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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

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Abstract The Arabidopsis genome encodes a family of inositol 1,3,4-trisphosphate 5/6-kinases which form a subgroup of a larger group of ATP-grasp fold proteins. An analysis of the inositol 1,3,4-trisphosphate 5/6-kinase family might, ultimately, be best rewarded by detailed comparison of related enzymes in a single genome. The enzyme encoded by At2G43980, AtITPK4; is an outlier to its family. At2G43980 is expressed in male and female organs of young and mature flowers. AtITPK4 differs from other family members in that it does not display inositol 3,4,5,6-tetrakisphosphate 1-kinase activity; rather, it displays inositol 1,4,5,6-tetrakisphosphate and inositol 1,3,4,5-tetrakisphosphate isomerase activity.

Keywords: ATP-grasp fold; Inositol polyphosphate kinase; Arabidopsis

1. Introduction

Among metazoan, plant and animal kingdoms; inositol polyphosphate kinases fall into a number of separate families, one of which, the inositol 1,3,4-trisphosphate 5/6-kinases, typified by the human enzyme hITPK1 [1], (recently reclassified as inositol 3,4,5,6-tetrakisphosphate 1-kinase/inositol 1,3,4,5,6-pentakisphosphate 1-phosphatase [2]), is absent from yeast. From a structural perspective, inositol 1,3,4-trisphosphate 5/6-kinases belong to a larger family of ATP-grasp fold proteins. They also show some functional and structural similarity with inositol polyphosphate multikinases, which include inositol hexakisphosphate kinase and inositol 1,4,5-trisphosphate 3-kinase. The ATP-grasp fold family was first identified in a comparison of D-ala-D-ala ligase (Ddl) and glutathione synthetase (GSHase) [3], which revealed the binding of ATP in a cleft formed between the β sheets of the central and C-terminal domain. Crystallographic studies have been made of human inositol 1,4,5-trisphosphate 3-kinase [4] and of an inositol 1,3,4-trisphosphate 5/6-kinase (EhIPK) from *Entamoeba histolytica* [5].

Within plants, the metabolic intermediates by which inositol hexakisphosphate, $InsP_6$, is synthesized may differ between plant organs and across species. Genetic evidence indicates that homologues of the yeast protein IPK2 (Arg82, ArgRIII; a multikinase), and of yeast IPK1, a family unrelated to the ATP-grasp fold family, contribute to $InsP_6$ synthesis in *Arabidopsis* [6]; while a homologue of hITPK1 contributes to $InsP_6$ synthesis in maize [7]. The Arabidopsis genome encodes a family of genes annotated as inositol 1,3,4-trisphosphate 5/6-kinases showing homology to hITPK1; only one, with GenBank Accession No. AF080173, has been characterized. The recombinant protein possesses inositol 1,3,4-trisphosphate 5/6-kinase activity [8]. We have characterized the remaining members of the family. Our analysis indicates a divergence of function not reported in other kingdoms.

2. Materials and methods

2.1. Bioinformatics

Using a previously identified inositol 1,3,4-trisphosphate 5/6-kinase from Arabidopsis, AF080173 At5G16760 [8], we searched the TAIR genome database at www.arabidopis.org using the WU-BLAST 2.0 program. This identified several candidates including AY050408 which were used as query for further searches. Three cDNAs were cloned from an Arabidopsis cDNA library by PCR; these correspond to genes At4G33770, At4G08170 and At2G43980. The cDNA cloned for At4G33770 encodes an N-terminally truncated protein corresponding to amino acids 44-391 of the current At4G33770.1 annotation of At4G33770, and that cloned for At4G08170 corresponds to the At4G08170.2 annotation of At4G08170. Current annotations identify a potential second, shorter splice variant for each gene, At4G33770.2 and At4G08170.1, respectively.

Alignments of conceptual translations of the full length genes were generated using the Needleman–Wunsch global alignment algorithm with default parameters in ClustalW (www.ebi.ac.uk). Individual pairwise alignments were performed with default parameters of the EMBOSS Pairwise Alignment Algorithms program (www.ebi.ac.uk).

2.2. Cloning

Primers used for cloning are described in the Supplementary Material. Reverse primers encoded a FLAG tag followed by a stop codon. At4G08170 was cloned with BamHI and NotI into pET28c (Novagen, Nottingham, UK). At2G43980 and At4G33770 were cloned using EcoRI and NotI into pET28a. Vector-encoded hexa-histidine (His) tags were added to all proteins.

