ITPK1 is an InsP₆/ADP phosphotransferase that controls systemic phosphate homeostasis in Arabidopsis

Esther Riemer¹, Debabrata Laha^{2,#}, Robert K. Harmel^{3,#}, Philipp Gaugler^{1,#}, Verena Pries¹, Michael Frei¹, Mohammad-Reza Hajirezaei⁴, Nargis P. Laha¹, Lukas Krusenbaum¹, Robin Schneider¹, Henning J. Jessen⁵, Adolfo Saiardi², Dorothea Fiedler³, Gabriel Schaaf^{1*}, Ricardo F.H. Giehl^{4*}

ORCID IDs:

DL: 0000-0002-7823-5489, RKH: 0000-0003-4413-7730, NPL: 0000-0002-7744-1408, HJJ: 0000-0002-1025-9484, AS: 0000-0002-4351-0081, DF: 0000-0002-0798-946X, GS: 0000-0001-9022-4515, RFHG: 0000-0003-1006-3163

¹ Department of Plant Nutrition, Institute of Crop Science and Resource Conservation, Rheinische Friedrich-Wilhelms-Universität Bonn, 53115 Bonn, Germany.

² Medical Research Council Laboratory for Molecular Cell Biology (MRC-LMCB), University College London, London WC1E 6BT, United Kingdom.

³ Leibniz-Forschungsinstitut für Molekulare Pharmakologie, 13125 Berlin, Germany and Department of Chemistry, Humboldt Universität zu Berlin, 12489 Berlin, Germany.

⁴ Department of Physiology & Cell Biology, Leibniz-Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany.

⁵ Department of Chemistry and Pharmacy and CIBSS-Centre for Integrative Biological Signalling Studies, Albert-Ludwigs University Freiburg, 79104 Freiburg, Germany.

[#] These authors contributed equally to this work.

^{*}To whom correspondence and material requests should be addressed: gabriel.schaaf@uni-bonn.de and giehl@ipk-gatersleben.de.

27 ABSTRACT

28 In plants, phosphate (P_i) homeostasis is regulated by the interaction of P_i starvation response
 29 transcription factors (PHRs) with stand-alone SPX proteins, which act as sensors for inositol
 30 pyrophosphates (PP-InsPs). Recently, ITPK1 was shown to generate the PP-InsP InsP₇ from
 31 InsP₆ *in vitro*, but the importance of this activity in P_i signaling remained unknown. Here, we
 32 show that uncontrolled P_i accumulation in ITPK1-deficient plants is accompanied by impaired
 33 P_i -dependent InsP₇ and InsP₈ synthesis. Reciprocal grafting demonstrates that P_i starvation
 34 responses are mainly controlled by ITPK1 activity in shoots. Nuclear magnetic resonance
 35 assays and PAGE analyses with recombinant protein reveal that besides InsP₆
 36 phosphorylation, ITPK1 is also able to catalyze ATP synthesis using 5-InsP₇ but not any other
 37 InsP₇ isomer as a P-donor when ATP is low. Additionally, we show that the dynamic changes
 38 in InsP₇ and InsP₈ to cellular P_i are conserved from land plant species to human cells,
 39 suggesting that P_i -dependent PP-InsP synthesis is a common component of P_i signaling across
 40 kingdoms. Together, our study demonstrates how P_i -dependent changes in nutritional and
 41 energetic states modulate ITPK1 activities to fine-tune the synthesis of PP-InsPs.

42

43

44

45

46

47

48

49 INTRODUCTION

50 In order to maintain cellular P_i homeostasis, plants have evolved complex sensing and
 51 signaling mechanisms that adjust whole-plant P_i demand with external P_i availability.
 52 Although many molecular players involved in these responses have been identified, the exact
 53 mechanism of P_i sensing in complex organisms, such as plants, still remains largely unknown.
 54 In the model species *Arabidopsis thaliana*, the MYB transcription factors PHOSPHATE
 55 STARVATION RESPONSE 1 (PHR1) and its closest paralog PHR1-LIKE1 (PHL1) control
 56 the expression of the majority of P_i starvation-induced (PSI) genes and influence numerous
 57 metabolic and developmental adaptations induced by P_i deficiency^{1, 2}. In agreement with their
 58 regulatory function, a subset of P_i deficiency-induced genes is deregulated in *phr1 phl1*
 59 mutants. However, since *PHR1* itself is not transcriptionally regulated by P_i deficiency, the
 60 existence of a post-translational control of PHR1 has been proposed¹. Emerging evidence
 61 indicates that a class of stand-alone SPX proteins negatively regulate the activity of PHR
 62 transcription factors in different plant species^{3, 4, 5, 6, 7, 8}. According to these studies, when
 63 plants have access to sufficient P_i, SPX proteins bind with high affinity to PHRs, thereby
 64 preventing binding of these transcription factors to DNA. Under low P_i, the affinity of SPX
 65 proteins towards PHRs is decreased, thus allowing these transcription factors to activate their
 66 transcriptional targets⁴.

