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

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The metabolic pathway(s) by which plants synthesize InsP_6 (inositol 1,2,3,4,5,6-hexakisphosphate) remains largely undefined [Shears (1998) Biochim. Biophys. Acta **1436**, 49–67], while the identities of the genes that encode enzymes catalysing individual steps in these pathways are, with the notable exception of *myo*-inositol phosphate synthase and ZmIpk [Shi, Wang, Wu, Hazebroek, Meeley and Ertl (2003) Plant Physiol. **131**, 507–515], unidentified. A yeast enzyme, ScIPK1, catalyses the synthesis of InsP_6 by 2-phosphorylation of Ins(1,3,4,5,6)P_5 (inositol 1,3,4,5,6-pentakisphosphate). A human orthologue, *HsIPK1*, is able to substitute for yeast *ScIPK1*, restoring InsP_6 production in a *Saccharomyces cerevisiae* mutant strain lacking the *ScIPK1* open reading frame (*ScIpk1*Δ). We have identified an *Arabidopsis* genomic sequence, *AtIPK1*, encoding an Ins(1,3,4,5,6)P_5 2-kinase. Inclusion of the AtIPK1 protein in alignments of amino acid sequences reveals that human and Arabidopis kinases are more similar to each other than to the *S. cerevisiae* enzyme, and further identifies an additional motif. Recombinant AtIPK1 protein expressed in *Escherichia coli* catalysed the synthesis of InsP_6 from Ins(1,3,4,5,6)P_5. The enzyme obeyed Michaelis–Menten kinetics with an apparent V_{max} of 35 nmol $\cdot$ min^{-1} $\cdot$ (mg of protein)$^{-1}$ and a K_m for Ins(1,3,4,5,6)P_5 of 22 μM at 0.4 mM ATP. RT (reverse transcriptase)–PCR analysis of *AtIPK1* transcripts revealed that *AtIPK1* is expressed in siliques, leaves and cauline leaves. *In situ* hybridization experiments further revealed strong expression of *AtIPK1* in male and female organs of flower buds. Expression of *AtIPK1* protein in an *ScIpk1*Δ mutant strain restored InsP_6 production and rescued the temperature-sensitive growth phenotype of the yeast.

Key words: *Arabidopsis*, AtIPK1, biochemical characterization, inositol polyphosphate, kinetic analysis, *Saccharomyces cerevisiae*.

INTRODUCTION

Inositol polyphosphates (Ins*Ps*) 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)P_3 (inositol 1,4,5-trisphosphate), generated from the membrane lipid PtdIns(4,5)P_2, is converted into InsP_6 (inositol 1,2,3,4,5,6-hexakisphosphate). Three genes, *PLC1*, *ScIPK2* and *ScIPK1*, 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 Ins*P* multikinase and an InsP_5 (inositol 1,3,4,5,6-pentakisphosphate) 2-kinase [2–5]. *ScIPK2* is identical with *ARG82* (also known as *ArgRIII*), 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 a minimal medium containing ornithine, a precursor of arginine [8]. Two *ScIPK2* orthologues, *AtIPK2*α and *AtIPK2*β, 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 [*ScIpk2*Δ] and restore wild-type Ins*P* 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 Ins*P* synthesis in plants, though this does not necessarily discount a role for the protein in cytosolic pathways.

ScIPK1 encodes an InsP_5 2-kinase [5]. Yeast strains lacking the *ScIPK1* open reading frame [*ScIpk1*Δ] are deficient in InsP_6 synthesis and accumulate mRNA in the nucleus [5]. IPK1 (InsP_5 2-kinase)-type proteins are not strongly conserved across species, with less than 24 % identity in pairwise combinations across the InsP_5 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 *ScIpk1*Δ strain and restore the Ins*P* profile to wild-type levels [10].

Little is known of the molecular identity of genes encoding enzymes that catalyse defined steps of InsP_6 synthesis in plants. Indeed, despite the recent identification of an insertion mutant in a maize Ins*P* kinase, *ZmIPK*, which reduces grain InsP_6 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)P_3 (inositol 1,3,4-trisphosphate) into Ins(1,3,4,5,6)P_5 as

