A *Solanum tuberosum* inositol phosphate kinase (StITPK1) displaying inositol phosphate–inositol phosphate and inositol phosphate–ADP phosphotransferase activities

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Abstract We describe a multifunctional inositol polyphosphate kinase/phosphotransferase from *Solanum tuberosum*, StITPKα (GenBank accession: EF362784), hereafter called StITPK1. StITPK1 displays inositol 3,4,5,6-tetrakisphosphate 1-kinase activity: $K_m = 27\ \mu M$, and $V_{max} = 19\ nmol\ min^{-1}\ mg^{-1}$. The enzyme displays inositol 1,3,4,5,6-pentakisphosphate 1-phosphatase activity in the absence of a nucleotide acceptor and inositol 1,3,4,5,6-pentakisphosphate–ADP phosphotransferase activity in the presence of physiological concentrations of ADP. Additionally, StITPK1 shows inositol phosphate-inositol phosphate phosphotransferase activity. Homology modelling provides a structural rationale of the catalytic abilities of StITPK1. Inter-substrate transfer of phosphate groups between inositol phosphates is an evolutionarily conserved function of enzymes of this class.
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Keywords: ATP-grasp fold; Inositol polyphosphate kinase; Phosphotransferase; *Solanum tuberosum*

1. Introduction

Recent characterization of human inositol tris/tetrakisphosphate kinase, hITPK1, an ATP-grasp fold protein, revealed an inositol phosphate–ADP phosphotransferase activity and an ADP-dependent inter-substrate inositol phosphate phosphotransferase activity that has been proposed [1,2] to represent an evolutionary point of distinction between animal enzymes and their plant [3–6] and protozoan counterparts [7].

While the physiological substrates and the catalytic mechanisms of hITPK [1] and a protozoan homolog [7,8] have been extensively studied, and for hITPK are well placed in an extensive biochemical literature [9–11], comparatively little is known of the plant enzymes. We have undertaken an in vitro analysis of the catalytic flexibility of a homolog from *Solanum tubero-*

sum (StITPK1), affording description of a range of phosphotransferase activities not previously elaborated for plant enzymes.

2. Materials and methods

2.1. Reagents

A potato tuber cDNA λZAP-based phagemid library (Stratagene) was supplied by Dr. J. Lloyd (Max Planck Institute for Molecular Plant Physiology, Gölm, Germany). Oligonucleotide primers were obtained from Sigma-Genosys (Haverhill, UK) and restriction enzymes from Invitrogen (Paisley, UK), Roche (Sussex, UK), New England Biolabs (Hitchin, UK) or Promega (Southampton, UK). Bacterial strains were obtained from Novagen (Nottingham, UK). Unlabelled inositol phosphates were obtained from Sigma–Aldrich and SiChem (Germany). [32P]ATP was obtained from Amersham.

2.2. Cloning

A fragment of StITPK1 was cloned from a tuber cDNA library by PCR using primers (5′- TCCTCCAAAACCCTAACCCT-3′ and 5′-TTTGTGCGACTTTGCACTTC-3′) designed from an EST sequence (SGN-E405384) identified in the Solanum Genomic Network's EST database (http://www.sgn.cornell.edu) by homology to AtITPK1 [3]. A labelled StITPK1 PCR fragment, labelled using an Amersham Ready-to-go DNA Labelling Kit and 32P dCTP, was used to screen the library in competent XL1-Blue *Escherichia coli* cells. Plaque lifts were performed (according to the Amersham Hy-Bond protocol). Positive plaques were confirmed by PCR using the original primers. A full length *StITPK1* cDNA was obtained with some sequence upstream of the predicted start codon, and a poly-A tail.

The cDNA was sub-cloned into the vector pET28a using mutagenic primers to add Nco1 and Xho1 restriction sites (5′-GAC-TCCATGGATGG-AAATGGCGGAGCCGA-3′ and 5′-CTGACTC-GAGCACGTGAATAGGGTTCTC-3′), to yield a construct in which StITPK1 is fused to a C-terminal six histidine tag.

2.3. Expression of protein

Expression of StITPK1-His in *E. coli* rosetta cells was induced overnight at 25 °C with 1 mM IPTG. Protein was purified by batch-elution from Ni-NTA affinity resin, and analysed by SDS–PAGE gel and Western Blot using an anti-His antibody after [12].

2.4. Enzyme assays

Kinase assays were conducted after [12], with ATP at either 0.4 μM or 0.4 mM concentration. For phosphatase assays, reactions contained: 20 mM HEPES, pH 7.5, 6 mM MgCl₂, 0.4 mM ADP, 1 mM DTT, approximately 10000 dpm of Ins(1[32P],3,4,5,6)P₅ (prepared using recombinant StITPK1-His), 100 μM unlabelled Ins(1,3,4,5,6)P₅

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                                                    3
ZmIPK    : MASDAAAEPSSGVTHPPRYVIGYALAPKKQQSFIQPSLVAQAASRGMDLVPVDASQPLAE : 60
OsIPK    : MAGD---EPLPGDGQRRRYLIGYALAPKKQQSFIQPSLVSRAAGRGMDLVPVDPSRPLPE : 57
AtITPK1  : ----------MSDSIQERYLVGYALAAKKQHSFIQPSLIEHSRQRGIDLVKLDPTKSLLE : 50
StITPK1  : ---------MKMAEPMRRYSVGYALAPKKQASFIQVSLVNLAKERGIDLIKIDTDKPLID : 51
MtITPK1  : -------MKLNGEEEEEKVVVGYALTSKKKKSFLKPNFIALARNKGIFFVAIDLNKPMLE : 53
HsITPK1  : -----------MQTFLKGKRVGYWLSEKKIKKLNFQAFAELCRKRGMEVVQLNLSRPIEE : 49
EhITPK1  : -----------MTTKQTVSLFIWLPESKQKTLFISTKNHTQFELNNIIFDVTLSTELPD : 48

             4                                                            A
             3
ZmIPK    : QGPFHLLIHKLY-----------GDDWRAQLVAFAARHPAVPIVDPPHAIDRLHNRISM : 108
OsIPK    : QGPFHLLIHKLY-----------GEEWRGQLDAFSAAHPAVPVVDPPHAIDRLHNRISM : 105
AtITPK1  : QGKLDCIIHKLY-----------DVYWKENLHEFREKCPGVPVIDLPEAIERLHNRVSM : 98
StITPK1  : QGPFDCVLHKMD-----------GDDWKRQLKEYGSEFPQALIIDSPEAIERLHNRISM : 99
MtITPK1  : QGPFDVVLHKLP-----------GKEWREILEDYRHKHPEVTILDPPDAIQHLLNRQSM : 101
HsITPK1  : QGPLDVIIHKLTDVILEADQNDSQSLELVHRFQEYIDAHPETIVLDPLPAIRTLLDRSKS : 109
EhITPK1  : KEPNAIITKRTHP----------VGKMADEMRKYEKDHPKVLFLESSAIHDMMSSREEI : 97

