

Research Article

Diversification in the inositol tris/tetrakisphosphate kinase (ITPK) family: crystal structure and enzymology of the outlier *AtITPK4*

Hayley L. Whitfield¹, Sining He^{1,2}, Yinghong Gu¹, Colleen Sprigg¹, Hui-Fen Kuo³,  Tzyy-Jen Chiou³,  Andrew M. Riley⁴, Barry V.L. Potter⁴, Andrew M. Hemmings^{1,5} and  Charles A. Brearley¹

¹School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.; ²Department of Biology, School of Life Sciences, Southern University of Science and Technology, Nanshan, Shenzhen 518055, China; ³Agricultural Biotechnology Research Centre, Academia Sinica, Taipei 115, Taiwan; ⁴Medicinal Chemistry & Drug Discovery, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, U.K.; ⁵College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China

Correspondence: Charles A. Brearley (c.brearley@uea.ac.uk) or Andrew M. Hemmings (a.hemmings@uea.ac.uk)



Myo-inositol tris/tetrakisphosphate kinases (ITPKs) catalyze diverse phosphotransfer reactions with *myo*-inositol phosphate and *myo*-inositol pyrophosphate substrates. However, the lack of structures of nucleotide-coordinated plant ITPKs thwarts a rational understanding of phosphotransfer reactions of the family. Arabidopsis possesses a family of four ITPKs of which two isoforms, ITPK1 and ITPK4, control inositol hexakisphosphate and inositol pyrophosphate levels directly or by provision of precursors. Here, we describe the specificity of Arabidopsis ITPK4 to pairs of enantiomers of diverse inositol polyphosphates and show how substrate specificity differs from Arabidopsis ITPK1. Moreover, we provide a description of the crystal structure of ATP-coordinated *AtITPK4* at 2.11 Å resolution that, along with a description of the enantiospecificity of the enzyme, affords a molecular explanation for the diverse phosphotransferase activity of this enzyme. That Arabidopsis ITPK4 has a K_M for ATP in the tens of micromolar range, potentially explains how, despite the large-scale abolition of InsP_6 , InsP_7 and InsP_8 synthesis in *Atitpk4* mutants, *Atitpk4* lacks the phosphate starvation responses of *Atitpk1* mutants. We further demonstrate that Arabidopsis ITPK4 and its homologues in other plants possess an N-terminal haloacid dehalogenase-like fold not previously described. The structural and enzymological information revealed will guide elucidation of ITPK4 function in diverse physiological contexts, including InsP_8 -dependent aspects of plant biology.

Introduction

Myo-inositol pyrophosphates (diphosphoinositol phosphates) are present in plants at levels that are a small fraction of their precursor, *myo*-inositol hexakisphosphate InsP_6 . Like inositol phosphates and nucleotides, they are responsive to phosphate resupply after chronic phosphate starvation [1]. The catalytic activities of inositol hydroxy- and phosphate kinases, IPMK, ITPK1, IPK1 and VIH1/2 (also known as VIP1/2) are responsible for InsP_6 and inositol pyrophosphate synthesis in Arabidopsis and contribute to the control of phosphate homeostasis [2–10] and the phosphate starvation response (PSR) whereby the Myb transcription factor PHR1 controls the expression of a host of genes regulating plant response to phosphate supply [6,11] (Supplementary Figure S1).

While early reports suggested that Pi is a ligand of the protein SPX1 whose interaction with PHR1 sequesters the latter from its activating interaction with the P1BS element of PSR genes [12], InsP_6 and inositol pyrophosphates are much tighter binding ligands of SPX1 [13]. In vitro studies of SPX1 orthologs show remarkably little discrimination in strength of binding between InsP_6 and inositol

Received: 1 December 2022
 Revised: 9 March 2023
 Accepted: 10 March 2023

Accepted Manuscript online:
 10 March 2023
 Version of Record published:
 29 March 2023

pyrophosphates [13], and some of the outputs proposed of inositol pyrophosphate interaction with SPX1 are met by InsP₆ [4,10,14]. Nonetheless, a current model of PSR attributes special function to InsP₈ [14]. A metabolic perspective is, nevertheless, provided by the global inositol phosphate response to resupply of phosphate after chronic starvation [1]. Here, the amplified response of InsP₈, relative to InsP₇ and InsP₆, points to metabolic flux from these precursors and possibly also from InsP₃, InsP₄ and InsP₅ species that are also increased on Pi resupply. That inositol pyrophosphate synthesis is coupled to the metabolism of lower inositol phosphates was proposed [9].

Even so, studies suggest that InsP₈ or the activity of the enzymes that make it do not necessarily correlate with PSR. Under Pi-limiting conditions *Vip1-2/vip2-2* seedlings lack PSR [15], showing quite different behaviour to reported *vih* mutants [4] [10] [14]. Kuo and coworkers also did not observe constitutive PSR in Pi-replete *vih1-1*, *vih2-3*, *vih2-4*, *vip1-1*, *vip1-2*, *vip2-1*, *vip2-2*, *vip1-2/vip2-1* mutants [6]. Here, the different alleles take different names depending on their source (Supplementary Table S1 [6]). Moreover, *itpk4* mutants, which show pronounced reductions in InsP₆ [6,16] are severely depleted in InsP₇ and InsP₈ [6] and lack the constitutive PSR established for *itpk1* and *ipk1* mutants under Pi-replete conditions [17]. These differences may arise from the pleiotropic consequences of disruption of inositol phosphate, including InsP₆ and inositol pyrophosphate, synthesis [18]. Inositol phosphates are participants in biotic and abiotic interactions of plants [18–21]. The effects of mutation of *Itpk1* and *Ipk1* extend to influence on plant immunity mediated by salicylic acid [19,20], a property shared by *Vih2* mutants [19] and other mutants of InsP₆ synthesis [20]. *Itpk1* mutants also show auxin-related phenotypes that may relate to combinatorial effect of altered inositol phosphate and inositol pyrophosphate species in these plants [21].

Here, we combine structural biology, enzyme assay and LC-ICP-MS to characterize AtITPK4 and *Atitpk4* mutants. We reveal novel facets of ITPK structure, substrate preference and kinetic behaviour that set ITPK4 apart from other inositol hydroxy- and phosphate kinases involved in inositol pyrophosphate synthesis in plants.

Results

The enantiomeric discrimination of AtITPK4 towards inositol phosphates is the opposite of ITPK1: ITPK4 prefers Ins(1,4,6)P₃

Despite a resurgence in interest in the role of inositol phosphates and pyrophosphates in plants, particularly in the context of Pi homeostasis, there are remarkably few studies of the isomeric complement of potential physiological substrates of inositol hydroxyl- or inositol phosphate-kinases of plants. Nonetheless, the dominant Ins(3,4,5,6)P₄ 1-kinase activity of Arabidopsis ITPK1 [9] fits well with historic analysis of inositol phosphate stereoisomerism and enantiomerism [22–24]. Previous characterization of the ITPK family highlighted that within Arabidopsis ITPK4 is an outlier to the family [25], and more broadly that ITPK's have diverse phosphotransfer capabilities [26–29]. The latter observation is important since phosphotransfer (from inositol phosphate or pyrophosphate) to water defines phosphatase activity, while transfer to ADP is a facet of reversibility exemplified by IPK1 and ITPK1 [1,9,30,31]. The ITPK family additionally shows 'phosphoisomerase' or 'phosphomutase' activity [25,26,29]. We therefore tested Arabidopsis ITPK4 for hydroxy-kinase and phosphate-kinase activity and for other phosphotransferase activities.

The substrate preference of Arabidopsis ITPK4 towards a variety of inositol phosphate substrates and the identities of products generated are shown (Table 1). The structures of these molecules are shown (Supplementary Figure S2). ITPK4 has preferential activity against 'lower' inositol phosphates (Figure 1 and Supplementary Figure S3). Like others [1] we found no evidence of inositol phosphate-kinase (pyrophosphorylating) activity (see below). Activity against Ins(1,4)P₂, Ins(1,4,6)P₃ and Ins(3,4,6)P₃ was tested with an ATP regenerating assay revealing product conversion activities of 6.8%, 10.4% and 0%, respectively (Figure 1). Consequently, the preference of ITPK4 for Ins(1,4,6)P₃ over Ins(3,4,6)P₃ is opposite to that of Arabidopsis ITPK1 [9] and we offer a structural explanation of this below. Nonetheless, extended incubation revealed ITPK4 could use Ins(3,4,6)P₃ as a weak substrate (Table 1). The products of phosphorylation of Ins(1,4)P₂, Ins(1,4,6)P₃ and Ins(3,4,6)P₃ were identified as Ins(1,3,4)P₃, Ins(1,3,4,6)P₄ and Ins(1,3,4,6)P₄, respectively, by spiking with standards and by comparison of previously reported separations of inositol phosphates [9,32]. The enantiomeric character of individual pairs of substrates is shown (Supplementary Figure S2). Early characterization of enzymes of this class in plants considered them as Ins(1,3,4)P₃ 5/6 kinases [25–29], here Ins(1,3,4)P₃ is a weak substrate for AtITPK4 when compared with Ins(1,4,6)P₃ (Supplementary Figure S3).

Table 1 Reactions catalyzed by AtITPK4

Kinase reaction		Assay type	
Substrate	Product	Regeneration	Standard
Ins1P		-	-
Ins3P			-
Ins(1,4)P ₂	Ins(1,3,4)P ₃	++	**
Ins(1,3,4)P ₃	Ins(1,3,4,5)P ₄	+	*
Ins(1,4,5)P ₃			-
Ins(1,4,6)P ₃	Ins(1,3,4,6)P ₄	+++	***
Ins(3,4,6)P ₃	Ins(1,3,4,6)P ₄	+	*
Ins(3,4,5)P ₃	Ins(3,4,5,6)P ₄		**
Ins(4,5,6)P ₃		-	
Ins(1,2,4,6)P ₄			-
Ins(2,3,4,6)P ₄			-
Ins(1,3,4,5)P ₄			-
Ins(1,3,5,6)P ₄			-
Ins(1,3,4,6)P ₄			-
Ins(1,4,5,6)P ₄	Ins(1,3,4,5,6)P ₅	+++	**
Ins(3,4,5,6)P ₄			-
Ins(1,2,3,4,5)P ₅		-	
Ins(1,2,3,5,6)P ₅		-	
Ins(1,2,3,4,6)P ₅		-	
Ins(1,3,4,5,6)P ₅		-	
Ins(1,2,4,5,6)P ₅		-	
Ins(2,3,4,5,6)P ₅		-	
Ins(1,2,3,4,5,6)P ₆		-	
Phosphotransfer to ADP			
Substrate	InsP product		
Ins(1,3,4)P ₃	Not verified	++	
Ins(1,4,6)P ₃	Not verified	++	
Ins(1,3,4,6)P ₄	Ins(1,4,6)P ₃ /Ins(3,4,6)P ₃	+++	
Ins(1,4,5,6)P ₄	Not verified	+	

For ATP-regeneration assays, -/+⁺/⁺⁺/⁺⁺⁺ indicates reaction strength within pair of enantiomers or classes (InsP₃, InsP₄) of compounds; - indicates absence of activity; otherwise, not tested. Without ATP regeneration (standard assay), reaction indicated -/*/**/****. Not verified, InsP₂s are poorly resolved on the column used and standards were not used. All reactions which failed to give products were analyzed beside positive controls that yielded products. Structures of substrates/products shown in Supplementary Figure S1.