2.3. At2G43980 promoter::reporter gene construct

A 2.0 kb fragment upstream of the initiation site was amplified by PCR from Arabidopsis DNA. The promoter was amplified using primers: forward, TGCACTGCAGCAAGAACTCCTGTCAAC; internal reverse primer, GTAGCAATGTGAGTGCTTCTGGTTC; internal forward primer, GAACCAGAAGCACTCACATTGCTAC; reverse, CCTAGATCTGAATAAATCAATACACG; in two fragments using an internal BsrD1site and then cloned into pGEM-T easy in a 3-way

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ligation, and sequenced. Subsequently, the promoter fragment was sub-cloned with PstI and BglII into the vector pEGFP (Clontech) and inserted with SalI and BglII into pBIN101 (Clontech, Basingstoke, UK) to obtain a transcriptional fusion of the promoter and the β-glucuronidase coding sequence.

2.4. Arabidopsis transformation

Transgenic plants harbouring the constructs were generated and GUS activity analysed according to Xiao et al. [9].

2.5. Expression analysis of inositol 1,3,4-trisphosphate 5/6-kinases

RT-PCR was performed according to Sweetman et al. [10] with the primers described in the original cloning. The amount of cDNA used in reactions was normalized using ubiquitin primers (Genbank Accession No. NM_105209: forward, GGCTAAGATCCAGGATAAGG; reverse, TCTGGATGTTGTAATCAGCC).

In situ hybridization was performed according to Sweetman et al. [10]. A DIG-labelled probe corresponding to nucleotides 1–160 of At2G43980, was amplified with primers: forward, ATAAAAGGGGTTCTACTT; reverse, CCTTGTTTTCCGGAAGATC.

2.6. Expression of proteins

His-tagged proteins were purified from *Escherichia coli* Rosetta transformed with pET28a and pET28c containing kinase cDNAs according to Sweetman et al. [10]. Coomassie staining of SDS–PAGE gels, and Western blots with an antibody to the T7 tag were performed to confirm successful inframe expression of protein produced from all three cDNAs; all according to Sweetman et al. [10].

2.7. Kinase assays

For TLC analysis, 5 μL (approximately 1–2 μg) of purified protein was assayed in 20 μL volume in 20 mM HEPES, pH 7.5, 6 mM $MgCl_2$, 10 mM LiCl, 0.4 μM ATP, 1 mM DTT with 1 μCi [γ-^{32}P]ATP and 20 μM inositol phosphate substrate, incubated at 30 °C for 2 h. The products were analysed according to Sweetman et al. [10].

2.8. Inositol phosphate standards

[^{3}H]Ins(1,3,4,6)P_4 was prepared by the phosphotransferase activity of human ITPK1 using [^{3}H]Ins(1,3,4,5)P_4 substrate [11]. [^{3}H]Ins(1,3,4,5)P_4 (18 Ci/mmol [740 MBq/mmol]) was obtained from DuPont/NEN (Stevenage, UK). [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,3,4,5,6)P_5, both of unknown specific activity, were prepared according to Brearley and Hanke [12].

2.9. HPLC

Adsorbosphere Strong Anion Exchange columns (Alltech, Carnforth, UK) were eluted with gradients derived from buffer reservoirs containing: A (water) and B (1.25 M $(NH_4)_2HPO_4$), pH 3.8, H_3PO_4, nominally at a gradient of 1 mM min^{-1}. Column fractions (0.2–0.5 mL) were mixed with 2 mL of Optima FloAP (Canberra Packard) scintillation fluid and radioactivity estimated.

3. Results

3.1. Sequence comparisons of inositol phosphate 5/6-kinases

An alignment of the predicted protein sequences for At5G16760, At4G33770, At4G08170 and At2G43980, human (hsITPK1) and *E. histolytica* IPK (EhIPK) enzymes is shown (Fig. 1A) and a phylogram (Fig. 1B). Hereafter, we refer to the proteins encoded by At5G16760, At4G33770, At4G08170 and At2G43980 as AtITPK1, AtITPK2, AtITPK3 and AtITPK4, respectively. Pairwise alignment showed that AtITPK3 and AtITPK2 share 54.4% sequence identity and 69.6% similarity. The phylogenetic analysis places these two proteins in one clade with AtITPK1 as an outlier. In pairwise alignment, AtITPK1 is most similar to AtITPK3 showing 39% identity and 54.1% similarity. AtITPK4 falls in a separate clade. Analysis of enzyme activity of the recombinant proteins (discussed below) distinguishes between AtITPK4 protein and the AtITPK2 and AtITPK3 pair, providing functional validation of our alignment.

AtITPK4 protein is distinguished from the other proteins by an extended N-terminal sequence (39 residues longer than that of AtITPK2). A blast search with amino acids 1–80 of AtITPK4 as query failed to identify significant homology to any proteins of other known functional annotation. In alignment, AtITPK4 has an extra 121 amino acids N-terminal to the start of the Entamoeba enzyme. AtITPK4 shows 14.9% identity and 25.7% similarity to the Entamoeba enzyme in a pairwise alignment.