                                                       H  A      44
ZmIPK    : LQVVSELDH--AADQDSTFGIPSQVVVYDAAALADF--GLLAA-LRFPLIAKPLVADGTA : 163
OsIPK    : LQVVSELDVPLHAHHHHTFGIPSQVVVYDAAALSDS--GLLAA-LRFPLIAKPLVADGTA : 162
AtITPK1  : LEVITQLRFP--VSDSERFGVPEQVVVMDSSVLSGG--GALGE-LKFPVIAKPLDADGSA : 153
StITPK1  : LQAVGEVEID---CENASFGIPKQTVIYDAKMVSAI--NLENEGLEFPVIAKPLVADGSA : 154
MtITPK1  : LQNVAELNLS---DCHGKVGPQQLVITKNASASTIPYEVTKAGMKLPLVAKPLVVDGSA : 158
HsITPK1  : YELIRKIEAY---MEDDRICSPPFMELTSLCGDDTMR-LLEKNGLTFPFICKTRVAHG-T : 164
EhITPK1  : NALLIKNNIP----------IPNSFSVKSKEEVIQL---LQSKQLIILPFIVKPENAQGTF : 144

              4                          A HH       6           1  H
ZmIPK    : KSHKMSLVYHREGLGKLRPPLVLQEFVNHGGVIFKVYVVGGHVTCVKRRSLPDVSPE--- : 220
OsIPK    : KSHKMSLVYHREGLRKLRPPLVLQEFVNHGGVIFKVYVVGAHVTCVKRRSLPDVSSDVL- : 221
AtITPK1  : KSHKMFLIYDQEGMKILKAPIVLQEFVNHGGVIFKVYVVGDHVQCVKRRSLPDISEE--- : 210
StITPK1  : KSHKMLLVFNKDGLRKLKPPIVLQEFVNHGAVIFKVYVVGDYVKCVKRKSLPDVKED--- : 211
MtITPK1  : KSHELCIAYDELSLLKLEPPLVLQEFVNHGGLLFKIYIVGETIKVVRRFSLPNVGKH--- : 215
HsITPK1  : NSHEMAIVFNQEGLNAIQPPCVVQNFINHNAVLYKVFVVGESYTVVQRPSLKNFSAG--- : 221
EhITPK1  : NAHQMKIVLEQEGIDDIHFPCLCQHYINHNNKIVKVFCIGNTLKWQTRTSLPNVHRCGIK : 204

ZmIPK    : ----DDASAQGSVSFSQ--VSNLPT-ERTAEEYYGEKSLEDAVV--PPAAFINQIAGGLR : 271
OsIPK    : ----QDASAEGSLSFSQ--VSNLPN-ERTAQEYYDDMRLEDAIM--PPTAFINDIAAALR : 272
AtITPK1  : ----KIGTSKGSLPFSQ--ISNLTAQEDKNIEYGEDRSLEKVEM--PPLSFLTDLAKAMR : 262
StITPK1  : ----GLGRLESYLPFSQ--VSNLNNFEKNDDKYYKLMNLENAEY--PPLSFLTNIARGLR : 263
MtITPK1  : ----ELLDVDGLFREPR--VSCAAASADEAD-----LDPNIAEH--PPKPLLERLARDLR : 262
HsITPK1  : -------TSDRESIFFN--SHNVSKPESSSVLTELDKIEGVFER--PSDEVIRELSRALR : 270
EhITPK1  : SVDFNNQHLEDILSWPEGVIDKQDIIENSANRFGSKILEDPILLNLTSEAEMRDLAYKVR : 264

