Characterization of an Arabidopsis inositol 1,3,4,5,6-pentakisphosphate 2-kinase (AtIPK1)

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INTRODUCTION

Inositol polyphosphates (InsPs) are essential regulators of diverse cellular processes in eukaryotic cells. The pathways by which these molecules are interconverted have been the subject of intense study (reviewed in [1]). Recent work in Saccharomyces cerevisiae [2,3] has defined a pathway by which Ins(1,4,5)P₃ (inositol 1,4,5-trisphosphate), generated from the membrane lipid PtdIns(4,5)P₂, is converted into InsP₆ (inositol 1,2,3,4,5,6-hexakisphosphate). Three genes, PLC1, ScIPK1 and ScIPK2, together rescue mRNA export from the nucleus of a temperature-sensitive gle1-2 mutant of yeast.

The three genes respectively encode a phospholipase C, an InsP₅ multikinase and an InsP₆ (inositol 1,3,4,5,6-pentakisphosphate) 2-kinase [2–5]. ScIPK2 encodes an identical with ARG82 (also known as ArgR111), a regulator of a transcription factor complex, ArgR-Mcm1, which controls arginine metabolism in yeast. The ability of ScIPK2 protein to regulate arginine metabolism may not, however, depend on the kinase activity of the protein as a mutant protein lacking kinase activity has been reported to rescue yeast growth on media lacking arginine [6]. Three ScIPK2 orthologues, AtIPK2α and AtIPK2β, have been characterized in Arabidopsis [7,8]. Moreover, despite their low levels of identity to yeast ScIPK2, both rescue the temperature-sensitive growth phenotype of an S. cerevisiae yeast strain lacking the ScIPK2 open reading frame [ScIPk2Δ] and restore wild-type InsPs profiles. These observations, together with the nuclear localization of green fluorescent protein-tagged AtIPK2β protein in plant cells [8], suggest that AtIPK2β participates in a nuclear pathway of InsP synthesis in plants, though this does not necessarily discount a role for the protein in cytosolic pathways.

ScIPK1 encodes an InsP₆ 2-kinase [5]. Yeast strains lacking the ScIPK1 open reading frame [ScIPk1Δ] are deficient in InsP₆ synthesis and accumulate mRNA in the nucleus [5]. IPK1 (InsP₆-2-kinase)-type proteins are not strongly conserved across species, with less than 24% identity in pairwise combinations across the InsP₆-2-kinase domain of fungal proteins [9]. A human orthologue of ScIPK1 was recently characterized, and despite low identity with ScIPK1 protein (less than 20%), human HsIPK1 was shown to rescue an ScIPk1Δ strain and restore the InsP₆ profile to wild-type levels [10].

Little is known of the molecular identity of genes encoding enzymes that catalyse defined steps of InsP₆ synthesis in plants. Indeed, despite the recent identification of an insertion mutant in a maize InsP₆ kinase, ZmIPK, which reduces grain InsP₆ by approx. 30% [11], the pathway disrupted remains unidentified. This is in part a consequence of the broad substrate specificity displayed by ZmIPK protein to a wide variety of inositol phosphate substrates in vitro. Our difficulty in ascribing physiological activity to the maize enzyme is also partly a consequence of our lack of complete knowledge of the identity of endogenous inositol phosphates in maize.

While some authors take the ability of ZmIPK protein to convert Ins(1,3,4)P₃ (inositol 1,3,4-trisphosphate) into Ins(1,3,4,5,6)P₅ as

Abbreviations used: DIG, digoxigenin; GroPIns(4,5)P₂, glycerophosphoinositol 4,5-bisphosphate; Ins3P, inositol 3-monophosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4,5,6)P₅, inositol 1,3,4,5,6-pentakisphosphate; IPK1, Ins(1,3,4,5,6)P₅-2-kinase; IPK2, inositol tetraakisphosphate; InsP₆, inositol 1,2,3,4,5,6-hexakisphosphate; IPK3, inositol 1,3,4,5,6-pentakisphosphate; IPK1, Ins(1,3,4,5,6)P₅-2-kinase; IPTG, isopropyl β-D-thiogalactoside; Ni-NTA, Ni²⁺-nitrilotriacetate; PP-InsP₄, diphospho InsP₄; RT, reverse transcriptase; SD–U, synthetic defined medium minus uracil.

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evidence of participation of a lipid-derived pathway that generates Ins(1,4,5)P$_3$, as a precursor of Ins(1,3,4,5)P$_4$ [12], we have described a soluble pathway in the duckweed Spirodea polyrhiza which proceeds from inositol to InsP$_5$ in a stepwise manner but crucially beginning with 3-phosphorylation of the inositol ring [13,14]. It is possible that multiple pathways are operative in the plant kingdom or that different pathways exist in aquatic and terrestrial plants. There is, however, a general consensus that the final step in InsP$_5$ synthesis is 2-phosphorylation of the inositol ring [15]. With this consensus in mind, we have cloned an InsP$_5$-2kinase from Arabidopsis. In the present study, we describe the heterologous expression of AtIPK1 in E. coli and characterization of its activity in vitro.