67 The *in vivo* interaction of PHR1 and SPX1 is influenced by P_i^{3, 4, 5}, suggesting that this
 68 mechanism could represent a direct link between P_i perception and downstream signaling
 69 events. However, the dissociation constants for P_i itself in a SPX-PHR complex ranged from
 70 10 mM to 20 mM^{3, 4, 5}, while P_i levels of only 60 μM to 80 μM have been recorded in the
 71 cytosol of plant cells⁹. A later study, demonstrated that SPX domains can actually act as
 72 receptors for inositol pyrophosphates (PP-InsPs) and isothermal titration calorimetry
 73 experiments revealed that 5PP-InsP₅ (hereafter referred to as 5-InsP₇) interacted more strongly

74 with SPX domains than P_i^{10} . In these assays, interaction of rice OsPHR2 and OsSPX4 was
 75 promoted at 5-InsP₇ concentrations as low as 20 μM^{10} . Although the isomeric nature of plant
 76 InsP₇ in vegetative tissues remains unknown, it has been proposed that the activity of PHRs is
 77 regulated by direct interaction with PP-InsPs. In support of this hypothesis, an Arabidopsis
 78 mutant for *INOSITOL PENTAKISPHOSPHATE 2-KINASE (IPK1)*, which is compromised in
 79 the synthesis of the PP-InsP precursor inositol hexakisphosphate (InsP₆), exhibits constitutive
 80 PSR and increased P_i accumulation when grown on sufficient P_i availability^{11, 12}. More
 81 recently, other mutants with compromised synthesis of PP-InsPs have been shown to exhibit
 82 disturbed PSR and P_i over-accumulation phenotypes^{13, 14, 15}, and it was observed that the
 83 levels of different inositol polyphosphates (InsPs) are significantly altered in P_i -deficient
 84 plants^{13, 14}. Interestingly, polyacrylamide gel electrophoresis (PAGE) analyses revealed that
 85 InsP₈ levels increase in P_i -sufficient plants and decrease as plants are exposed to P_i
 86 deficiency¹⁴, suggesting that the enzymes involved in the synthesis of PP-InsPs could act as
 87 regulators of P_i homeostasis in plants. This regulation was first discovered in the yeast
 88 *Saccharomyces cerevisiae* in which cellular levels of PP-InsPs decrease when exposed to P_i -
 89 deficient medium^{10, 16}. However, the biosynthetic pathway resulting in P_i -dependent PP-InsP
 90 synthesis still remains largely unresolved and it also remains unclear if this response is
 91 conserved in the plant lineage and across kingdoms.

92 In plants, synthesis of InsP₈ is mediated by VIH1 and VIH2¹⁷, a class of bifunctional
 93 kinase/phosphatase enzymes¹⁵ sharing homology to the yeast and animal Vip1/PPIP5Ks^{17, 18},
 94 ¹⁹. Although *vih1* and *vih2* single mutants do not exhibit impaired P_i accumulation¹³, deletion
 95 of both VIHs results in severe growth defects and P_i overaccumulation^{14, 15}. Since plant
 96 genomes do not encode homologues of the metazoan and yeast InsP₆ kinases IP6K/Kcs1, it
 97 has since long remained elusive how plants synthesize InsP₇. Using a yeast complementation
 98 assay, we recently identified Arabidopsis ITPK1 and ITPK2 as novel plant InsP₆ kinases and