Abbreviations used: DIG, digoxigenin; GroPIns(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate; Ins3*P*, inositol 3-monophosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5,6)P_5, inositol 1,3,4,5,6-pentakisphosphate; InsP_3, inositol trisphosphate; InsP_4, inositol tetrakisphosphate; InsP_6, inositol 1,2,3,4,5,6-hexakisphosphate; InsP_5, inositol (1,3,4,5,6)-pentakisphosphate; IPK1, Ins(1,3,4,5,6)P_5 2-kinase; IPTG, isopropyl β-D-thiogalactoside; Ni-NTA, Ni^{2+}-nitrilotriacetate; *PP*-InsP_4, diphospho InsP_4; 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)P_3 [12], we have described a soluble pathway in the duckeed *Spirodela polyrhiza* which proceeds from inositol to InsP_6 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_6 synthesis is 2-phosphorylation of the inositol ring [15]. With this consensus in mind, we have cloned an InsP_5 2-kinase from *Arabidopsis*. In the present study, we describe the heterologous expression of *AtIPK1* in *E. coli* and characterization of its activity *in vitro*. We describe kinetic parameters for the His-tagged AtIPK1 protein and show by functional complementation of a mutant *S. cerevisiae ScIpk1Δ* strain that the *AtIPK1* gene rescues the temperature-sensitive growth phenotype and restores the Ins*P* profile of this strain. Moreover, we describe the organ-specific expression pattern of *AtIPK1* transcripts. In characterizing AtIPK1 as an InsP_5 2-kinase, we identify amino acid motifs highly conserved between *Arabidopsis* and human InsP_5 2-kinase.

Since the paper was originally submitted, our attention has been drawn to a recently published paper by Stevenson-Paulik et al. [45], demonstrating that *AtIPK1* encodes an InsP_4 (inositol tetrakisphosphate) and InsP_5 2-kinase.

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. Ins*P* substrates were obtained from Sigma (Poole, Dorset, U.K.).

Bacteria

E. coli strain DH5α (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 *Crepidotus thermophilus* RT (reverse transcriptase) from Roche according to the manufacturer's instructions using a reverse primer specific to the cDNA. PCRs were performed with Pfu polymerase (Stratagene, Amsterdam, The Netherlands) with restriction sites for cloning (underlined) inserted at the 5′-end of the primers. Reverse primers also contained a 5′-sequence encoding a FLAG tag followed by a stop codon.

At5G 42810 was cloned with BamHI and NotI: forward primer, ACGTGGATCCTAATGGAGATGATTTTGGAGGAG; reverse primer, ACGTGCGGCCGCTTATTTGTCATCGTCATCTTTGTAATCGCTGTGGGAAGGTTTTGAGTTG.

At5G 59900 was cloned using EcoRI and NotI: forward primer, ACGTGAATTCATGAAGCTCCCTTGCACGATTC; reverse primer, ACGTGCGGCCGCTTATTTGTCATCGTCATCTTTGTAATCGGATTTAGAACTAGTGTCATTC.

At5G 42810 cDNA was cloned into pET28c and At5G 59900 was cloned into pET28a (Novagen). Both cDNAs were also cloned into pYES2 (Invitrogen).

RT–PCR

Total RNA was extracted from stems, leaves, flowers, siliques and cauline leaves of *Arabidopsis* using TRI reagent following the manufacturer's instructions. Then, 200 ng of RNA was reverse transcribed with *C. thermophilus* RT using oligo(dT)$_{17}$ according to the manufacturer's instructions. The amounts of cDNA template used for subsequent PCR were normalized with respect to ubiquitin product (GenBank® accession no. NM_105209: forward primer, GGCTAAGATCCAGGATAAGG; reverse primer: TCTGGATGTTGTAATCAGCC).

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_2PO_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-NTA (Ni^{2+}-nitrilotriacetate) 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_2PO_4, 300 mM NaCl and 20 mM imidazole (pH 8) to elute non-specifically bound proteins, and washed with 500 μl of NaH_2PO_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 (20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.1 % Tween 20 and 1 % BSA). Horseradish peroxidase-conjugated secondary antibody was used at 1:1000 dilution in TBST. Blots were developed by enhanced chemiluminescence.

Ins*P* 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 dithiothreitol with 1 μCi (37 kBq) [γ-^{32}P]ATP and 20 μM Ins*P* 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 on to POLYGRAM® CEL 300 PEI TLC plates (Macherey-Nagel, Düren, Germany). Plates were developed in 0.5 M HCl to separate InsP_3 (inositol trisphosphate) from unreacted ATP and duplicate plates were run in 1.08 M KH_2PO_4, 0.64 M K_2HPO_4 and 1.84 M HCl to separate InsP_4, InsP_5 and InsP_6. 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 [^{32}P]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. [^{32}P]Ins(1,3,4,5,6)P_5 substrate was prepared with a recombinant plant protein. The

[^{32}P]Ins(1,3,4,5,6)P_5 co-eluted with [^{3}H]Ins(1,3,4,5,6)P_5, but not D/L-Ins(1,2,3,4,5)P_5, D/L-Ins(1,2,3,4,6)P_5 or D/L-Ins(1,2,4,5,6)P_5. The protein content of the assay was 200 ng: the extent of reaction was less than 20 %. TLC plates were developed in 1.08 M KH_2PO_4, 0.64 M K_2HPO_4 and 1.84 M HCl and quantified using a Molecular Dynamics phosphorimager.