**MATERIALS AND METHODS**

Restriction enzymes were purchased from Roche (Lewes, East Sussex, U.K.). Oligonucleotides were obtained from Invitrogen (Paisley, U.K.). DNA sequencing was performed by Dundee University Sequencing Service. InsP substrates were obtained from Sigma (Poole, Dorset, U.K.).

**Bacteria**

*E. coli* strain DH5$\alpha$ (Invitrogen) was used for vector construction. *E. coli* strain Rosetta(DE3) (Novagen, Nottingham, U.K.) was used for protein induction and purification.

**Isolation of AtIPK1**

DNA manipulations were performed by standard procedures [16]. RNA was extracted from *Arabidopsis* leaves and roots using Sigma TRI reagent (Sigma). Tissues were homogenized in liquid N$_2$ and 500 µl of TRI reagent was added. Samples were centrifuged at 2000 g and RNA precipitated from the upper aqueous layer with propan-2-ol. Then, 250 ng of RNA was reverse transcribed with M-MLV reverse transcriptase (from Roche according to the manufacturer’s instructions). PCRs were centrifuged at 2000 g for 15 min and the supernatant of the pellet was used for subsequent PCR were normalized with respect to ubiquitin product (GenBank® accession no. NM_105209: forward primer, GGCTAGATCCAGGATAAG; reverse primer: TCTGGATGGTGAATCAGCC).

**Expression and purification of proteins in E. coli**

pET28 vectors containing kinase cDNAs were transformed into chemically competent *E. coli* Rosetta cells and selected with 50 µg/ml kanamycin. Colonies were picked into 50 ml of Luria-Bertani broth and grown to an A$_{600}$ (absorbance) of 1. IPTG (isopropyl β-D-thiogalactoside) was added to a final concentration of 100 µM and the culture was grown at 25 °C for 16 h. Cells were pelleted, resuspended in 5 ml of 50 mM NaH$_2$PO$_4$, 300 mM NaCl and 10 mM imidazole (pH 8), sonicated for 3 × 30 s, centrifuged at 2000 g for 15 min and the supernatant of the lysate was retained. Qiagen (Crawley, W. Sussex, U.K.) Ni$_2$NTA (Ni$^{2+}$-nitrilotriacetic) resin (200 µl) prewashed twice in 1 M NiSO$_4$ was added to the lysate and incubated for 1 h at 4 °C. The resin was subsequently washed twice with 10 ml of 50 mM NaH$_2$PO$_4$, 300 mM NaCl and 20 mM imidazole (pH 8) to elute non-specifically bound proteins, and washed with 500 µl of NaH$_2$PO$_4$, 300 mM NaCl and 250 mM imidazole (pH 8) to elute AtIPK1 protein. Bacterial lysates and protein fractions from the purification were analysed by SDS/PAGE [17]. Protein concentrations were determined by the Bradford method using BSA as a standard [18].

**SDS/PAGE and protein blotting**

Proteins were analysed on SDS/12 % polyacrylamide gels. Protein gel blotting was performed essentially as described in [19]. Anti-FLAG antibody (Sigma Anti-FLAG M-2 monoclonal; F 3165) or anti T7 antibody was used at 1:1000 dilution in TBST. Blots were washed in 20 and 1 % BSA. Horseradish peroxidase-conjugated secondary antibody was used at 1:1000 dilution in TBST. Blots were developed by enhanced chemiluminescence.

**InsP kinase assays**

Purified protein (0.2 µg) was assayed in 20 µl volume of Hepes (pH 7.5), 6 mM MgCl$_2$, 10 mM LiCl, 0.4 µM ATP and 1 mM diithiothreitol with 1 µCi (37 kBq) [γ-32P]ATP and 20 µM InsP$_5$ substrate. Reaction mixtures were incubated at 30 °C for 2 h. The reactions were stopped by the addition of 2 µl of 2 M HCl and 2 µl of the products were spotted onto POLYGRAM® CEL 300 PEI TLC plates (Macherey-Nagel, Düren, Germany). Plates were developed in 0.5 M HCl to separate InsP$_5$ (inositol trisphosphate) from unreacted ATP and duplicate plates were run in 1.08 M KH$_2$PO$_4$, 0.64 M K$_2$HPO$_4$ and 1.84 M HCl to separate InsP$_5$, InsP$_4$ and InsP$_3$. Plates were exposed to a Fuji X-ray film.

**Kinetic analysis of AtIPK1 (At5G 42810) at high (0.4 mM) ATP concentration**

Reactions were performed for 15 min with [32P]Ins(1,3,4,5,6)P$_5$ substrate at Ins(1,3,4,5,6)P$_5$ concentrations of 5, 10, 20, 50 and 100 µM in a final volume of 20 µl. [32P]Ins(1,3,4,5,6)P$_5$ substrate was prepared with a recombinant plant protein. The
Yeast complementation and phenotypic assay

ScIpk1Δ strain BWY1228, MATa ipk1::kanMX4 [20], was provided by Adolfo Saiardi (John Hopkins University School of Medicine, Baltimore, MD, U.S.A.). The ScIpk1Δ strain was transformed with a number of putative InsP5 kinase genes from Arabidopsis in the plasmid vector pYES2 (Invitrogen) using the LiAc method [21]. Transformants were selected on SD–U (synthetic defined medium minus uracil; BD Biosciences) with glucose (Clontech, Basingstoke, Hampshire, U.K.). To compare growth rates, cells were streaked on to SD–U containing galactose and raffinose without glucose and grown at 37°C for 3 days.