Substrate inhibition of AtITPK4 by ATP

Kinetic assays of ITPK4 against Ins(1,4,6)P₃, limited to less than 10% substrate turnover, revealed a K_M for ATP of $52 \pm 7 \mu\text{M}$ and V_{max} $8.64 \pm 0.33 \text{ nmol min}^{-1} \text{ mg}^{-1}$ when fitted to the Michaelis–Menten equation (Figure 1D). This reveals a striking difference between Arabidopsis ITPK1 and Arabidopsis ITPK4 for their preferred substrates. The latter has a 15 fold lower K_M for ATP and a 1000 fold lower V_{max} . ITPK1 prefers Ins(3,4,5,6)P₄, and displays K_M for ATP of 1.22 mM, V_{max} $8640 \text{ nmol min}^{-1} \text{ mg}^{-1}$ [9]. Riemer et al. [1] reported

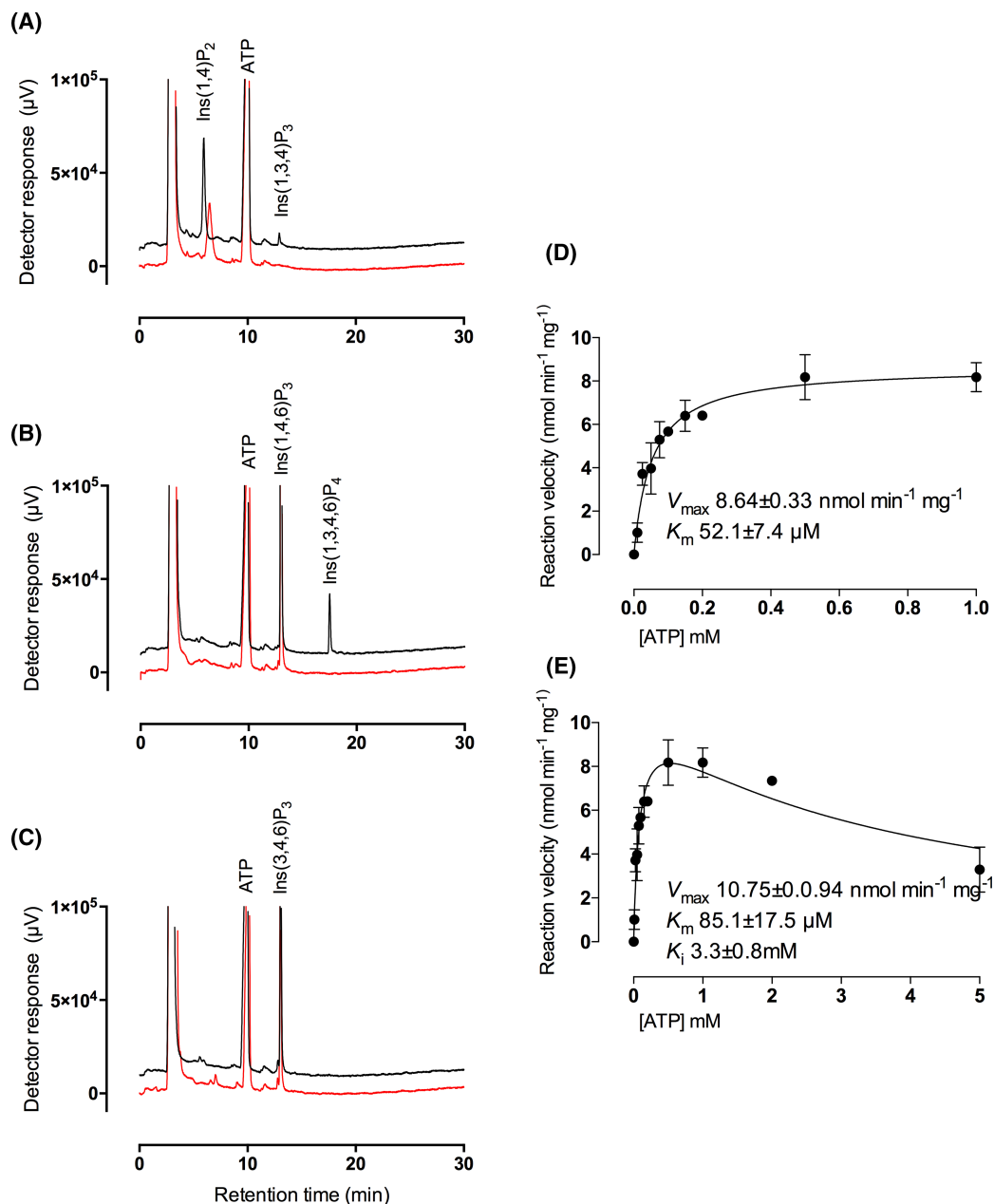


Figure 1. Hydroxy-kinase reactions catalyzed by AtITPK4.

(A) Ins(1,4)P₂, (B) Ins(1,4,6)P₃ and (C) Ins(3,4,6)P₃ incubated with (black line) or without protein (red line). Products of 20 min reactions were analyzed by HPLC. These assays were set up to distinguish the use of different substrates. (D and E) Michaelis–Menten kinetic parameters for hydroxy-kinase action of AtITPK4 on Ins(1,4,6)P₃. Reaction conditions were set to limit substrate consumption to <10%. Analysis of the kinase activity of AtITPK4 to these substrates has been performed on at least five separate occasions by these methods.

kinetic parameters for AtITPK1 of K_M for ATP with InsP₆ of 0.52 mM and V_{max} 18 nmol min⁻¹ mg⁻¹. Thus, Arabidopsis ITPK4 activity towards Ins(1,4,6)P₃ is comparable to Arabidopsis ITPK1 activity towards InsP₆ and this is several orders of magnitude less than ITPK1 activity towards Ins(3,4,5,6)P₄. A recent report afforded K_M for InsP₆ of 0.025 mM and K_M for ATP of 0.21–36 mM, with V_{max} in the low tens of nmol min⁻¹ mg⁻¹, for *Zea mays* inositol tris/tetrakisphosphate kinase 1, *ZmITPK1* [33]. Activity against ‘lower’ inositol phosphates was not described.

As well as a low K_M for ATP, Arabidopsis ITPK4 showed inhibition at high ATP concentrations where data fitted to a substrate inhibition model in GraphPad gave a K_i of 3.3 mM (Figure 1E). Such inhibition was not seen for AtITPK1, tested up to 10 mM ATP [9], though inhibition is seen at 10 mM in the data of Riemer et al. [1]. It is tempting to speculate that the difference in K_M for ATP for ITPK1 and ITPK4 underlies the difference in PSR of the respective mutants, since both are essential for inositol pyrophosphate synthesis either directly from InsP₆ (ITPK1 [1,9]) or from their contribution to InsP₆ synthesis (ITPK1 and ITPK4 [1,6,9,16]).

AtITPK4 does not phosphorylate IP₅ or IP₆

We tested a range of substrates under ATP-regenerating and non-regenerating assay conditions. Unlike Arabidopsis ITPK1, which converts Ins(1,2,3,4,5)P₅ to putative 5PP-Ins(1,2,3,4)P₄ [9], Arabidopsis ITPK4 did not phosphorylate IP₆ (Supplementary Figure S4) nor did it phosphorylate any of the six InsP₅ isomers (Table 1). The putative 5PP-Ins(1,2,3,4)P₄ product (of AtITPK1) may be the novel unidentified PP-InsP₄ detected in Arabidopsis [1]. ZmITPK1, however, converts Ins(1,2,3,4,5)P₅ to 3PP-Ins(1,2,4,5)P₄ with ca. 25% of the activity towards InsP₆. It also displays apparent pyrophosphorylating activity against multiple InsP₅ isomers in a coupled phosphate-releasing assay [33]. Interestingly, 3PP-Ins(1,2,4,5)P₄ elutes just before 5-InsP₇, which elutes after InsP₆ on Partisphere SAX HPLC (see Figures 1,2 of [33]), while the putative 5PP-Ins(1,2,3,4)P₄ elutes before InsP₆ on the same column (see Supplementary Figure S7 of [9]). ZmITPK1 shares less identity with the ATP Grasp fold of Arabidopsis ITPK4 than does HsITPK1. These observations highlight the importance of effective means of discriminating products, which can be achieved in a simple manner by HPLC and post-column detection with ferric nitrate [9] or HPLC and radio-detection [9,33].

Arabidopsis ITPK4 was however able to use Ins(1,4)P₂, Ins(1,4,6)P₃ and Ins(3,4,5)P₃ as substrates and additionally Ins(1,3,4)P₃, Ins(3,4,6)P₃ and Ins(1,4,5,6)P₄ as weak substrates. It did not use Ins1P, Ins3P, Ins(1,4,5)P₃, Ins(1,3,4,6)P₄ or Ins(3,4,5,6)P₄ under non-regenerating assay conditions when incubated for 2 h (Table 1). Again, the preference of Arabidopsis ITPK4 for enantiomers is opposite to that of Arabidopsis ITPK1, it prefers Ins(1,4,6)P₃ over Ins(3,4,6)P₃ and Ins(1,4,5,6)P₄ over Ins(3,4,5,6)P₄. We caution against assertions of activity of the ITPK family within and between species without exhaustive testing, the ITPK family is remarkable for its catalytic flexibility.

