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Metabolic and Signaling Properties of an *Itpk* Gene Family in *Glycine max*[1]

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Abstract

We have cloned and characterized four *Itpk* genes from soybean. All four recombinant Itpk proteins showed canonical Ins(1,3,4)P_3 5/6-kinase activity, but a kinetic analysis raised questions about its biological significance. Instead, we provide evidence that one alternative biological role for soybean Itpks is to interconvert the Cl^- channel inhibitor, Ins(3,4,5,6)P_4, and its metabolic precursor, Ins (1,3,4,5,6)P_5, within a substrate cycle. The soybean Itpks also phosphorylated Ins(3,4,6)P_3 to Ins (1,3,4,6)P_4 which was further phosphorylated to Ins(1,3,4,5,6)P_5 by soybean Ipk2. Thus, soybean Itpks may participate in an inositol lipid-independent pathway of $InsP_6$ synthesis.

1. INTRODUCTION

$InsP_6$, the most abundant inositol phosphate in plants, has several cell-signaling functions (1), and is also a structural cofactor for the auxin receptor (2). Additionally, $InsP_6$ is a phosphate storage compound, so it accumulates to considerable levels in plant seeds (1). This has a number of nutritional, agricultural and environmental consequences. For example, the inability of humans and other monogastrics to digest dietary $InsP_6$ has anti-nutrient effects due to its chelation of mineral cations such as calcium, iron, zinc, and potassium (1). Another important problem is that phytate-rich soybeans, grains and other plant seeds are widely used as the primary protein source in feed for monogastric farm animals (3). Undigested $InsP_6$ phosphorus is excreted in manure, which is applied to pastures and croplands. Runoff from agricultural fields can result in environmental phosphorus pollution, eutrophication and impaired water quality (4;5). Therefore, there is considerable interest in using molecular genetics to generate crops in which $InsP_6$ synthesis during seed development is disrupted (1). However, it is first necessary to identify the enzymes in this biosynthetic pathway. There is general agreement that the final metabolic step in the pathway of $InsP_6$ synthesis in plants (and other organisms) is the phosphorylation of Ins(1,3,4,5,6)P_5 by a 2-kinase (6;7). Thus, a major challenge for this

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field is to identify the pathway(s) by which Ins(1,3,4,5,6)P_5 is synthesized, and to characterize the enzymes that are involved.

Proposed routes of Ins(1,3,4,5,6)P_5 synthesis in plants involve “lipid-dependent” and “lipid-independent” pathways. The former involves the phosphorylation of the Ins(1,4,5)P_3 that is formed by PLC-mediated hydrolysis of the inositol phospholipid, PtdIns(4,5)P_2 (1). In this case, Ins(1,4,5)P_3 is phosphorylated to Ins(1,3,4,5,6)P_5 in a two-step reaction catalyzed by a single enzyme, Ipk2 (7). With regards to the PtdIns(4,5)P_2-independent pathway, there is continuing debate concerning the nature of both the metabolic intermediates and the enzymes involved (6;8). A [^{32}P]-labeling strategy led to the following proposed pathway in duckweed: Ins3P -> Ins(3,4)P_2 -> Ins(3,4,6)P_3 -> Ins(1,3,4,6)P_4 -> Ins(1,3,4,5,6)P_5 (9). However, this experimental approach can give misleading information if there is substrate cycling between metabolic intermediates (10).

A low InsP_6 phenotype has been observed in a line of maize with a mutation in an *Itpk* gene (11). This was one of the reasons that we focused on Itpk in the current study, using soybean as a model system because of its agronomic significance. Several Itpk proteins have previously been identified in *Arabidopsis* (12–14) and rice (13). This enzyme family is best known for its Ins(1,3,4)P_3 5/6-kinase and Ins(3,4,5,6)P_4 1-kinase activities (12;14), but it has been unclear how these might be relevant to putative “lipid-independent” pathways of InsP_6 synthesis (1; 7;9). An examination of the kinetic parameters of these two metabolic reactions should help determine possible biological significance; this information is mostly absent from previous studies in this field and so is a particular focus of the current study. The role of the Itpk family in regulating Ins(3,4,5,6)P_4 metabolism in plants is also useful to understand because this polyphosphate inhibits transmembrane chloride flux, thereby regulating pollen growth (15).

2. MATERIALS AND METHODS

2.1 Gene Identification and Recombinant Protein Expression

We performed a BLAST database search of the soybean genome using as queries the Itpk sequences previously identified in *Arabidopsis thaliana* (AF080173) and *Zea mays* (AY172635). Putative EST clones of soybean Itpk were obtained from Biogenetic Services (Brookings, South Dakota) and were sequenced. Complete coding regions for four Itpk genes (which we named type 1 through type 4) were amplified using the following primer pairs (restriction sites underlined): *Gm*Itpk1, 5′-GATCCCCGGGAATGGCGGAGAAGAGATTCG-3′ and 3′-CCTACTCGAGTCAAGCTTGAAGAGATTCCTCTT-5′; *Gm*Itpk2, 5′-GATCGAATTCATGTCCGAGTCGGAAGTAGCA-3′ and 3′-CACACTCGAGCTACGCAGTCTTGGAGCGTA-5′; *Gm*Itpk3, 3′-GATCGAATTCATGAGGTTGAGGGAGGAGGTAG-5′ and 5′-GATCCTCGAGCTATTTTTTCTTGTACTTCCCCTGC-3′; *Gm*Itpk4, 5′-GATCCCCGGGAATGAGGCTAAACGGTGAAATCTC-3′ and 3′-GGCCCTCGAGTTAGGCAGCAAGTTTCTTATTA-5′. The genes were cloned into the protein expression vector pGEX4T-1 (GE Healthcare, Waukesha, WI), and the proteins were expressed in *E. coli* and purified using GST-sepharose beads (GE Healthcare, Waukesha, WI) as previously described (16). The soybean Ipk2 gene (accession EU033957) was identified based on a search of the soybean database using known Arabidopsis sequences (17). *Gm*Ipk2 was amplified and cloned into pGEX4T-1 using the following primer pair: 5’-GACTGGATCCATGCTCAAGATCCCGGAGC-3′ and 5’-ATGATCGCGGCCGCTCAGTCACTTGTGAAGACATGCTAC-3′. Recombinant protein was prepared as described above, and showed canonical Ins(1,3,4,5)P_4 6-kinase activity (data not shown).