For InsP5 analysis, cells were grown in YPD medium (1%, w/v, yeast extract, 2%, w/v, peptone and 2%, w/v, glucose; wild-type and ScIpk1Δ) or SD–U (transformed yeast) at 30°C to an A600 of 0.6 and then resuspended in minimal medium without inositol and containing galactose. Cells were grown overnight at 30°C in 10 ml of medium in the presence of 50 µCi (1.85 MBq) myo-[2-3H]inositol (20 Ci/mmol; Amersham Biosciences). To extract inositol phosphates, cells were pelleted at 1000 g for 5 min, resuspended in 100 µl of water, and 400 µl of methanol/HC1 (100:1, v/v) was added. The cells were vortex-mixed for 30 s and 0.8 g of glass beads (Sigma, G-8772) were added and vortex-mixed. The cells were frozen in liquid nitrogen and thawed and this process was repeated five times. Then, 400 µl of methanol/HCl (100:1, v/v) and 2 ml of chloroform were added and vortex-mixed. The mixture was vortex-mixed and spun briefly to separate the phases. The upper aqueous phase was retained and 500 µl of cold 5% (v/v) HClO4 was added and the mixture was incubated on ice for 10 min. After centrifugation at 10000 g for 5 min in a cooled centrifuge, the resulting supernatant was neutralized with 2 M KOH, 50 mM Mes buffer and 10 mM EDTA. The supernatant obtained after centrifugation at 10000 g for 5 min was analysed by HPLC.

HPLC

InsP5s were resolved by HPLC on a 25 cm Partisphere Strong Anion Exchange column (Whatman, Maidstone, Kent, U.K.). [3H]InsP5 (18 Ci/mmol, 740 MBq/mmol) was obtained from NEN Dupont (Stevenage, Herts., U.K.). The column was eluted with a gradient derived from buffers A (water) and B [1.25 M (NH4)2HPO4, adjusted to pH 3.8 with H3PO4] mixed as follows: 0 min, 0% B; 5 min, 0% B; 65 min, 100% B; 75 min, 100% B. Radioactivity was determined on-line with a Canberra Packard (Pangbourne, Berkshire, U.K.) A510 Radiochemical Flo-Detector fitted with a 0.5 ml flow-cell, either by Cerenkov counting or by admixture of Flo-Scint AP (Canberra Packard) scintillation cocktail. Radioactivity was estimated with an integration interval of 12 s.

In situ mRNA hybridization

In situ hybridization of RNA transcripts was performed with modifications to the method of Welham et al. [22]. Formamide (50%, v/v) was added to post-RNAse washed, DIG (digoxigenin)-11-UTP-labelled probes [Boehringer Mannheim (Roche), Lewes, East Sussex, U.K.] were generated from AtIPK1 cDNA in the vector pGEM-T Easy (Promega, Southampton, U.K.). Primers were designed to amplify the region corresponding to residues 880–1080 of the cDNA in pET28α: forward primer, GCTGTCTATGGCTCAGGAG; reverse primer, ATACTCCTTCAATCTTCTT. The PCR product was cloned into pGEM-T Easy and verified by sequencing. Antisense probe was produced by linearizing the resultant pGEM-T Easy plasmid with SacII and transcribing with T7 RNA polymerase. Sense probe was produced by linearizing the plasmid with SacI and transcribing with SP6 RNA polymerase. Sense (control) and antisense probes were tested on all samples analysed.

RESULTS

Identification and cloning of Arabidopsis thaliana InsP5 2-kinases

To identify InsP5 2-kinases from Arabidopsis, we used a bioinformatics based approach. Using a previously identified InsP5 2-kinase from human (GenBank accession no. NP_073592) [10], we searched the TAIR (The Arabidopsis Information Resource) Arabidopsis genome database at www.arabidopsis.org using the WU-BLAST 2.0 program [23]. This identified several candidates for further study with locus identifiers At1G 22100, At1G 58936, At5G 42810, At4G 08145 and At5G 59900. Of these, At5G 42810 and At5G 59900 were successfully amplified from Arabidopsis cDNA and the others were not pursued further at the time.

To clone cDNAs for these loci, primers were designed corresponding to the 5'- and 3'-ends of the predicted coding sequences. RNA was extracted from leaf and root tissue and RT–PCR was performed. To facilitate cloning and further study, restriction sites were added to all four proteins, suggesting that they may have an important functional role (Figure 1).