AtITPK4 regulates InsP₆, InsP₇ and InsP₈ synthesis

Atitpk4 mutants have been characterized in detail. They lack the phosphate over-accumulation of Atitpk1 and Atitpk1. They lack PSR, and they show reduced seed InsP₆ [6,16]. TiO₂-PAGE analysis also showed greatly reduced vegetative tissue InsP₆, InsP₇ and InsP₈ [17]. To test for effect of Itpk4 mutation on inositol phosphates, we extracted inositol phosphates in perchloric acid, concentrated them on TiO₂ and analyzed by LC-ICP-MS (Figure 2). This approach retains the considerable resolving power of HPLC on acid-eluted CarboPac PA200 both for inositol phosphates and inositol pyrophosphates. It resolves 5-InsP₇, 4/6-InsP₇ and 1/3-InsP₇ [9]. We did not detect InsP₇ or InsP₈ with column-loading of extracts equivalent to ~160 mg of soil-grown itpk4-1, ITPK4-OE line or ITPK4-YFP-complemented itpk4-1 [6,17]. The levels of InsP₆ in itpk4-1 were significantly different ($P < 0.05$) from that of Col0 and ITPK4-OE, with mean and S.D. of 26 ± 1 , 153 ± 39 and 181 ± 43 pmol g⁻¹ f. wt., respectively. A single ITPK4-YFP-complemented itpk4-1 gave a value of 118 pmol g⁻¹ f. wt. The reduction in InsP₆ content is consistent with [³H]-myo-inositol- and [³²P]-P_i-labelling of seedlings [6], with TiO₂-PAGE analysis of mature vegetative tissues [17] and with measurements in seeds [6]. Moreover, the absolute levels in vegetative tissues match that measured by CE-MS in P_i-deplete, hydroponically grown Col0, ~150 pmol g⁻¹ f. wt. [1]. These data validate LC-ICP-MS for measurement of inositol phosphates in vegetative tissues, the site of PSR, without recourse to radiolabeling. They further draw attention to the lack of PSR in itpk4 mutants [6,17].

Reduction in InsP₆ levels in itpk4-1 was accompanied by increases in a peak with the chromatographic properties of an InsP₃ (Figure 2E,F). These increases were reversed by complementation with ITPK4 (Figure 2D,F) and absent from the ITPK4-overexpression line (Figure 2B,F).

The crystal structure of AtITPK4

The foregoing enzymological and physiological characterization of ITPK4 and itpk4 mutants again sets ITPK4 apart from other ITPK family members. To provide a structural context for these observations, we sought crystal structures of AtITPK4 and AtITPK1. We anticipated that these structures would help facilitate understanding of the variation in substrate preference between the two. No crystal structure for an ITPK4 exists but

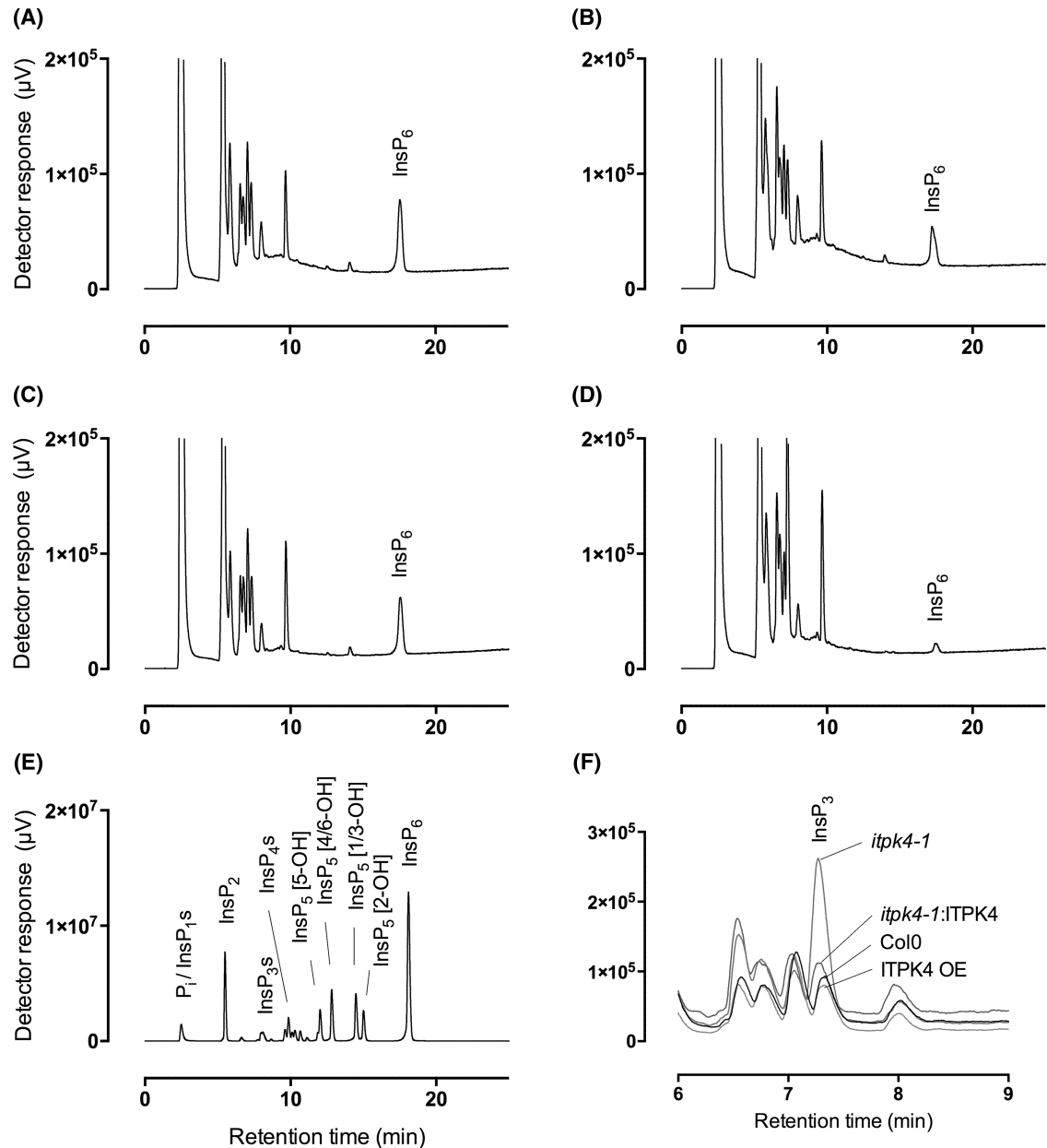


Figure 2. Inositol phosphates in mature vegetative tissue.

Extracts of (A) Col0, (B) ITPK4-YFP-complemented *itpk4-1*, (C), ITPK4 overexpression line, (D), *itpk4-1*, were analyzed by HPLC-ICP-MS. (E) acid-hydrolysed InsP_6 standards. (F) Comparison of resolved peaks in the InsP_3 region of chromatograms (A–D). The traces shown are representative of triplicate determinations for this experiment. Individual genotypes have been analyzed on at least one other occasion by this method.

crystal structures of wild-type ITPK1 enzymes from *Entamoeba histolytica* (*EhITPK1*) [34] and *Homo sapiens* (*HsITPK1*) [27] are available. For plant enzymes, a medium-resolution (2.9 Å) structure of the wild-type apo enzyme from maize (*ZmITPK1*) has been reported along with a 2.6 Å resolution structure of an InsP_6 -coordinated H192A mutant [33]. These structures of *ZmITPK1* lack electron density for groups of residues that we show below form part of the binding pocket for nucleotide in *AtITPK4*.

We were unable to crystallize *AtITPK1*, whose enzymology is described [9], but were successful in obtaining a crystal structure of *AtITPK4* in complex with ATP using the structure of *EhITPK1* (PDB entry 1Z2N) as a

search model in molecular replacement. The structure was solved in space group $P2_12_12_1$ with a monomer of the enzyme in the asymmetric unit. Refined against all data to 1.91 Å resolution, this gave a final model with an R-factor of 21.0% (R_{free} 24.1%) (Table 2).

The structure consists of a canonical C-terminal ATP Grasp kinase domain (residues 151–423) and an N-terminal domain (residues 1–150) which adopts a haloalkane dehalogenase (HAD)-like fold (Figure 3). This latter domain is not conserved in any other ITPK family member and its structure was built in stages by careful manual fitting to difference electron density maps interspersed with rounds of refinement. Despite this, the quality of the fit of the final refined model of this domain to difference electron density maps was generally lower than that seen in the kinase domain. The average refined atomic temperature factors for the HAD-like and kinase domains are 76.7 Å² and 36.1 Å², respectively, reflecting a generally higher degree of flexibility in the former. Nevertheless, the topology of the domain is clearly indicated by the corresponding electron density maps (Supplementary Figure S5) and geometrical indicators are in accordance with correct chain tracing. This degree of inherent mobility localized to a domain within an otherwise ordered protein structure is not unusual e.g. ([35] and [36]) and may indicate either a degree of intrinsic disorder in the domain or the absence of a stabilizing binding partner in the crystal, be it a lower molecular weight ligand or other protein(s) that would otherwise participate as part of either a homo- or heteroprotein complex. In this respect, we found no evidence that *AtITPK4* engages in homo-oligomerization, a property described for other HAD superfamily proteins (e.g. [37]).

Comparison of the kinase domains of *AtITPK4* and *AtITPK1* suggests a structural basis for enantiospecificity

The kinase domain of *AtITPK4* possesses the familiar ATP Grasp fold found in all previously described ITPK family members (Supplementary Figure S6) and features the expected highly positively charged active site (Figure 3C). The overall structure is made up of three conserved subdomains which we shall refer to as N-terminal, central and C-terminal following the nomenclature previously applied in descriptions of the crystal structures of ITPK1 orthologs [27,33,34] (Figure 3). Comparative analysis using all-by-all flexible pairwise structure alignments between the kinase domain of *AtITPK4* and the available ITPK1 crystal structures revealed human ITPK1 to be the most similar (PDB entry 2QB5; RMSD 2.95 Å; 21.0% sequence identity for 306 structurally equivalenced residues) (Figure 4). While the enzyme from *E.histolytica* had a slightly lower RMSD (PDB entry 1Z2N; 2.85 Å) it showed both a lower number of structurally equivalenced residues (280) and a lower percentage identity (15.9%). The maize ITPK1 structure shows the lowest structural and sequence homology (PDB entry 7TN5; RMSD 3.04 Å; 264 equivalenced residues; 16.2% identity). Unsurprisingly, the predicted structure of *AtITPK1* taken from the AlphaFold Protein Structure Database [38] also has an ATP Grasp fold. AlphaFold predictions of protein structures have been shown to be of high accuracy [39]. Indeed, we note an RMSD of 0.64 Å over 287 aligned C α atoms between AlphaFold-predicted and observed structures of the kinase domain of *AtITPK4* demonstrating the predictive power of the method. However, despite this, we have chosen not to compare directly the predicted structure of *AtITPK1* with the crystal structure of *AtITPK4* but rather to draw on it only for qualitative indications of overall fold and sequence alignment.

