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Arabidopsis ITPK1 and ITPK2 Have an Evolutionarily Conserved Phytic Acid Kinase Activity

Debabrata Laha,[*,†,‡] Nargis Parvin,[†] Alexandre Hofer,[§] Ricardo F. H. Giehl,[∥] Nicolas Fernandez-Rebollo,[⊥] Nicolaus von Wirén,[∥] Adolfo Saiardi,[‡] Henning J. Jessen,[#,@] and Gabriel Schaaf[*,†]

[†]Institute of Crop Science and Resource Conservation, Department of Plant Nutrition, Rheinische Friedrich-Wilhelms-University Bonn, 53115 Bonn, Germany

[‡]Medical Research Council Laboratory for Molecular Cell Biology (MRC-LMCB), University College London, London WC1E 6BT, United Kingdom

[§]Department of Chemistry, University of Zurich, 8057 Zurich, Switzerland

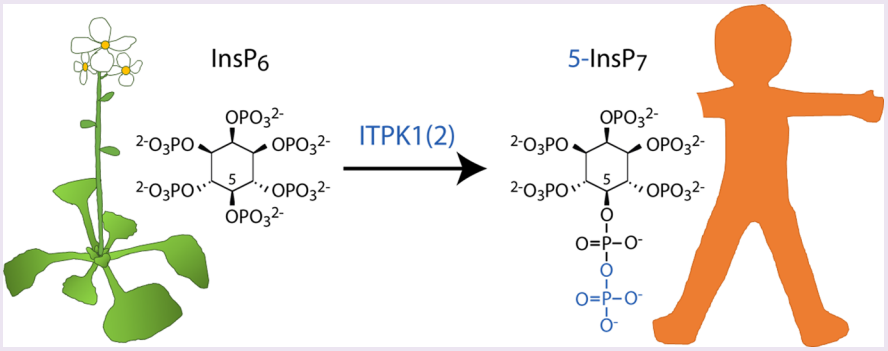
[∥]Department of Physiology & Cell Biology, Leibniz-Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany

[⊥]Center for Plant Molecular Biology, Department of Plant Physiology, Eberhard Karls University Tübingen, 72076 Tübingen, Germany

[#]Department of Chemistry and Pharmacy, Albert-Ludwigs University Freiburg, 79104 Freiburg, Germany

[@]CIBSS-Centre for Integrative Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany

Supporting Information



ABSTRACT: Diphospho-*myo*-inositol polyphosphates, also termed inositol pyrophosphates, are molecular messengers containing at least one high-energy phosphoanhydride bond and regulate a wide range of cellular processes in eukaryotes. While inositol pyrophosphates $InsP_7$ and $InsP_8$ are present in different plant species, both the identity of enzymes responsible for $InsP_7$ synthesis and the isomer identity of plant $InsP_7$ remain unknown. This study demonstrates that *Arabidopsis* ITPK1 and ITPK2 catalyze the phosphorylation of phytic acid ($InsP_6$) to the symmetric $InsP_7$ isomer 5-$InsP_7$ and that the $InsP_6$ kinase activity of ITPK enzymes is evolutionarily conserved from humans to plants. We also show by ^{31}P nuclear magnetic resonance that plant $InsP_7$ is structurally identical to the *in vitro* $InsP_6$ kinase products of ITPK1 and ITPK2. Our findings lay the biochemical and genetic basis for uncovering physiological processes regulated by 5-$InsP_7$ in plants.

Phytic acid ($InsP_6$) is an important storage molecule for phosphorus, inositol, and di- and trivalent metal ions and also serves as a precursor for molecular messengers termed diphospho-*myo*-inositol polyphosphates or inositol pyrophosphates. These messengers contain at least one high-energy phosphoanhydride bond and regulate diverse processes in metazoans, including ribosome biogenesis, DNA repair, telomere length maintenance, immune response, spermiogenesis, insulin signaling, and blood clotting.[1,2] In plants, inositol pyrophosphates are involved in the defense against necrotrophic fungi and insect herbivores and regulate nutrient sensing and lipid metabolism.[3−7]