Pairwise alignments of predicted protein sequences of At5G 42810 with both human and S. cerevisiae InsP5 2-kinases showed only limited conservation overall with translations of At5G 42810 showing 27.4% identity to human InsP5 2-kinase and 17.8% identity to S. cerevisiae InsP5 2-kinase: the human and S. cerevisiae sequences show 10.18% identity. Despite this low level of conservation, there are several residues conserved across all four proteins, suggesting that they may have an important functional role (Figure 1).

Remarkably, the cysteine in ScIPK1 at amino acid position 139, conserved among yeasts and corresponding to Cys58 in previous alignments with human IPK1 [10] and essential for ScIPK1 activity [9], is not present in protein encoded by At5G 42810. Interestingly, the corresponding amino acid in our alignment is a lysine residue.

In addition to the sequence motifs, BOX A and B, identified previously in amino acid position 139, conserved among yeasts and corresponding to Cys162 in previous alignments [10,11] and the endogenous stop codon was replaced with sequence encoding a FLAG tag prior to a stop codon. Products of the predicted size were obtained for both At5G 42810 and At5G 59900 (results not shown) and these were cloned into the bacterial expression vector pET28 and their identities confirmed by sequencing.

In situ mRNA hybridization of RNA transcripts was performed with modifications to the method of Welham et al. [22]. Formamide (50%, v/v) was added to post-RNAse washed, DIG (digoxigenin)-11-UTP-labelled probes [Boehringer Mannheim (Roche), Lewes, East Sussex, U.K.] were generated from AtIPK1 cDNA in the vector pGEM-T Easy (Promega, Southampton, U.K.). Primers were designed to amplify the region corresponding to residues 880–1080 of the cDNA in pET28α: forward primer, GCTGTCTATGGCTCAGGAG; reverse primer, ATACTCCTTCAATCTTCTT. The PCR product was cloned into pGEM-T Easy and verified by sequencing. Antisense probe was produced by linearizing the resultant pGEM-T Easy plasmid with SacII and transcribing with T7 RNA polymerase. Sense probe was produced by linearizing the plasmid with SacI and transcribing with SP6 RNA polymerase. Sense (control) and antisense probes were tested on all samples analysed.
aligns with the original BOX B motif from yeast [9] and we have named this motif BOX B. Additionally, the old human BOX B motif (residues 330–337) LDLLDIEG [10] now aligns with Arabidopsis residues 310–317 LDKLDIEG and we have called this motif BOX E. The yeast kinase shares little homology in this region and appears to lack a BOX E. The new human BOX B sequence shares greater homology with the new yeast BOX B sequence and the Arabidopsis BOX B sequence, than did the original [10]. Thus we propose that the inclusion of significantly disparate sequences [10] caused some uncertainty in the alignment of yeast and human motifs.

Additionally, extension of BOX D to include human InsP5 2-kinase residues 452–458, reveals striking similarity of amino acid sequence YKLD(G/K)KI between AtIPK1, residues 420–426, and human InsP5 2-kinase. Similarly, the BOX A motif VEIKPKCGF is identical in human InsP5 2-kinase, residues 135–143, and AtIPK1, residues 165–173. In general, within BOXES A–D, AtIPK1 and human InsP5 2-kinase are more similar to each other than they are to yeast proteins. Nevertheless, our phylogenetic analysis shows that human InsP5 2-kinase, ScIPK1 and AtIPK1 form a related group, while protein encoded by At5G 59900 is a more divergent member of the family.

Expression analysis of Arabidopsis InsP5 kinases

To determine in which tissues these genes are expressed, RT–PCR analysis was performed. RNA was isolated from Arabidopsis stems, leaves, flowers, siliques and cauline leaves and reverse-transcribed using oligo(dT)<sub>17</sub>. As a control, PCR primers were designed to ubiquitin and cDNA levels were normalized against this product (Figure 2). At5G 42810 transcripts were detected most strongly in cDNA reverse-transcribed from leaf and cauline leaf RNA, but with some signal from siliques. A smeared signal was obtained for flowers though the in situ hybridization conducted subsequently (Figure 3) shows that the transcript is expressed in flowers. At5G 59900 transcripts were detected most strongly in cDNA from stem and flower.

Expression of At5G 42810 transcripts in floral tissues

Additional analysis of the pattern of expression of At5G 42810 transcripts was performed by mRNA in situ hybridization. For this, antisense and sense (control) DIG-labelled probes were generated from At5G 42810 cDNA and cloned into pGEM-T Easy (see the Materials and methods section). Focusing on flower development, we observed mRNA hybridization of the antisense probe to all flower organs (Figure 3). Expression of At5G 42810 transcripts was observed in the major organs of developing flower buds. Thus the sepals and petals were strongly labelled (Figures 3A and 3B). We similarly observed strong staining of the male and female organs of immature and mature flower buds: particularly strong expression was observed in the gynoecium and carpels which are fused to form the gynoecium (Figure 3A). The anthers of stamens were labelled most markedly in tissues
in the bud in male (anthers) and female (ovary) tissues. (B) Transverse section through a mature floral bud. At5G 42810 is expressed throughout the bud in male (anthers) and female (ovary) tissues. (C) In situ mRNA hybridization of sense (control) probe in transverse section through a mature floral flower.