demonstrated that ITPK1 generates the symmetric InsP₇ isomer 5-InsP₇, the major form identified in seed extracts²⁰. More recently, we further showed that InsP₇ and InsP₈ levels are compromised in TPK1-deficient plants, demonstrating that ITPK1 functions as an InsP₆ kinase *in planta*²¹. Considering that InsP₇ is the precursor for InsP₈ synthesis, the next challenge is to determine how InsP₇ levels respond to the plant's P_i status. A recent study reported that InsP₇ levels increase in shoots of P_i-deficient Arabidopsis plants as determined by HPLC analysis of [³²P]-P_i-labeled plant extracts¹³. However, this response is opposite to what has been described in yeast¹⁶ and mammalian cells²² and is inconsistent with increased InsP₈ levels detected by PAGE in P_i-sufficient Arabidopsis plants¹⁴. Considering that ³²P-labeling entails complicated molecule assignment and does not provide a mass assay of the inositol backbone but a readout for pyrophosphate moiety turnover, these results await confirmation via alternative approaches. Importantly, it remains unclear whether ITPK1 and ITPK2 contribute to PP-InsP synthesis in a P_i- and/or cellular energy status-dependent manner and how the proposed regulatory activity of InsP₈ to suppress PSR might be deactivated once plants experience P_i deficiency.

Here, we combine strong anion exchange chromatography (SAX)-HPLC analyses of [³H]-inositol-labeled seedlings and PAGE to investigate P_i-dependent changes in the levels of InsP₆, InsP₇ and InsP₈ across diverse species and several *Arabidopsis* mutants. We demonstrate that specifically in shoots, PP-InsPs decrease during P_i starvation and strongly increase after P_i resupply. P_i-dependent regulation of PP-InsPs was also highly conserved from diverse plant species to human cells. We find that ITPK1 is critical for the synthesis of PP-InsPs involved in the regulation of P_i homeostasis. In addition, we demonstrate that ITPK1-mediated conversion of InsP₆ to 5-InsP₇ requires high ATP concentrations and that ITPK1 has ADP phosphotransferase activity under conditions of decreased adenylate energy charge. These results provide a further link between P_i-dependent changes in nutritional and

energetic states with the synthesis of regulatory PP-InsPs. Finally, our study demonstrates that ITPK1 activity in shoots regulates PSR responses in a PHR1/PHL1-dependent manner, revealing that ITPK1 is a critical component of the systemic P_i sensing and signaling mechanism in plants.

RESULTS

Loss of ITPK1 but not ITPK2 affects P_i homeostasis in Arabidopsis

Previously, it has been reported that shoot P_i concentrations are significantly changed in a number of InsP biosynthesis mutants grown in hydroponics¹³. We assessed the growth of several of these mutants on soil and compared total P_i levels in shoots (measured with ICP-OES) with that of WT and the P_i -overaccumulator *pho2-1*. We confirmed that *ipk1-1* and *itpk1* plants accumulated significantly more total P_i in shoots, reaching levels comparable to those detected in *pho2-1* (Fig. 1a and 1b). To a much lesser degree, shoot P_i levels were also significantly increased in shoots of *mips1* and *vih2-4* plants, whereas P_i concentrations in all other mutants assessed, including *itpk2-2*, were similar to their respective WT. A full elemental analysis indicated that the concentrations of other nutrients were largely unaffected in shoots of *itpk1* plants as compared to WT (Suppl. Fig. 1). Total P_i levels were also significantly increased in flowers and seeds of *itpk1* plants, although the relative changes were less marked as those detected in rosette leaves (Suppl. Fig. 2). Excessive P_i accumulation could be almost completely complemented in transgenic lines expressing the genomic *ITPK1* fragment in the *itpk1* background (Fig. 1c and 1d), showing that P_i overaccumulation phenotype was indeed associated with the loss of ITPK1.

To investigate whether P_i accumulation is dependent on external P_i availability, we cultivated plants on agar plates supplemented with sufficient or insufficient P_i . A root phenotypical

analysis revealed that under sufficient P_i supply *itpk1* plants had shorter roots than WT plants, a phenotype that was also largely rescued in the recomplemented lines (Fig. 1e and 1f)²¹. The short-root phenotype of *itpk1* plants was also observed when plants were exposed to low P_i supply in agar plates (Fig. 1e and 1f) or cultivated in hydroponics (Suppl. Fig. 3), and was likely not associated with P_i overaccumulation, as the length of primary roots of *pho2-1* plants was similar to WT (Suppl. Fig. 3). Furthermore, we found that increased total P_i accumulation of *itpk1* plants was not detected in roots, while in shoots it was dependent on P_i availability (Fig. 1g and 1h). The shoot P_i overaccumulation phenotype of *itpk1* has been associated with defective down-regulation of PSRs in roots¹³. Our qPCR analysis confirmed that the expression of many PSI genes was up-regulated in *itpk1* roots but only when plants were grown under sufficient P_i or when P_i -deficient plants were resupplied with P_i for 6 hours (Suppl. Fig. 4). Together, these results demonstrate that loss of ITPK1 but not ITPK2 affects the regulation of PSR in plants.