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2.2 Enzyme Assays

[^{3}H]-Ins(1,3,4)P_3 was synthesized by incubating [^{3}H]-Ins(1,3,4,5)P_4 (Perkin Elmer, Wellesley, MA) with recombinant Ins(1,3,4,5)P_4 5-phosphatase (provided by Dr. Gillaspy at Virginia Tech). [^{3}H]-Ins(3,4,5,6)P_4 and [^{3}H]-Ins(1,3,4,5,6)P_5 were prepared as previously described (16). Non-radioactive Ins(1,3,4)P_3, Ins(3,4,6)P_3 and Ins(3,4,5,6)P_4 were purchased from CellSignals (Columbus, OH). [^{32}P]-ATP was purchased from Perkin Elmer (Perkin Elmer, Wellesley, MA).

To study the metabolism of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4, each *Gm*ITPK isoform was incubated with radiolabeled substrate (1000 to 3000 D.P.M), plus the appropriate concentration of non-radiolabled substrate. Incubations were performed for various times (<1 hour) at 37°C in 100 µl assay buffer containing 20 mM HEPES pH 7.2, 100 mM KCl, 6 mM $MgSO_4$, 5 mM ATP, 10 mM phosphocreatine, 2 units/ml creatine-phosphokinase, 0.3mg/ml BSA. For Ins (1,3,4,5,6)P_5 metabolism, ATP was replaced by ADP. For Ins(3,4,6)P_3 metabolism, the reaction volume was reduced to 20 ul and the non-radiolabeled ATP was replaced with 100,000 D.P.M. [^{32}P]-ATP.

Reactions were quenched and neutralized as previously described and analyzed by either gravity-fed ion-exchange columns, or by HPLC (using either a 4.6 × 125 mm Partisphere SAX column, or a 250 × 4.6 mm Q100 column (Thompson Instruments, Clear Brook, VA) (16). The assays containing [^{32}P] were analyzed using an on-line scintillation counter.

2.3 Real-Time PCR Expression Analysis

Soybean seeds were sorted into four different stages of development according to seed size (0–4 mm, 5–6 mm, 7–8 mm, 9–10 mm). Two biological replicates were from the soybean cultivar "Jack" and the third from Virginia experimental line V71-370. RNA was extracted using Tri-Reagent according to manufacturer's instructions (MRCgene, Cincinnati, OH) and then treated with DNase I using a Turbo DNA-free kit per manufacturer's instructions (Ambion, Austin, TX). cDNA first strand synthesis was generated using oligo dT primers (12–18 bp) (Invitrogen, Carlsbad, CA) and the Omniscript cDNA kit (Qiagen, Valencia, CA). The absence of residual contaminating DNA was verified prior to cDNA synthesis using RNA as the template for the PCR reaction. The following primer pairs were designed for the 3' untranslated region: *Gm*Itpk1, 5"-CTGCGAAGTAATGCTCAAGA-3" and 3"-GCAACTCGTGCCAACC-5"; *Gm*Itpk2, 5"-TGAGGACGCTGAAATGCC-3" and 3"-AGACAACAGTGTAAATGTGTAATAACATC-5" *Gm*Itpk3, 5"-CCTACTGTTGCTGAGCTTC-3" and 3"-GGGCTTACGTCATGTGGG-5" *Gm*Itpk4, 5"-ATGCCAGGCTATGAGCAC-3" and 3"-ACAGACCCTATTTCCACCTT-5". To check for equivalent synthesis of cDNA we used primers (5"-AGCGTGGTTATGTTGCCTCAAACT-3" and 3"-CTTGATGACTCCCACAGCAACAGT-5") for a soybean housekeeping gene, elongation factor 1A (18;19). Amplification and analysis were conducted using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

3. RESULTS AND DISCUSSION

3.1 Identification of Four Soybean Itpk Genes

We sequenced four complete *Gm*Itpk genes (types 1–4; Fig. 1A) which can be grouped into two pairs (Fig. 1B). *Gm*Itpk1 and *Gm*Itpk2 share 46% identity. *Gm*Itpk3 and *Gm*Itpk4 are 67% identical. Alignments of the soybean sequences with homologues from other organisms reveal several highly conserved regions, including 16 residues previously shown to be catalytically important in *Hs*ITPK (Fig. 1A). A phylogenetic tree indicates that the soybean enzymes belong

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to two major branches, along with all but one member of the *Arabidopsis* and rice gene families (Fig. 1B).

Using real-time PCR (Fig. 2) and Northern analysis (data not shown) we quantified the expression of the four soybean *Itpk* genes in seeds at several developmental stages. *Gm*Itpk3 was much more abundantly expressed, especially during the early stages of seed development.