                M H             HM M    1
ZmIPK    : RALGLQLFNFDMIRDVRAGDRYLVIDINYFPG-YAKMPGYETVLTDFFWEMVHKDGVGNQ : 330
OsIPK    : RALGLHLFNFDMIRDARAGDRYLVIDINYFPG-YAKMPGYETVLTDFFWEMVHKD-DDTP : 330
AtITPK1  : ESMGLNLFNFDVIRDAKDANRYLIIDINYFPG-YAKMPSYEPVLTEFFWDMVTKK----- : 316
StITPK1  : RVTKLHLFNFDVIRDDRVGNRYLIIDINYFPG-FAKMPNYERVLTDFFWDVLNQNDKSLG : 322
MtITPK1  : RRLGLHLFNIDMIREHGTKDVFYVIDINYFPGKLGRSDRLDREPVVYPVRAVYQIVHTIE : 322
HsITPK1  : QALGVSLFGIDIIINNQTG-QHAVIDINAFPG-YEGVSEFFTDLLNHIATVLQGQSTAMA : 328
EhITPK1  : CALGVQLCGIDFIKENEQG-NPLVVDVNVFPS-YGGKVDFDWFVEKVALCYTEVAKI--- : 319
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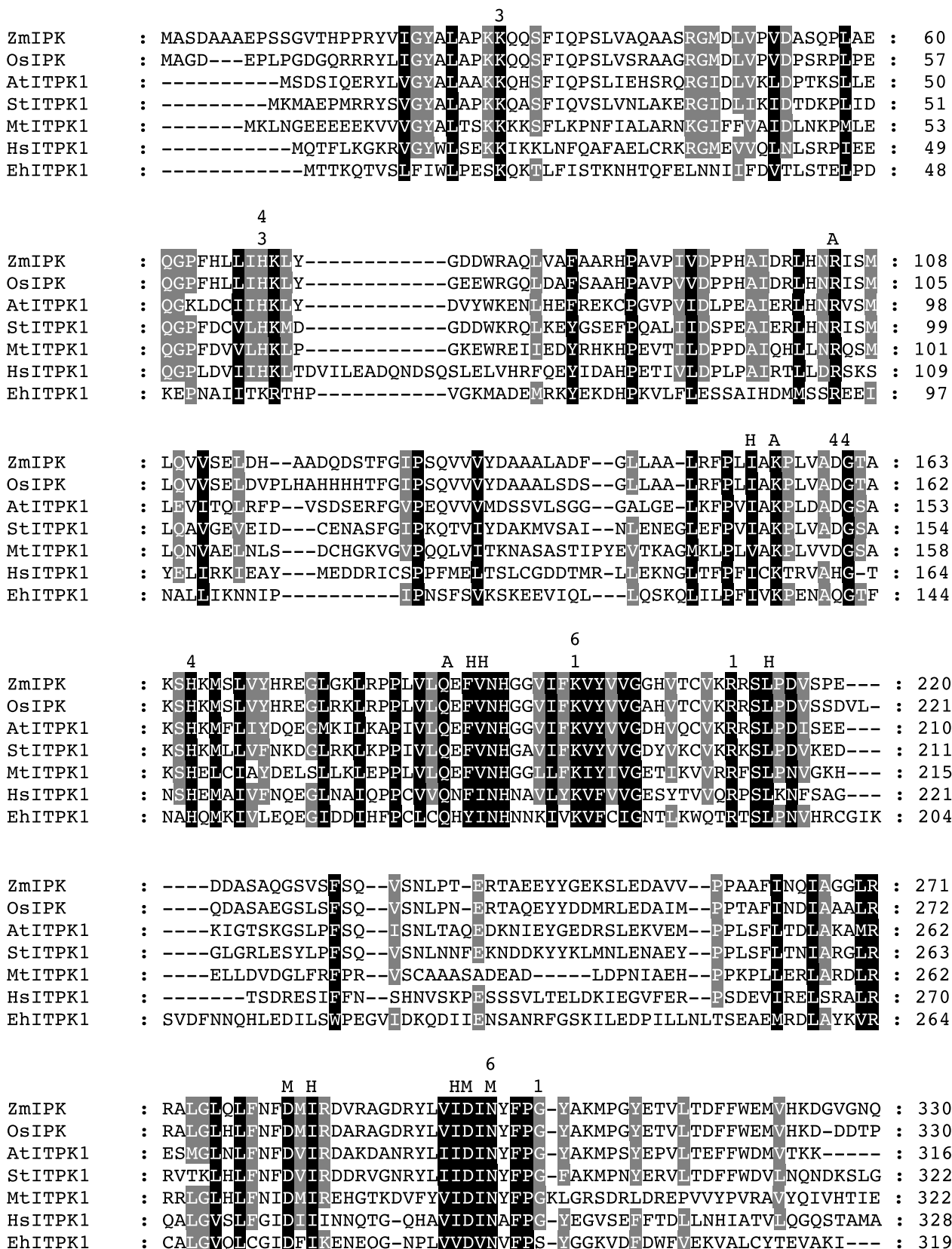
Fig. 1. Alignment (CLUSTAL W) of full length sequence of *Solanum tuberosum* protein accession EF362784, StITPK1; *Medicago truncatula* protein accession ABE89774, MtITPK1; *Arabidopsis thaliana*, protein accession AAC28859, AtITPK1; *Zea mays* protein accession AAO17299, ZmIPK; *Oryza sativa* protein accession AM410634, OsIPK; *Homo sapiens* protein accession NP_055031, HsITPK1, commonly referred to as hITPK1; *Entamoeba histolytica* protein accession AAD22969, EhITPK1. Extreme C-terminal sequences are not shown. Residues coordinating individual phosphates of substrates are numbered according to the phosphate coordinated, or lettered, M, A or H, to indicate magnesium-, ATP-binding or hydrophobic interactions with ATP. Conservation of residues at 100% is indicated in black, and at 80% in grey.

and recombinant StITPK1-His protein. Reactions were performed for 1 h at 30 °C and stopped by heating to 95 °C for 5 min. We estimate that the purity of our protein is >98% estimated by SDS–PAGE. We have found that *E. coli* extracts display trivial phosphatase activity against inositol phosphates and nucleotides, but we have found no evidence of phosphotransfer from inositol phosphate to inositol phosphate, from inositol phosphate to ADP, or from ATP to inositol phosphate.

2.5. Preparation of inositol phosphate standards

[^{3}H]inositol-labeled substrates were prepared according to Sweetman et al. [6].

2.6. HPLC

Separations were performed on Partisphere and Adsorbosphere SAX columns [6,12]. Reverse phase (RP-HPLC) separations were performed on a 25 cm × 4.6 mm Phenomenex Synergi 4 μ Hydro-RP column (Phenomenex, Macclesfield, UK). The column was eluted at a flow rate of 1 mL min^{-1} with a solvent mixture derived from buffer reservoirs containing: A (50 mM NaH_2PO_4, 5 mM tetrabutyl ammonium hydroxide) and B (ACN/MeOH/H_2O [40/50/10 v/v/v] containing 5 mM tetrabutyl ammonium hydroxide) mixed as follows: 70% A, 30% B.

2.7. Kinetic analysis of StITPK1

Approximately 2.8 μg of StITPK1 was incubated with 15 μM, 10 μM, 5 μM and 2 μM Ins(3,4,5,6)P_4 in the presence of 37 kBq [γ-^{32}P]ATP and 0.4 mM ATP. Reactions were allowed to proceed for 20 min at 30 °C and stopped by heating to 95 °C for 5 min. The reaction time and enzyme concentration were chosen to limit ATP consumption to <10%. Reactions were performed in triplicate.

2.8. Homology modelling

An alignment of *Entamoeba histolytica* inositol 1,3,4-triphosphate 5/6-kinase (EhITPK1) with StITPK1 for homology modelling utilised the structural information available in the PDB entry 1Z2P [8] which contains coordinates for EhITPK1 in complex with 1,3,4-triphospho-*myo*-inositol, Mg^{2+} and the ATP mimic, 5′-adenosyl-methylene-triphosphate (AMPPCP). A model was generated by threading using FUGUE [13] employing default parameters. MODELLER (version 8.2) [14] was employed to generate homology models of StITPK1 plus magnesium ions and ADP or ATP cofactors. The models were subjected to stereochemical validation using appropriate routines in MODELLER. Models for the complexes of inositol polyphosphate substrates with the StITPK1 used the 1,3,4-triphospho-*myo*-inositol complex with EhITPK1 as a starting point.

To investigate the structural basis for the observed phosphotransferase activity of StITPK1, PDB entry 1Z2O [8], containing coordinates for the complex of EhITPK1 in complex with Mg^{2+}, ADP and *myo*-inositol 1,3,4,6-tetrakisphosphate, was employed as a template together with MODELLER to generate models for StITPK1 plus ADP in complex with *myo*-inositol 1,3,4,5,6-pentakisphosphate. All figures were prepared using PyMOL [15].