We have compared residues forming the active site of ATP-grasp fold inositol 1,3,4-trisphosphate 5/6-kinases. The Arabidopsis proteins and AtITPK4 in particular have a few notable substitutions among amino acids which, in the crystal structures of the Entamoeba enzyme-Ins(1,3,4)P_3-5′-adenosyl-methylene-triphosphate (AMPPCP)–Mg^{2+}; and enzyme–Ins(1,3,4,6)P_4–ADP–Mg^{2+} complexes; hydrogen bond or form salt bridges to the 1-, 3-, 4- and 6-phosphates [5]. For the 1-phosphate, AtITPK4 alone replaces Arg192 with Lys373; Ser295 of the Entamoeba enzyme is conserved in AtITPK4, but replaced by a Gly in AtITPK1,2, and -3; while Lys179 is conserved throughout AtITPK1,2,3 and -4. Of the residues coordinating the 3-phosphate of the inositol ring, the Lys17 equivalent is variant among all AtITPKs; while the K57 equivalent is substituted by a His in AtITPK1,2,3 and -4, though we note an adjacent Lys in all. Of the residues coordinating the 4-phosphate, Gln141 is replaced by an Asp conserved in AtITPK1,2 and -3, but replaced by Cys320 in AtITPK4; while His147 and Gly142 are conserved in equivalent positions throughout AtITPK1,2,3 and -4. Considering the coordination of the 6-phosphate of Ins(1,3,4,6)P_4, Entamoeba Lys179 and Asn291 are conserved in equivalent positions throughout AtITPK1,2,3 and -4.

Generally, the hydrophobic residues lining the cofactor adenine ring binding site are identical or replaced by other hydrophobic residues, e.g. AtITPK1,2,3 and -4 substitute Val for Ile171. The magnesium-binding aspartates and asparagine and other fingerprint ATP-grasp fold residues (Asp275, Asp289, Asn291, Arg94, Lys136, and Gln168 of the Entamoeba enzyme) are identical in AtITPK1,2,3 and -4.

3.2. Expression analysis

AtITPK3 and AtITPK2 transcripts were expressed in all tissues examined (Fig. 2). AtITPK4 transcript was relatively weakly expressed in leaf, including cauline leaf, and siliques, but not detected in stem or flowers.

Promoter-glucuronidase reporter fusions were used to investigate further the tissue distribution of AtITPK4 transcripts (Fig. 3). No staining was observed with the wild-type plants. For AtITPK4 transcript-expressing seedlings, staining was intense in roots, leaf vasculature and was also evident in young flower buds and ovules. AtITPK4 transcript expression was not detected by RT-PCR in mature flowers; nevertheless, the expression of transcript in these tissues was confirmed by mRNA in situ analysis.

AtITPK4 transcript is expressed throughout early flower development which culminates [13] in the opening of the flower bud; stage 13. Fig. 3B(iv) shows a flower bud at stage 12 with petals extended and level with the long stamens. Expression of

