5 mM $MgCl_2$ (**A**), and with PtdIns4*P* in the presence of 1.5 mM $MgCl_2$ or 0.5 mM $MnCl_2$ (**B**). Lipid products were separated by TLC and detected with a PhosphorImager. In (**B**) duplicates were performed and separated side by side on the TLC plate. As a control, recombinant GST was also tested with PtdIns4*P* as substrate [GST in (**A**)]. Note that no PtdIns*P* band, which migrates towards the top of the TLC section presented, is detected.

obtained from PtdIns3*P* and PtdIns4*P* were resolved as distinct bands when separated by TLC in the region where PtdIns$P_2$ isomers were recovered in this TLC solvent system (Figure 3A), suggesting that different PtdIns$P_2$ isomers were formed from these two lipids. When PtdIns4*P* was the substrate, a minor band migrating exactly like the product obtained with PtdIns3*P* was also detected in some cases (compare Figures 3A and 3B). Both $Mg^{2+}$ and $Mn^{2+}$ could serve as the metal ion in the reaction (Figure 3B), with $Mn^{2+}$ being slightly more efficient.

To identify the PtdIns$P_2$ isomers formed by AtPIP5K1, the lipid products obtained from PtdIns3*P*, PtdIns4*P* and PtdIns5*P* were deacylated, spiked with an aliquot of one of two [$^3$H]inositol-labelled total yeast lipid deacylates of known composition, one containing [$^3$H]Gro*P*Ins(3,5)$P_2$ and [$^3$H]Gro*P*Ins(4,5)$P_2$, the other [$^3$H]Gro*P*Ins(3,4)$P_2$ and [$^3$H]Gro*P*Ins(4,5)$P_2$ (see the Materials and methods section) and then analysed by HPLC. After deacylation, the major [$^{32}$P]Gro*P*Ins$P_2$ isomer obtained from the lipid products generated from PtdIns4*P* was eluted at exactly the same place as [$^3$H]Gro*P*Ins(4,5)$P_2$ (Figure 4, upper panel). The elution profile of [$^3$H]Gro*P*Ins(4,5)$P_2$ on this column has been presented earlier [14] and was confirmed by deacylating [$^3$H]PtdIns(4,5)$P_2$ (from NEN Life Science) and using the resulting [$^3$H]Gro*P*Ins(4,5)$P_2$ as an internal standard in some samples. The deacylation product obtained from the lipid synthesized by GST–AtPIP5K1 from PtdIns3*P* was eluted at exactly the same place as [$^3$H]Gro*P*Ins(3,4)$P_2$ (Figure 4, lower panel). The same analysis protocol showed that when PtdIns5*P* was the substrate, the lipid product generated consisted mainly of PtdIns(4,5)$P_2$ (results not shown). These results indicate that, *in vitro*, AtPIP5K1 is a dual substrate-specific lipid kinase, phosphorylating PtdIns3*P* to PtdIns(3,4)$P_2$, and PtdIns4*P* to PtdIns(4,5)$P_2$.

### Phosphorylation of AtPIP5K1 by PKA results in inhibition of activity

We tested whether GST–AtPIP5K1 could be phosphorylated *in vitro* by commercially available protein kinases. GST–AtPIP5K1 bound to glutathione–Sepharose resin was incubated with the catalytic subunit of PKA in the presence of [$\gamma$-$^{32}$P]ATP and $MgCl_2$ and subsequently resolved by SDS/PAGE and analysed by PhosphorImaging (Figures 5A and 5B). As shown in Figure 5, PKA actively phosphorylated GST–AtPIP5K1, whereas no autophosphorylation was detected in the absence of exogenous protein kinase. GST alone was not significantly phosphorylated by PKA (Figure 5D), demonstrating that AtPIP5K1 itself was the target of phosphorylation by PKA. Phosphorylation of GST–AtPIP5K1 by PKA resulted in an inhibition of its lipid kinase activity, as measured with PtdIns4*P* as substrate. The inhibition reached approx. 40 % after 30 min of phosphorylation with PKA (Figure 5C). Treatment of PKA-phosphorylated GST–AtPIP5K1 with alkaline phosphatase resulted in a significant decrease in the $^{32}$P-labelling of GST–AtPIP5K1 (Figure 5B). The dephosphorylation of the recombinant protein was accompanied by a full recovery of lipid kinase activity (Figure 5C). This suggests that several sites were phosphorylated by PKA and that not all of them were involved in inhibition of the catalytic activity of the enzyme. It also indicates that the synthesis of at least some of the phosphoinositides might be regulated by protein phosphorylation in plant cells.