Kinetic analysis of AtIPK1 (At5G 42810) was also performed at low (0.4 μM) ATP concentration: reactions were performed for 1 h with Ins(1,3,4,5,6)P_5 substrate at 5, 10, 20, 50 and 100 μM concentration in a final volume of 20 μl. The protein content of the assay was 20 ng: the extent of reaction was less than 10 %. TLC plates were developed in 1.08 M KH_2PO_4, 0.64 M K_2HPO_4, 1.84 M HCl and quantified using a Molecular Dynamics PhosphorImager.

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 Ins*P* 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 Ins*P* 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 A_{600} 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-^{3}H]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/HCl (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 along with 450 μl of water. The mixture was vortex-mixed and spun briefly to separate the phases. The upper aqueous phase was retained and 500 μl of cold 5 % (w/v) $HClO_4$ 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

Ins*P*s were resolved by HPLC on a 25 cm Partisphere Strong Anion Exchange column (Whatman, Maidstone, Kent, U.K.). [^{3}H]InsP_6 [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 $(NH_4)_2HPO_4$ adjusted to pH 3.8 with H_3PO_4] 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 admixure 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 washes. DIG (digoxigenin)-11-rUTP-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 pET28c: forward primer, GCTGTCTATGGCTCAGGAG; reverse primer, ATACTCCTTC-ACAATCTTC. 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* InsP_5 2-kinases

To identify InsP_5 2-kinases from *Arabidopsis*, we used a bioinformatics based approach. Using a previously identified InsP_5 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 incorporated at the 5′- and 3′-ends and the endogenous stop codons were 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.

Pairwise alignments of predicted protein sequences of At5G 42810 with both human and *S. cerevisiae* InsP_5 2-kinases showed only limited conservation overall with translations of At5G 42810 showing 27.4 % identity to human InsP_5 2-kinase and 17.8 % identity to *S. cerevisiae* InsP_5 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 Cys^{162} in previous alignments with human IPK1 [10] and essential for ScIPK1 activity [9], is not present in protein encoded by At5G 42810. In At5G 42810, the corresponding amino acid in our alignment is a lysine residue.

In addition to the sequence motifs, BOX A and B, identified previously in yeast IPK1s [9], and BOX C and D, identified when human InsP_5 2-kinase was included [10], our comparison of AtIPK1 with *S. cerevisiae* and human IPK1s reveals strong conservation of residues in additional motifs and some differences in comparison with previous alignments [10,11].

Thus the AtIPK1 sequence TAKDCSIMI, residues 365–373, is identical with residues 399–407 in human InsP_5 2-kinase and