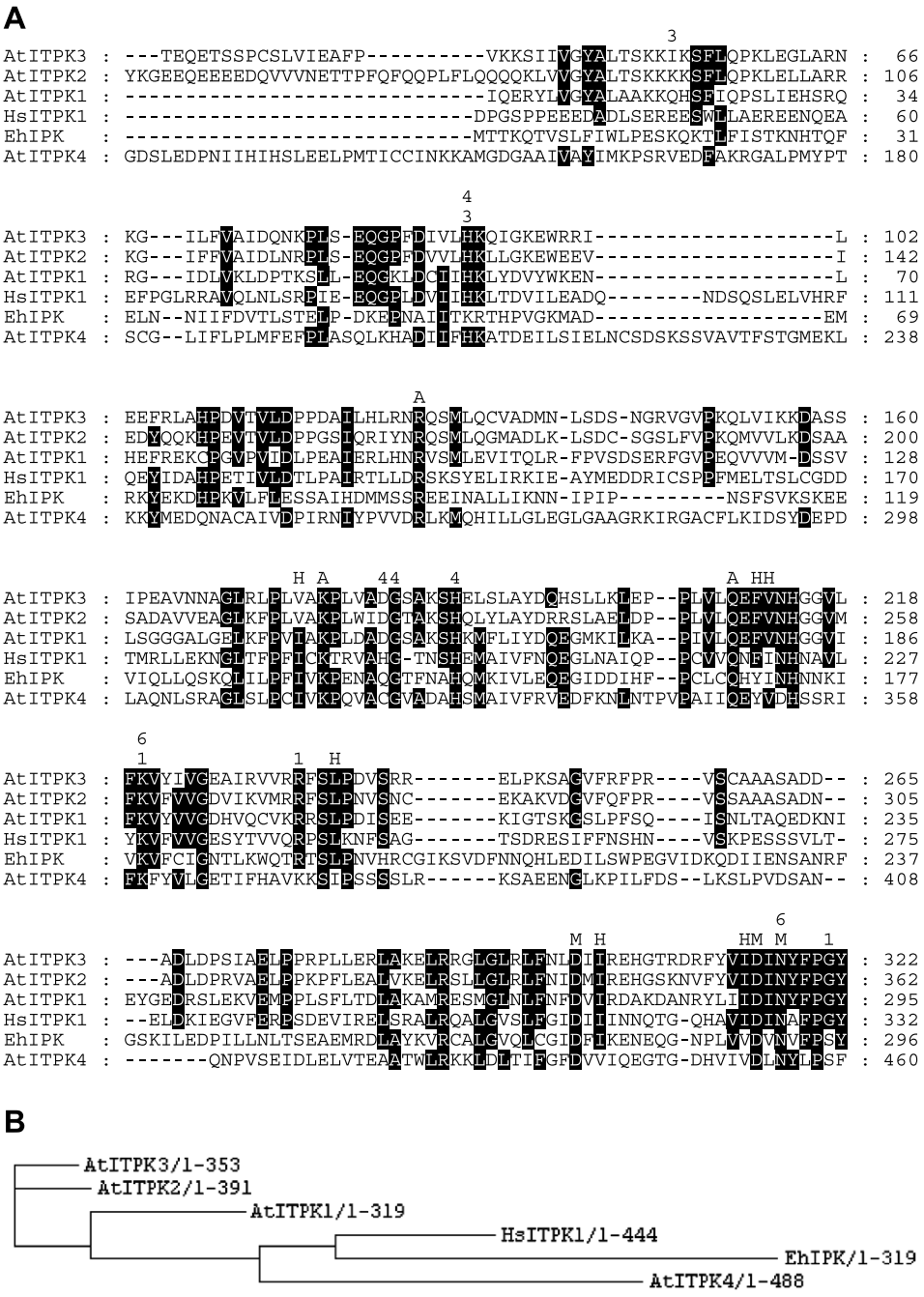

Fig. 1. (A) Alignment of full length sequences of AtITPK1, AtITPK2, AtITPK3, AtITPK4, hITPK1 and EhIPK; extreme N- and C-terminal sequences not displayed. Residues identical in four or more sequences are highlighted. Residues indicated above the alignment denote residues referred to in the text by reference to the crystal structures of complexes of substrate and product of EhIPK: residues coordinating different phosphates of the substrate or product are numbered 1, 3, 4 and 6 according to the phosphate coordinated; magnesium- and ATP-binding residues are indicated, M and A, respectively; hydrophobic residues binding the adenine ring are indicated, H. (B) Phylogenetic tree of full length sequences of AtITPK1, AtITPK2, AtITPK3, AtITPK4, hITPK1 and EhIPK.

AtITPK4 transcript is strongly localized to the ovary at this stage. The flower buds (iii–i) are at progressively earlier stages (iii < stage 6, when sepals enclose the bud). Expression of AtITPK4 transcript is evident in the inner organs of early flower buds. Fig. 3C shows expression of AtITPK4 transcript in mature siliques (>stage 18). Thus, AtITPK4 transcript is expressed throughout flower development in Arabidopsis. Fig. 4D shows expression in leaf vasculature.

In corroboration, we observed hybridization of the AtITPK4 antisense probe to all flower organs (Fig. 4A). The sepals and petals were strongly labelled, as were the male and female organs of immature and mature flower buds: particularly strong expression was observed in the carpels. The anthers and stamens were labelled, as were pollen grains, the transmitting tissue and ovules. Sense probes did not result in signals greater than the background (data not shown). A transverse section of a silique

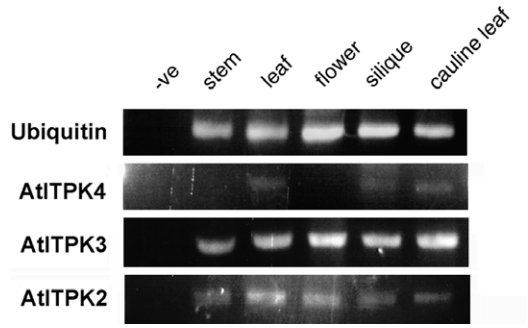

Fig. 2. Expression of *Arabidopsis* inositol 1,3,4-trisphosphate 5/6-kinases: RT-PCR of cDNA derived from stem, leaf, flower, silique, cauline leaf; with primers to ubiquitin, AtITPK2, AtITPK3 and AtITPK4. A negative control lacking template is included.