Figure 3  In situ mRNA hybridization analysis of At5G 42810 transcripts

(A) Oblique section through an immature floral bud. At5G 42810 is expressed in sepals, anthers, gynoecium and carpels of gynoecium. (B) Transverse section through a mature floral bud. At5G 42810 is expressed throughout the bud in male (anthers) and female (ovary) tissues. (C) In situ mRNA hybridization of sense (control) probe in transverse section through a mature flower.

Biochemical characterization of A. thaliana InsP$_5$ 2-kinases

To determine the activities of the putative InsP$_5$ kinases, cDNAs were cloned into the bacterial expression vector pET28 in frame with N-terminal His and T7 tags. The C-terminal His tag encoded in this vector was replaced with a FLAG tag. Following induction in the E. coli Rosetta strain with IPTG and purification with Ni-NTA resin, protein expression was confirmed by Western-blot analysis using both anti-T7 and anti-FLAG antibodies (Figure 4). Protein expression was highest from the plasmid encoding AtIPK1 and this protein was selected for further analysis. At5G 59900 expression in E. coli containing pET28 with either AtIPK1 or At5G 59900 after induction with IPTG. Total bacterial protein extract was blotted and detected with antibodies to the N-terminal T7 tag (α-T7) or the C-terminal FLAG tag (α-FLAG). The smaller labelled bands present in both Western blots are likely to be degradation products from the breakdown of the recombinant proteins.

Figure 4  Expression of recombinant InsP kinase proteins

(A) Coomassie Blue-stained gel of protein extracted from E. coli containing pET28 with either AtIPK1 (At5G 42810) or At5G 59900 inserted. Uninduced: total protein from uninduced bacteria; Induced: total protein from bacteria after induction with IPTG; Lysate: soluble protein fraction following lysis of bacteria; Purified: protein after purification with Ni-NTA resin. The estimated molecular mass of At5G 42810 is 50.5 kDa, and that of At5G 59900 is 101.6 kDa. (B) Western blot of total protein extract from E. coli containing pET28 with either AtIPK1 or At5G 59900 after induction with IPTG. Total bacterial protein extract was blotted and detected with antibodies to the N-terminal T7 tag (α-T7) or the C-terminal FLAG tag (α-FLAG). The smaller labelled bands present in both Western blots are likely to be degradation products from the breakdown of the recombinant proteins.

In vitro activity of purified AtIPK1

To determine the substrate range of AtIPK1, kinase assays were performed. Purified protein was incubated with the following substrates: Ins(1,3,4,5,6)$P_5$, a racemic mixture of Ins(1,4,6)$P_5$ and Ins(3,4,6)$P_5$, In$[3^H]$Ins(1,4,6)$P_5$, Ins(3,4,5,6)$P_5$, Ins(1,3,4,6)$P_5$, Ins(4,5)$P_5$, GroPIns(4,5)$P_5$. (glycerophospho-inositol 4,5-bisphosphate), Ins$P_5$ and Ins(1,4,5)$P_3$. Radiolabelled products of the assay were identified by autoradiography following separation on TLC plates: positive results were confirmed with HPLC analysis. No products were observed in the absence of protein (Figure 5A, lane 1). The strongest activity was observed against Ins(1,3,4,5,6)$P_5$ substrate, yielding Ins$P_5$e (Figure 5A, lane 5). Identification of this product as Ins$P_5$e was verified by anion-exchange chromatography on a Partisphere SAX HPLC column eluted with (NH$_4$)$_2$HPO$_4$: a $[^{3}P]$Ins$P_5$e standard eluted at a retention time of 65 min on this column and gradient (inset to Figure 5B). The other radiolabelled peaks in the chromatogram are ATP substrate and Pi. Comparison of Figure 5 with Figure 8(B) reveals our ability to resolve Ins$P_5$e from PP-Ins$P_4$ (diphospho Ins$P_3$), present in yeast, discounting the possibility that AtIPK1 catalyses synthesis of a diphospho Ins$P_5$ from Ins(1,3,4,5,6)$P_5$; diphospho Ins$P_5$ elutes after Ins$P_5$e on Partisphere SAX columns [20]. In addition to the IPK1 activity characterized in the foregoing, we observed weak activity against Ins(1,3,4,6)$P_5$ generating Ins$P_5$e (Figure 5A, lane 6). A low level activity was seen with Ins$P_5$e as a substrate producing a product with the mobility of Ins$P_3$e (Figure 5A, lane 9). This may reflect phosphatase and kinase activities of the protein, as suggested for Ins(3,4,5,6)$P_5$, 1-kinase [24] and ZmIPK, or the presence of some contaminating Ins$P_3$ in the Ins$P_5$e substrate.
Figure 5  Substrate range of AtIPK1 (At5g 42810-encoded protein)
(A) Purified recombinant AtIPK1 was incubated at 30°C with [γ-32P]ATP and the following substrates: no inositol phosphate substrate (−ve), Ins(1,3,4,5,6)P5, an acyclic mixture of Ins(1,4,6)P3 and Ins(3,4,6)P3 [−Ins(1,4,6)P3], Ins(3,4,5,6)P4, Ins(1,3,4,6)P4, Ins(1,3,4,5,6)P5, GroPIns(4,5)P2, InsP6 or Ins(1,4,5)P3. 10 % of total reaction products were spotted on to the bottom of a TLC plate and chromatography was conducted with 1.8 M HCl. Note that products were observed with Ins(1,3,4,5,6)P5, Ins(1,3,4,6)P4 and InsP6. (B) The [32P]-labelled products obtained on incubation of recombinant At5G 42810-encoded protein with Ins(1,3,4,5,6)P5 and [γ-32P]ATP were resolved by HPLC on a Partisphere SAX column. The inset in (B) shows the elution of a [3H]InsP6 standard under identical chromatographic conditions.