In metazoa, amoebae, and yeast, two distinct kinases, IP6K/Kcs1 and PPIP5K/Vip1, mediate the stepwise biosynthesis of $InsP_7$ (PP-$InsP_5$) and $InsP_8$ ($[PP]_2$-$InsP_4$), respectively.[1,8] On the basis of the biochemical activities of the involved kinases, the isomer identity of yeast and mammalian inositol

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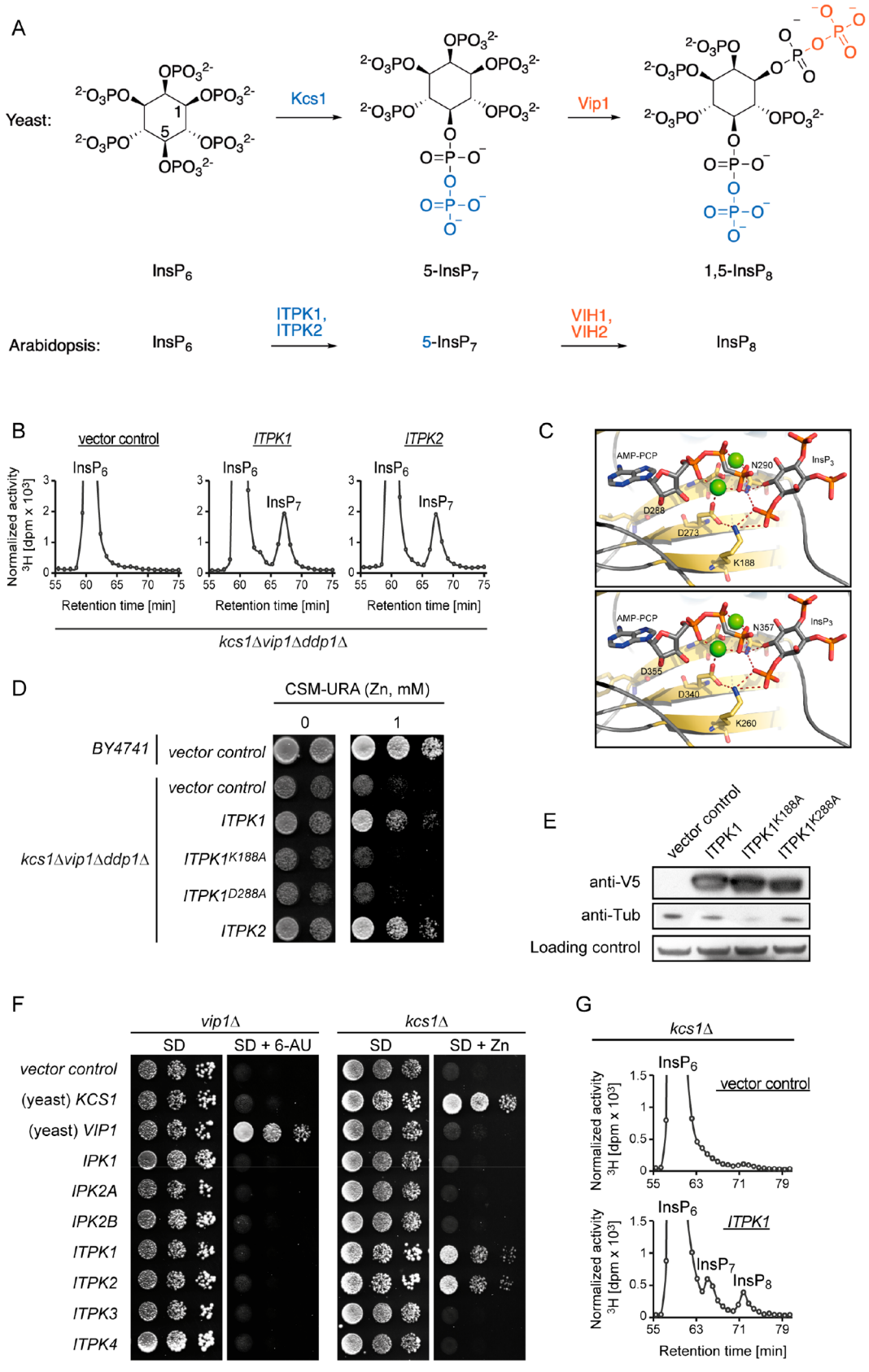