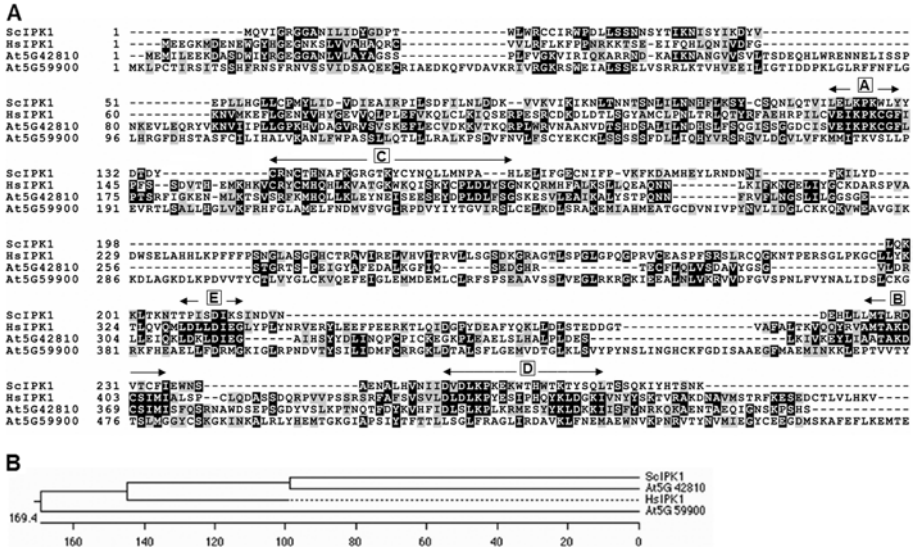

Figure 1 Comparison of IPK1s

(**A**) Clustal W alignments of the amino acid sequences of human (HsIPK1), yeast (ScIPK1) and Arabidopsis (At5G 42810, AtIPK1) IPK1s and Arabidopsis At5G 59900-encoded protein. Residues that are identical and shared by half the sequences are shaded in black. Residues which are similar and shared by half the sequences are shaded in grey. Boxed letters A–E indicate the motifs conserved between human IPK1 and AtIPK1. At5G 59900 sequence is truncated to 570 amino acids since there is no apparent homology with the other sequences beyond this point. BOXES A–D have been previously identified [10,11]. The AtIPK1 sequence TAKDCSIMI, residues 365–373, is identical with residues 399–407 in human InsP_5 2-kinase and aligns with yeast BOX B sequence and these residues have been identified as the Arabidopsis and reassigned human BOX B motifs. The old human BOX B motif (residues 330–337) LDLLDIEG [10] now aligns with Arabidopsis residues 310–317 LDKLDIEG and we have called these motifs BOX E, a motif the yeast kinase appears to lack. (**B**) Phylogenetic tree of HsIPK1, ScIPK1, AtIPK1 (At5G 42810) and At5G 59900-encoded protein.

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 InsP_5 2-kinase residues 452–458, reveals striking similarity of amino acid sequence YKLD(G/K)KI between AtIPK1, residues 420–426, and human InsP_5 2-kinase. Similarly, the BOX A motif VEIKPKCGF is identical in human InsP_5 2-kinase, residues 135–143, and AtIPK1, residues 165–173. In general, within BOXES A–D, AtIPK1 and human InsP_5 2-kinase are more similar to each other than they are to yeast proteins. Nevertheless, our phylogenetic analysis shows that human InsP_5 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 Arabidopis InsP_5 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)$_{17}$. 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

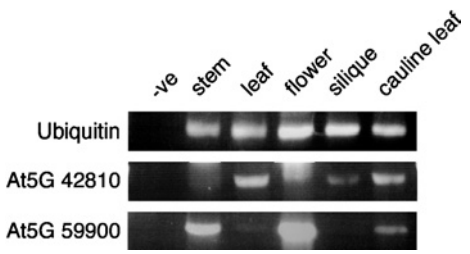

Figure 2 RT–PCR analysis of IPK1 expression

RT–PCR of cDNA derived from *A. thaliana* stem, leaf, flower, silique and cauline leaf with primers specific to ubiquitin (top panel), *AtIPK1* (middle panel) or *At5G 59900* (bottom panel). The lane titled '– ve' is a negative control PCR reaction without template added. The products for At5G 42810 'stem' and 'leaf' samples were masked by the presence of a larger, smeared band.

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

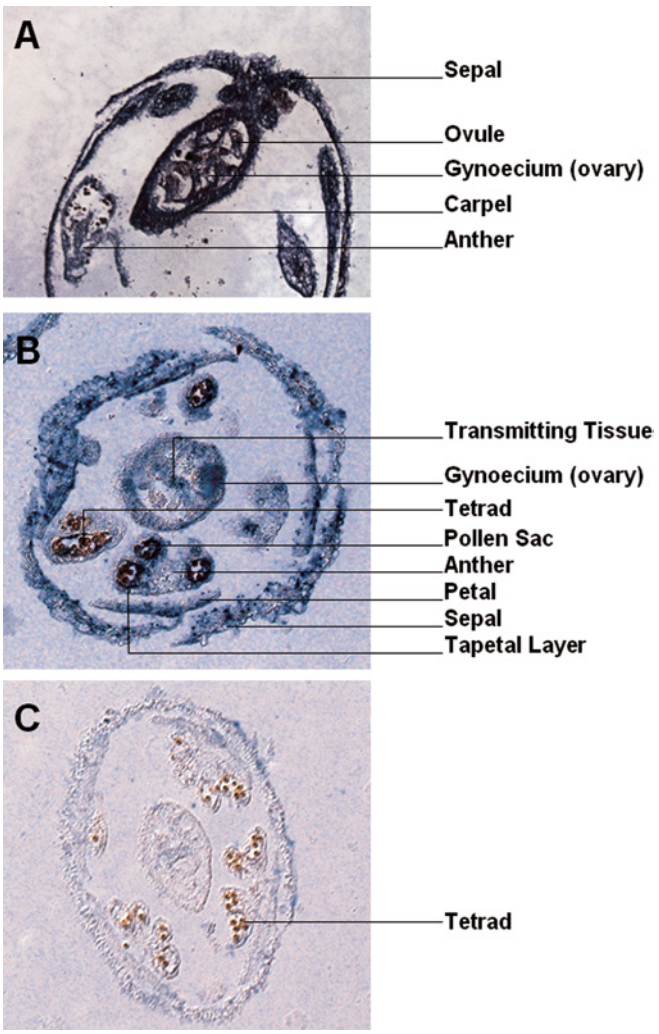

Figure 3 ***In situ*** **mRNA hybridization analysis of At5G 42810 transcripts**

(**A**) Oblique section through an inmmature 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.

surrounding the pollen sac, with the tapetal layer strongly stained (Figure 3B). Individual tetrads can be observed within pollen sacs. The transmitting tissue (Figure 3B) and ovules (Figure 3A) were labelled also. Significantly, the use of sense probes did not result in signals greater than the background (Figure 3C).