3.2 Phosphorylation of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 by Soybean Itpks

The canonical catalytic activity of all Itpk proteins is the phosphorylation of Ins(1,3,4)P_3 to both Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 (12;14;20;21). We found that all four soybean Itpks phosphorylated Ins(1,3,4)P_3 (Fig. 3A;Table 1). The two InsP_4 products were initially identified by HPLC co-elution with standards (Fig. 3B). Additionally, we noted that *Arabidopsis* Ipk2 has previously been shown to convert Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 to Ins(1,3,4,5,6)P_5 (17). Therefore, to further confirm the nature of the two InsP_4 products made by the soybean Itpks, the InsP_4s were incubated with recombinant *Gm*Ipk2 and were both found to be completely phosphorylated to Ins(1,3,4,5,6)P_5 (data not shown).

Itpks typically have K_m values for their substrates that are around 1 μM or less (21). Two of the soybean proteins (types 2 and 3) showed much lower affinities for Ins(1,3,4)P_3 (Table 1), which raises questions concerning the physiological relevance of Ins(1,3,4)P_3 phosphorylation by these enzymes *in vivo*. We therefore turned our attention to an alternate substrate, namely, Ins(3,4,5,6)P_4. We found that Ins(3,4,5,6)P_4 was phosphorylated by all four soybean Itpks with K_m values of 0.3 – 1 μM (Fig 4;Table 1). HPLC analysis revealed that the product of Ins (3,4,5,6)P_4 phosphorylation co-eluted with a standard of [^{3}H]-Ins(1,3,4,5,6)P_5 and not standards of either D/L-[^{3}H]-Ins(2,3,4,5,6)P_5 or [^{3}H]-InsP_6 (Fig. 4). These data verify that soybean Itpks specifically phosphorylate the 1-position of Ins(3,4,5,6)P_4.

A comparison of the catalytic efficiencies (V_{max}/K_m) of the Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 substrates revealed that the latter is the preferred substrate of all four isoforms (Table 1). The type 4 enzyme was the least catalytically efficient, by a factor of 35 to 100 (Table 1).

3.3 Synthesis of Ins(3,4,5,6)P_4 by Soybean Itpks

As well as exhibiting Ins(3,4,5,6)P_4 1-kinase activity, mammalian Itpk1 has been shown to dephosphorylate Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4 (16;22). We recently demonstrated that *Gm*Itpk2 can also dephosphorylate Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4 (2932 ± 50 μmol/mg protein/min) (16). We now report that the three other *Gm*Itpk isoforms also show this activity: type 1 = 403 ± 4, type 3= 487 ± 47, type 4 = 88 ± 16 (μmol/mg protein/min), albeit at rates 6 to 33-fold lower than that of the type 2 enzyme. Substrate cycling between Ins(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 may serve a signaling role in plants, in which Ins(3,4,5,6)P_4 has been shown to regulate Cl^- channel conductance (15), similar to the role of Ins(3,4,5,6)P_4 in animal cells (22).

3.4 Phosphorylation of Ins(3,4,6)P_3 by Soybean Itpks

It has been speculated that Ins(3,4,6)P_3, a known constituent of plants (23), participates in a "phospholipid-independent" pathway of InsP_6 synthesis, with both Ins(1,3,4,6)P_4 and Ins (3,4,5,6)P_4 being offered as candidate products of Ins(3,4,6)P_3 phosphorylation (6;8). Little work has been done to identify enzyme(s) that might catalyze these reactions, with the exception of a recent report showing that a racemic mixture of Ins(3,4,6)P_3/Ins(1,4,6)P_3 was phosphorylated by the recombinant Itpk from *Arabidopsis* (14). The use of a substrate mixture prevents us from knowing which of the two were phosphorylated. In the current study we used pure Ins(3,4,6)P_3 as a substrate. We did not have radiolabeled Ins(3,4,6)P_3, so we instead added [^{32}P]-ATP to our assays and measured [^{32}P]-InsP_4 formation. All four isoforms phosphorylated

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$Ins(3,4,6)P_3$ (Fig. 5). The $[^{32}P]$-$InsP_4$ that was formed was separated from a standard of $[^3H]$-$Ins(3,4,5,6)P_4$ (Fig. 5). This leaves just two alternative structures for the $[^{32}P]$-$InsP_4$ that was formed, namely, $Ins(2,3,4,6)P_4$ or $Ins(1,3,4,6)P_4$. The $Ins(2,3,4,6)P_4$ can be excluded, because only one class of enzymes can phosphorylate the 2-position of inositol (the $Ins(1,3,4,5,6)P_5$ 2-kinases; (7)). We therefore conclude that soybean Itpks can phosphorylate $Ins(3,4,6)P_3$ to $Ins(1,3,4,6)P_4$. Since the latter can be further phosphorylated to $Ins(1,3,4,5,6)P_5$ by *Gm*Ipk2 (Section 3.2), our data indicate that soybean have the enzymatic machinery for the "phospholipid-independent" pathway shown in Fig. 5C. The significance of this information lies in it defining a role for Itpk in $InsP_6$ synthesis in plants. Our data also indicate that it would now be profitable for this field of research to determine the nature of the enzymes that synthesize $Ins(3,4,6)P_3$.