Figure 1. *Arabidopsis* ITPK1 and ITPK2 are $InsP_6$ kinases. (A) Schematic representation of the known inositol pyrophosphate synthesis in yeast. In *Arabidopsis*, only the identity of $InsP_7$ kinases was known,[4] whereas this study demonstrates that ITPK1 and ITPK2 function as $InsP_6$ kinases. (B) SAX-HPLC analyses of extracts from designated [^{3}H]inositol-labeled *kcs1Δvip1Δddp1Δ* yeast transformants. Activities of fractions containing the $InsP_6$–$InsP_8$ peaks are shown. Experiments were repeated twice with similar results. (C) Structural models (close-up of the substrate binding pocket) of *Arabidopsis* ITPK1 (top) and ITPK2 (bottom) in complex with Ins(1,3,4)P_3 and the ATP analogue AMP-PCP. Models were obtained by the PHYRE2 web portal and built on the *Entamoeba histolytica* ITPK1 structure (Protein Data Bank entry 1Z2P) in complex with the

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Figure 1. continued

nonhydrolyzable ATP analogue AMP-PCP, Mg^{2+} ions (green), and Ins(1,4,5)P_3. (D) Growth assays of *kcs1*Δ*vip1*Δ*ddp1*Δ yeast transformants expressing *Arabidopsis ITPK1* and *ITPK2* and designated catalytic mutants of *ITPK1* spotted in serial dilutions on selective minimal medium. Wild-type yeast (BY4741) transformed with vector control pDR195 and mutant yeast transformed with pDR195(*KCS1*) served as positive controls, while mutant yeast transformed with pDR195 served as a negative control. (E) Stability of mutant *Arabidopsis* ITPK1 proteins. Immunoblots of soluble lysates were prepared from *kcs1*Δ*vip1*Δ*ddp1*Δ yeast transformants expressing ITPK1 mutants (as designated) in translational fusion with a C-terminal V5 tag. Tagged proteins were detected with antibodies against V5 (Invitrogen). Tubulin (anti-Tub) and a representative unspecific band (loading control) served as controls for proper normalization. (F) *Arabidopsis* homologues encoding lower inositol polyphosphate kinases as indicated were expressed from pDR195 in a *vip1*Δ (left) and a *kcs1*Δ (right) yeast strain. Transformants were spotted in serial dilutions on selective minimal medium with appropriate supplements or SD medium with supplements containing 1.5 mM 6-azauracil (depicted as 6-AU) and 1.5 mM $ZnSO_4$. Cells ectopically expressing yeast *KCS1* (left) or yeast *VIP1* (right) served as positive controls, and cells transformed with pDR195 (vector control) served as negative controls. (G) Extracts of [^{3}H]inositol-labeled designated yeast transformants were resolved by SAX- HPLC. Activities of fractions containing the $InsP_6$–$InsP_8$ peaks are shown. Heterologous expression of *Arabidopsis* ITPK1 in a *kcs1*Δ yeast strain results in the robust appearance of $InsP_7$ and also the $InsP_8$ peak. The $InsP_8$ peak was absent in the HPLC analysis described in panel B as the *kcs1*Δ*vip1*Δ*ddp1*Δ strain lacks the Vip1 kinase activity that is responsible for $InsP_8$ production *in vivo*.[4,8]

pyrophosphates was proposed to represent 5-$InsP_7$ and 1,5-$InsP_8$, respectively[1,2] (Figure 1A). While plant genomes encode PPIP5K/Vip1 homologues,[4,9] $InsP_6$ kinases have not yet been identified, and therefore, the function and isomeric nature of plant $InsP_7$ and $InsP_8$ remain elusive.[1,4]