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* Rosetta strains yielded little protein, and we were unable to detect enzyme activity for the recombinant protein. In both Western blots, what we assume to be degradation products are visible as smaller bands below the recombinant proteins.

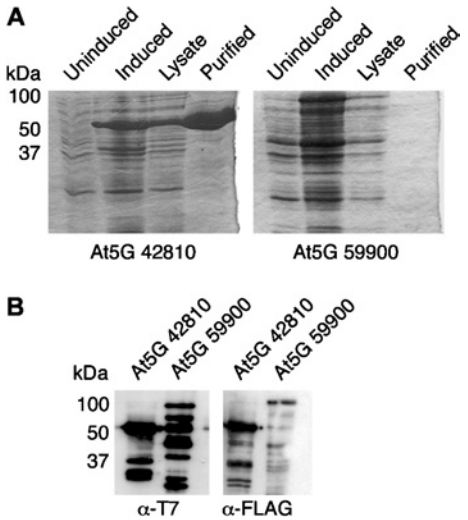

Figure 4 **Expression of recombinant Ins*P* kinase proteins**

(**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)P_3, a racemic mixture of Ins(1,4,6)P_3 and Ins(3,4,6,)P_3 [L-Ins(1,4,6)P_3], Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5, Ins(1,3,4,6)P_4, Ins(4,5)P_2, GroPIns(4,5)P_2 (glycerophosphoinositol 4,5-bisphosphate), InsP_6 and Ins(1,4,5)P_3. ^{32}P-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 InsP_6 (Figure 5A, lane 5). Identification of this product as InsP_6 was verified by anion-exchange chromatography on a Partisphere SAX HPLC column eluted with $(NH_4)_2HPO_4$: a [^{3}H]InsP_6 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 P_i. Comparison of Figure 5 with Figure 8(B) reveals our ability to resolve InsP_6 from *PP*-InsP_4 (diphospho InsP_4), present in yeast, discounting the possibility that AtIPK1 catalyses synthesis of a diphospho Ins*P* from Ins(1,3,4,5,6)P_5: diphospho InsP_5 elutes after InsP_6 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_4 generating InsP_5 (Figure 5A, lane 6). A low level activity was seen with InsP_6 as a substrate producing a product with the mobility of InsP_6 (Figure 5A, lane 9). This may reflect phosphatase and kinase activities of the protein, as suggested for Ins(3,4,5,6)P_4 1-kinase [24] and ZmIPK, or the presence of some contaminating InsP_5 in the InsP_6 substrate.

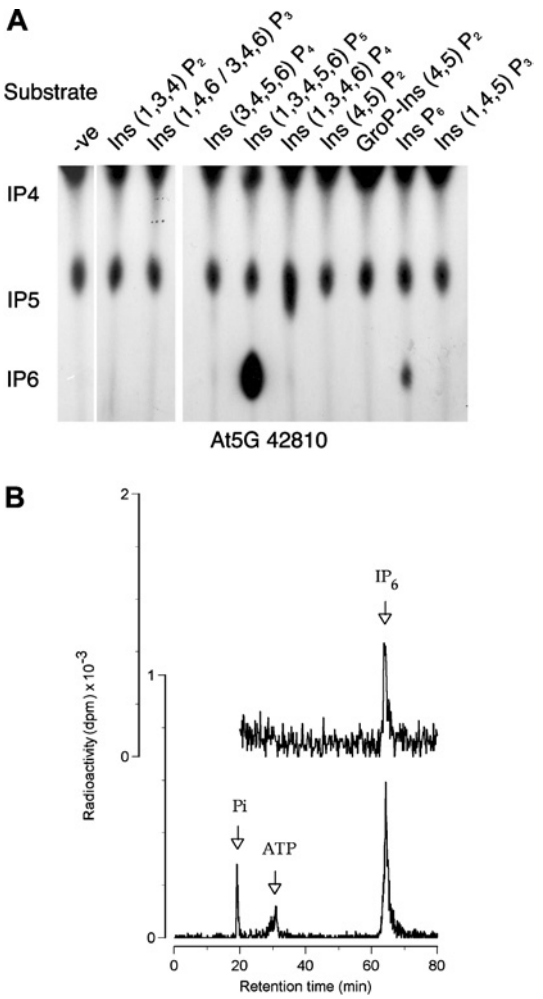

Figure 5 Substrate range of AtIPK1 (At5g 42810-encoded protein)