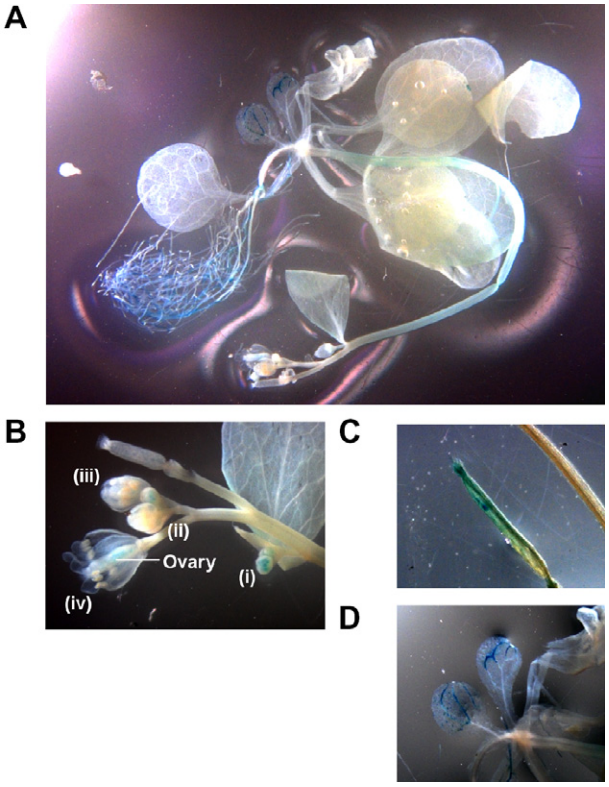

Fig. 3. Promoter::GUS analysis of AtITPK4: (A) whole plant; (B) developing (i–iv) flower buds; (C) mature siliques; (D) leaf. Reporter expression is indicated by blue-staining of the β-glucuronidase product.

revealed expression in the seed and pod wall (Fig. 4B), consistent with AtITPK4 promoter::reporter analysis.

3.3. Activity of Arabidopsis inositol phosphate kinases

Kinase assays were performed with AtITPK2, AtITPK3 and AtITPK4 proteins (Fig. 5). We have also confirmed (data not shown) the use of Ins(1,3,4)P_3 substrate by AtITPK1, as reported [8]. For AtITPK2, AtITPK3 and AtITPK, the substrates were Ins(1,3,4)P_3, a mixture of Ins(1,4,6)P_3 and Ins(3,4,6,)P_3, Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5 and Ins(1,3,4,6)P_4. Radioactive products were identified with TLC and positive results confirmed with HPLC analysis.

AtITPK2 showed activity against Ins(1,3,4)P_3 (Fig. 5A). In this assay the substrate was converted to InsP_4 but not to InsP_5. In addition, activity was also observed against the racemic mixture of Ins(1,4,6)P_3 and Ins(3,4,6)P_3 producing InsP_4, and Ins(3,4,5,6)P_4 produced InsP_5. AtITPK3 yielded InsP_4 from Ins(1,3,4)P_3 (Fig. 5B); InsP_4 from a racemic mixture of Ins(1,4,6)P_3 and Ins(3,4,6)P_3; and InsP_5 from Ins(3,4,5,6)P_4. AtITPK4 yielded InsP_4 from Ins(1,3,4)P_3 (Fig. 5C); InsP_4 from a racemic mixture of Ins(1,4,6)P_3 and Ins(3,4,6)P_3; and InsP_4 from Ins(1,3,4,6)P_4.

Given the production of ^{32}P-labelled InsP_4 from Ins(1,3,4,6)P_4, we tested AtITPK4 against other inositol tetrakisphosphates including three recognized substrates of this family (Fig. 5D): Ins(1,3,4,5)P_4; Ins(1,3,4,6)P_4; Ins(1,4,5,6)P_4, which is not a physiological substrate of hITPK1 [14]; and Ins(3,4,5,6)P_4. Ins(1,3,4,6)P_4 was strongly preferred. InsP_5 products were not detected.

Fig. 6A shows HPLC separation of InsP_4 standards. Fig. 6B–F shows, respectively; the chromatography of the ^{32}P products of reaction of AtITPK4 with Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4)P_3 and Ins(1,4,6)P_3. These samples were spiked with [^{3}H]Ins(1,3,4,6)P_4 and [^{3}H]Ins(1,3,4,5)P_4 prior to HPLC. [^{32}P]Ins(1,3,4,6)P_4 was produced in all cases. Thus, with InsP_4 substrates, the enzyme displays an isomerase activity, that, in the absence of InsP_5 product, we interpret as a phosphatase and kinase activity.