In terms of the overall structures of their kinase domains, *AtITPK4* and the ITPK1 (including *AtITPK1*) have two major areas of divergence; the first is an insertion in the central subdomain of the ITPK1s relative to *AtITPK4*, and the second is an insertion in the N-terminal subdomain of *AtITPK4* relative to ITPK1s (Figures 3,4). The insertion in the ITPK1s is found in the polypeptide connection between the C-terminal β -strand of the central domain (strand β 20) and a helix (α 15) of the C-terminal domain, the former domain known to adjust its position on nucleotide and substrate binding [40]. This polypeptide lies across the top of the active site cavity linking the two domains and for this reason we refer to it as the ‘tether’. In all the ITPK1s of known molecular structure where this polypeptide is resolved it provides residues which help bind and orient ATP and are positioned to interact with a bound substrate molecule. This region is disordered in the crystal structure of *ZmITPK1*, however, sequence alignment indicates an insertion relative to *AtITPK4* at this site (Figure 4). The insertion in the tether polypeptide of *AtITPK1* is predicted to be eight amino acids in length relative to *AtITPK4* and potentially provides residues such as Asn234, glutamates at 236 and 239, and Arg240 to line the active site cleft. The second divergent feature is a unique insertion in *AtITPK4* which folds to form a two-stranded β -sheet and connecting polypeptide loop which we refer to as the ‘tab’ lying on the domain surface (Figure 3). This feature is well conserved in ITPK4-like proteins from Brassicaceae (Supplementary Figure S7) (however, we acknowledge the lack of experimental verification of the substrate specificity of the

Table 2 Data collection and refinement statistics^a

DATA COLLECTION	
Wavelength/Å	0.9795
Resolution range	44.17–1.91 (1.98–1.91)
Space group	P 2 ₁ 2 ₁ 2 ₁
Unit cell	45.62 61.8 176.47 90 90 90
Total reflections	519 292 (50 938)
Unique reflections	39 633 (3892)
Multiplicity	13.1 (13.1)
Completeness (%)	99.90 (99.67)
Mean I/sigma(I)	10.14 (0.87)
Wilson B-factor	33.32
R_{merge}	0.1853 (2.965)
R_{meas}	0.1928 (3.084)
R_{pim}	0.05281 (0.8422)
$CC_{1/2}$	0.998 (0.376)
CC^*	1.0 (0.739)
REFINEMENT	
Reflections used in refinement	39 622 (3887)
Reflections used for R_{free}	1952 (185)
R_{work}	0.2097 (0.3150)
R_{free}	0.2409 (0.3190)
$CC(\text{work})$	0.957 (0.683)
$CC(\text{free})$	0.933 (0.613)
Number of non-hydrogen atoms	3981
Macromolecules	3733
Ligands	43
Solvent	217
Protein residues	479
RMS(bonds)	0.009
RMS(angles)	1.15
Ramachandran favoured (%)	94.90
Ramachandran allowed (%)	4.03
Ramachandran outliers (%)	1.06
Rotamer outliers (%)	2.38
Clashscore	18.89
Average B-factor	48.44
Macromolecules	48.93
Ligands	26.70
Solvent	43.13
Number of TLS groups	4

^aStatistics for the highest-resolution shell are shown in parentheses.

homologues we identified). Compared with the ITPK1s, it reorganizes one end of the active site cleft and introduces a highly conserved salt bridge between Asp211 and Arg165, the latter of these residues pointing towards the catalytic centre. Both the tab and tether insertions help shape the active site cleft and contribute residues to

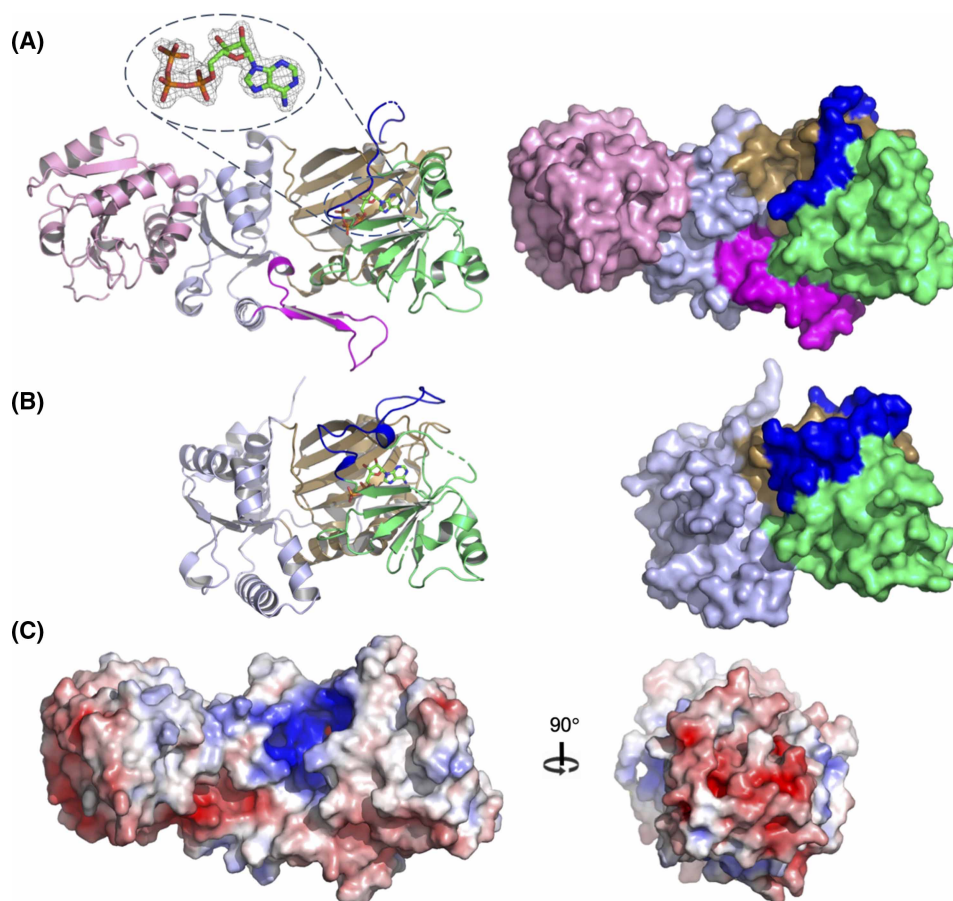


Figure 3. An overview of the crystal structure of AtITPK4.

(A) Left panel, cartoon representation of structure of AtITPK4 coloured by domain: HAD domain (pink), kinase N-terminal domain (light blue), kinase central domain (lime green) and kinase C-terminal domain (sand). The residues of the tab insertion in AtITPK4 are coloured magenta while those of the tether are shown in blue. Broken lines in the backbone trace indicate residues unresolved in the model due to disorder. The sidechains of residues forming the unique ion pair in AtITPK4 are shown in stick format with interactions indicated by dotted black lines. Bound ATP is shown in stick format with atom colouring as follows: carbon-green, oxygen-red, nitrogen-blue and phosphorus-orange. Inset: the $2F_o - F_c$ electron density map (grey mesh) in the region of ATP is contoured at 1.5σ . Right panel, molecular surface representation of the structure of AtITPK4 coloured by domain and insertion. (B) Left panel, cartoon representation of the structure of human ITPK1 (HsITPK1). Bound ADP is shown in stick format. The orientation of view, and atom and domain colouring follows that shown in panel (a). Right panel, molecular surface representation of the HsITPK1 structure coloured by domain and insertion. (C) Left panel, a view of the molecular surface of AtITPK4 coloured by electrostatic potential (red-acidic, blue-basic). The orientation of the molecule is the same as that in panel (A). Right panel, view of the electrostatic potential of the HAD-like domain. The view represents a 90° rotation to that shown the left panel such that the HAD-like domain is viewed head on.

substrate specificity pockets, suggesting they may contribute to differential substrate recognition. The net result of these differences is that AtITPK4 has an active site which is more open than that of *Eh*ITPK1, and particularly so compared with the very enclosed pocket of *Hs*ITPK1 (Supplementary Figure S8). The narrow active site in the predicted structure of AtITPK1 continues this trend.

Molecular modelling identifies substrate specificity pockets and highlights roles of tab and tether insertions in determining hydroxy-kinase specificity

The structures of substrates and products of AtITPK4 are shown (Supplementary Figure S2). The crystal structure of AtITPK4 lacks a bound inositide substrate and so attempts were made through molecular modelling to

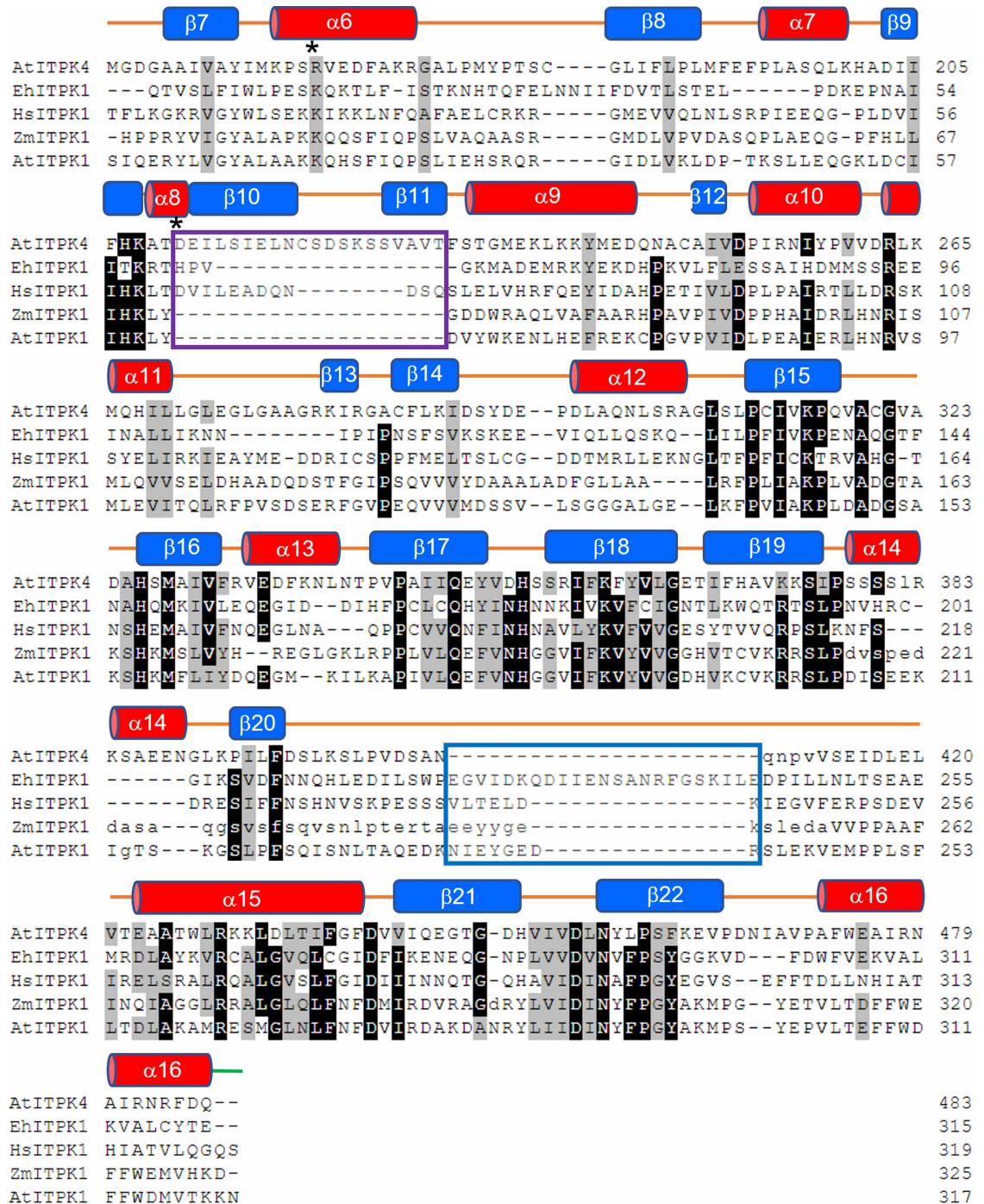


Figure 4. Structure-based sequence alignment of ITPKs.