ITPK1 is required for P_i -dependent synthesis of PP-InsPs

Recently, two studies have raised strong evidence for the role of InsP₈ in controlling P_i homeostasis in plants^{14, 15}. To further investigate which InsPs respond to rapid changes in the plant's P_i status, we first performed SAX-HPLC analyses of extracts from [³H]-inositol-labeled WT seedlings. Of all InsPs detected, only one InsP₃ isomer, InsP₆ and the PP-InsPs InsP₇ and InsP₈ decreased in response to P_i starvation and increased again when P_i was resupplied to P_i -starved seedlings for 6 hours (Fig. 2a and 2b). Notably for InsP₈, P_i resupply increased levels beyond those detected in seedlings continuously grown with sufficient P_i (Fig. 2b).

In order to increase the throughput of InsP₆, InsP₇ and InsP₈ analysis and to allow assessing fully developed plants under more physiological conditions, we grew plants in hydroponics with aerated solution. Highly anionic InsPs and PP-InsPs extracted from plant tissues were purified by titanium dioxide (TiO₂)-based pulldown followed by separation via PAGE and subsequent visualization by Toluidine Blue and 4',6-diamidino-2-phenylindole (DAPI) based on previously established protocols^{23, 24, 25}. In agreement with our HPLC analyses, InsP₆, InsP₇ and InsP₈ signals in shoots of WT plants were strongly decreased when plants were deprived of P_i for 4 days (Fig. 2c and 2d). However, only InsP₇ and especially InsP₈ level were quickly restored by refeeding plants with P_i for 12 hours. Compared to WT, InsP₇ and InsP₈ levels were significantly lower in shoots of *itpk1* plants especially after P_i resupply (Fig. 2c and 2d). These results confirmed that ITPK1 functions as a cellular InsP₆ kinase *in planta* and suggested that an ITPK1-dependent InsP₇ pool is required and rate limiting for the efficient synthesis of InsP₈ under conditions of high P_i availability. In contrast to *itpk1*, PP-InsP levels were not compromised in the *itpk2-2* mutant (Suppl. Fig. 5a), further suggesting that ITPK1 can compensate for the loss of *ITPK2*. Notably, in roots of both WT and *itpk1* plants, InsP₇ and InsP₈ were barely detected by PAGE (Fig. 2e).

To further address the role of different InsPs in PSR, we analyzed the *itpk4-1* mutant, which was recently reported to display reduced levels of InsP₅[1/3-OH], InsP₆ and InsP₇¹³. Whereas HPLC analysis of [³H]-inositol-labeled seedlings confirmed the reported defects in InsP₅ [1/-OH] synthesis²¹, our PAGE analyses did not detect any changes in InsP₆, InsP₇ nor InsP₈ levels in this mutant as compared to WT irrespective of the imposed P_i regime (Suppl. Fig. 5b and 5c). Thus, the undisturbed synthesis of PP-InsPs in *itpk4-1* plants may explain why this mutant shows no PSR phenotypes (Fig. 1b)¹³.

Considering that only InsP₇ and InsP₈ responded to P_i, we then investigated the accumulation of these PP-InsPs in the P_i overaccumulator mutant *pho2-1*. We found that, compared to WT,

pho2-1 plants accumulated much higher levels of InsP₇ and InsP₈, even when grown on low P_i for 4 days (Fig. 2f). Notably, elemental analysis revealed that P_i levels in shoots of *pho2-1* plants exposed to low P_i were still significantly higher than those detected in WT plants grown under sufficient P_i (Fig. 2g). Together, these results indicate that the synthesis of PP-InsPs is dependent on the internal, cellular P_i levels rather than external P_i availability.