(**A**) Purified recombinant AtIPK1 was incubated at 30 °C with [γ-^{32}P]ATP and the following substrates: no inositol phosphate substrate (− ve), Ins(1,3,4)P_3, a racemic mixture of Ins(1,4,6)P_3 and Ins(3,4,6,)P_3 [L-Ins(1,4,6)P_3], Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5, Ins(1,3,4,6)P_4, Ins(4,5)P_2, GroPIns(4,5)P_2, InsP_6 or Ins(1,4,5)P_3. 10 % of total reaction products were spotted on to the bottom of a TLC plate and chromatography was conducted with 1.84 M HCl. Note that products were observed with Ins(1,3,4,5,6)P_5, Ins(1,3,4,6)P_4 and InsP_6. (**B**) The ^{32}P-labelled products obtained on incubation of recombinant At5G 42810-encoded protein with Ins(1,3,4,5,6)P_5 and [γ-^{32}P]ATP were resolved by HPLC on a Partisphere SAX column. The inset in (**B**) shows the elution of a [^{3}H]InsP_6 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 InsP_6 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 ($r^2 = 0.99$) from which K_m and V_{max} were determined (Figure 6). An apparent K_m for Ins(1,3,4,5,6)P_5 of 22 μM and a V_{max} of 35 nmol $\cdot$ min^{-1} $\cdot$ 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 K_m for Ins(1,3,4,5,6)P_5 of 176 μM and V_{max} of 1.5 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}.

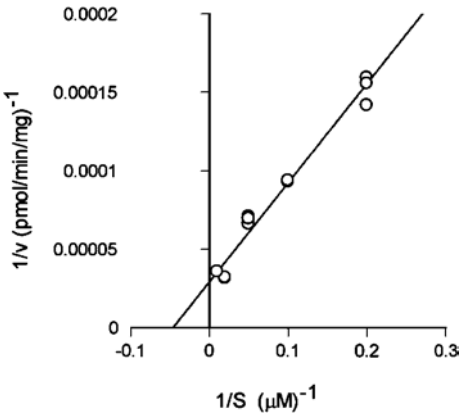

Figure 6 Kinetic analysis of AtIPK1

Purified recombinant His-tagged AtIPK1 was assayed at 0.4 mM ATP concentration and various Ins(1,3,4,5,6)P_5 concentrations. A Lineweaver–Burk transformation of the data is shown, $1/v$ in (pmol $\cdot$ min^{-1} $\cdot$ mg^{-1})$^{-1}$ and $1/S$ in μM^{-1}.

In vivo analysis of *A. thaliana* InsP_5 2-kinases in *S. cerevisiae*

To determine if the AtIPK1 protein was able to phosphorylate InsP_5 *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, *ScIpk1*Δ, lacking the *ScIPK1* open reading frame (strain BWY1228; MATa ipk1::kanMX4). The yeast strain is unable to synthesize InsP_6: the cells accumulate InsP_5 and the diphosphoinositol polyphosphate *PP*-InsP_4 [20]. These observations are consistent with original observations with yeast mutants [9]. *ScIpk1*Δ 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 *ScIpk1*Δ, 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 *ScIpk1*Δ yeast and *ScIpk1*Δ 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 *ScIpk1*Δ yeast.

To measure directly the ability of AtIPK1 to rescue InsP_6 synthesis in this yeast strain, liquid cultures were grown in either YPD medium (wild-type and *ScIpk1*Δ) or synthetic defined medium lacking uracil (pYES2-transformed strains) to an A_{600} of 0.6 and then resuspended in a medium containing galactose and *myo*-[2-^{3}H]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], *ScIpk1*Δ yeast accumulated InsP_5 and *PP*-InsP_4 (Figure 8A). Production of InsP_6 was evident in wild-type and *ScIpk1*Δ yeast expressing *AtIPK1*, indicated by a ^{3}H-labelled product that eluted with identical retention time to [^{3}H]InsP_6 (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 *ScIpk1*Δ yeast is associated with a functional IPK1 activity of the gene