Kinetic analysis of purified AtIPK1 protein
Kinetic parameters were estimated for the IPK1 activity of AtIPK1. Reaction products were resolved by TLC and the extent of conversion into InsPs products was determined after exposure of the products to a phosphorimager screen. Reaction times and the amount of enzyme were set to limit the consumption of ATP to < 10%. A plot of reaction velocity against substrate concentration showed apparent Michaelis–Menten kinetics, and a double reciprocal plot of 1/v against 1/S gave a straight line (r² = 0.99) from which Kₘ and Vₘₐₓ were determined (Figure 6). An apparent Kₘ for Ins(1,3,4,5,6)P₅ of 22 µM and a Vₘₐₓ of 35 nmol · min⁻¹ · mg⁻¹ were determined at 0.4 mM ATP. Kinetic analysis was also performed at a lower ATP concentration (0.4 µM), yielding an apparent Kₘ for Ins(1,3,4,5,6)P₅ of 176 µM and Vₘₐₓ of 1.5 nmol · min⁻¹ · mg⁻¹.

In vivo analysis of A. thaliana InsP₅ 2-kinases in S. cerevisiae
To determine if the AtIPK1 protein was able to phosphorylate InsPs in vivo, the AtIPK1 cDNA was cloned into the yeast expression vector pYES2 under the control of a galactose-inducible promoter and transformed into an S. cerevisiae mutant strain, ScIpk1Δ, lacking the Sipk1 open reading frame (strain BWY1228; MATa ipk1::kanMX4). The yeast strain is unable to synthesize InsP₅, the cells accumulate InsP₆ and the diphosphoinositol polyphosphate PP-InsP₆ [20]. These observations are consistent with original observations with yeast mutants [9]. ScIpk1Δ yeast were transformed independently with pYES2 vector and pYES2-AtIPK1 (harbouring AtIPK1) and selected on defined media lacking uracil. Colonies were picked and expression of the AtIPK1 protein product was confirmed by Western-blot analysis (Figure 7A).

To determine if expression of AtIPK1 could rescue the temperature-sensitive growth arrest of ScIpk1Δ, cells were streaked on to plates containing galactose, but lacking uracil and glucose, and grown at 37°C for 3 days (Figure 7B). As expected, untransformed ScIpk1Δ yeast and ScIpk1Δ transformed with the empty vector showed no or limited growth at this temperature, undergoing only a few cell divisions [5]. In contrast, in the presence of pYES2-AtIPK1, numerous larger colonies were observed, showing that the expression of this gene can rescue the growth defect of ScIpk1Δ yeast.

To measure directly the ability of AtIPK1 to rescue InsP₆ synthesis in this yeast strain, liquid cultures were grown in either YPD medium (wild-type and ScIpk1Δ) or synthetic defined medium lacking uracil (pYES2-transformed strains) to an A₆₀₀ of 0.6 and then resuspended in a medium containing galactose and myo-[2-3H]inositol but lacking glucose or supplemental inositol. After 24 h at 30°C, inositol phosphates were extracted and analysed by HPLC on a Partisphere SAX column (Figure 8). As described previously [10,11,20], ScIpk1Δ yeast accumulated InsPs and PP-InsP₄ (Figure 8A). Production of InsPs was evident in wild-type and ScIpk1Δ yeast expressing AtIPK1, indicated by a [3H]-labelled product that eluted with identical retention time to [3H]InsP₆ (compare Figures 8B, 8C and 5B). Similar observations were reported in the original identification of ScIpk1 [5] and subsequently confirmed for orthologues from other yeasts and human [10,11,20]. Our experiments confirm that the ability of the AtIPK1 to rescue the growth phenotype of ScIpk1Δ yeast is associated with a functional IPK1 activity of the gene.

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expression in Scipk1Δ yeast, revealed by Western blotting, and in some instances confirmation of catalytic activity for recombinant protein expressed in E. coli, we did not observe restoration of the InsP profile (results not shown) with Arabidopsis accessions, At4G 08170, At2G 43980, At4G 33770, At4G 59900 and At4G 55030.