AtITPK4- *Arabidopsis thaliana* ITPK4 (PDB entry 7PUP), *EhITPK1*- *Entamoeba histolytica* ITPK1 (PDB entry 1Z2P), *HsITPK1*- *Homo sapiens* ITPK1 (PDB entry 2QB5), *ZmITPK1*- *Zea mays* ITPK1 (PDB entry 7ZN5), *AtITPK1*- *Arabidopsis thaliana* ITPK1 (AlphaFold Protein Structure Database entry Q9SBA5). Residue single letter codes shown in lower case for residues unresolved in structures of *AtITPK4* and *ZmITPK1*. Note that the alignment of the unresolved residues in *ZmITPK1* follows that predicted by the corresponding AlphaFold model. Residues with at least 80% homology are shown with black backgrounds, while those conserved by residue class in at least four of the five sequences are shown with light grey backgrounds. Secondary structural elements of *AtITPK4* are indicated by red cylinders (α-helices) or by blue boxes (β-strands) and labelled. The regions of the tab and tether insertions are enclosed in magenta and blue boxes, respectively. Residues forming the unique active site ion pair in *AtITPK4* are indicated by asterisks (*).

predict the likely residue composition of specificity pockets. By so doing it was hoped to provide insights into the possible roles of the tab and tether insertions on substrate specificity. Consequently, models of the complexes formed by *At*ITPK4 with the inositol trisphosphates $\text{Ins}(1,4,6)\text{P}_3$ or $\text{Ins}(3,4,6)\text{P}_3$ (Figure 5) and with the tetraphosphates $\text{Ins}(1,4,5,6)\text{P}_4$ or $\text{Ins}(3,4,5,6)\text{P}_4$ (Supplementary Figure S9) were constructed and relaxed by energy minimization. Critically, these potential substrates were manually docked to the active site of *At*ITPK4 and oriented and positioned for stereochemically favoured hydroxy-kinase action at the C3 or C1 hydroxyl of the substrate, as appropriate [41]. The consequence of this constraint is that, if the two faces of the inositol ring of the substrate are termed obverse and reverse (where the obverse face is viewed looking down onto the unique axial phosphate at the 2-carbon position), then for enzymatic phosphorylation at the 1-, 3- or 5-hydroxyl

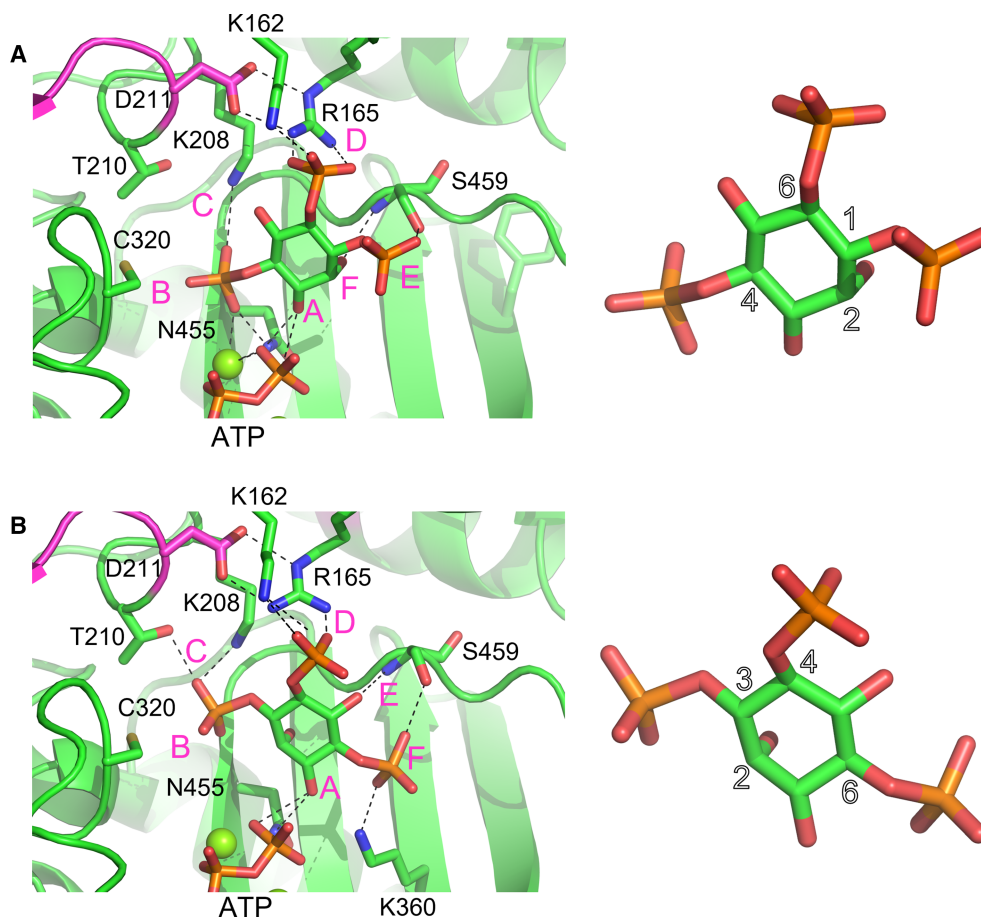


Figure 5. Prediction of the binding modes of the enantiomeric substrate pair $\text{Ins}(1,4,6)\text{P}_3$ and $\text{Ins}(3,4,6)\text{P}_3$ to the active site of *At*ITPK4.

(A) Left panel, Close-up view of the energy minimized predicted binding mode of the good substrate, $\text{Ins}(1,4,6)\text{P}_3$, in the kinase domain active site. Enzyme shown in cartoon format and coloured green except for the residues of the tab insertion which are coloured magenta. The substrate and active site residues (labelled) with which it forms polar interactions are shown in stick format with carbon coloured green, oxygen red, nitrogen blue and phosphorus orange. Polar interactions are indicated by dashed lines. Specificity subsites are labelled A-F such that the hydroxyl group positioned to accept the γ -phosphate of ATP by in-line transfer (the hydroxyl attached to carbon 3 of the inositol ring, in this case) occupies subsite A and the remaining subsites are arrayed in a clockwise sense when observed from the viewpoint adopted in this figure. Right panel, close-up of the docked $\text{Ins}(1,4,6)\text{P}_3$ substrate with a selection of the carbon atom positions of the inositol ring numbered. (B) Left panel, View of the energy minimized predicted binding mode of the poor substrate, $\text{Ins}(3,4,6)\text{P}_3$, in the kinase domain active site. The hydroxyl (attached to carbon 1 of the inositol ring, in this case) occupies subsite A. Display format and colouring as in panel (A). Right panel, closeup of the docked $\text{Ins}(3,4,6)\text{P}_3$ substrate with a selection of the carbon atom positions of the inositol ring numbered.

positions of the ring, the reverse face of the ring should be oriented towards an observer sitting on the γ -phosphate of the ATP coenzyme. Conversely, the obverse orientation will be favoured for phosphorylation at the 4- or 6-hydroxyl positions and indeed this is what is observed in the complex of *Eh*ITPK1 with Ins(1,3,4)P₃ and the non-hydrolyzable ATP analogue, AMP-PCP [34]. While the resulting molecular models of the complexes are speculative and should be interpreted with caution, they do at least suggest features of the active site of *At*ITPK4 which may help provide a molecular context for the observed substrate preference of the enzyme.

To describe best the predicted differential interactions of the enzyme with enantiomeric substrate pairs, we adopt the specificity subsite nomenclature of Miller et al. [34]. In this scheme, subsite A is the site of phosphoryl transfer and constitutes the catalytic centre. From a vantage point positioned on the γ -phosphate of ATP looking at the reverse face of the substrate, specificity subsites available to bind phosphates attached at the inositol ring carbon positions are then labelled B-F in a clockwise fashion, following the order of increasing carbon number of the *myo*-inositol ring (Figure 5 and Supplementary Figure S9). The residues contributing to the specificity subsites in the relaxed models of the inositol tris- and tetrakisphosphates with *At*ITPK4 are summarized in (Supplementary Table S1). Residues of the N-terminal domain tab insertion contribute principally to specificity pockets C and D. Conserved in ITPK4-like sequences, residue Cys320 in the central domain contributes to pocket B. This residue is replaced by an aspartate in *Zm*ITPK1 and *At*ITPK1. On the other hand, specificity pockets E and F are underpopulated in ITPK4s due to the absence of the insertion in the tether polypeptide. The tether insertion in the central domain of ITPK1s contributes residues to specificity pockets E and F, as seen, for example, in the structures of the complexes of *Eh*ITPK1 with Ins(1,3,4)P₃ and Ins(1,3,4,6)P₄ (PDB entries 1Z2P and 1Z2O, respectively). In plant ITPK1 an asparagine residue (Asn280 in *Zm*ITPK1 and Asn271 in *At*ITPK1) contributes to the F-pocket. This residue becomes a glycine in ITPK4s (Gly437 in *At*ITPK4). Potential poses that would allow phosphorylation of Ins(1,3,4)P₃ on the 5-hydroxyl and Ins(3,4,5)P₃ on the 6-hydroxyl (Table 1) are illustrated (Supplementary Figure S10), with the latter requiring obverse orientation of the substrate.