Reversible phosphoisomerase activity between Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 has been reported for hITPK1 [2]; for the Entamoeba enzyme which converts Ins(1,3,4,5)P_4 to Ins(1,3,4,6)P_4, but does not convert Ins(1,3,4,6)P_4 to Ins(1,3,4,5)P_4 [5]; and for a recently characterized barley enzyme [15]. AtITPK4 does not display Ins(1,3,4,6)P_4 to Ins(1,3,4,5)P_4 isomerase activity (Fig. 6B), but shows isomerase activity against other substrates (Fig. 6C and D). Removal of the 1- or 3-phosphate of Ins(1,3,4,6)P_4 and subsequent re-phosphorylation at the same position could generate [^{32}P]Ins(1,3,4,6)P_4 from Ins(1,3,4,6)P_4. Phosphorylation of Ins(1,4,6)P_3 at the 3-position (Fig. 6F) supports this possibility. Interestingly, AtITPK4 is more active against Ins(1,4,5,6)P_4 than against Ins(3,4,5,6)P_4 (Fig. 5D), an observation that further distinguishes AtITPK4 from other family members in Arabidopsis (this study) and across kingdoms [14,15].

Accordingly, we estimated kinetic parameters for the activity of AtITPK4 against its preferred substrate ie. the reaction that yielded [^{32}P]Ins(1,3,4,6)P_4 from [γ-^{32}P]ATP and Ins(1,3,4,6)P_4. The reaction was performed at 0.4 mM ATP and the products were analysed by reverse phase HPLC. Conversion of ATP to InsP_4 products was limited to less than 1% of radioactivity in the assay. A double reciprocal plot of the averages of triplicate measurements yielded an apparent K_m for Ins(1,3,4,6)P_4 of 42 μM and a V_{max} of 4.4 nmol min^{-1} mg^{-1} (Fig. 7).

4. Discussion

Arabidopsis possesses a family of genes annotated as inositol 1,3,4-trisphosphate 5/6-kinases after the first molecular description of enzymes of this class in plants [8]. We propose the use of the name inositol tris/tetrakisphosphate kinase (ITPK) as applied to hITPK1 [14], to distinguish enzymes of this family from enzymes of the multikinase family. We find

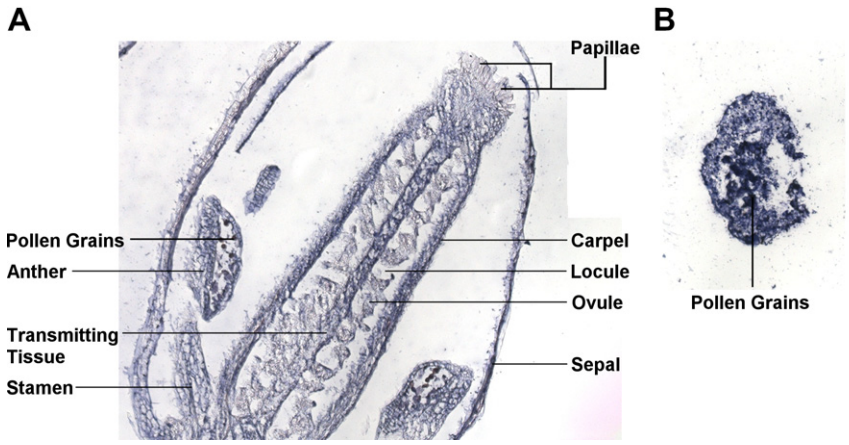

Fig. 4. mRNA hybridization analysis of *Arabidopsis* AtITPK4: (A) longitudinal section of floral bud and (B) transverse section of mature silique. Expression of transcripts is indicated by blue staining of the DIG-labelled probe.