### AtPIP5K1 is also phosphorylated by an *A. thaliana* protein kinase

*A. thaliana* contains several hundred different protein kinases but very little is known about their site specificity. Therefore, to investigate whether AtPIP5K1 might be regulated *in vivo* by

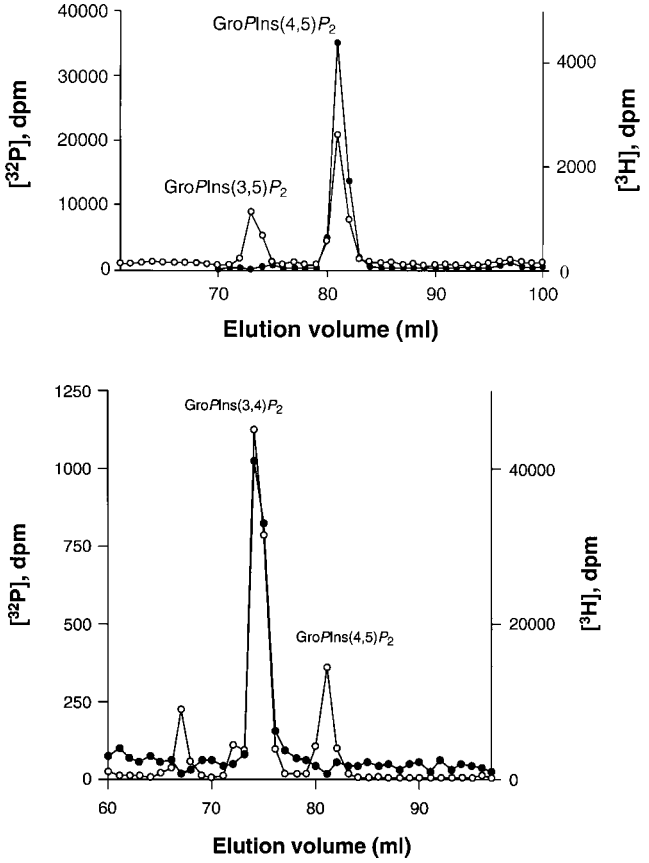


**Figure 4 Identification of the products of the lipid kinase activity of GST–AtPIP5K1**

The activity of GST–AtPIP5K1 was assayed with PtdIns4*P* (upper panel) or PtdIns3*P* (lower panel) as substrate. The $^{32}$P-labelled lipid products (●) were deacylated and analysed by HPLC on a Partisphere SAX column. The deacylation products were spiked with an internal standard consisting of the total lipid deacylate obtained from [$^3$H]inositol-labelled yeast cells (○) that had been shocked with NaCl to stimulate the production of PtdIns(3,5)$P_2$ (**A**), and [$^3$H]inositol-labelled yeast cells overexpressing the mouse inositol lipid 3-kinase p110$\gamma$ and hence generating substantial amounts of PtdIns(3,4)$P_2$ (**B**).