3. Results

3.1. Cloning and expression of StITPK1

Solanum tuberosum inositol polyphosphate kinase-1 (StITPK1, GenBank accession: EF362784, originally called StITPKα) was cloned from a potato tuber cDNA library. StITPK1 is aligned with other plant, human and protozoan sequences in Fig. 1. StITPK1 shares 54% identity with AtIPK from Arabidopsis [3], also known as AtITPK1 [6]; 52% identity with ZmIPK from maize [4] and 21% with EhITPK1 [8]. All of the sequences aligned in Fig. 1 contain the conserved domain: pfam05770.3, Ins134_P3_kin; an IPK domain identified in the Protein Families database (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF05770).

We have identified residues that occupy positions equivalent to a number of amino acids that coordinate the inositol phos-

phate substrates of EhITPK1 [8]. These include a conserved lysine, K20 of StITPK1, K17 of EhITPK1, that coordinates the 3-phosphate of Ins(1,3,4)P_3 and Ins(1,3,4,6)P_4 substrates. A histidine, H60, conserved between plant and human enzymes, replaces K57 of EhITPK1 that coordinates both the 3- and 4-phosphates of inositol phosphate substrates. A number of residues identical in StITPK1 and EhITPK1 include (StITPK1) K146, G152, H157, Q178, K189, R202 and L205; the EhITPK1 equivalents of which respectively bind ATP, the 4-phosphate of substrates, the 4-phosphate again, ATP, the 6- and 1-phosphates, the 1-phosphate, and contribute to hydrophobic interactions with ATP. D274, D289, and N291 are conserved metal-binding residues. Similarly, a number of hydrophobic residues including F273, instead of I274, and I288 are highly conserved residues that line a hydrophobic pocket that binds the adenine ring of the nucleotide co-substrate.

3.2. StITPK1 catalytic activity

Of the substrates tested, which included Ins1P, Ins(1,4)P_2, Ins(4,5)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4; only Ins(3,4)P_2, Ins(1,3,4)P_3, Ins(1,4,6)P_3, Ins(3,4,5)P_3, Ins(3,4,6)P_3 and Ins(3,4,5,6)P_4 generated products. Inositol tetrakisphosphate products were identified by coelution with ^{3}H-labelled standards and by lack of coelution with other potential products on Adsorbosphere SAX columns [3,6–10].

At low ATP (0.4 μM), conditions favouring catalysis with poor substrates [16], and 20 μM inositol phosphate substrate (InsP$_4$:ATP ratio of ≈50:1); the enzyme displays Ins(3,4,6)P_3 1-kinase activity (Fig. 2A). In contrast, at 0.4 mM ATP (InsP$_4$:ATP ratio of ≈1:20) 'phosphomutase' activity was evident generating Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 (Fig. 2F). Similar observation were made with Ins(1,3,4)P_3 (data not shown).

Threading of StITPK1 onto the EhITPK1 structure confirms the conservation of the structural elements of the ATP-grasp fold in StITPK1 (Fig. 6A). A number of substitutions of residues that interact with inositol phosphate substrates are identified in Figs. 1 and 6B: including (StITPK1/EhITPK1) N272G; D151Q, the aspartate replacing Q141 of EhITPK1 that forms a hydrogen bond with the 4-phosphate of Ins(1,3,4,6)P_4; and G295S, the glycine replaces the serine, S295, of EhITPK1 that hydrogen bonds to the 1-phosphate of Ins(1,3,4)P_3 in the enzyme–substrate–AMPPCP complex. S295 contributes to the Ins(1,4,5)P_3 3-kinase activity of EhITPK1, an activity lacking in StITPK1. Other active site residues are indicated in Fig. 6B–D: H157 corresponds to H147; K61 corresponds to R58; and Y191 corresponds to F181. Binding of Ins(3,4,6)P_3 to StITPK1 in a mode consistent with phosphorylation at the 1-position is indicated in Fig. 6B. The model reveals the close approach (less than 3 Å) of the oxygen atom of the 1-OH of Ins(3,4,6)P_3 and the phosphorus atom of the γ-phosphate of ATP. StITPK1 displays Ins(1,4,6)P_3 3-kinase activity (Fig. 2B). The enantiomeric nature of Ins(1,4,6)P_3 and Ins(3,4,6)P_3 requires the binding (Fig. 6B and C) of enantiomers in different modes to effect phosphorylation on enantiotopic positions.

Ins(1,3,4,5)P_4 was obtained as product from Ins(1,3,4)P_3 (Fig. 2C), and Ins(3,4,5,6)P_4 was obtained from Ins(3,4,5)P_3 (Fig. 2D). Ins(1,3,4,6)P_4 was obtained from Ins(3,4)P_2 (Fig. 2E).