We employed a yeast (*Saccharomyces cerevisiae*) *kcs1*Δ*vip1*Δ*ddp1*Δ strain devoid of inositol pyrophosphates and with reduced inositol pyrophosphatase activity to identify *Arabidopsis* $InsP_6$ kinases by heterologous expression. To assess whether kinases that phosphorylate the hydroxyl group of inositol phosphates might also exert $InsP_6$ kinase activity, we tested all *Arabidopsis* proteins with known or predicted inositol phosphate kinase activity, including members of the inositol tetra/pentaphosphate 2-kinase (IPK1) family, the inositol polyphosphate kinase (IPK2) family, and the inositol 1,3,4-trisphosphate 5/6-kinase (ITPK) family, for their ability to complement growth defects of the *kcs1*Δ*vip1*Δ*ddp1*Δ strain. To this end, we screened suitable growth conditions and determined an increased Zn^{2+} concentration to cause severe growth defects of this strain as compared to its isogenic wild-type control (Figure S1A). Notably, *kcs1*Δ*vip1*Δ*ddp1*Δ-associated growth defects were rescued by inositol 1,3,4-trisphosphate 5/6-kinases ITPK1 and ITPK2 (Figure S1A), which are not related to yeast Kcs1 or mammalian IP6K enzymes in terms of sequence. *Arabidopsis* and maize ITPKs have been shown to phosphorylate the 1-, 5-, and 6-OH positions of the myo-inositol ring *in vitro*.[10−13] The fact that they can catalyze the formation of a phosphoanhydride bond was hitherto unknown.[11,14,15]

Strong anion exchange (SAX)-HPLC analyses revealed the appearance of a robust $InsP_7$ peak in extracts from yeast transformants expressing ITPK1 or ITPK2 but not ITPK3 or ITPK4 (Figure 1B and Figure S1B). The inability of ITPK3 and ITPK4 to display $InsP_6$ kinase activity was most likely not caused by the instability of these kinases in yeast as ectopic expression of both homologues had a profound effect on $InsP_{5a}$ [the $InsP_5$ (2-OH) isomer] and, in the case of ITPK4, also on two different $InsP_3$ isomers (Figure S1B). Structural models based on the crystal structure of *Entamoeba histolytica* ITPK [Protein Data Bank entry 1Z2P (Figure 1C and Figure S1C,D)] enabled us to generate stable catalytic dead versions of ITPK1 that failed to rescue growth defects and $InsP_7$ synthesis of the *kcs1*Δ*vip1*Δ*ddp1*Δ strain (Figure 1D,E and Figure S1E), indicating that rescue depends on their kinase activities. We also found that whereas ITPK1 and ITPK2 were not able to complement growth defects or defects in inositol pyrophosphate synthesis of the *vip1*Δ yeast strain (Figure 1F and Figure S1F), both rescued *kcs1*Δ-associated defects in growth and inositol pyrophosphate synthesis (Figure 1F,G and Figure S1G). Together, these results suggest that plant ITPK1 and ITPK2 execute Kcs1/IP6K-like, but not Vip1/PPIP5K-like, pyrophosphate synthase activities.

In agreement with the function of ITPK1 and ITPK2 as $InsP_6$ kinases, incubation of the purified recombinant enzymes with $InsP_6$ and ATP resulted in the robust synthesis of an $InsP_7$ isomer as determined by polyacrylamide gel electrophoresis (PAGE) (Figure 2A,B and Figure S2A). To investigate whether we can further reconstitute $InsP_8$ synthesis *in vitro*, we added recombinant VIH2 (the *Arabidopsis* $InsP_7$ kinase) to the ITPK1 kinase reaction mixture and indeed observed a strong signal with the chromatographic mobility of $InsP_8$ (Figure 2B). Mass spectrometry (MALDI-ToF-MS) analyses of the PAGE-purified ITPK1 and ITPK2 products revealed a major signal at m/z 738.8 that corresponds to the mass of deprotonated $InsP_7$ (Figure S2B−E), further indicating that both ITPKs can synthesize $InsP_7$ from $InsP_6$ *in vitro*.