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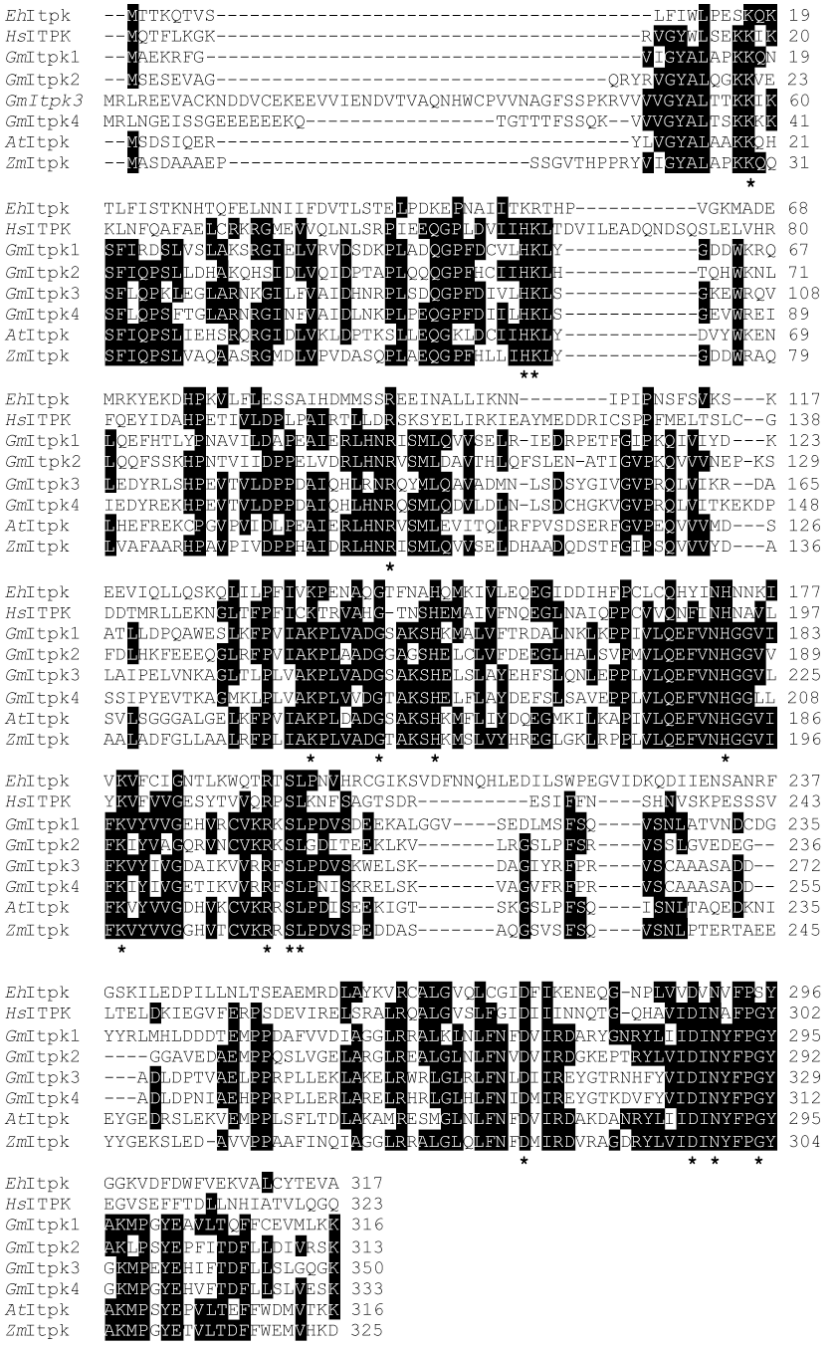

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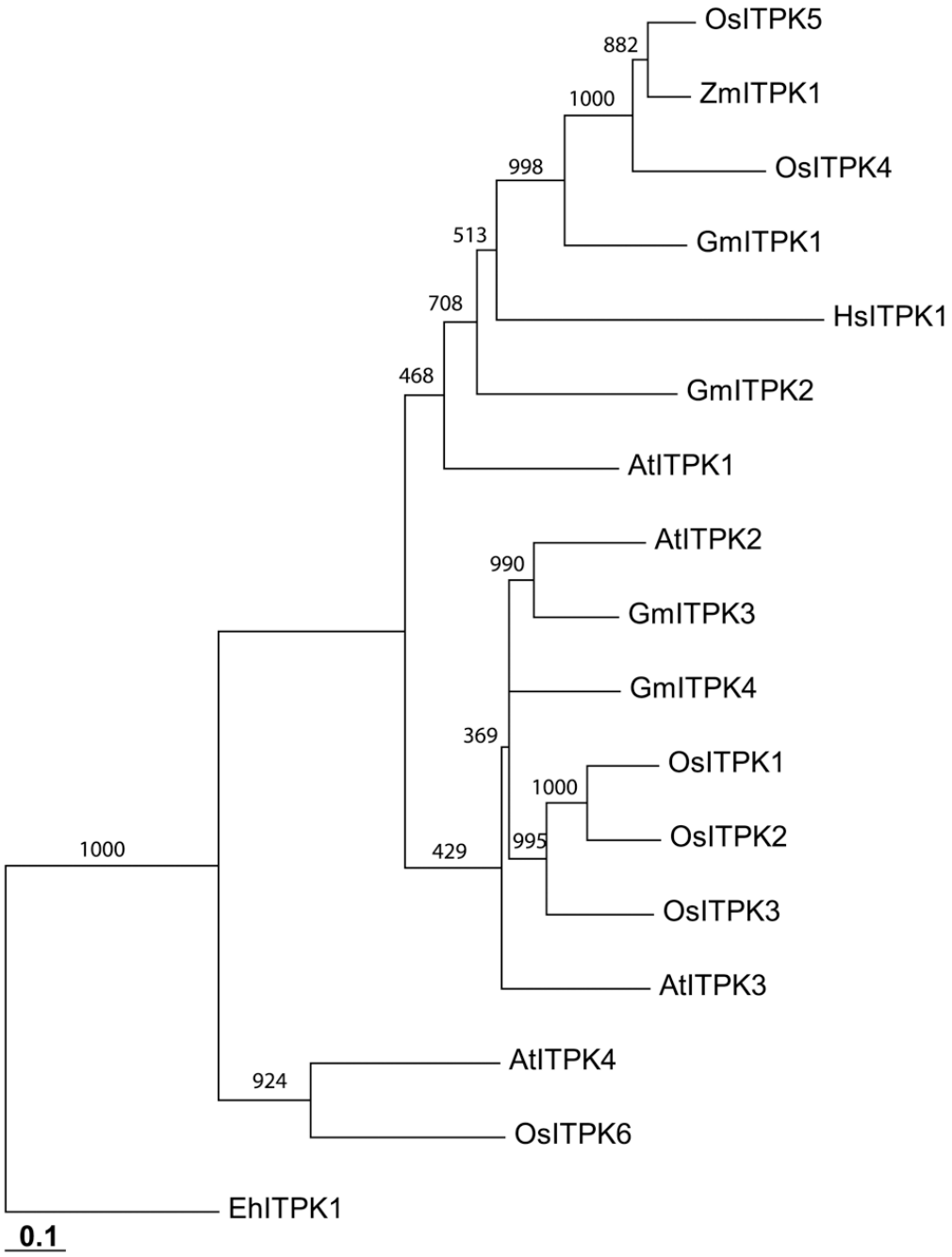