Given the importance of Ins(1,3,4)P_3 to InsP$_6$ synthesis in animals, we sought to establish whether StITPK1 has a greater affinity for either Ins(1,3,4)P_3 or Ins(3,4,5,6)P_4 as substrate.
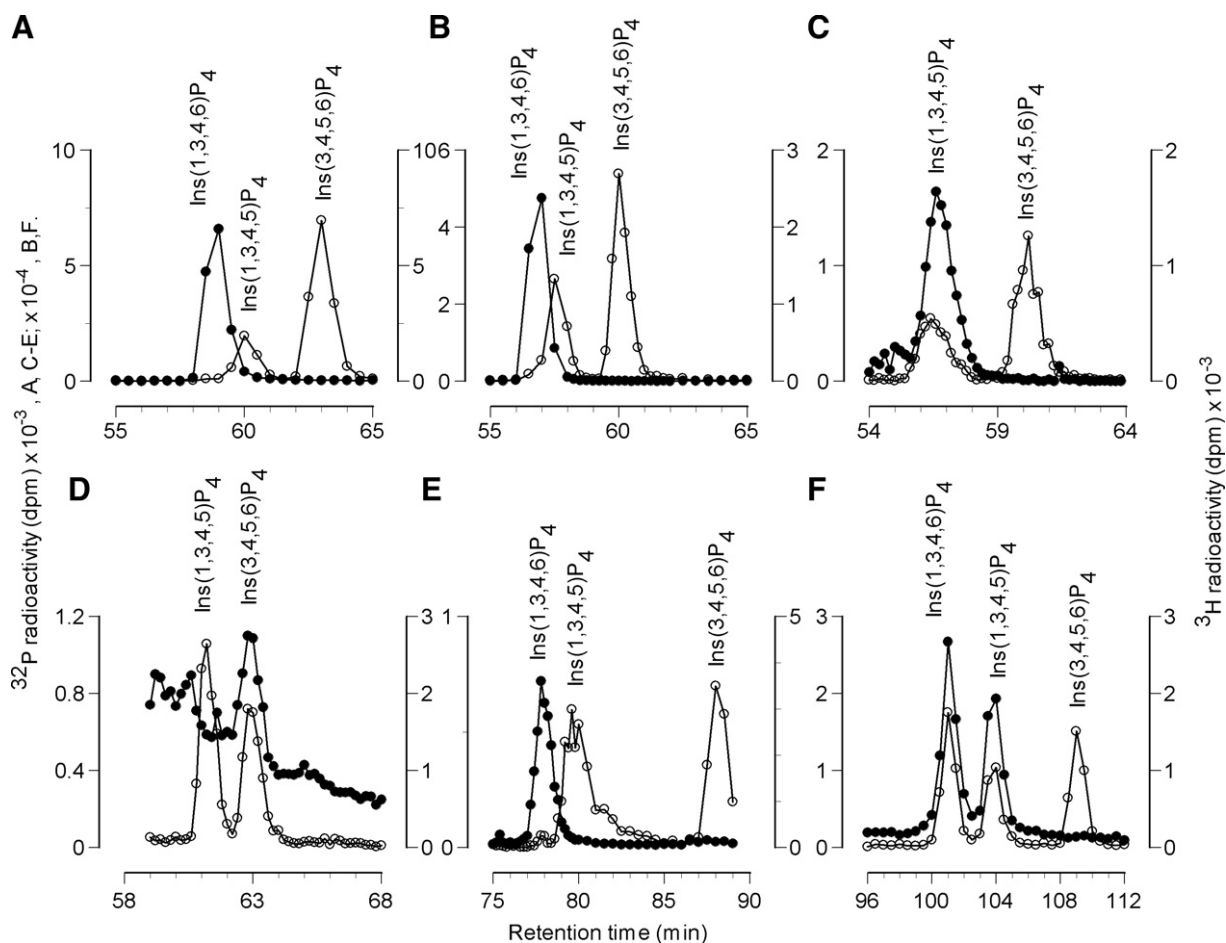
Fig. 2. Products of reactions catalyzed by StITPK1-His. Adsorbosphere SAX HPLC of ^{32}P products (●), left Y-axis; mixed with standards (○) of [^{3}H]Ins(1,3,4,5)P$_4$ and [^{3}H]Ins(3,4,5,6)P$_4$, right Y-axis of panels A–E, and additionally containing a [^{3}H]Ins(1,3,4,6)P$_4$ standard, panel F. The substrates used were: (A) Ins(3,4,6)P$_3$; (B) Ins(1,4,6)P$_3$; (C) Ins(1,3,4)P$_3$; (D) Ins(3,4,5)P$_3$ and (E) Ins(3,4)P$_2$, all at 0.4 μM ATP; and (F) Ins(3,4,6)P$_3$ at 0.4 mM ATP. For panels A, C–E, the units are dpm × 10^{-3}; for panels B and F, the units are dpm × 10^{-4}. Confirmation of the identity of products was performed on more than three occasions with different preparations of protein in assays of typically 15–60 min duration.

Recombinant StITPK1-His generated more InsP$_5$ product from Ins(3,4,5,6)P$_4$, than it did InsP$_4$ product from Ins(1,3,4)P$_3$ (Fig. 3A). The substrate preference of the enzyme was: Ins(3,4,5,6)P$_4$ ≈ Ins(1,4,6)P$_3$ ≈ Ins(3,4,6)P$_3$ ≫ Ins(1,3,4)P$_3$ > Ins(3,4,5)P$_3$ (data not shown).

StITPK1-His displays apparent Michaelis–Menten kinetics (Fig. 3B) with Ins(3,4,5,6)P$_4$: K_m = 27 μM, and V_{max} = 19 nmol min^{-1} mg^{-1}. The product coeluted with [^{3}H]Ins(1,3,4,5,6)P$_5$ (Fig. 4C), but not D/L-[^{3}H]Ins(1,2,4,5,6)P$_5$, D/L-[^{3}H]-Ins(1,2,3,5,6)P$_4$ nor Ins(1,2,3,4,6)P$_5$. The result of molecular modeling of Ins(3,4,5,6)P$_4$ substrate is shown in Fig. 6D, with Ins(3,4,5,6)P$_4$ bound in the same mode as Ins(3,4,6)P$_3$, that is with the 2-OH projecting into the active site.

3.3. InsP$_5$/ADP phosphotransferase activity of StITPK1

With Ins(1[^{32}P],3,4,5,6)P$_5$ (20 μM Ins(1,3,4,5,6)P$_5$) substrate and 0.1 mM ADP, Ins(1[^{32}P],3,4,5,6)P$_5$ was consumed in a time-dependent manner yielding [^{32}P]ATP (Fig. 4A): supported by a coeluting UV absorbance at 258 nm (Fig. 4C and D) that was not present in samples lacking ADP and/or enzyme. Little inorganic phosphate is visible in Fig. 4C and D; thus, the predominant activity of StITPK1 at physiological ADP and Ins(1,3,4,5,6)P$_5$ is that of an InsP$_5$-ADP phosphotransferase.

Remarkably, inclusion of 5 μM Ins(1,3,4)P$_3$ in assays with StITPK1-His, ADP, and Ins(1[^{32}P],3,4,5,6)P$_5$ also generated a [^{32}P]InsP$_4$ (Fig. 4E) that was absent from assays lacking Ins(1,3,4)P$_3$ (Fig. 4C and D) and increased Ins(1[^{32}P],3,4,5,6)P$_5$ dephosphorylation (Fig. 4E). Confirmation that the [^{32}P]InsP$_4$ was the product of phosphotransfer to Ins(1,3,4)P$_3$ was provided in other assays by the inclusion of [^{3}H]Ins(1,3,4)P$_3$ acceptor, yielding [^{3}H^{32}P]InsP$_4$ (data not shown). We conclude that inter-substrate phosphotransfer is evolutionarily conserved between plants and animals. In the absence of ADP, dephosphorylation of [^{3}H]Ins(1,3,4,5,6)P$_5$ yielded a [^{3}H]InsP$_4$ which eluted in the position of Ins(3,4,5,6)P$_4$ (compare Fig. 5 with standards shown in Fig. 2F). Dephosphorylation of [^{3}H]Ins(1,3,4,5,6)P$_5$ was increased by the inclusion of 0.4 mM ADP (data not shown).