Synthesis of InsP₇ and InsP₈ relies on ITPK1 and VIH2 and on InsP₆ compartmentalization

As suggested in previous studies, the presence of InsP₇ and InsP₈ is likely central for P_i sensing in cells, as they promote the interaction of PHR with SPX proteins^{10, 14, 15}. Intriguingly, our elemental analysis revealed that total P_i levels in shoots were not altered to the same degree in *vih2-4*, although previous HPLC analyses of [³H]-inositol-labeled seedlings indicated that InsP₈ levels are strongly decreased in this mutant¹⁷. We further confirmed by PAGE the strong decrease of InsP₈ in shoots of P_i-resupplied *vih2-4* plants (Fig. 3a and 3b). PAGE analysis also detected a significant increase in InsP₇ levels in *vih2-4* shoots after P_i resupply (Fig. 3a and 3b), suggesting that a certain InsP₇ pool is regulated by VIH2-dependent conversion to InsP₈ and that an increase of this pool in *vih2* mutant plants appears to partially compensate for the loss of InsP₈ in regulating P_i signaling.

ITPK1, *VIH1* and *VIH2* are not only expressed in shoots but also in roots^{13, 15}. However, since little to no InsP₇ and InsP₈ could be detected by PAGE in roots of Arabidopsis and rice plants (Fig. 2e and Suppl. Fig. 6), we wondered whether the availability of cytosolic InsP₆ could determine the amount of InsP₇ and InsP₈ that can be synthesized. To test this hypothesis, we assessed these PP-InsPs in shoots and roots of *mrp5*, a mutant defective in vacuolar loading of InsP₆²⁶. In contrast to WT, we could detect InsP₇ and InsP₈ both in shoots and in roots of

mrp5 plants (Fig. 3c and 3d). Lack of *MRP5* resulted in higher levels of InsP₇ and InsP₈ as compared to WT but InsP₈ was still clearly responsive to P_i starvation and P_i resupply in this mutant. However, increased levels of these PP-InsPs in roots did not significantly affect P_i accumulation in *mrp5* shoots (Fig. 1b). Altogether, these results demonstrate that P_i-dependent synthesis of InsP₇ and InsP₈ relies on stepwise phosphorylation reactions mediated by ITPK1 and VIHs, and on the cytosolic/nucleoplasmic availability of InsP₆.

ITPK1 has InsP₆ kinase and ATP synthase activities

Recently, we demonstrated that recombinant ITPK1 phosphorylates InsP₆ *in vitro* at position 5 thereby generating 5-InsP₇, which is the main InsP₇ isomer detected in seeds²⁰. Considering that ITPK1 is required for the robust increase in InsP₈ when P_i-deficient plants are resupplied with P_i (Fig. 2c and 2d), we asked whether ITPK1 is able to function as an InsP₇ kinase. As shown in Suppl. Fig. 7a, neither of the natural occurring isomers, 1-InsP₇ and 5-InsP₇, appears to be a substrate for ITPK1 kinase activity suggesting that the ITPK1-dependent increase in InsP₈ after P_i resupply is caused by an increase in a rate limiting pool of InsP₇. To further investigate the enzymatic properties of ITPK1, we performed nuclear magnetic resonance (NMR) assays with the recombinant protein taking advantage of [¹³C₆]-labelled InsP₆. First, InsP₆ kinase reaction conditions were analyzed with respect to magnesium ion (Mg²⁺) concentration and temperature dependencies as well as to quenching efficiency by EDTA (Suppl. Fig. 7b-d). Using 2.5 mM ATP, optimal enzyme activity was observed with as little as 2.5 mM Mg²⁺ and conversion could be fully quenched by a surplus of EDTA exceeding the Mg²⁺ concentration by 1.36 mM. Even though the protein behaved well at 37°C and increased kinase activities were observed at this elevated temperature (Suppl. Fig. 7d), we decided to carry out subsequent experiments at 25°C to more closely reflect temperatures in unstressed plants. Subsequent kinetic analysis revealed that ITPK1 exhibits a surprisingly high K_M for

ATP of approximately 520 μ M (Fig. 4a and 4b). Unlike VIHs, the kinase activity of ITPK1 was largely insensitive to P_i and was also not affected by the non-metabolizable P_i analog phosphite (Suppl. Fig. 8). When InsP₅ [2-OH] was presented as substrate to ITPK1, no conversion could be detected (Suppl. Fig. 7e), suggesting that ITPK1 has no IPK-like activity to generate InsP₆ from InsP₅ [2-OH]. Furthermore, in contrast to InsP₆ kinases of the IP6K/Kcs1 family, no activity was observed when 1-InsP₇ was used as a substrate (Suppl. Fig. 7f and 7g), thus confirming our PAGE analysis (Suppl. Fig. 7a).