The ability of AtIPK1 protein to rescue the growth and InsP profile of Scipk1Δ yeast is intriguing given the low similarity of AtIPK1 to ScIPK1. However, even within yeasts there is remarkably little similarity between IPK1 orthologues: human IPK1 shares less than 20% identity with ScIPK1 overall, yet all these proteins rescue the growth and InsP profiles of Scipk1Δ yeast [10,11,20]. ScIPK1 cDNA, in concert with PLC1 and IPK2 cDNAs, not only complements lesions in mRNA export in a temperature-sensitive glel-2 mutant [5], but also participates in InsP6 synthesis [3–6]. This observation was prosayed by descriptions of hyperosmotic stress-induced increases in InsP6 in Saccharomyces cerevisiae and partial purification of enzyme activity(ies) capable of converting Ins1,4,5P3 into InsP6 [25]. While the subcellular localization of IPK2 in the nucleus of yeast [5], human [26] and plant [8] cells, and of IPK1 in the nucleus and nuclear membrane of yeast cells [3,4], suggests that IPK2 and IPK1 participate in nuclear InsP6 synthesis, it is not clear whether IPK2 and IPK1 orthologues participate in extranuclear InsP6 synthesis also. This question is likely to be of great significance in the context of massive InsP6 accumulation as phytic acid (metal chelates of inositol hexakisphosphate) not only in plants [27], but also in the extracellular hydatid cyst wall of the parasitic cestode Echinococcus granulosus [28]. It is not clear at present whether AtIPK1 specifically contributes to nuclear or cytosolic pathways of InsP6 synthesis.

**DISCUSSION**

Although early studies have indicated that the final step in InsP6 biosynthesis in S. polyrhiza is 2-phosphorylation of Ins(1,3,4,5,6)P5 [13,14], the molecular identities of plant genes encoding enzymes capable of catalysing this reaction have not been established. Nevertheless, since the first description of a cytosolic pathway to InsP6 in Dictyostelium discoideum [29], it has become apparent that IPK1s have a common function in InsP6 synthesis across kingdoms [15]. In yeast, InsP6 2-kinase (IPK1) contributes to a pathway of InsP6 synthesis, which ‘begins’ with lipid-derived Ins(1,4,5)P3. Although this pathway is intimately associated with regulation of mRNA export from the nucleus [3,4,6], it is not clear whether the catalytic reactions are restricted exclusively to the nucleus. Human InsP6 2-kinase was recently shown to complement the lesion in InsP6 production in an S. cerevisiae Scipk1Δ, and to complement the synthetic lethality of the glel-1 ipk1-4 mutant strain [10]. While the subcellular localization of human InsP6 2-kinase remains undefined, it is clear that, despite low overall similarity between human IPK1 and yeast IPK1s, the human enzyme can substitute for yeast protein.

In the present paper, we confirm that AtIPK1 can substitute for ScIPK1 to restore the growth phenotype of an Scipk1Δ strain. Moreover, we show that this rescue is associated with the restoration of the InsP profile of the Scipk1Δ mutant. We have not experimentally verified that our functional complementation rescues mRNA export, but others [3,4] have indicated that Scipk1 mutants are defective in mRNA export. In the context of InsP6, it will be particularly interesting to establish if AtIPK1 contributes to nuclear or cytosolic pathways, or whether it participates in a lipid-derived or exclusively ‘soluble’ pathway [12]. Such an

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**Figure 7** Complementation of temperature-sensitive growth phenotype of an Scipk1Δ strain by expression of AtIPK1

The S. cerevisiae Scipk1Δ strain (BWY1128) was transformed with empty pYES2 plasmid or pYES2 harbouring At5G 42810. (A) Western blot of total yeast protein extracted from Scipk1Δ yeast after induction with galactose: an antibody to the C-terminal FLAG tag was used. Lane 1: Scipk1Δ transformed with empty vector; lane 2: Scipk1Δ transformed with pYES2-AtIPK1. (B) Rescue of the temperature-sensitive growth defect of Scipk1Δ yeast grown at 37°C expressing: left – untransformed Scipk1Δ; top – Scipk1Δ transformed with empty pYES2; middle – Scipk1Δ transformed with pYES2 containing AtIPK1 (At5G 42810). Colonies were grown on a defined medium lacking uracil at 37°C.

**Figure 8** Expression of AtIPK1 restores InsP6 production in an S. cerevisiae Scipk1Δ strain

The Scipk1Δ strain, BWY1128, expressing empty pYES2 plasmid (A), the pYES2 plasmid harbouring At5G 42810 (B), and wild-type (WT) yeast (C), was grown in medium containing [3H]inositol. Inositol phosphates were extracted and resolved by HPLC on a Partisphere SAX column as described in the Materials and methods section. Inositol phosphate peaks are identified with arrows.