Because MS and PAGE analyses are not suitable for discriminating $InsP_7$ isomers, we employed nuclear magnetic resonance (NMR) spectroscopy to unambiguously define the isomeric nature of ITPK1- and ITPK2-derived $InsP_7$. $InsP_6$ (like *myo*-inositol) is a *meso* compound with a plane of symmetry dissecting the 2 and 5 positions (Figure S3A). As a consequence, the 1/3 and 4/6 phosphates reside in an identical chemical environment and thus must have identical chemical shifts in the ^{31}P NMR spectrum. The ^{31}P[^{1}H] NMR spectrum of $InsP_6$ therefore shows four resonances and a ratio of the integrals of 1 (C2):2 (C1 and C3):2 (C4 and C6):1 (C5) (Figure S3B). The spectrum of ITPK1-derived $InsP_7$ also shows a symmetrical peak pattern, but it consists of three single resonances with an integral ratio of 2:1:2 and two doublets with P−P coupling (ratio of 1:1) representing the α- and β-phosphates of the pyrophosphate moiety (Figure 2C). The symmetry of the resonances in the NMR spectrum indicated that ITPK1 generates either 5-$InsP_7$ or 2-$InsP_7$ as phosphorylation at any of the other possible positions would break the symmetry of the resulting $InsP_7$ and thus lead to several additional resonances of the phosphate monoesters. To further investigate the molecular structure of the ITPK1 product, we performed spiking experiments using different synthetic $InsP_7$ standards.[16−18] Addition of 5-$InsP_7$ to the ITPK1 $InsP_6$ kinase reaction mixture did not lead to the appearance of additional peaks (Figure 2C), besides minor $InsP_6$ resonances (around 1.5 ppm, ∼10%), which is present as an impurity in the synthetic material. In contrast, addition of 2-$InsP_7$ caused the

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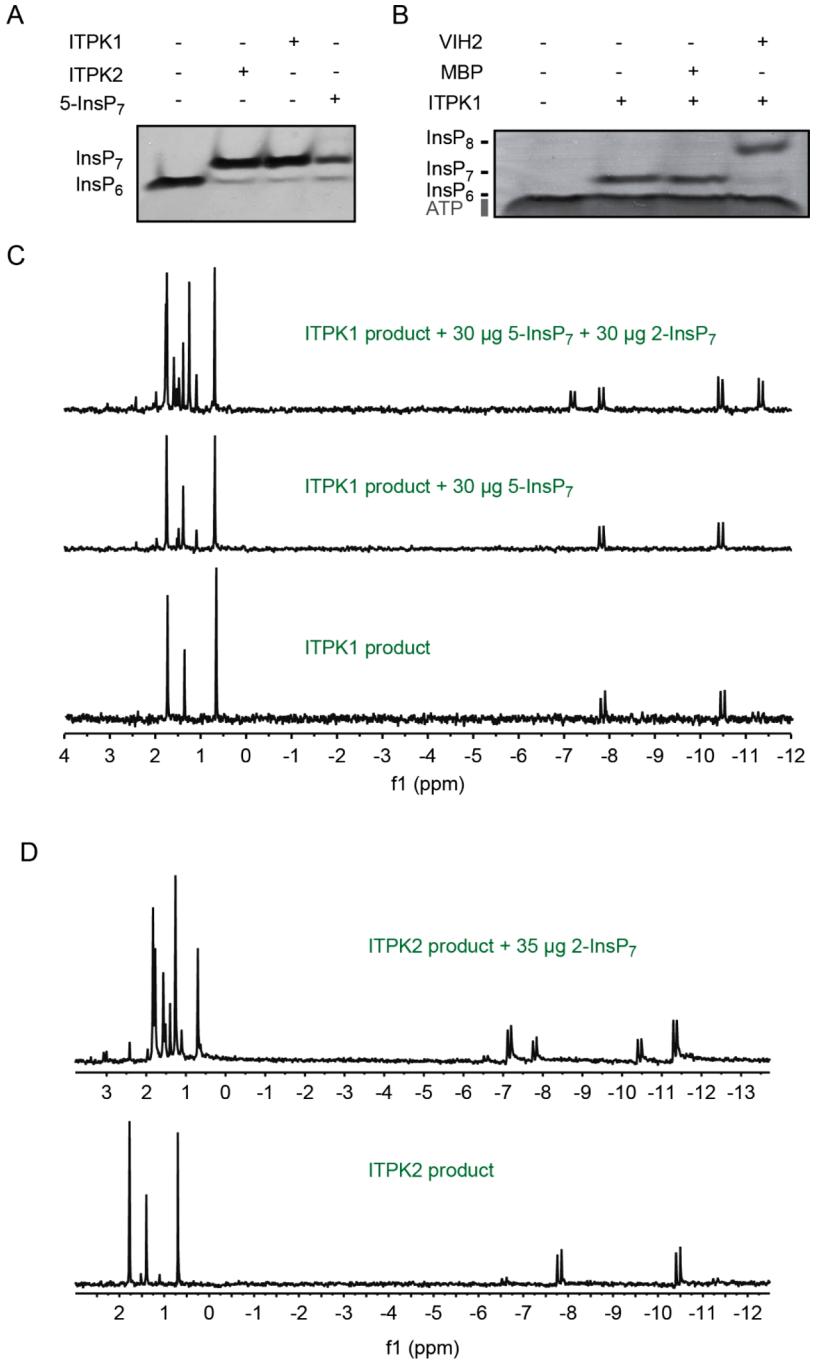