The predicted productive binding poses (i.e. required for hydroxy-kinase activity) of the poor substrates Ins(3,4,6)P₃ and Ins(3,4,5,6)P₄ with *At*ITPK4 notably lack a phosphate in the B-subsite. Indeed, phosphorylation may be disfavoured by the poorer solvation of the axial 2-hydroxyl group of the substrate found in this pocket. Conversely, the 4-phosphate in this pocket as found in the predicted complexes with the better substrates Ins(1,4,6)P₃ (Figure 5) and Ins(1,4,5,6)P₄ (Supplementary Figure S9) is predicted to make a hydrogen bond with the thiol group of Cys320 and also coordinate a magnesium ion. If accurately predicted, these interactions are likely crucial for specific hydroxy-kinase activity by *At*ITPK4. The arguments we have used to predict the docking poses of potential inositol phosphate substrates to *At*ITPK4 will also be applicable to *At*ITPK1. It follows that the predicted docking poses of Ins(3,4,6)P₃ and Ins(3,4,5,6)P₄ to *At*ITPK1 will result in the occupation of the F-subsite by the 6-phosphate of the substrate. In *Zm*ITPK1s this phosphate is predicted to interact with the sidechain of an Asn280. The presence of Gly437 at this site in *At*ITPK4 and the absence of the tether insertion may help to explain the poor activity of this enzyme towards these potential substrates.

***At*ITPK4 does not phosphorylate Ins(1,2,3,4,5)P₅**

It is tempting to compare the crystal structures of InsP₆-coordinated *Zm*ITPK1 H192A variant (PDB entry 7TN8) and ATP-coordinated *At*ITPK4 (this study; PDB entry 7PUP) to investigate the documented lack of InsP₆-kinase activity of *At*ITPK4 [1,8]. However, the usefulness of the comparison is additionally limited by the paucity of information relating to the inositol phosphate hydroxy-kinase activity of *Zm*ITPK1 [33]. Nevertheless, we note that among substrates tested in the first report of this enzyme, these did not include InsP₆ or enantiomeric pairs, Ins(3,4,5,6)P₄ was the best substrate [42]. The product of *Zm*ITPK1-catalyzed phosphorylation of InsP₆ is reported to be 5-InsP₇ by virtue of it being a substrate for DIPP1 (Figure 1 [33]). We note, however, that the DIPP1-coupled phosphate release ‘pyrophosphatase’ assay indicated phosphorylation of all six InsP₅s, while a specific 3-pyrophosphorylation of Ins(1,2,3,4,5)P₅ was reported. Set against this remarkably promiscuous apparent pyrophosphate-synthesizing ability of *Zm*ITPK1, the inability of *At*ITPK4 to phosphorylate InsP₅ isomers is a significant departure, potentially explained by the replacement of the 5-phosphate-coordinating asparagine residue (Asn280) of *Zm*ITPK1 with a glycine residue (Gly437) in *At*ITPK4. The remaining 5-phosphate-coordinating residues in the *Zm*ITPK1 structure, Lys198, Tyr200 and Lys306 are conserved in *At*ITPK4. However, without structures for Ins(1,2,3,4,5)P₅-coordinated *Zm*ITPK1 or *At*ITPK4, the explanation of apparent different pyrophosphate products awaits further structural studies.

AtITPK4 shares the reversibility of phosphotransferase activity of ITPK1

The absence of coordinated nucleotide in crystal structures of plant ITPKs compromises molecular interpretation of the broad range of phosphotransfer catalyzed by this family of enzymes. Nonetheless, we tested the ability of AtITPK4 to execute phosphotransfer to ADP using the HPLC-based assay of Whitfield et al. [9]. Dual UV detection at 290 nm and 254 nm detects inositol phosphate (as complex with post-column ferric ion) at 290 nm and simultaneously detects nucleotide at 254 nm. HPLC analysis of products of assays in which AtITPK4 was incubated with inositol phosphate substrate and ADP overnight showed that AtITPK4 most efficiently uses Ins(1,3,4,6)P₄ (the product of its preferred kinase substrate) in a phosphotransferase reaction to ADP (Table 1). An ADP-dependent phosphatase activity against Ins(1,3,4,5,6)P₅ was reported for ZmITPK1 [33], but neither a nucleotide nor specific InsP₄ product were identified. Nonetheless, the precedent of earlier work on HsITPK1 [43], AtITPK1 and other plant ITPKs [25,26,28,29] make it likely that the ZmITPK1 product is Ins(3,4,5,6)P₄. AtITPK4 also showed ATP-generating phosphotransferase activity with Ins(1,3,4)P₃, Ins(1,4,6)P₃, Ins(1,4,5,6)P₄ and Ins(1,3,4,6)P₄ (Table 1). For the latter substrate, it is unlikely that the product is a racemic mixture of Ins(1,4,6)P₃/Ins(3,4,6)P₃ (the two are unresolvable) but rather a mixture of unequal amounts — because in the forward direction the Ins(1,4,6)P₃ enantiomer is preferred.

Nucleotide binding in the plant ITPK family

The reversal of enantiospecificity for inositol phosphate hydroxy-kinase activities of AtITPK4 (this study) compared with AtITPK1 [9] led us to revisit potential phosphotransfer (including phosphatase) reactions — here with InsP₃ and InsP₄ substrates. AtITPK4 shows much lower inositol phosphate hydroxy-kinase activity (several orders of magnitude) than AtITPK1, like AtITPK1 [9] it shows phosphotransfer to ADP (Table 1) and in the absence of inositol phosphate AtITPK4 has lower phosphatase activity than AtITPK1 (Supplementary Figure S11). These reactions are afforded a structural perspective through our crystal structure of ATP-coordinated AtITPK4. Unlike in the structure of ZmITPK1, the nucleotide-binding region of the ATP Grasp fold of AtITPK4 closely resembles that seen in HsITPK1 and EhITPK1 and is formed from two 4-stranded antiparallel β -sheets. The equivalent pattern in ZmITPK1 is four plus three, where the edge strand of the sheet in the central domain is unresolved. This missing strand is found as β 20 in AtITPK4 (Figure 4) and corresponds spatially to β 12 in HsITPK1 and EhITPK1. Residues of the polypeptide following β 20 (or its equivalent) form specific interactions with bound ATP in AtITPK4 (Supplementary Figure S12) and in the human and entamoeba ITPKs. The presence of disordered residues in ZmITPK1, particularly the subset equivalent to those coordinating ATP in AtITPK4, is consistent with the absence of bound nucleotide in the structure of the maize enzyme. The authors [33] speculate that the density-lacking residues of ZmITPK1 constitute a hinge and catalytic specificity element that somehow optimizes InsP₆ kinase activity. While the equivalent of ZmITPK1 His192, first described as nucleotide coordinating residue in EhITPK1 and HsITPK1 [34], also contacts nucleotide in AtITPK4, in H192A-mutated ZmITPK1 the mutated residue and its neighbours adopt poses facing away from the nucleotide as it is bound in AtITPK4.

A haloacid dehydrogenase (HAD)-like N-terminal domain of the ITPK4 family

Among the ITPK family in Arabidopsis, AtITPK4 is the only member with an N-terminal domain of ~150 residues preceding the kinase domain. Searches of the PDB revealed structural similarity of this domain to a broad range of haloacid dehydrogenase superfamily (HAD) proteins. A family of phosphohydrolases found in all organisms, HAD domain-containing proteins act on a broad variety of metabolites including nucleotides, sugars and phosphorylated amino acids. The function of this domain in AtITPK4 is unknown. Using as query the full-length amino acid sequence of AtITPK4, a search against non-redundant sequences revealed this domain to be found only in ITPK4 proteins from plants. That this domain is present in a broad range of species including soybean, oilseed rape and castor bean (Supplementary Figure S13) suggests that it may have a regulatory or catalytic function.

The AtITPK4 HAD-like domain is formed from a 3-layered α/β sandwich with repeating β - α units adopting the Rossmannoid topology characteristic of the HAD superfamily (HADSF) [44]. While the minimal canonical HAD domain has a parallel central β -sheet of five strands in the order 54123, the parallel sheet of AtITPK4 has a sixth peripheral β -strand in order 654123 which we refer to as S1–S6 (Supplementary Figure S5). HADSF proteins characteristically harbour the so-called squiggle and flap structural signatures that allow the enzyme to adopt distinct conformational states and that contribute to substrate specificity. The squiggle comprises a

nearly complete single helical turn immediately following S1, whilst the flap is formed from a β -hairpin turn downstream of the squiggle and is often, but not exclusively, formed by two strands projecting from the core of the domain. The *AtITPK4* HAD-like domain possesses the first of these but in place of the β -hairpin flap an unstructured 14 residue loop (residues 12–25 inclusive) is found placing it in the C0 family of capless HAD domains. The active site of HADSF enzymes is partly covered by the β -hairpin flap occurring after S1. Additional inserts occurring between the two strands of the flap or in the region immediately after S3 provide extensive shielding for the catalytic cavity. These inserts, termed caps, often contribute residues required for specificity or auxiliary catalytic functions and play a central role in the reactions catalyzed by most HAD hydrolases e.g. [44]. Although the β -hairpin flap feature which is responsible for substrate selectivity is absent from *AtITPK4*, the loop in this position (residues 15–22) is flexible and possibly able to fold over the active site to form a lid [44].

Despite possessing the appropriate structural characteristics of the superfamily, some sequence elements considered essential for HAD domain function are absent from *AtITPK4* (Supplementary Figure S14). The catalytic core residues are highly conserved in HADSF proteins and found in four signature motifs (Motifs I–IV) in the amino acid sequence [45]. These motifs are spatially arranged around a single binding cleft at the C-terminal end of the strands of the central sheet and form the active site of HAD superfamily enzymes. Found at the C-terminal end of strand S1, the first aspartate residue of Motif I (DxD) is required as a catalytic nucleophile. The carboxylate group of this aspartate and the backbone carbonyl of the second coordinate an Mg^{2+} cofactor. However, despite the protein being crystallized from a solution containing 100 mM $MgCl_2$ no evidence for a magnesium ion was identified in electron density maps. In *AtITPK4* this motif is replaced with the sequence DES where the aspartate and serine residues are well-conserved across ITPK4-like sequences. The absence of the second aspartate from the consensus motif sequence may not in itself preclude enzymatic activity as *DehRhb*, the L-haloacid dehalogenase gene from a marine member of the Rhodobacteraceae, is a functional HAD that possesses only the first aspartate residue in Motif I [46]. Motif II is found at the end of the S2 strand and contains a conserved serine or threonine residue which helps to orient the substrate for nucleophilic attack by forming a hydrogen bond with its transferring phosphoryl group. While a serine is found in *AtITPK4* at position –1 relative to the consensus, its sidechain points in the other direction and the residue is not conserved in close homologues. Motif III centres on a conserved lysine residue found on the loop following S3 or on the helix to which this loop is connected. The function of this lysine is to stabilize the negative charge of the reaction intermediate together with Ser/Thr of motif II, however, no lysine or other basic residue is found either in this loop or on the following helix in *AtITPK4*. Finally, Motif IV maps to the loop immediately following strand S4 and contains acidic residues typically exhibiting the signature (G/S)(D/S)x3-4(D/E) (where x is any amino acid). Together with the aspartate residues of motif I, the conserved motif IV acidic Asp or Glu residues are involved in the coordination of Mg^{2+} in HADSF family members. In *AtITPK4* the corresponding sequence is ASSRKEE but neither of the glutamate residues are oriented appropriately to serve this function, albeit we note that the quality of the electron density in this region is relatively poor. Y95, presented from strand S4, is a possible alternative, as demonstrated in the Arabidopsis VSP1 HAD domain [47].