A number of other putative inositol phosphate kinases from Arabidopsis were tested for their ability to rescue the growth and InsP profile of Scipk1Δ yeast. Despite successful
The biochemical characterization of inositol (1,3,4,5,6)pentakisphosphate 2-kinase (IPK1) from human (K<sub>M</sub> = 0.4 µM and V<sub>max</sub> = 20 nmol·min<sup>-1</sup>·mg<sup>-1</sup>) has been studied in detail. The enzyme displays a broad substrate specificity and is able to phosphorylate a variety of inositol phosphate substrates, including Ins(1,3,4,5,6)P<sub>5</sub>, though the stereoisomerism of the products was not established. ZmIPK shows homology to the Ins(1,3,4)P<sub>3</sub> 5/6-kinases first identified in humans [33], and also in Arabidopsis [34]. Although the physiological substrates of these enzymes are not clear, the identification of genes encoding enzymes with this catalytic flexibility was paralleled also in Ins(3,4,5)P<sub>3</sub> 6-kinase and Ins(1,3,4,5)P<sub>4</sub> 6-kinase activities in immature soya bean [35].

More recently, the human enzyme was reclassified as dual specificity Ins(1,3,4,5,6)P<sub>5</sub> 6/kinase/Ins(3,4,5,6)P<sub>3</sub> 1-kinase [36] and further studies have established that the human enzyme is also an Ins(1,3,4,5,6)P<sub>5</sub> 1-kinophosphatase [24]. It has been suggested that the maize protein may display phosphatase or phosphomutase activity. The broad in vitro substrate specificity of ZmIPK makes possible a variety of routes to InsP<sub>5</sub> [12]. In the context of InsP<sub>5</sub> synthesis, a number of biochemical studies performed on plant enzyme activities are pertinent. Inositol phosphate kinase activities capable of phosphorylating Ins(3)P<sub>3</sub> (inositol 3-monophosphate) to InsP<sub>3</sub> (stereoisomer undefined) were described in germinating mung bean [37–39]. Moreover, that InsP<sub>5</sub> was produced from Ins2P<sub>2</sub> (inositol 2-monophosphate) substrate suggests perhaps that with InsP<sub>5</sub> substrate the final product is Ins(1,3,4,5,6)P<sub>5</sub>. Similarly, two inositol phosphate kinase activities were described in the duckweed (Lemna gibba) by Bollmann et al. [40]; one converting Ins3P<sub>3</sub> into InsP<sub>5</sub>, and a second converting InsP<sub>5</sub>, into InsP<sub>6</sub>. The existence of these enzyme activities, and the identification of Ins(3,4,5,6)P<sub>4</sub> 1-kinase activity in permeabilized mesophyll protoplasts [41], give a strong indication that pathways, exclusive of lipid-derived Ins(1,4,5)P<sub>3</sub>, i.e. those which add the 3-phosphate before the 1-, 4- and 5-phosphates, operate widely across the plant kingdom.

In the context of IPK1, the biochemical studies of Phillippy et al. [42] are especially pertinent. These authors characterized IPK1 activity from immature soya bean seeds, obtaining kinetic parameters K<sub>M</sub>(IP<sub>6</sub>) = 2.5 µM and V<sub>max</sub>(IP<sub>6</sub>) = 243 nmol·min<sup>-1</sup>·mg<sup>-1</sup> respectively. These results are most similar to the kinetic parameters described for a C-terminal fragment of the S. pombe enzyme (K<sub>M</sub>(IP<sub>6</sub>) = 5.9 µM and V<sub>max</sub>(IP<sub>6</sub>) = 240 nmol·min<sup>-1</sup>·mg<sup>-1</sup>). The parameters derived for AtIPK1 at 0.4 mM ATP (K<sub>M</sub>(IP<sub>6</sub>) = 22 µM, and V<sub>max</sub>(IP<sub>6</sub>) = 35 nmol·min<sup>-1</sup>·mg<sup>-1</sup>) fall between those described for the soya bean activity and those obtained for recombinant IPK1 from human (K<sub>M</sub>(IP<sub>6</sub>) = 0.4 µM and V<sub>max</sub>(IP<sub>6</sub>) = 30 nmol·min<sup>-1</sup>·mg<sup>-1</sup>) and S. cerevisiae (K<sub>M</sub>(IP<sub>6</sub>) = 0.64 µM and V<sub>max</sub>(IP<sub>6</sub>) = 20 nmol·min<sup>-1</sup>·mg<sup>-1</sup>). The higher value for K<sub>M</sub>(IP<sub>6</sub>) (176 µM) obtained for AtIPK1 at low (0.4 µM) ATP concentration may indicate allosteric influence of ATP, affording a mechanism by which the Arabidopsis enzyme could respond to cellular ATP, which is likely to be an important regulator of phytic acid synthesis.

Our identification of differential organ-specific patterns of expression of AtIPK1 and At4G 59900 transcripts may indicate different functions for AtIPK1 and At4G 59900-encoded protein. Though we were unable to demonstrate catalytic activity for At4G 59900 protein, it is possible that At4G 59900 encodes an inositol phosphate kinase. Whether AtIPK1, or At4G 59900 protein, prove to modulate the signalling functions of InsP<sub>n</sub>, as a mobilizer of calcium in stomatal guard cells [43,44], or as a regulator of mRNA export processes like those described in yeast [5], or not, the expression of AtIPK1 in siliques probably indicates a role for IPK1 in seed phytate synthesis. Identification of Arabidopsis gene(s) encoding InsP<sub>n</sub> 2-kinase will enable molecular genetic approaches to the study of inositol phosphate function in this model system.

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