Figure 2. ITPK1 and ITPK2 catalyze the synthesis of the 5-$InsP_7$ isomer. (A) *In vitro* $InsP_6$ kinase assay with recombinant *Arabidopsis* ITPK1 and ITPK2. Reaction products were separated via PAGE and stained with toluidine blue. $InsP_6$ served as a control. (B) *In vitro* reconstitution of plant $InsP_8$ synthesis from $InsP_6$. *In vitro* kinase assay similar to panel A with the addition of VIH2 to the ITPK1 reaction mixture. Depending on the incubation time for destaining, the ATP/ADP band disappears or is visible as a smear below $InsP_6$. (C) Proton-decoupled $^{31}P[^1H]$ NMR analyses of PAGE-purified ITPK1 products reveal a symmetrical $InsP_7$ resonance pattern (bottom spectrum). Two signals with the integral 2 correspond to the phosphates in mirror-symmetry related positions 1/3 and 4/6 (δ 1.7, 0.7). One signal corresponds to a single phosphate at position 2 or 5 (δ 1.3), and resonances at δ −10.5 and −7.9 (doublets due to ^2J P−P coupling) represent the α-P and β-P, respectively, of a P-anhydride. Addition of 30 μg of synthetic 5-$InsP_7$ does not lead to the appearance of significant additional peaks (middle spectrum), while addition of 30 μg of synthetic 2-$InsP_7$ does so (top spectrum). (D) $^{31}P[^1H]$ NMR analyses of PAGE-purified ITPK2 products reported symmetrical $InsP_7$ (bottom). Resonances with the integral 2 at δ 1.7 and 0.7 correspond to the phosphates at enantiomeric positions 1/3 and 4/6, respectively. One signal corresponds to a single phosphate at the non-enantiomeric 2 or 5 position (δ 1.3), and resonances at δ −10.55 and −8.0 (each of which generates a phosphorus−phosphorus doublet) represent the α-P and β-P of a diphospho moiety, respectively. Addition of 35 μg of synthetic 2-$InsP_7$ caused the appearance of additional resonances (top).

appearance of several additional peaks. The most obvious differences stem from the P-anhydride resonances at δ −7.5 and −11.5 of 2-$InsP_7$ (Figure 2C). These results unambiguously show that ITPK1 phosphorylates $InsP_6$ at position 5 and not at position 2. ITPK2 behaved identically in these experiments and also generated a symmetric product distinct

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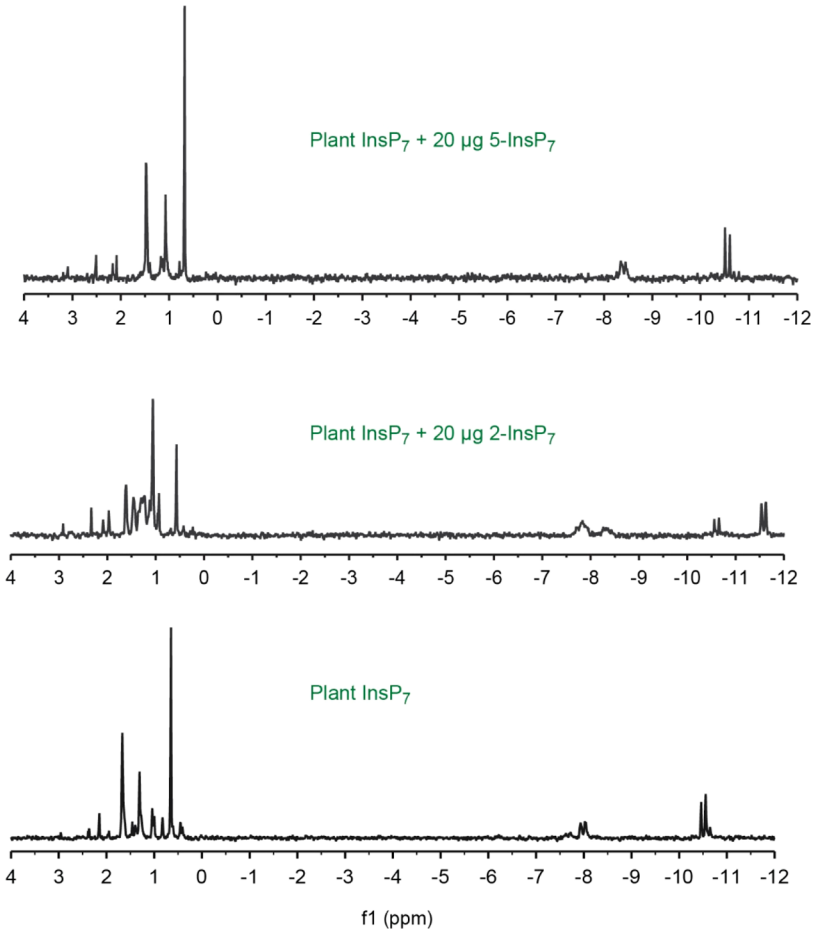