Figure 1. Soybean Itpks: Amino-acid sequences and phylogenetic relationships

Panel A: Alignment (using ClustalW) of Itpks from the following species (Genbank accession numbers in parentheses): *Entamoeba histolytica* (*Eh*; AF118848), *Homo sapiens* (*Hs*; NP_055031), Soybean (*Gm*; type 1, EU033958, type 2, EU033959, type 3, EU033960, type 4, EU033961), *Arabidopsis thaliana* (*At*; type 1, Q9SBA5) and *Zea mays* (*Zm*; type 1, Q84Y01). The recent publication of the *Arabidopsis Itpk* gene family (14) led us to renumber the previously designated soybean *Gm*Itpk4 sequence (16) as *Gm*Itpk2. A small number of C-terminal residues (which show no sequence similarity) are omitted for clarity. Highlighted residues are conserved in at least four of the aligned proteins. Asterisks denote residues in *Hs*ITPK which, when mutated, decrease catalytic activity at least 20% (24;25). Panel B shows

a phylogram generated with Treeview (26) from a 1000 iteration bootstrap analysis using the sequences described in Panel A, plus additional sequences from *Arabidopsis* (14) and rice (8). Scale bar indicates nucleotide substitutions per site.

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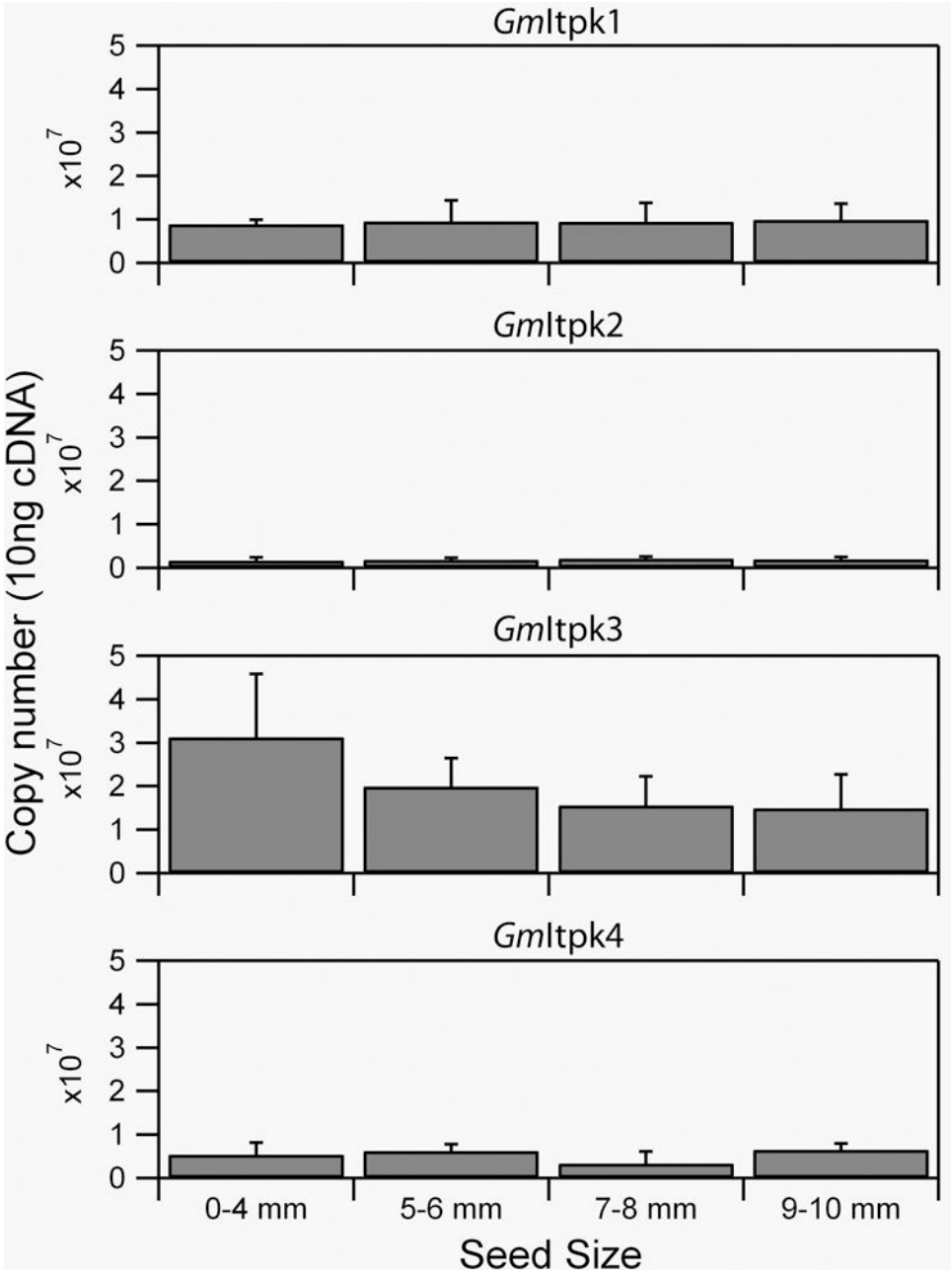