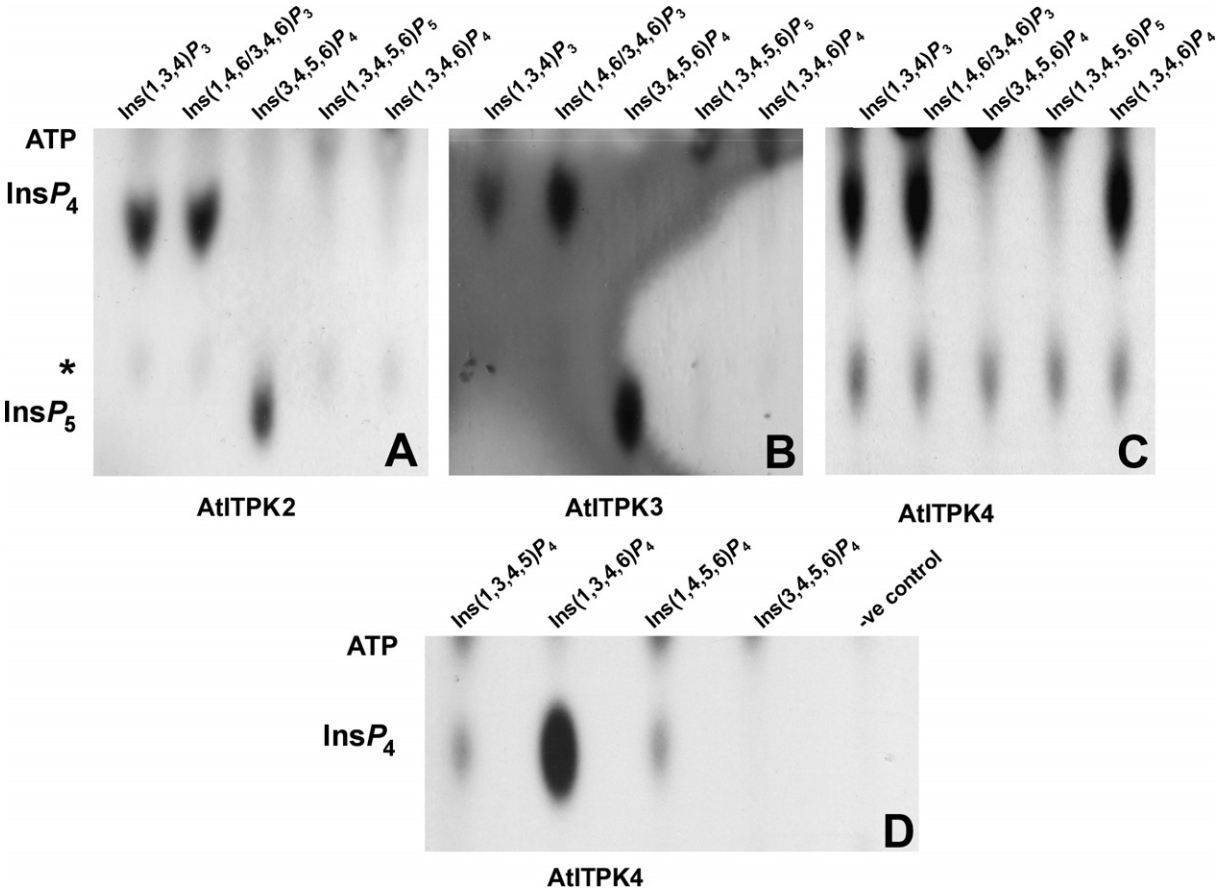

Fig. 5. Substrate specificity of recombinant proteins: (A) AtITPK2; (B) AtITPK3 and (C, D) AtITPK4. The mobilities of $InsP_4$s, $InsP_5$s, ATP, and contaminants (*) in the ATP are indicated.

that the expression of AtITPK isoforms are dissimilar indicating that the isoforms have functions specific to the organs in which they are expressed.

AtITPK2 and AtITPK3 have similar substrate specificities. In their usage of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 as kinase substrates, these isoforms are typical of the family [1,7,11,15]. AtITPK1 also possesses inositol 3,4,5,6-tetrakisphosphate 1-kinase activity (data not shown). We find additionally that AtITPK2, AtITPK3 and AtITPK4, and AtITPK1 (for which the data is not shown), all utilize D/L-Ins(3,4,6)P_3 as kinase substrate.

AtITPK4 differs from the other isoforms; in sequence, expression pattern and substrate specificity and reactions catalyzed. The kinetic parameters that we have derived for AtITPK4 are not greatly dissimilar to values for Arabidopsis kinases of other classes, against their substrates. One