Figure 3. Structural elucidation of plant $InsP_7$ by NMR analyses. $^{31}P[^1H]$ NMR analyses of *mrp5* seed-purified $InsP_7$ reveal a symmetrical $InsP_7$ isomer (bottom) with resonances at δ 1.7 and 0.7 typical for the 1/3 and 4/6 phosphate esters, respectively, a resonance at δ 1.3 characteristic of a single phosphate ester in the mirror plane (i.e., 2 or 5 position), and phosphoanhydride resonances appearing as doublets at δ −10.55 and −8.0. Addition of 20 μg of 5-$InsP_7$ did not lead to the appearance of any additional resonances (top), while addition of the same amount of 2-$InsP_7$ to PAGE-purified plant $InsP_7$ resulted in resonances that were different from resonances of 5-$InsP_7$ (middle).

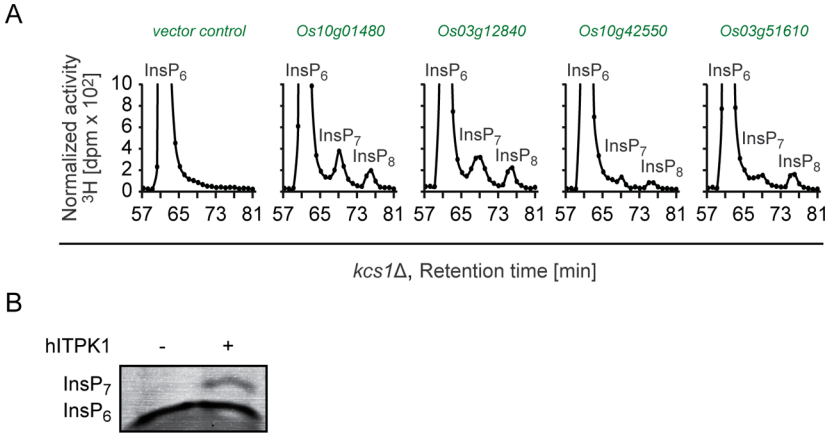

Figure 4. $InsP_6$ kinase activity of ITPK1 is evolutionarily conserved. (A) Four rice ITPK homologues as indicated possess $InsP_6$ kinase activity. SAX-HPLC analyses of designated [^{3}H]inositol-labeled yeast transformants. Cells transformed with pDR195 (vector control) served as negative controls. (B) Recombinant human ITPK1 phosphorylates $InsP_6$ *in vitro*. The kinase reaction was resolved by PAGE and visualized with toluidine blue.

from 2-$InsP_7$ (Figure 2D). Furthermore, mixing of ITPK1 and ITPK2 $InsP_6$ kinase reaction mixtures led to the same pattern of resonances in the ^{31}P NMR spectrum (Figure S3C). Taken together, these results show that both ITPK1 and ITPK2 synthesize 5-$InsP_7$ from $InsP_6$ *in vitro*.