Figure 2. Expression of soybean *Itpk* genes in developing seeds

The expression of the *GmItpk* genes was determined by real-time PCR as described in the Methods Section.

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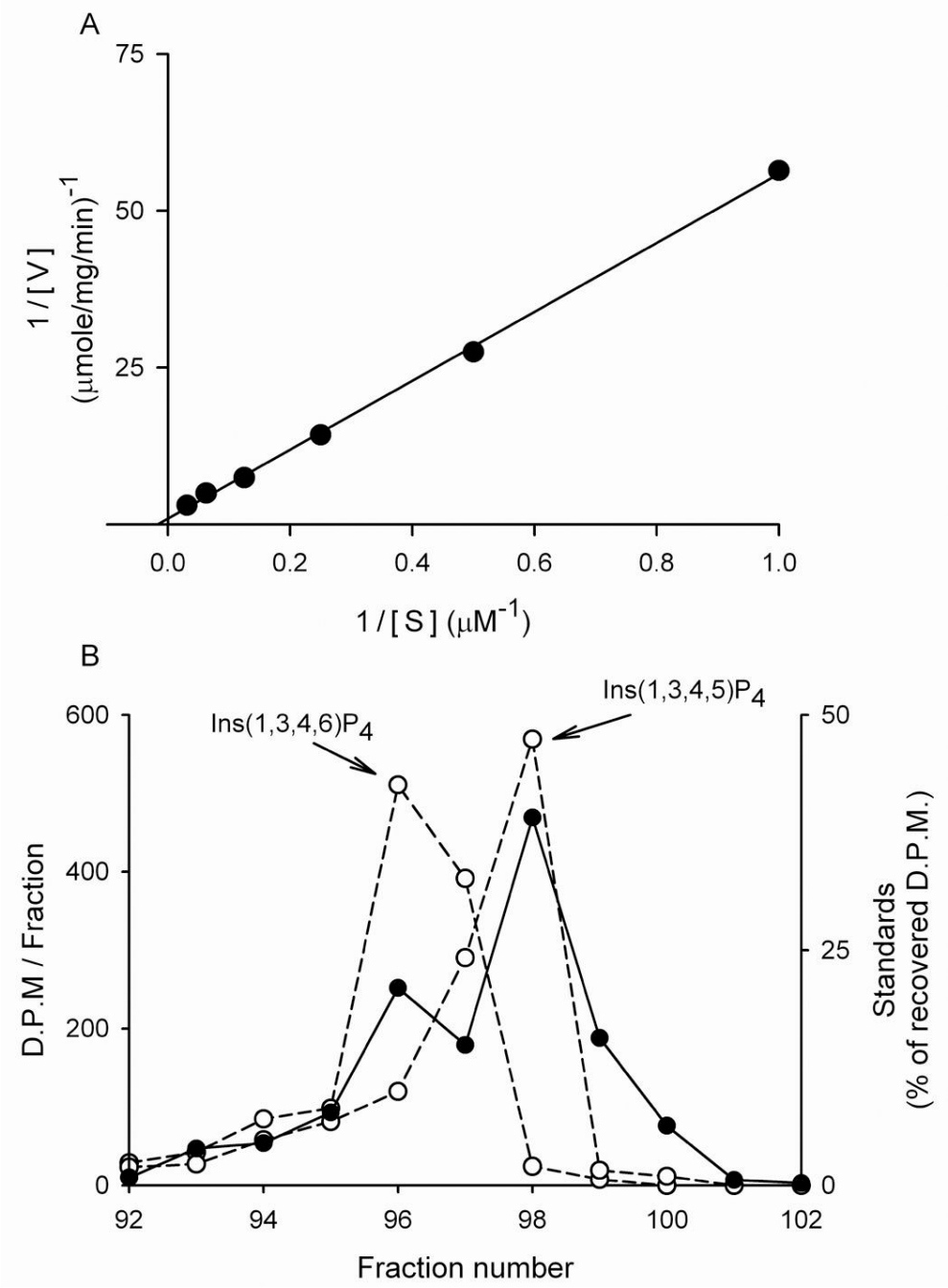

Figure 3. Ins(1,3,4)P_3 phosphorylation by soybean Itpk

Panel A shows a representative kinetic plot for the phosphorylation of [^{3}H]-Ins(1,3,4)P_3 by *Gm*Itpk3 (see also Table 1). Panel B describes the analysis of the products of [^{3}H]-Ins(1,3,4)P_3 phosphorylation by 2 μg *Gm*Itpk2 (filled circles) using Q100 HPLC chromatography as described in Methods. The open circles denote the elution of [^{14}C]-labeled standards of Ins(1,3,4,5)P_4 (added in the same HPLC run) and Ins(1,3,4,6)P_4 (added in a parallel run). Similar data were obtained in two additional experiments.