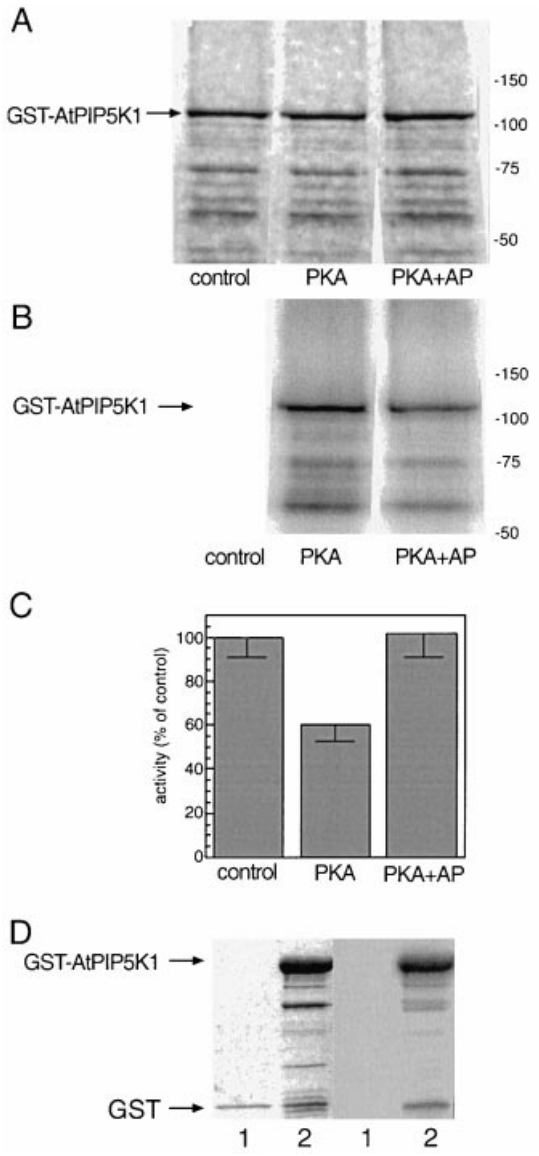


**Figure 5 AtPIP5K1 is phosphorylated and inhibited by PKA**

GST [lane 1 in (**D**)] and GST–AtPIP5K1 [(**A**–**C**), and lane 2 in (**D**)] were incubated with the catalytic subunit of PKA in the presence of [$\gamma$-$^{32}$P]ATP and $MgCl_2$ (PKA), or with PKA, [$\gamma$-$^{32}$P]ATP and $MgCl_2$ followed by alkaline phosphatase (PKA + AP), as described in the Materials and methods section. In a control experiment, GST–AtPIP5K1 was incubated in the same conditions without PKA or AP (control). The samples were then separated by SDS/PAGE and stained with Coomassie Brilliant Blue (**A**) followed by drying and PhosphorImaging (**B**). The migration of molecular mass markers is indicated (in kDa) at the right. In parallel, samples treated in the same way were assayed for lipid kinase activity, with PtdIns4*P* as substrate in the presence of 1.5 mM $MgCl_2$. Lipid products were analysed by TLC and quantified (means ± S.D.) from the PhosphorImage (**C**). (**D**) Left panel, Coomassie staining; right panel, PhosphorImage. Lanes 1, GST; lanes 2, GST–AtPIP5K1.

phosphorylation, we tested subcellular fractions from *A. thaliana* plants for their ability to phosphorylate GST–AtPIP5K1 loaded on glutathione–Sepharose resin. As shown in Figure 6, the cytosolic fraction was able to phosphorylate GST–AtPIP5K1, although relatively weakly. We did not detect any phosphorylation with the microsomal fraction (results not shown). To demonstrate that the incorporation was in AtPIP5K1, we treated glutathione–Sepharose loaded with $^{32}$P-labelled GST–AtPIP5K1 with thrombin. As expected, the GST–AtPIP5K1 protein band disappeared completely, whereas a smaller phosphorylated protein of approx. 72 kDa could be detected that was not present before treatment with thrombin (Figure 6). This is smaller than the calculated mass of intact AtPIP5K1 (86 kDa) and it probably reflects cleavage by thrombin at site(s) present in AtPIP5K1 in addition to the site present between the GST and AtPIP5K1 sequences. The major Coomassie-stained band after cleavage with thrombin (asterisk in Figure 6), approx. 50 kDa in size, probably represents a further degradation of AtPIP5K1. This band is only very weakly phosphorylated. AtPIP5K1 does indeed contain several putative thrombin cleavage sites. It is also worth noting that after cleavage with thrombin, no radioactivity was detected in the GST band, confirming that the $^{32}$P labelling present in GST–AtPIP5K1 was not in GST itself. Similar results were obtained whether $Ca^{2+}$ was present or not during phosphorylation with plant subfractions, suggesting that the protein kinase responsible was not $Ca^{2+}$-dependent. We were unable to detect any effect of phosphorylation of GST–AtPIP5K1 by the cytosol on its activity, measured with PtdIns4*P* as substrate (results not shown).