Next, we elucidated the molecular structure of plant $InsP_7$ generated *in vivo*. To obtain sufficient material for NMR spectroscopy, plant $InsP_7$ was enriched using a TiO_2 pull-down protocol coupled with PAGE purification.[19,20] Because species more anionic than $InsP_6$ were not detected by PAGE when

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working with wild-type *Arabidopsis* extracts, we used *Arabidopsis mrp5* seeds, which are defective in vacuolar $InsP_6$ loading, thus increasing the substrate concentration for cyto- and/or nucleoplasmic $InsP_6$ kinases[9,21] (Figure S4A). PAGE analyses indicated that 100−200 nmol of $InsP_7$ with the chromatographic mobility of 5-$InsP_7$ was purified from *mrp5* seeds (Figure S4B). MALDI-MS of PAGE-purified material revealed a peak at m/z 738.8 corresponding to the mass of monodeprotonated $InsP_7$ (Figure S4C). The symmetrical ^{31}P NMR spectrum of plant-purified $InsP_7$ (Figure 3) appeared to be similar to those of the reaction products of ITPK1 and ITPK2 (Figure 2C,D). Spiking with 2-$InsP_7$ caused the appearance of two additional doublet signals (P−P coupling) around δ −8 and −12 and several other resonances in the phosphate monoester region (0−3 ppm). In contrast, addition of 5-$InsP_7$ did not lead to significant additional resonances (Figure 3). Overall, our ^{31}P NMR analyses confirm that plant $InsP_7$ is structurally identical to the $InsP_6$ kinase product of ITPK1 and ITPK2 and is 5-$InsP_7$.

Considering the prevalence of ITPKs throughout higher plants and metazoans (Figure S5), we explored whether the $InsP_6$ kinase function of these enzymes is evolutionarily conserved. To this end, we performed growth complementation and HPLC analyses with rice (*Oryza sativa*) ITPKs and identified four homologues with $InsP_6$ kinase function (Figure 4A and Figure S6). Notably, also purified recombinant human ITPK1 catalyzed the synthesis of $InsP_7$ from $InsP_6$ in the presence of ATP (Figure 4B). Collectively, these results suggest that *Arabidopsis* ITPK1 and ITPK2 possess an $InsP_6$ kinase function that is evolutionarily conserved throughout different kingdoms of higher eukaryotes.

The isomer identity and physiological roles of plant inositol pyrophosphates have remained largely enigmatic despite the clear presence of these diphosphoinositol polyphosphates in extracts of various plant species.[4,22] The inability to analyze these molecules was in part due to the absence of genes in plant genomes encoding homologues of canonical $InsP_6$ kinases such as yeast Kcs1 and metazoan IP6Ks. Here, we provide evidence that ITPKs have a high catalytic flexibility and not only phosphorylate lower inositol polyphosphates[13,23,24] but also can phosphorylate $InsP_6$. Our work opens new avenues for manipulating and better understanding inositol pyrophosphate signaling in eukaryotic cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.9b00423.

Additional data on heterologous expression of *Arabidopsis* inositol polyphosphate kinases in yeast, MS and NMR spectra of ITPK1- and ITPK2-dependent $InsP_7$, MS analysis of plant $InsP_7$, phylogenetic tree of ITPK proteins, evolutionary conservation of $InsP_6$ kinase function of rice ITPKs, and information about reagents and experimental procedures (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: gabriel.schaaf@uni-bonn.de.
*E-mail: d.laha@ucl.ac.uk.

ORCID

Debabrata Laha: 0000-0002-7823-5489
Nargis Parvin: 0000-0002-7744-1408
Alexandre Hofer: 0000-0001-6396-3689
Ricardo F. H. Giehl: 0000-0003-1006-3163
Nicolaus von Wirén: 0000-0002-4966-425X
Adolfo Saiardi: 0000-0002-4351-0081
Henning J. Jessen: 0000-0002-1025-9484
Gabriel Schaaf: 0000-0001-9022-4515

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Notes

The authors declare no competing financial interest.
During the revision of this manuscript, a study by Adepoju et al. (2019) also reported on the ability of ITPK1 and ITPK2 to catalyze the phosphorylation of $InsP_6$ to $InsP_7$ in vitro (https://www.biorxiv.org/content/10.1101/724914v1, DOI: https://doi.org/10.1101/724914).

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