The characterization of structurally and sequence-unrelated mammalian InsP₆ kinases of the IP6K family has demonstrated that these enzymes can shift their activities from kinase to ADP phosphotransferase at low ATP-to-ADP ratios^{27, 28}. This prompted us to assess if also ITPK1 possesses such activity. *In vitro* reactions with unlabeled 5-InsP₇ and subsequent PAGE analyses revealed that ITPK1 indeed mediates 5-InsP₇ hydrolysis and that this activity only occurs in the presence of ADP (Fig. 4c). A similar activity using any other InsP₇ isomer as phosphoryl donor could not be detected (Fig. 4d), suggesting a high degree of substrate specificity not only for ITPK1-mediated kinase activity but also for the reverse reaction (i.e., InsP₇ dephosphorylation / ATP synthesis). To determine the kinetic parameters of this reaction, we subsequently incubated ITPK1 with [¹³C₆]-labelled 5-InsP₇ in the presence of ADP and detected the formation of ATP and InsP₆ (Suppl. Fig. 9a and 9b). No ATP formation was detected when the enzyme was incubated without 5-InsP₇ (Suppl. Fig. 10c). Interestingly, the velocity of the reverse reaction was almost two times faster than the forward reaction, whereas the K_M for ADP and ATP was relatively similar for ITPK1 (Fig. 4b, 4e and 4f). Taken together, these results demonstrate that ITPK1-mediated InsP₆ kinase and ADP phosphotransferase activities are regulated by adenylate energy charge. In agreement with results obtained in agar plate-grown seedlings¹⁵, we observed that ATP levels and ATP/ADP ratios dropped significantly in response to P_i deficiency in shoots of hydroponically-grown

WT plants but rapidly increased after P_i resupply (Suppl. Fig. 10). Thus, the P_i -dependent changes in energy status of plants may ultimately regulate the synthesis of InsP₇ by shifting the activity of ITPK1.

ITPK1 activity in shoots controls PHR1- and PHL1-regulated PSRs

To directly address whether ITPK1-dependent synthesis of PP-InsPs in shoots or roots is responsible for P_i accumulation, we performed grafting experiments. As expected, shoot P_i overaccumulation was also detected when roots and shoots of *itpk1* plants were self-grafted (Fig. 5a). However, shoot P_i was almost back to WT levels when Col-0 shoots were grafted onto *itpk1* roots, while remaining approximately 75% higher when *itpk1* shoots were grafted onto Col-0 roots (Fig. 5a). Shoot dry weight or shoot levels of other nutrients were not significantly altered by the different graft combinations (Suppl. Fig. 11). These results suggest that ITPK1 activity in shoots is more determinant for the regulation of PSR and P_i accumulation.

We then addressed the putative involvement of ITPK1 in P_i signaling by analyzing the genetic interaction between ITPK1 and the transcription factors PHR1 and PHL1, which together control most of the transcriptional responses induced by P_i starvation². Shoot growth was not significantly altered in homozygous double or triple mutants as compared to single or double mutants (Fig. 5b and 5c). Although *phr1 itpk1* and *phr1 phl1 itpk1* plants still accumulated significantly more P_i than *phr1* and *phr1 phl1*, respectively, the relative increments were smaller than in the presence of functional PHR1 and PHL1 (Fig. 5d). In turn, the short-root phenotype caused by *ITPK1* mutation could not be restored by knocking out these transcription factors (Suppl. Fig. 12). While most P_i starvation-induced transcriptional

responses were also suppressed in the triple mutant, absence of ITPK1 maintained *PHT1*;8 up-regulated in the *phr1 phl1* background (Fig. 5e) potentially suggesting a PHR1/PHL1-independent regulation of *PHT1*;8 expression. Finally, PAGE analysis revealed that InsP₈ levels remained very low when these two transcriptional regulators were knocked out (Fig. 5f and 5g). In contrast, P_i resupply-induced InsP₇ accumulation was largely independent of PHR1 and PHL1. Collectively, these observations demonstrate that ITPK1-dependent PP-InsP synthesis in shoots is required for undisturbed coordination of systemic P_i signaling by PHR1 and PHL1.