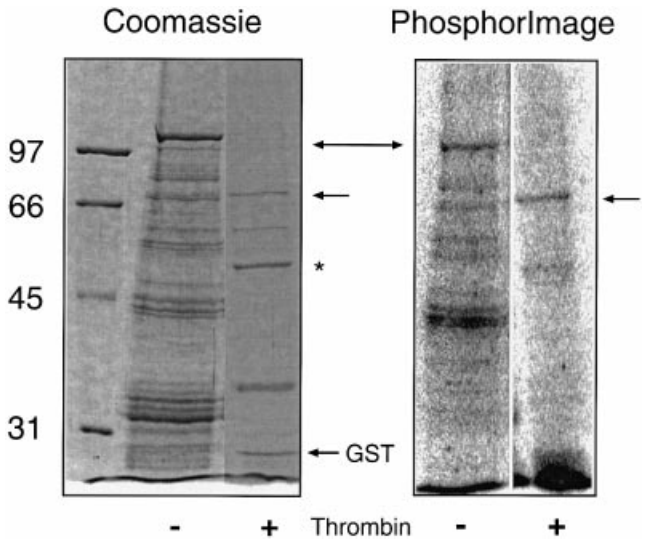


**Figure 6 AtPIP5K1 is phosphorylated by an *A. thaliana* protein kinase**

GST–AtPIP5K1 bound to glutathione–Sepharose beads was incubated with a cytosolic fraction obtained from *A. thaliana* plants in the presence of [$\gamma$-$^{32}$P]ATP and $MgCl_2$ and separated by SDS/PAGE followed by PhosphorImaging (−). GST was released from phosphorylated GST–AtPIP5K1 fusion protein by incubation of resin with thrombin, followed by SDS/PAGE and PhosphorImaging (+). The double-headed arrow indicates GST–AtPIP5K1; the single arrow indicates the major phosphoprotein obtained after treatment of phosphorylated GST–AtPIP5K1 with thrombin. The positions of molecular mass markers (in the leftmost lane) are indicated in kDa at the left.

## DISCUSSION

We demonstrate that AtPIP5K1 has dual substrate specificity *in vitro*, catalysing the synthesis of PtdIns(4,5)$P_2$ and PtdIns(3,4)$P_2$ from PtdIns4*P* and PtdIns3*P* respectively. This substrate specificity resembles that of type I PIPkins, but the latter can also generate PtdIns(3,5)$P_2$ and PtdIns(3,4,5)$P_3$ from PtdIns3*P* [19,35], which AtPIP5K1 cannot. However, it is the *S. cerevisiae* PIPkin Mss4p that shows the most similar substrate specificity [36]. Indeed, *in vitro*, the substrate specificities of these two enzymes are almost indistinguishable.

Further investigations will be required to determine whether AtPIP5K1 participates in the synthesis of PtdIns(4,5)$P_2$ and/or PtdIns(3,4)$P_2$ *in vivo*. Because *AtPIP5K1* is constitutively expressed in the plant, although at a low level in some tissues [27], it is most probable that it is involved in the synthesis of PtdIns(4,5)$P_2$ *in vivo*. Alternatively, AtPIP5K1 could be involved in the response to stress conditions and hormones because *AtPIP5K1* expression is stimulated by such factors [27]. We have recently demonstrated that *A. thaliana* cultured cells subjected to salt or hyperosmotic stress respond by a marked increase in the synthesis of PtdIns(4,5)$P_2$ [14]. This increase in PtdIns(4,5)$P_2$ could be accounted for by the stimulation of the expression of a gene encoding a PIPkin generating PtdIns(4,5)$P_2$ or by the stimulation of such an enzyme already present in cells. AtPIP5K1 would obviously be a good candidate for this response. Stimulation of pre-existing AtPIP5K1 enzymes could involve dephosphorylation by a specific protein phosphatase, because we show here that phosphorylation of AtPIP5K1 inhibits its activity and that full activity is restored by treatment of phosphorylated AtPIP5K1 with a protein phosphatase.