Comparison of binding of Ins(1,3,4,5,6)P$_5$, in the presence of ADP or ATP, reveal that the active site cannot accommodate Ins(1,3,4,5,6)P$_5$ and ATP. Modelling of Ins(1,3,4,5,6)P$_5$ in two modes, with the 2-OH projecting into (Fig. 6E) or out (Fig. 6F) of the active site, reveals the juxtaposition of the γ-phosphate of ATP and the 1- and 3-phosphates of Ins(1,3,4,5,6)P$_5$, respectively. The model predicts that the 1-phosphate of Ins(1,3,4,5,6)P$_5$ is lost on phosphotransfer to ADP (Fig. 6E), consistent with data of Figs. 4 and 5.
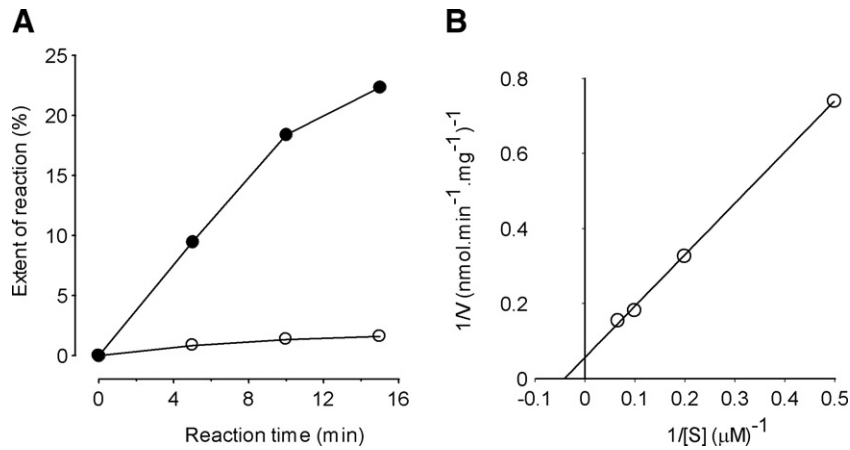
Fig. 3. StITPK1 substrate preference and kinetics. (A) StITPK1-HIS was incubated with inositol phosphate, 40 μM ATP and [γ-^{32}P]ATP at 30 °C. Products were resolved by RP-HPLC; peak area expressed as a percentage of the total radioactivity recovered, Ins(1,3,4)P$_3$ (○), Ins(3,4,5,6)P$_4$ (●). (B) Lineweaver–Burk plot for Ins(3,4,5,6)P$_4$ substrate.
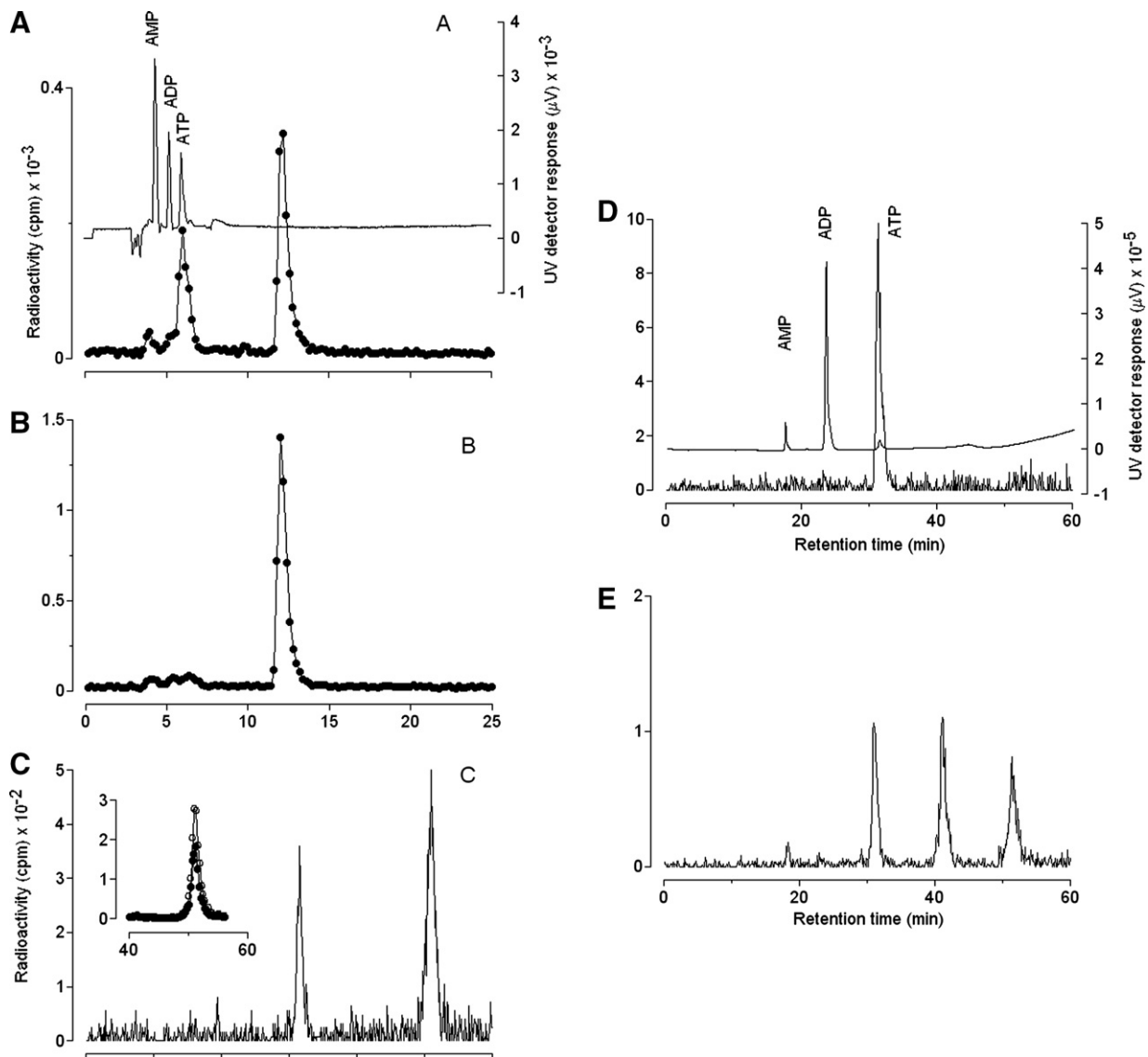