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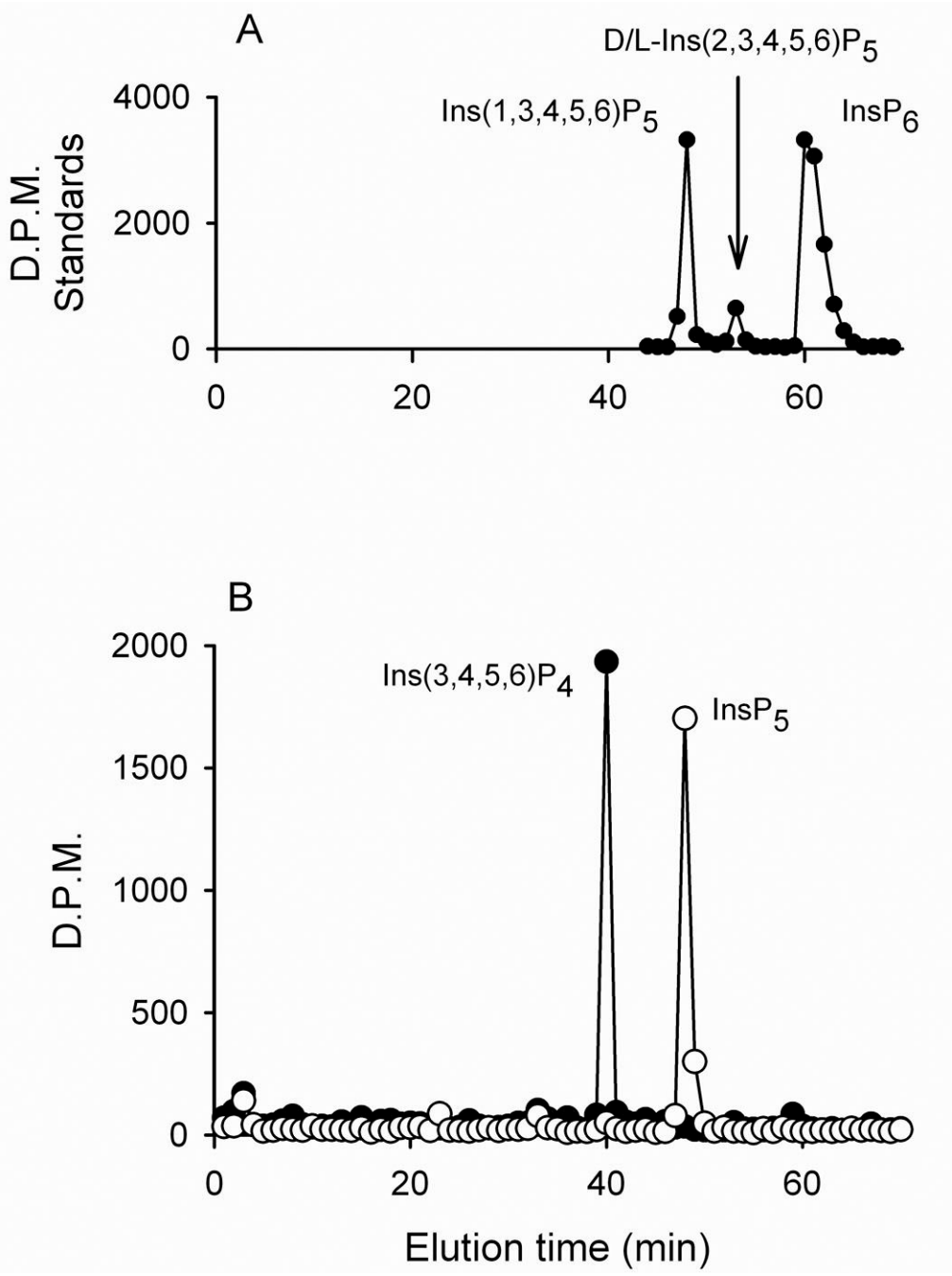

Figure 4. Ins(3,4,5,6)P_4 phosphorylation by soybean Itpk

Panel A: A Partisphere SAX HPLC column was calibrated with a mixture of [^{3}H]-labeled standards of Ins(1,3,4,5,6)P_5, D/L-Ins(2,3,4,5,6)P_5 and InsP_6. Panel B: [^{3}H]-Ins(3,4,5,6)P_4 was incubated with (closed circles) and without (open circles) 2 μg of *Gm*Itpk2 for 20 min (see Methods) and the products were analyzed by Partisphere SAX HPLC. Similar data were obtained with the other three *Gm*Itpk isoforms.