PtdIns(3,4)$P_2$ is present in yeast cells in trace amounts and much more is made when *MSS4* is overexpressed [36]. It is not clear whether this lipid has a relevant function in yeast [36]. No PtdIns(3,4)$P_2$ was detected in *A. thaliana* cultured cells either, under the various experimental conditions tested [14]. However, significant amounts of this phosphoinositide have been detected in plant cells, for example in guard cells of *C. communis* [22] and in *Spirodela polyrhiza* [37]. It thus seems that plant cells synthesize this phosphoinositide in unstimulated conditions. The plant PIPkin(s) responsible for the synthesis of PtdIns(3,4)$P_2$ *in vivo* have not been identified; an involvement of AtPIP5K1 is plausible but remains to be demonstrated. This hypothesis is in line with the work of Brearley and Hanke [37] in which the synthesis of PtdIns(3,4)$P_2$ in *S. polyrhiza* was shown to proceed via the 4-phosphorylation of PtdIns3*P*. However, the *A. thaliana* genome contains several other genes encoding proteins very similar to AtPIP5K1 and Mss4p (C. Pical, unpublished work), adding other potential candidates that might be involved in the synthesis of this phosphoinositide. In platelets it has been shown that the stimulation of PtdIns(3,4)$P_2$ generation via the thrombin receptor is due to a new type of PIPkin, namely an enzyme that phosphorylates PtdIns3*P* on the 4-OH group distinct from any type I–III PIPkins identified so far [38]. It is not yet known whether such an enzyme is also present in plants and yeast. As far as *A. thaliana* is concerned, we can safely say that PtdIns(3,4)$P_2$ is not generated by the only PtdIns 3-kinase present in *A. thaliana*, because this enzyme, AtVps34, belongs to the family of PtdIns 3-kinase that uses only PtdIns as a substrate, both *in vitro* and *in vivo* [39].

We also demonstrate that AtPIP5K1 is inhibited by PKA-catalysed phosphorylation. Two PIPkin activities have recently been shown to be negatively regulated by phosphorylation. Mouse PIPKI$\alpha$ was phosphorylated on a Ser residue by PKA, resulting in a 50% inhibition of its activity [40]. A similar result was obtained with a PIPkin partly purified from *Saccharomyces pombe* plasma membranes on phosphorylation with the *S. pombe* casein kinase I orthologue Cki1 [41]. The PKA phosphorylation motif RRXS (single-letter amino acid codes) containing the Ser residue believed to be the target of PKA in mouse PIPKI$\alpha$ [38] is conserved in other type I PIPkins from animals but is absent from *A. thaliana* PIPkins. The genome of *A. thaliana* does not contain any gene encoding a protein with significant similarity to the catalytic subunit of PKA, but it is possible that one of the many protein kinases present in plants has a sequence recognition motif similar to that of PKA. AtPIP5K1 was weakly phosphorylated by a cytosolic fraction prepared from *A. thaliana* plants in a $Ca^{2+}$-independent manner. However, no change in lipid kinase activity was observed. This is most probably due to the poor phosphorylation level achieved with the crude cytosol. For the preparation of plant cytosolic fractions, plant material is homogenized with a volume of buffer equivalent to 2–3-fold the weight of fresh tissue, resulting in an extensive dilution of the soluble proteins.

In conclusion, our results suggest that plants contain at least one potential homologue of yeast Mss4p, although it still remains to be proved that AtPIP5K1 is able to replace Mss4p functionally, for example by complementing an *MSS4* deletion mutant. We also demonstrate for the first time that a plant enzyme involved in the metabolism of phosphoinositides can be regulated by phosphorylation and dephosphorylation. Identification of the phosphorylated residue(s) in AtPIP5K1 as well as the plant protein kinase and phosphatase responsible for the phosphorylation and dephosphorylation of this enzyme is now necessary for a resolution of its role in the regulation of phosphoinositide metabolism.

We thank Kazuo Shinozaki (Riken, Ibaraki, Japan) for providing the *AtPIP5K1* cDNA. Grants from the Swedish Council for Forestry and Agricultural Research (C.P.), the Lund Fysiografiska Sällskapet (C.P.) and the Crafoord Foundation (C.P.), the Swedish Natural Science Research Council (M.S.), and the Swedish Foundation for Strategic Research (M.S.) are acknowledged. S.K.D. is a Royal Society University Research Fellow; this work was funded by The Royal Society, the MRC and the Wellcome Trust.