Fig. 4. Phosphotransferase activities of StITPK1. (A) RP-HPLC of the products of a 40 min reaction with Ins(1[^{32}P],3,4,5,6)P$_5$ and ADP: Left Y-axis, ^{32}P; Right Y-axis, UV 258 nm. (B) products of a 0 min reaction. (C–E) Partisphere SAX HPLC of ^{32}P products of (C) 20 min reaction with an internal standard (inset) of [^{3}H]Ins(1,3,4,5,6)P$_5$, ^{32}P (●) ^{3}H (○); (D) 120 min reaction, Left Y-axis, ^{32}P; right Y-axis, UV 258 nm. (E) 20 min reaction with Ins(1[^{32}P],3,4,5,6)P$_5$, ADP and Ins(1,3,4)P$_3$.
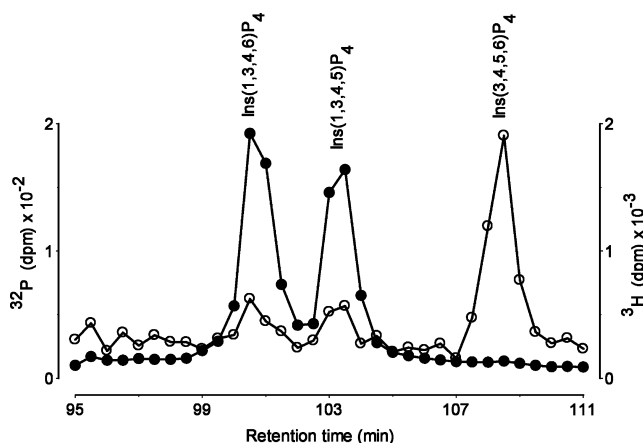
Fig. 5. Phosphatase activity of StITPK1. Adsorbosphere SAX HPLC of products of assay with [³H]Ins(1,3,4,5,6)P₅ resolved with Ins(1[³²P],3,4,6)P₄ and Ins(1[³²P],3,4,5)P₄ standards, [³H]; (○); [³²P], (●). The elution of inositol phosphates can be directly compared with that of ³H and ³²P standards in Fig. 2F.

4. Discussion

StITPK1, like hITPK1, possesses phosphatase and inter-substrate phosphotransferase activity. Phosphotransfer to ADP affords a potential point of control for InsP₆ synthesis in plants where massive accumulation of InsP₆ in storage tissues is the developmental norm. The accumulation of InsP₆ is a hugely energetic process and it is plausible that this process is governed in part not just by ATP supply, but by ADP/ATP balance. The corollary of this is that phosphotransfer to ADP may be physiologically relevant.

Our data begin to provide a molecular explanation of a number of reports of inositol polyphosphate-nucleotide phosphotransferase activities from plants. Activities transferring phosphate from InsP₆ to nucleotide have been described in mung bean [17] and soybean [18]. We note that ADP-phosphotransferase activities can be attributed to a diverse family of inositol phosphate kinases, including, in addition to ITPKs, InsP₆ kinase [19], and IPK1-like activities [17,18].
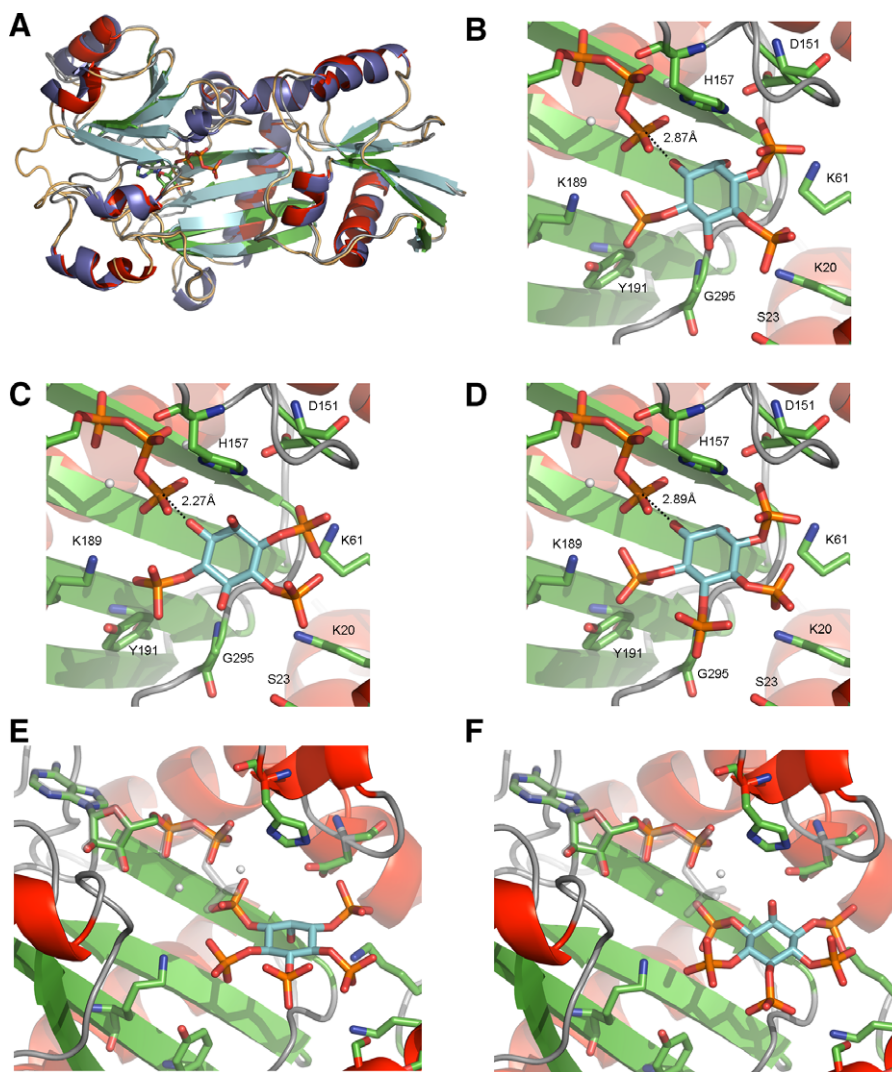

Fig. 6. Homology modelling of StITPK1 and its complexes with inositol polyphosphate substrates (A) overlay of the crystal structure of EhITPK1 with bound Mg²⁺, AMPPCP and Ins(1,3,4)P₃; and the threaded structure of the StITPK1. (B–F) docking of substrates to StITPK1 in the presence of ATP and magnesium ions: (B) Ins(3,4,6)P₃; (C) Ins(1,4,6)P₃; (D) Ins(3,4,5,6)P₄; (E) Ins(1,3,4,5,6)P₅; and (F) Ins(1,3,4,5,6)P₅. In E and F, the predicted position of the γ-phosphate of ATP is superimposed in grayscale.

Our demonstration of phosphotransfer to ADP also affords potential insight into the reaction mechanism of plant ITPKs. While H162 is important for phosphotransfer reactions of hITPK1 [1], it is not critical for enzymes of this class because StITPK1, with an aspartate, D151, in the equivalent position, displays all the phosphotransfer reactions reported for hITPK1.