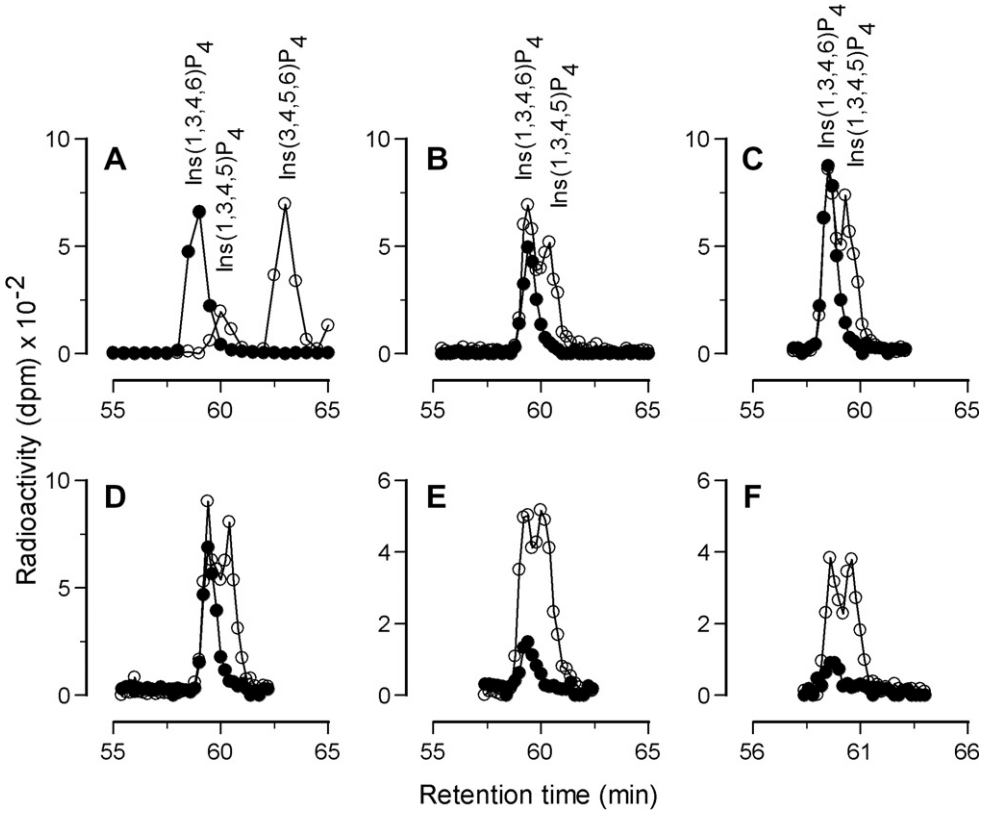

Fig. 6. Products of reaction of AtITPK4 with $InsP_3$ and $InsP_4$ substrates. (A) ^3H (○) and ^{32}P (●) standards. (B–F) ^{32}P (●) reaction products for Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4)P_3 and Ins(1,4,6)P_3 substrates, respectively. ^3H standards (○) of Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 were added post reaction and prior to HPLC.

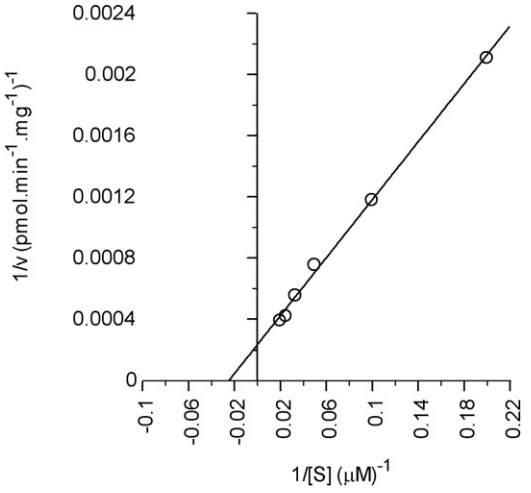

Fig. 7. Kinetic analysis of Ins(1,3,4,6)P_4 'isomerase' activity of AtITPK4. Error bars not shown. The regression coefficient of the line of best fit was 0.997.

of the inositol polyphosphate 6-/3-/5-kinases has K_m 15 μM, and V_{max} 50 nmol min^{-1} mg^{-1} for Ins(1,4,5)P_3 [16]; while the Ins(1,3,4,5,6)P_5 2-kinase activity of the unrelated protein AtIPK1 was estimated at K_m 22 μM, and V_{max} 35 nmol min^{-1} mg^{-1} [10]. Consequently, the isomerase activity of AtITPK4 is not trivial compared to the activities of two proteins that have been demonstrated to contribute to $InsP_6$ synthesis in Arabidopsis [6].

AtITPK4 may have a function in the $InsP_6$ synthesis that is associated with seed development, but its contribution cannot be mediated via Ins(3,4,5,6)P_4 because it lacks the Ins(3,4,5,6)P_4 1-kinase activity that is the prominent activity of this class of enzyme in plants [7,15]. Ins(3,4,5,6)P_4 was identified as a 1-kinase substrate in *Spirodela polyrhiza* [12,17]. Although we did not detect Ins(1,3,4,6)P_4 in these studies, we noted the possibility that an unobserved Ins(1,3,4,6)P_4 might participate in $InsP_6$ synthesis as for *Dictyostelium discoideum* [18]. Clearly, the lack of Ins(1,3,4,6)P_4 or Ins(3,4,5,6)P_4 kinase activity for AtITPK4 precludes direct participation as a tetrakisphosphate kinase in $InsP_6$ synthesis. In this case, activity against lower inositol phosphates might be relevant. It is possible that the isomerase activity, like the kinase/phosphatase activity of hITPK1, may have a regulatory role; perhaps related to other physiological substrates/products which remain as yet unidentified in plants. Other than a metabolic role as in higher inositol polyphosphate synthesis of animals [19], it is not obvious what the function of Ins(1,3,4,6)P_4 might be. Nevertheless, the localization of a somewhat specialized inositol phosphate kinase to floral tissues may indicate a specific function in flower development mediated through inositol tris- and tetrakisphosphates. The results of this study will allow closer examination of the contribution of AtITPK4 to such processes and additionally provides the first family-wide analysis of ITPK function within a single genome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.046.

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