## REFERENCES

1 Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. Nature (London) **361**, 315–325
2 Toker, A. (1998) The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. Curr. Opin. Cell Biol. **10**, 254–261
3 Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S. and Takenawa, T. (2001) Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science **291**, 1047–1051
4 Ford, M. G., Pearse, B. M. F., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R. and McMahon, H. T. (2001) Simultaneous binding of PtdIns(4,5)$P_2$ and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science **291**, 1051–1055
5 De Camilli, P., Emr, S. D., McPherson, P. S. and Novick, P. (1996) Phosphoinositides as regulators in membrane traffic. Science **271**, 1533–1539
6 Wurmser, A. E., Gary, J. D. and Emr, S. D. (1999) Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. J. Biol. Chem. **274**, 9129–9132
7 Alexandre, J., Lassalles, J. F. and Kado, R. D. (1990) Opening of $Ca^{2+}$ channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. Nature (London) **343**, 567–570
8 Blatt, M. F., Thiel, G. and Trentham, D. R. (1990) Reversible inactivation of $K^+$ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. Nature (London) **346**, 766–769
9 Gilroy, S., Read, N. D. and Trewavas, A. J. (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. Nature (London) **346**, 769–771
10 Yang, W., Burkhart, W., Cavallius, J., Merrick, W. C. and Boss, W. F. (1993) Purification and characterization of a phosphatidylinositol 4-kinase activator in carrot cells. J. Biol. Chem. **268**, 392–398
11 Drøbak, B. K., Watkins, P. A. C., Valenta, R., Dove, S. K., Lloyd, C. W. and Staiger, C. J. (1994) Inhibition of plant plasma membrane phosphoinositide phospholipase C by the actin-binding protein, profiling. Plant J. **6**, 389–400
12 Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C. and Chua, N. H. (1999) Rac homologues and compartmentalized phosphatidylinositol 4, 5-bisphosphate act in a common pathway to regulate polar pollen tube growth. J. Cell Biol. **145**, 317–330
13 Bunney, T. D., Watkins, P. A. C., Beven, A. F., Shaw, P. J., Hernandez, L. E., Lomonossoff, G. P., Shanks, M., Peart, J. and Drøbak, B. K. (2000) Association of phosphatidylinositol 3-kinase with nuclear transcription sites in higher plants. Plant Cell **12**, 1679–1688
14 Pical, C., Westergren, T., Dove, S. K., Larsson, C. and Sommarin, M. (1999) Salinity and hyperosmotic stress induce rapid increases in phosphatidylinositol 4,5-bisphosphate, diacylglycerol pyrophosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells. J. Biol. Chem. **274**, 38232–38240
15 DeWald, D. B., Torabinejad, J., Jones, C. A., Shope, J. C., Cangelosi, A. R., Thompson, J. E., Prestwich, G. D. and Hama, H. (2001) Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. Plant Physiol. **126**, 759–769
16 Meijer, H. J. G., Divecha, N., van den Ende, H., Musgrave, A. and Munnik, T. (1999) Hyperosmotic stress induces rapid synthesis of phosphatidyl-D-inositol 3,5-bisphosphate in plant cells. Planta **208**, 294–298
17 Hinchliffe, K. A., Ciruela, A. and Irvine, R. F. (1998) PIPkins, their substrates and their products: new functions for old enzymes. Biochim. Biophys. Acta **1436**, 87–104
18 McEwen, R. K., Dove, S. K., Cooke, F. T., Painter, G. F., Holmes, A. B., Shisheva, A., Ohya, Y., Parker, P. J. and Michell, R. H. (1999) Complementation analysis in PtdInsP kinase-deficient yeast mutants demonstrates that *Schizosaccharomyces pombe* and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. J. Biol. Chem. **274**, 33905–33912
19 Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W. and Anderson, R. A. (1997) Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. J. Biol. Chem. **272**, 17756–17761
20 Rameh, L. E., Tolias, K. F., Duckworth, B. C. and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature (London) **390**, 192–196
21 Carpenter, C. L. and Cantley, L. C. (1996) Phosphoinositide kinases. Curr. Opin. Cell Biol. **8**, 153–158