Searches for a catalytic activity of the HAD-like N-terminal domain of *AtITPK4*

Taken together, the preceding evidence suggests that the HAD domain in *AtITPK4* is atypical. Similarity searches using DALI [48] and the coordinates of the HAD domain as query revealed a range of structural homologues. Unsurprisingly, functionally-characterized enzymes with the highest Z-scores included dehalogenases e.g. an L-2-haloacid dehalogenase (1JUD; $Z = 12.6$) [49]. Hits were retrieved also to HAD domain proteins with verified or inferred phosphatase or phosphomutase activities e.g. a bifunctional epoxide hydrolase (4HAI; $Z = 10.5$) [50] where the HAD domain displays lipid phosphate phosphatase activity, a β -phosphoglucosyltransferase (4UW9; $Z = 10.2$) [51] and a phosphoserine phosphatase (5JLP, $Z = 10.1$) [52]. To test for catalytic activity, we sought to express and purify the separate N-terminal and C-terminal domains of *AtITPK4*. However, these efforts were unsuccessful, producing only insoluble protein. Therefore, activity was probed in the full-length enzyme alongside the close homologue, *AtITPK1*, which lacks the HAD-like domain, as a control. Tests for dehalogenase activity used chloropropionic acid revealed no activity for either protein. To determine whether the *AtITPK4* enzyme had enzymatic properties typical of a HAD phosphatase, the enzymes were incubated with ATP, PNPP or G6P and phosphate release measured. *AtITPK1* showed much greater phosphate release from ATP in the absence of inositol phosphate substrate, but neither enzyme released phosphate from G6P or PNPP in standard assay conditions for HAD proteins (Supplementary Figure S11A).

In the presence of inositol substrate both enzymes stoichiometrically transferred phosphate from ATP to inositol (Supplementary Figure S11B,C).

Discussion

Phylogenetic analysis identifies ITPK4 to be an outlier to the ITPK family in plants. Its possession of a HAD-like domain sets it apart from all other inositol phosphate kinases of IPK1, IP3-3K, ITPK, IPMK (IPK2) and VIH/PP5K classes. Whether the *AtITPK4* HAD-like domain is functional is yet to be determined, but a lack of catalytic activity does not rule out other modulating functions, for example metabolic sensors are known to involve catalytically inactive enzymes through transcriptional activation [53]. There are few examples in the literature involving the presence of a HAD domain in addition to another functional active site. The mammalian soluble epoxide hydrolase (sEH) detoxification enzyme C-terminal region contains the catalytic region responsible for its characterized activity. Also present is an N-terminal HAD phosphatase, not conserved in the plant homologue, which acts independently of the C-terminal EH activity [54]. The authors speculate that this HAD domain synergistically regulates the physiological process, perhaps by acting as a serine phosphatase to down-regulate an opposing pathway.

What is clear, however, is that *itpk4* mutants that are blocked in InsP_6 , InsP_7 and InsP_8 synthesis lack PSR [17]. We may assume, therefore, that the activities of neither the ATP-Grasp nor HAD-like domains of *AtITPK4* are essential for PSR, whereas the ATP-Grasp kinase activity of *AtITPK1* is. Without a much better understanding of the inositol phosphate profile of Arabidopsis, particularly the discrimination of enantiomers, it is difficult to be categorical about the role of individual inositol phosphate species. This applies as much to inositol pyrophosphates as it does inositol phosphates. Nevertheless, we remain intrigued that the two gene families with undisputed influence on PSR (*Itpk1* and *Ipk1*) both accumulate $\text{Ins}(1,4,5,6)\text{P}_4$ and/or $\text{Ins}(3,4,5,6)\text{P}_4$, that the latter enantiomer is predominant in *ipk1* [2] and, moreover, that *AtITPK1* favours $\text{Ins}(3,4,5,6)\text{P}_4$ as substrate, massively over InsP_6 . In contrast, *AtITPK4* has a fraction of the enzyme activity of *AtITPK1*, lacks InsP_6 kinase activity [8] and this study, shows an opposite enantiospecificity to the $\text{Ins}(1,4,5,6)\text{P}_4/\text{Ins}(3,4,5,6)\text{P}_4$ pair, but mutants thereof have pronounced effect on InsP_6 , InsP_7 and InsP_8 levels without effect on PSR [6,17]. It is tempting to speculate again [9] that $\text{Ins}(3,4,5,6)\text{P}_4$ is coupled metabolically through *AtIPK1* to control PSR. Whether inositol pyrophosphates and the VIH enzymes that make InsP_8 [3,7] are the exclusive agents of PSR is coming under increased scrutiny [15], since as reported by others [6] *vih* mutants do not necessarily show PSR.

These data point to distinct roles of *AtITPK1* and *AtITPK4*, reflected also in substantive differences in kinetic parameters. *AtITPK1* K_M displays ATP in the range 0.52–1.22 mM [1,9], while *AtITPK4* displays K_M for ATP of 0.052 mM (this study). Even in phosphate-deprived plants, it seems likely that ITPK4 is saturated with ATP — since phosphate starvation only reduces adenine nucleotide levels by a factor of two [1]. The levels of adenine nucleotides are quite poorly described in plants, though the work of Straube and coworkers [55] estimates ATP levels at 750 μM ($\sim 120 \text{ nmol g}^{-1}$, f. wt) in rosette leaves of 33 d-old Arabidopsis, an order of magnitude greater than that reported for hydroponic grown Arabidopsis [1]. An exhaustive study of metabolite levels in eight *Flaveria* species varying in C3 to C4 photosynthetic character measured ATP between 71 and 154 nmol g^{-1} f. wt [56]. These data suggest that *AtITPK4* activity is metabolically isolated, protected from excursions in nucleotide levels that might be expected to influence inositol pyrophosphate levels through effect on ITPK1 and IPK1 and VIH2 [1,31,33] and VIH1/2 [4,10]. Put another way, much of cellular InsP_8 synthesis, that arising from *AtITPK4* activity, is likely protected from excursions in energy charge. One counter argument requires compartmentation of inositol phosphate and inositol pyrophosphate synthesis, as invoked [1] to explain the altered inositol phosphate and inositol pyrophosphate profile of mutants of the InsP_6 transporter *mrp5* [57]. But, again, *mrp5* does not show PSR [1,6]. We speculate that ITPK4 retains its function in nucleotide-compromising physiological situations.

Whatever the contribution of ITPK4 to plant physiology, it is ancestral. Both ITPK1 and ITPK4 have orthologs in ancestral aquatic vascular plants. One estimate for the stem age of Lemnaceae is ca. 103.6 Ma [58]. ITPK1 and ITPK4 also have homologues in the liverwort *Marchantia polymorpha*, an ancestral terrestrial plant [59]. One estimate places divergence of the crown group (of *Marchantia polymorpha*) comprising the Ricciaceae and Oxymitracae at ca. 115 Ma [60]. Study of the duckweed taxa identified pathways of InsP_6 synthesis from inositol that are lipid-independent [22,23,61], proceeding from inositol and/or (via) Ins3P and $\text{Ins}(3,4,5,6)\text{P}_4$, the preferred substrate of ITPK1 [9]. Others have shown that ITPK1 enzymes accept inositol monophosphates as substrates [62]. As we have shown, ITPK4 shows opposite enantiospecificity in choice of InsP_3

and InsP₄ substrates compared with ITPK1. Since ITPK4 lacks activity against inositol monophosphate, it seems likely that the contribution of ITPK4 to InsP₆ and inositol pyrophosphate synthesis is epistatic to inositol monophosphate production.

Methods

Reagents

Inositol phosphates and assay reagents for kinase assays were obtained from commercial sources described [9], or, for Ins(1,4,6)P₃/Ins(3,4,6)P₃ and Ins(1,4,5,6)P₄/Ins(3,4,5,6)P₄ enantiomeric pairs, as described (Supplementary Figures S2, S3).

Protein purification

AtITPK4 was cloned essentially as described for AtITPK1 [9] (except that a pOPINE rather than pOPINF plasmid was employed. The primers used were 5'-AGGAGATATACCATGAAAGGGGTTCTACTTGGACGA-3' and 5'-GTGATGGTGATGTTTATGCTTCTCTTGGACAT-3' (where italics denote the pOPINE specific sequence required for recombination). Purification was also carried out according to the previously published protocol [9] but without 3C cleavage and additional Ni-NTA affinity purification steps since the C-terminal 6 × His tag is not cleavable in pOPINE constructs.

X-ray crystal structure determination

Purified AtITPK4 was concentrated to 10 mg/ml and single crystals grown using the sitting drop vapour diffusion method by equilibration at 16°C against a crystallization solution containing 20% PEG 4000, 0.1 M MgCl₂, 0.1 M Tris.HCl pH 7.8. A single crystal was harvested into a cryoprotect solution containing 30% (v/v) ethylene glycol and X-ray diffraction data collected at 100°C on beamline I04 at the Diamond Light Source (Oxford). Molecular replacement phasing was performed with Phaser [63] and the crystal structure of *Entamoeba histolytica* inositol 1,3,4-trisphosphate 5/6-kinase in complex with ADP and Mg²⁺ (PDB entry 1Z2N) [34] as a search model. Extensive manual rebuilding using Coot [64] interspersed with restrained refinement with Phenix.refine [63,65,66] as necessary to complete the final structure including the 150 amino acid N-terminal domain not present in the molecular replacement search model. Refinement employed a TLS model generated with the TLSMD web server [67].

Protein structure and sequence analysis

The predicted structure model for AtITPK1 was taken from the AlphaFold Protein Structure Database (AlphaFold DB, <https://alphafold.ebi.ac.uk>) [38,39]. Structure-based sequence alignments were calculated using POSA (Partial Order Structure Alignment) [68,69]. Other sequence alignments were performed using Cobalt [70]. Identification of ITPK4-like sequences from the UNIREF90 database, their alignment and subsequent derivation of estimates of amino acid conservation were carried out using CONSURF [71]. The list of homologues was manually edited to retain only those which spanned at least 95% of the amino acid sequence of AtITPK4 and for which the expectation value (E-value) was less than or equal to 1×10^{-130} . A total of 95 such sequences were retained. Position-specific conservation scores were then computed using a Bayesian algorithm [72] and these scores were divided into a discrete scale of nine grades for visualization.