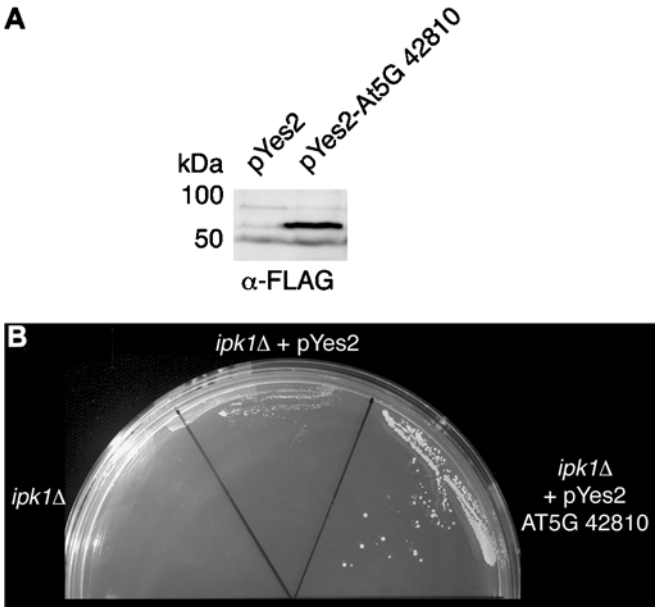

Figure 7 Complementation of temperature-sensitive growth phenotype of an *Sclpk1 Δ* strain by expression of *AtIPK1*

The *S. cerevisiae Sclpk1Δ* strain (BWY1228) was transformed with empty pYES2 plasmid or pYES2 harbouring At5G 42810. (**A**) Western blot of total yeast protein extracted from *Sclpk1Δ* yeast after induction with galactose: an antibody to the C-terminal FLAG tag was used. Lane 1: *Sclpk1Δ* transformed with empty vector; lane 2: *Sclpk1Δ* transformed with pYES2-*AtIPK1*. (**B**) Rescue of the temperature-sensitive growth defect of *Sclpk1Δ* yeast grown at 37°C expressing: left – untransformed *Sclpk1Δ*; top – *Sclpk1Δ* transformed with empty pYES2.1 vector; right – *Sclpk1Δ* transformed with pYES2.1 vector containing *AtIPK1* (At5G 42810). Colonies were grown on a defined medium lacking uracil at 37°C.

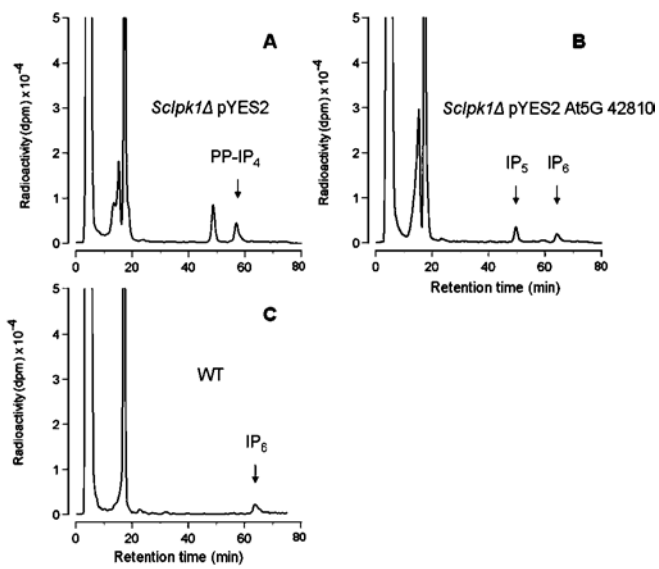

Figure 8 Expression of AtIPK1 restores InsP_6 production in an *S. cerevisiae Sclpk1Δ* strain

The *Sclpk1Δ* strain, BWY1228, expressing empty pYES2 plasmid (**A**), the pYES2 plasmid harbouring At5G 42810 (**B**), and wild-type (WT) yeast (**C**), was grown in medium containing [^{3}H]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.

product. A number of other putative inositol phosphate kinases from *Arabidopsis* were tested for their ability to rescue the growth and Ins*P* profile of *ScIpk1Δ* yeast. Despite successful 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 Ins*P* 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 Ins*P* 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 Ins*P* 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 *gle1-2* mutant [5], but also participates in InsP_6 synthesis [3–6]. This observation was presaged by descriptions of hyperosmotic stress-induced increases in InsP_6 in *Saccharomyces pombe* and partial purification of enzyme activity(ies) capable of converting Ins(1,4,5)P_3 into InsP_6 [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 InsP_6 synthesis, it is not clear whether IPK2 and IPK1 orthologues participate in extranuclear InsP_6 synthesis also. This question is likely to be of great significance in the context of massive InsP_6 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 InsP_6 synthesis.