22 Parmar, P. N. and Brearley, C. A. (1993) Identification of 3- and 4-phosphorylated phosphoinositides and inositol phosphates in stomatal guard cells. Plant J. **4**, 255–263
23 Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J. and Michell, R. H. (1997) Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature **390**, 187–192
24 Sommarin, M. and Sandelius, A. S. (1988) Phosphatidylinositol and phosphatidylinositolphosphate kinases in plant plasma membranes. Biochim. Biophys. Acta **958**, 268–278
25 Hong, Z. and Verma, D. P. S. (1994) A phosphatidylinositol 3-kinase is induced during soybean nodule organogenesis and is associated with membrane proliferation. Proc. Natl. Acad. Sci. U.S.A. **91**, 9617–9621
26 Welters, P., Takegawa, K., Emr, S. D. and Chrispeels, M. J. (1994) AtVPS34, a phosphatidylinositol 3-kinase of *Arabidopsis thaliana*, is an essential protein with homology to a calcium-dependent lipid binding domain. Proc. Natl. Acad. Sci. U.S.A. **91**, 11398–11402
27 Mikami, K., Katagiri, T., Iuchi, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) A gene encoding phosphatidylinositol-4-phosphate 5-kinase is induced by water stress and abscisic acid in *Arabidopsis thaliana*. Plant J. **15**, 563–568
28 Chang, K. J., Marcus, N. A. and Cuatrecasas, P. (1974) The effect of dichloroacetate on the phosphorylation of mitochondrial proteins. J. Biol. Chem. **249**, 6854–6865
29 Painter, G. F., Grove, S. J. A., Gilbert, I. H., Holmes, A. B., Raithby, P. R., Hill, M. L., Hawkins, P. T. and Stephens, L. R. (1999) General synthesis of 3-phosphorylated myo-inositol phospholipids and derivatives. J. Chem. Soc. Perkin Trans. **1**, 923–935
30 Satterlee, J. S. and Sussman, M. R. (1997) An *Arabidopsis* phosphatidylinositol-4-phosphate 5-kinase homolog with seven novel repeats rich in aromatic and glycine residues (Accession no. AF019380) (PGR 97–150). Plant Physiol. **115**, 864
31 Cooke, F. T., Dove, S. K., McEwen, R. K., Painter, G., Holmes, A. B., Hall, M. N., Michell, R. H. and Parker, P. J. (1998) The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in *S. cerevisiae*. Curr. Biol. **8**, 1219–1222
32 Dove, S. K. and Michell, R. H. (1999) Analysis of polyphosphorylated inositol lipids of *S. cerevisiae*. In Signal Transduction: A Practical Approach (Milligan, G., ed.), pp. 255–281, Oxford University Press, Oxford
33 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. Nature (London). **227**, 680–685
34 Bearden, Jr, J. C. (1978) Quantification of submicrogram quantities of protein by an improved protein-dye binding assay. Biochim. Biophys. Acta **533**, 525–529
35 Tolias, K. F., Rameh, L. E., Ishihara, H., Shibasaki, Y., Chen, J., Prestwich, G. D., Cantley, L. C. and Carpenter, C. L. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases synthesize the novel lipids phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 5- phosphate. J. Biol. Chem. **273**, 18040–18046
36 Desrivières, S., Cooke, F. T., Parker, P. J. and Hall, M. N. (1998) *MSS4*, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. J. Biol. Chem. **273**, 15787–15793
37 Brearley, C. A. and Hanke, D. E. (1993) Pathway of synthesis of 3,4- and 4,5-phosphorylated phosphatidylinositols in the duckweed *Spirodela polyrhiza* L. Biochem. J. **290**, 145–150
38 Banfic, H., Downes, C. P. and Rittenhouse, S. E. (1998) Biphasic activation of PKBα/Akt in platelets. Evidence for stimulation both by phosphatidylinositol 3,4-bisphosphate, produced via a novel pathway, and by phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. **273**, 11630–11637
39 Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. **253**, 239–254
40 Park, S. J., Itoh, T. and Takenawa, T. (2001) Phosphatidylinositol 4-phosphate 5-kinase type I is regulated through phosphorylation response by extracellular stimuli. J. Biol. Chem. **276**, 4781–4787
41 Vancurova, I., Choi, J. H., Lin, H., Kuret, J. and Vancura, A. (1999) Regulation of phosphatidylinositol 4-phosphate 5-kinase from *Schizosaccharomyces pombe* by casein kinase I. J. Biol. Chem. **274**, 1147–1155

Received 24 April 2001/26 July 2001; accepted 24 August 2001