Enzymes of both ITPK and inositol phosphate multikinase (IPMK) classes have been shown to contribute to $InsP_6$ synthesis in plants: mutation of ZmIPK reduced kernel phyate in maize [4], while mutation of AtIPK2β, an IPMK, reduced seed phyate in Arabidopsis [20]. While the in vivo substrates of these enzymes have not been formally identified, it has been suggested that AtIPK2β contributes to a pathway downstream of $Ins(1,4,5)P_3$ [20] as has been established in yeast. It is less clear what physiological substrate ZmIPK, as an exemplar of plant ITPKs, might access; but the substrate is not $Ins(1,4,5)P_3$. Most reasonably, in the direction of phytic acid synthesis the substrate is likely to be $Ins(3,4,5,6)P_4$. For the moment, we do not consider that the demonstration of phosphotransfer to $Ins(1,3,4)P_3$ is physiologically relevant to plants, given the absence in this kingdom, and in yeast, of the $Ins(1,4,5)P_3$ 3-kinases of the animal kingdom that generate the $Ins(1,3,4,5)P_4$ precursor of $Ins(1,3,4)P_3$. Nevertheless, it seems plausible that other inositide – inositide transferase activities may be physiologically relevant. In summary, given the range of activities displayed by ITPKs it is possible that ITPKs afford higher eukaryotes a level of control of $InsP_6$ synthesis that is not evident in yeast; but which is superimposed upon the activity of IPMKs which are common to plants, yeast and animals. Such considerations may not be well answered until we have a more complete understanding of the biochemistry of ITPKs and IPMKs in other lower eukaryotes.

References

[1] Chamberlain, P.P., Qian, X., Stiles, A.R., Cho, J., Jones, D.H., Lesley, S.A., Grabau, E.A., Shears, S.B. and Spraggon, G. (2007) Integration of inositol phosphate signaling pathways via human ITPK1. J. Biol. Chem. 282, 28117–28251.

[2] Saiardi, A. and Cockcroft, S. (2008) Human ITPK1: a reversible inositol phosphate kinase/phosphatase that links receptor-dependent phospholipase C to Ca^{2+}-activated chloride channels. Science Signal. (1), 5.

[3] Wilson, M.P. and Majerus, P.W. (1997) Characterization of a cDNA Encoding *Arabidopsis thaliana* Inositol 1,3,4-trisphosphate 5/6-kinase. Biochem. Biophys. Res. Commun. 232, 678–681.

[4] Shi, J., Wang, H., Wu, Y., Hazebrock, J., Meeley, R.B. and Ertl, D.S. (2003) The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. Plant Physiol. 131, 507–515.

[5] Josefsen, L., Bohn, L., Sorensen, M.B. and Rasmussen, S.K. (2007) Characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfamily. Gene 397, 114–125.

[6] Sweetman, D., Stavridou, I., Johnson, S., Green, P., Caddick, S.E.K. and Brearley, C.A. (2007) *Arabidopsis thaliana* inositol 1,3,4-trisphosphate 5/6-kinase 4 (AtITPK4) is an outlier to a family of ATP-grasp fold proteins from Arabidopsis. FEBS Lett. 581, 4165–4171.

[7] Field, J., Wilson, M.P., Mai, Z., Majerus, P.W. and Samuelson, J. (2000) An *Entamoeba histolytica* inositol 1,3,4-trisphosphate 5/6-kinase has a novel 3-kinase activity. Mol. Biochem. Parasitol. 108, 119–123.

[8] Miller, G.J., Wilson, M.P., Majerus, P.W. and Hurley, J.H. (2005) Specificity determinants in inositol polyphosphate synthesis: crystal structure of inositol 1,3,4-trisphosphate 5/6-kinase. Mol. Cell 18, 201–212.

[9] Balla, T., Guillemette, G., Baukal, A.J. and Catt, K.J. (1987) Metabolism of inositol 1,3,4-trisphosphate to a new tetrakisphosphate isomer in angiotensin-stimulated adrenal glomerulosa cells. J. Biol. Chem. 262, 9952–9955.

[10] Shears, S.B. (1989) The pathway of myo-inositol 1,3,4-trisphosphate phosphorylation in liver. Identification of myo-inositol 1,3,4-trisphosphate 6-kinase, myo-inositol 1,3,4-trisphosphate 5-kinase, and myo-inositol 1,3,4,6-tetrakisphosphate 5-kinase. J. Biol. Chem. 264, 19879–19886.

[11] Shears, S.B. (2004) How versatile are inositol phosphate kinases? Biochem. J. 377, 265–280.

[12] Sweetman, D., Johnson, S., Caddick, S.E., Hanke, D.E. and Brearley, C.A. (2006) Characterization of an Arabidopsis inositol 1,3,4,5,6-pentakisphosphate 2-kinase (AtIPK1). Biochem. J. 394, 95–103.

[13] Shi, J.Y., Blundell, T.L. and Mizuguchi, K. (2001) FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J. Mol. Biol. 310, 243–257.

[14] Marti-Renom, M.A., Stuart, A.C., Fiser, A., Sanchez, R., Melo, F. and Sali, A. (2000) Comparative protein structure modeling of genes and genomes. Annu. Rev. Biophys. Biomol. Struct. 29, 291–325.

[15] DeLano, W.L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA.

[16] Poinas, A., Backers, K., Riley, A.M., Mills, S.J., Moreau, C., Potter, B.V. and Erneux, C. (2005) Interaction of the catalytic domain of inositol 1,4,5-trisphosphate 3-kinase A with inositol phosphate analogues. ChemBiochem. 6, 1449–1457.

[17] Biswas, S., Maity, I.B., Chakrabarti, S. and Biswas, B.B. (1978) Purification and characterization of myo-inositol hexaphosphate-adenosine diphosphate phosphotransferase from *Phaseolus aureus*. Arch. Biochem. Biophys. 185, 557–566.

[18] Phillippy, B.Q., Ulla, A.H. and Ehrlich, K.C. (1994) Purification and some properties of inositol 1,3,4,5,6-pentakisphosphate 2-kinase from immature soybean seeds. J. Biol. Chem. 269, 28393–28399.

[19] Voglmaier, S.M., Bembenek, M.E., Kaplin, A.I., Dorman, G., Olszewski, J.D., Prestwich, G.D. and Snyder, S.H. (1996) Purified inositol hexakisphosphate kinase is an ATP synthase: diphosphoinositol pentakisphosphate as a high-energy phosphate donor. Proc. Natl. Acad. Sci. USA 93, 4305–4310.

[20] Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., Frye, R.A. and York, J.D. (2005) Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc. Natl. Acad. Sci. USA 102, 12612–12617.