DISCUSSION

Although early studies have indicated that the final step in InsP_6 biosynthesis in *S. polyrhiza* is 2-phosphorylation of Ins(1,3,4,5,6)P_5 [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 InsP_6 in *Dictyostelium discoideum* [29], it has become apparent that IPK1s have a common function in InsP_6 synthesis across kingdoms [15]. In yeast, InsP_5 2-kinase (IPK1) contributes to a pathway of InsP_6 synthesis, which 'begins' with lipid-derived Ins(1,4,5)P_3. 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 InsP_5 2-kinase was recently shown to complement the lesion in InsP_6 production in an *S. cerevisiae ScIpk1Δ*, and to complement the synthetic lethality of the *gle1-2 ipk1-4* mutant strain [10]. While the subcellular localization of human InsP_5 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 Ins*P* 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 InsP_6, 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

analysis relies heavily on an understanding of the profile of inositol phosphates present in the two cellular compartments. At present, our knowledge of inositol phosphates in plants is limited to a few examples including *S. polyrhiza* [13,14], barley aleurone [30,31] and maize [32]. It is noteworthy that analysis of the short-term non-equilibrium labelling approach of the former study indicated that phosphate addition to the 1-position of the inositol ring occurred after addition of the 3-phosphate. This observation precludes a lipid-derived route of InsP_6 synthesis in the duckweed *Spirodela*. At present, there is considerable conjecture regarding earlier steps of InsP_6 synthesis in plants [12]. A recent report described *Mutator* (transposable element) insertions in an inositol phosphate multikinase from maize, *ZmIPK* [11]. These were responsible for 30 % reduction in seed phytate. *ZmIpk* loss-of-function mutants are allelic to the low-phytic acid mutant *lpa2* [32]. ZmIPK protein showed *in vitro* activity against a number of inositol phosphate substrates including Ins(1,3,4)P_3, a racemic mixture of D/L-Ins(1,4,6)P_3, Ins(3,5,6)P_3 and Ins(3,4,5,6)P_4, though the stereoisomerism of the products was not established. ZmIPK shows homology to the Ins(1,3,4)P_3 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 by the biochemical characterization of Ins(1,3,4)P_3 5-kinase and Ins(1,3,4,5)P_4 6-kinase activities in immature soya bean [35].

More recently, the human enzyme was reclassified as dual specificity Ins(1,3,4)P_3 5/6-kinase/Ins(3,4,5,6)P_4 1-kinase [36] and further studies have established that the human enzyme is also an Ins(1,3,4,5,6)P_5 1-phosphatase [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_6 [12]. In the context of InsP_6 synthesis, a number of biochemical studies performed on plant enzyme activities are pertinent. Inositol phosphate kinase activities capable of phosphorylating Ins3*P* (inositol 3-monophosphate) to InsP_5 (stereoisomer undefined) were described in germinating mung bean [37–39]. Moreover, that InsP_6 was produced from Ins2*P* (inositol 2-monophosphate) substrate suggests perhaps that with Ins3*P* substrate the final product is Ins(1,3,4,5,6)P_5. Similarly, two inositol phosphate kinase activities were described in the duckweed (*Lemna gibba*) by Bollmann et al. [40]: one converting Ins3*P* into InsP_3, and a second converting InsP_3 into InsP_6. The existence of these enzyme activities, and the identification of Ins(3,4,5,6)P_4 1-kinase activity in permeabilized mesophyll protoplasts [41], give a strong indication that pathways, exclusive of lipid-derived Ins(1,4,5)P_3, 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 $K_{m\,(IP5)} = 2.5\ \mu M$ and $V_{max\,(IP5)} = 243\ nmol \cdot min^{-1} \cdot mg^{-1}$ respectively. These results are most similar to the kinetic parameters described for a C-terminal fragment of the *S. pombe* enzyme ($K_{m\,(IP5)} = 5.9\ \mu M$ and $V_{max\,(IP5)} = 240\ nmol \cdot min^{-1} \cdot mg^{-1}$). The parameters derived for AtIPK1 at 0.4 mM ATP ($K_{m\,(IP5)} = 22\ \mu M$, and $V_{max\,(IP5)} = 35\ nmol \cdot min^{-1} \cdot mg^{-1}$) fall between those described for the soya bean activity and those obtained for recombinant IPK1 from human ($K_{m\,(IP5)} = 0.4\ \mu M$ and $V_{max\,(IP5)} = 30\ nmol \cdot min^{-1} \cdot mg^{-1}$) and *S. cerevisiae* ($K_{m\,(IP5)} = 0.64\ \mu M$ and $V_{max\,(IP5)} = 20\ nmol \cdot min^{-1} \cdot mg^{-1}$). The higher value for $K_{m\,(IP5)}$ (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_6, 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_5 2-kinase will enable molecular genetic approaches to the study of inositol phosphate function in this model system.

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