Molecular modelling

Molecular models of the complex between inositol tris- and tetrakisphosphates with AtITPK4 were generated with reference to the crystal structures of EhITPK1 in complex with Mg²⁺/AMP-PCP/Ins(1,3,4)P₃ (PDB entry 1Z2P) and with Mg²⁺/ADP/Ins(1,3,4,6)P₄ (PDB entry 1Z2O), respectively. Least squares superposition of the β-sheet residues of the C-terminal domains of EhITPK1 from PDB entry 1Z2P and AtITPK4 produced, by direct transfer of atomic coordinates, a draft docking pose of Ins(1,3,4)P₃ along with ATP and two magnesium ions to the AtITPK4 crystal structure. Subsequent *in silico* substitution of phosphate groups of the inositol polyphosphate ligand generated models of, in turn, Ins(1,4,6)P₃ or Ins(3,4,6)P₃. Rotation of the ligands 180° about the C2-C5 axis (where C_n indicates the carbon number, n, of the inositol ring) followed by rotation about an axis normal to the ring produced models of Ins(1,4,6)P₃ or Ins(3,4,6)P₃ bound to AtITPK4 positioned for stereochemically-favoured hydroxy-kinase action at the C3 or C1 hydroxyl of the substrate, respectively [37]. A similar process involving PDB entry 1Z2O produced models of Ins(1,4,5,6)P₄ or Ins(3,4,5,6)P₄ along with ATP

and magnesium ions docked to *AtITPK4*. All models of the complexes were subsequently energy minimized to convergence using the Prime module of Schrodinger (Schrödinger Release 2022-2: Prime, Schrödinger, LLC, New York, NY, 2021) employing the all-atom OPLS force field [73,74]. Conserved water molecules identified in high resolution crystal structures have been frequently linked to enzyme function [75,76] but their positions are unreliable at the resolution of the crystal structure of *AtITPK4* and so they were discarded and the VSGB 2.1 implicit solvent model [77] employed. Illustrations were generated using the PyMOL Molecular Graphics System, Version 2.5 (Schrödinger, LLC).

HPLC assays

Both Standard and ATP Regeneration assays were performed in 20 mM HEPES pH 7.3, 6 mM $MgCl_2$, 10 mM $LiCl_2$ and 1 mM DTT. Regeneration assays contained 5 mM phosphocreatine, 3 U creatine kinase and 1 mM ATP [9]. To identify substrates of *AtITPK4*, regeneration assays were incubated overnight with 3 μM *AtITPK4*. Specifically, for kinetic comparisons of $Ins(1,4,6)P_3$, $Ins(3,4,6)P_3$ and $Ins(1,4)P_2$, regeneration assays were set up as described using 3 μM *AtITPK4* and incubated for 20 min at 25°C. For determination of K_M and V_{max} , 3 μM *AtITPK4* was incubated with 1 mM $Ins(1,4,6)P_3$ and ATP at 0–5 mM for 1 hour at 25°C. For ATP phosphatase activity assays, 1 mM ATP with or without 1 mM substrate was incubated with 10 μM *AtITPK1* or *AtITPK4* at 25°C for 2 h. HPLC analysis and detection of inositol phosphates by complexation with ferric ion was performed as described [9]. Data were analyzed using GraphPad software (GraphPad Software Inc., San Diego, U.S.A.) with Michaelis–Menten or substrate inhibition fit.

HPLC-ICP-MS

Whole soil-grown plants (triplicates) of Col0, *itpk4-1*, an ITPK4 overexpression line and a single ITPK4-YFP-complemented *itpk4-1* plant (ITPK4-OE) [5] were frozen in LN_2 , ground in a mortar and pestle and extracted with 1 M perchloric acid. The extract was treated with TiO_2 [78], recovered in 300 μl water and aliquots injected onto a CarboPac PA200 column. Inositol phosphates were eluted with a gradient of HCl [9] and detected as PO^+ , m/z 47, using a Thermo Icap-TQ (Thermo Scientific) triple quadrupole Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) used as a HPLC detector.

Phosphate release assays

Performed as described [9] using 1 mM substrate (ATP, PNPP or G6P) and 10 μM *AtITPK4* or *AtITPK1*.

HAD assay with chloropropionic acid

A colorimetric assay was performed as described by Hou et al. [79] with *AtITPK4* concentrations 0 μM , 1 μM , 2 μM and 4 μM . Additionally NaCl, $MgCl_2$ and $LiCl_2$ were tested for effects on activity. Assays were monitored at 540 nm for 1 h and then a single plate read performed after overnight incubation.

Data Availability

Coordinates and diffraction data for the crystal structure of *AtITPK4* in complex with ATP have been deposited in the PDB with accession code 7PUP [80].

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was funded by the BBSRC through IPA award BB/M022978/1. SH was funded by a UEA-Southern University of Science and Technology (SUSTech, Shenzhen, China) Doctoral Training Studentship. This work was funded in part by The Wellcome Trust. BVLP is a Wellcome Trust Senior Investigator (grant 101010).

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Open access for this article was enabled by the participation of University of East Anglia in an all-inclusive Read & Publish agreement with Portland Press and the Biochemical Society under a transformative agreement with JISC. This research was funded in part by The Wellcome Trust. For the purpose of open access the authors have applied a CC BY public copyright licence to any Author-Accepted Manuscript version arising from this submission.

CRedit Author Contribution

Charles A. Brearley: Conceptualization, Formal analysis, Supervision, Funding acquisition, Investigation, Methodology, Writing — original draft, Project administration, Writing — review and editing. **Hayley Whitfield:** Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing. **Sining He:** Investigation. **Yinghong Gu:** Investigation. **Colleen Sprigg:** Investigation. **Hui-Fen Kuo:** Investigation. **Tzyy-Jen Chiou:** Funding acquisition, Project administration. **Andrew M. Riley:** Investigation, Writing — review and editing. **Barry V.L. Potter:** Funding acquisition, Investigation, Supervision, Writing — review and editing. **Andrew M. Hemmings:** Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing.

Acknowledgements

The authors would like to thank Diamond Light Source for beamtime under proposals MX18565 and the staff of beamline i04 for assistance with crystal testing and data collection.

Abbreviations

AMP-PCP, 5'-adenylyl methylenediphosphonate; *AtIPK1*, *Arabidopsis thaliana* inositol pentakisphosphate 2-kinase; *AtITPK1*, *Arabidopsis thaliana* inositol tris/tetrakisphosphate kinase 1; BSTFA, bis-trifluoroacetamide; DTT, reduced dithiothreitol; EDTA, ethylenediamine tetra-acetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; His, histidine; HPLC, high-pressure liquid chromatography; *EhITPK1*, *Entamoeba histolytica* inositol tris/tetrakisphosphate kinase 1; *HsITPK1*, *Homo sapiens* inositol tris/tetrakisphosphate kinase 1; IP3-3K, inositol 1,4,5-trisphosphate 3-kinase; IP6K, inositol hexakisphosphate kinase; IPMK (IPK2), inositol polyphosphate multikinase; Ins(1,3,4)P₃, 1D-*myo*-inositol 1,3,4-trisphosphate; Ins(1,4,6)P₃, 1D-*myo*-inositol 1,4,6-trisphosphate; Ins(3,4,6)P₃, 1D-*myo*-inositol 3,4,6-trisphosphate; Ins(4,5,6)P₃, *myo*-inositol 4,5,6-trisphosphate; Ins(1,2,4,6)P₃, 1D-*myo*-inositol 1,2,4,6-tetrakisphosphate; Ins(1,3,4,6)P₄, *myo*-inositol 1,3,4,6-tetrakisphosphate; Ins(1,4,5,6)P₄, 1D-*myo*-inositol 1,4,5,6-tetrakisphosphate; Ins(1,3,5,6)P₄, 1D-*myo*-inositol 1,3,5,6-tetrakisphosphate; Ins(2,3,4,6)P₄, 1D-*myo*-inositol 2,3,4,6-tetrakisphosphate; Ins(3,4,5,6)P₄, 1D-*myo*-inositol 3,4,5,6-tetrakisphosphate; Ins(1,2,3,4,5)P₅, 1D-*myo*-inositol 1,2,3,4,5-pentakisphosphate; Ins(1,2,3,4,6)P₅, *myo*-inositol 1,2,3,4,6-pentakisphosphate; Ins(1,2,3,5,6)P₅, 1D-*myo*-inositol 1,2,3,5,6-pentakisphosphate; Ins(1,2,4,5,6)P₅, 1D-*myo*-inositol 1,2,4,5,6-pentakisphosphate; Ins(1,3,4,5,6)P₅, *myo*-inositol 1,3,4,5,6-pentakisphosphate; Ins(2,3,5,4,6)P₅, 1D-*myo*-inositol 2,3,4,5,6-pentakisphosphate; Ins(1,2,3,4,5,6)P₆, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate; 3-PP-Ins(1,2,4,5)P₄, 1D-3-diphospho-*myo*-inositol 1,2,4,5-tetrakisphosphate; 5-PP-Ins(1,2,3,4)P₄, 1D-5-diphospho-*myo*-inositol 1,2,3,4-tetrakisphosphate; 1-InsP₇ 1-PP-InsP₅, 1D-1-diphospho-*myo*-inositol 2,3,4,5,6-pentakisphosphate; 3-InsP₇ 3-PP-InsP₅, 1D-3-diphospho-*myo*-inositol 1,2,4,5,6-pentakisphosphate; 4-InsP₇ 4-PP-InsP₅, 1D-4-diphospho-*myo*-inositol 1,3,5,6-pentakisphosphate; 5-InsP₇ 5-PP-InsP₅, 5-diphospho-*myo*-inositol 1,2,3,4,6-pentakisphosphate; 6-InsP₇ 6-PP-InsP₅, 1D-6-diphospho-*myo*-inositol 1,2,3,4,5-pentakisphosphate; 1,5-InsP₈, 1D-1,5-bis-diphospho-*myo*-inositol 2,3,4,6-tetrakisphosphate; LC-ICP-MS, liquid chromatography inductively coupled plasma mass spectrometry; Ni-NTA, nickel-nitriloacetic acid; PCR, polymerase chain reaction; PDB, Protein DataBank; P_i, orthophosphate; PHR1, Arabidopsis Phosphate Starvation Response 1; PPIP5K, diphosphoinositol pentakisphosphate kinase; SPX1, SYG1/Pho81/XPR1 domain-containing protein 1; Tris, tris (hydroxymethyl)aminomethane; VIH1, *Arabidopsis thaliana* diphosphoinositol pentakisphosphate kinase 1; VIH2, *Arabidopsis thaliana* diphosphoinositol pentakisphosphate kinase 2; *ZmITPK1*, *Zea mays* inositol tris/tetrakisphosphate kinase 1.

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