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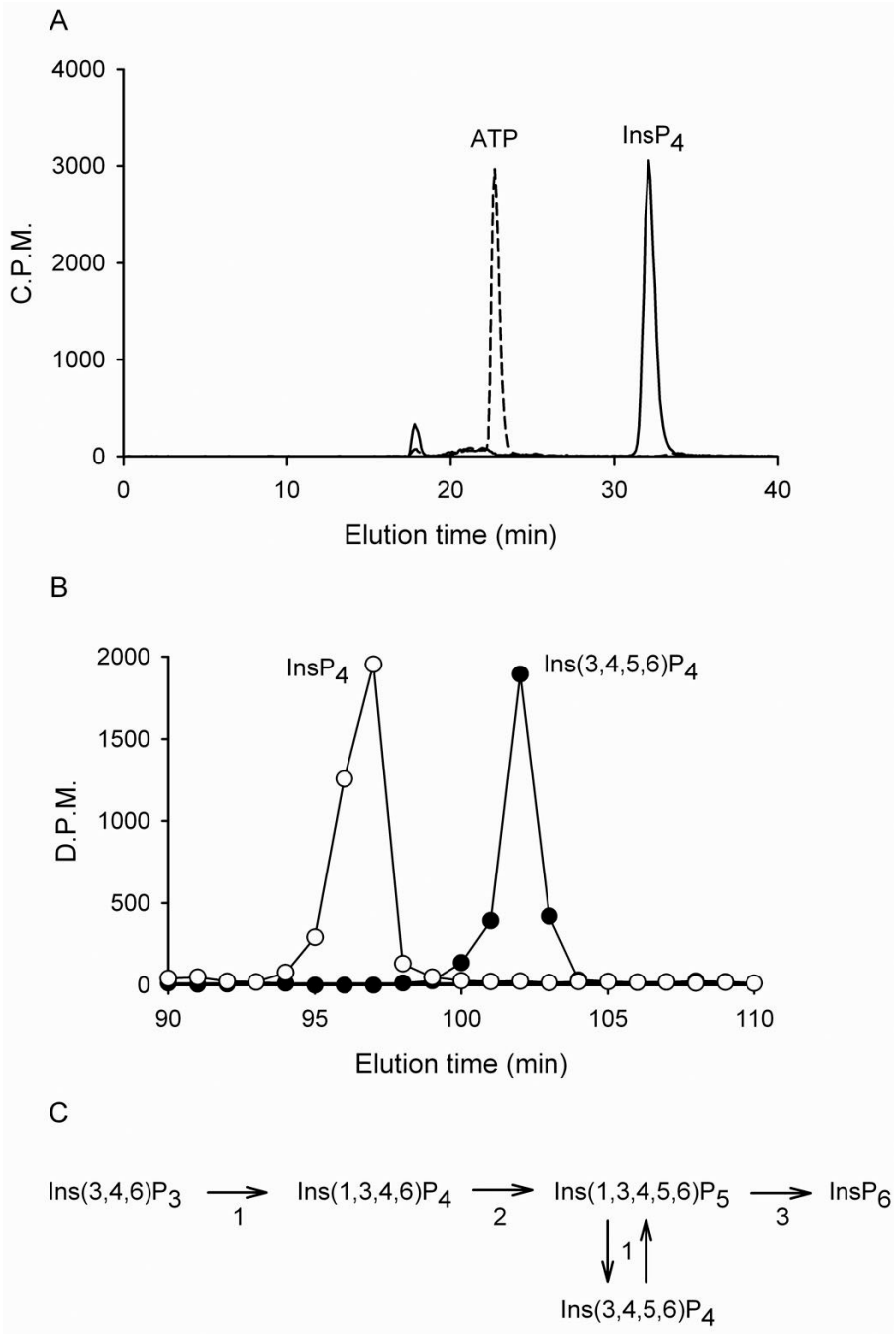

Figure 5. Ins(3,4,6)P_3 phosphorylation by soybean Itpk and its contribution to InsP_6 synthesis

Panel A: [^{32}P]-ATP and 50 μM Ins(3,4,6)P_3 were incubated with (solid line) and without (broken line) 2 μg *Gm*Itpk1 for 30 min and the products were resolved by Q100 HPLC. Panel B: An aliquot of the [^{32}P]-InsP_4 product described in panel A (open circles) was chromatographed with an [^{3}H]-Ins(3,4,5,6)P_4 standard (closed circles) using a Partisphere SAX HPLC. Similar data were obtained with the other three *Gm*Itpk isoforms. Panel C: Proposed metabolic pathway from Ins(3,4,6)P_3 to InsP_6 in soybean. Numbers adjacent to the arrows represent the enzymes that are responsible for each reaction: 1 = *Gm*Itpk1-4 (for evidence of Ins(3,4,6)P_3 phosphorylation see Figs 5A,B and Section 3.4; for Ins(3,4,5,6)P_4 /

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Ins(1,3,4,5,6)P_5 interconversion see Section 3.2 and Section 3.3), 2 = *Gm*Ipk2 (see Section 3.2), 3 = Ins(1,3,4,5,6)P_5 2-kinase (see ref. (7)).

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Table 1

Kinetic parameters of soybean Itpks

	K_m (μM)		V_{max} (μmol/mg/min)		V_{max}/K_m	
	Ins(1,3,4)P_3	**Ins(3,4,5,6)P_4**	**Ins(1,3,4)P_3**	**Ins(3,4,5,6)P_4**	**Ins(1,3,4)P_3**	**Ins(3,4,5,6)P_4**
*Gm*Itpk1	3.7 ± 0.4	0.28 ± 0.004	0.5 ± 0.1	0.083 ± 0.02	0.135	0.3
*Gm*Itpk2	46.2 ± 6.1	0.78 ± 0.04	1.97 ± 0.3	0.76 ± 0.06	0.043	0.97
*Gm*Itpk3	43.4 ± 8.3	1.05 ± 0.16	0.86 ± 0.1	0.22 ± 0.1	0.02	0.21
*Gm*Itpk4	4.4 ± 0.6	0.7 ± 0.03	0.007±0.001	0.004±0.0003	0.002	0.006

Data are means and standard errors from 3–4 experiments.