P_i-dependent synthesis of PP-InsPs is conserved across species

Next, we assessed whether P_i-dependent regulation of InsP₇ and InsP₈ is evolutionarily conserved across species. Similar to Arabidopsis, we observed that both InsP₇ and InsP₈ levels decreased strongly in response to P_i-deficiency in shoots of hydroponically-grown rice plants and were quickly restored by refeeding plants with P_i (Fig. 6a and 6b). Notably, in this species, a clear InsP₇ signal could be observed as soon as 30 min after P_i resupply and appeared before substantial changes in InsP₈ could be detected by PAGE. We also detected increased accumulation of PP-InsPs in P_i-replete and P_i-refed gametophores of the moss *Physcomitrella patens* (Fig. 6c-6e), suggesting that P_i-dependent InsP₇ and InsP₈ synthesis is also conserved in non-vascular land plants.

Since we recently showed that purified recombinant human ITPK1 is also able to catalyze 5-InsP₇ synthesis²⁰, we assessed P_i-dependent synthesis of PP-InsPs in the human HCT116 cell line. We found that while InsP₆ levels remained largely unaffected by P_i conditions, both InsP₇ and InsP₈ decreased in cells after removing P_i from the culture and sharply increased again after P_i resupply (Fig. 6f). Altogether, these results indicate that P_i-dependent synthesis of InsP₇ and InsP₈ seems to be evolutionary conserved across a range of different multicellular species.

319

320 DISCUSSION

321 Previously, isothermal titration calorimetry experiments demonstrated that 5-InsP₇ interacted
 322 more strongly with SPX domains than P_i and that 5-InsP₇ concentrations as low as 20 μM can
 323 effectively promote the interaction of OsSPX4 with OsPHR2¹⁰, suggesting that InsP₇ can
 324 affect P_i signaling. The finding that an *itpk1* mutant in Arabidopsis over-accumulates P_i and
 325 constitutively express P_i starvation-induced genes under sufficient P_i (Fig. 1b; Suppl. Figs. 2
 326 and 4)¹³ provided further evidence that ITPK1 function is required for proper regulation of P_i
 327 homeostasis. In this work, we demonstrate that InsP₇ and InsP₈ most closely mirror P_i levels
 328 in different organisms as they decrease in response to P_i deficiency and quickly increase after
 329 P_i resupply (Fig. 2a-2c; Fig. 6). In *A. thaliana*, we show that both InsP₇ and InsP₈ levels are
 330 compromised in shoots of *itpk1* plants, especially when P_i-deficient plants are resupplied with
 331 P_i (Fig. 2c and 2d). Since we detected only InsP₆ kinase but no InsP₇ kinase activity with
 332 purified recombinant ITPK1 (Fig. 4c; Suppl. Fig. 7a)²⁰, the decreased InsP₈ levels in *itpk1*
 333 plants likely resulted from the diminished 5-InsP₇ available for the subsequent
 334 phosphorylation at the C1 position by VIH1 and VIH2. Our results also indicate that ITPK1
 335 can shift its activity and become an ADP phosphotransferase that specifically hydrolyses 5-
 336 InsP₇ when adenylate energy charges are low, such as in P_i-deficient cells (Fig. 4; Suppl. Fig.
 337 10). Thus, our results with ITPK1 and those of Zhu et al.¹⁵ with VIH1 and VIH2 provide a
 338 detailed view on how changes in cellular P_i status alter the activity of these enzymes to
 339 efficiently produce and eliminate the signaling molecules InsP₇ and InsP₈ in plants (Fig. 7).

340 Although both ITPK1 and ITPK2 possess InsP₆ kinase activity²⁰, PSR seems only disturbed in
 341 *itpk1* but not *itpk2* mutants (Fig. 1b)¹³. PP-InsPs are not significantly altered in *itpk2* seedlings
 342 (Suppl. Fig. 5a)²¹, suggesting that ITPK1 can fully compensate for the loss of ITPK2.

Furthermore, since only *ITPK2* transcription is induced by P_i deficiency (Suppl. Fig. 13), it is likely that ITPK1 and ITPK2 play independent roles in P_i signaling. In a previous study, the putative role of InsP₇ in plant P_i signaling was excluded because HPLC analysis of [³²P]- P_i -labeled plants showed that InsP₇ levels were similarly compromised in *itpk1* and *itpk4* plants¹³, whereas only *itpk1* displays obvious PSR defects (Fig. 1b)¹³. However, our PAGE analyses showed that InsP₇ and InsP₈ levels are not compromised in the *itpk4-1* mutant (Suppl. Fig. 5b and 5c). Therefore, a role for InsP₇ and/or InsP₈ cannot be excluded based on the comparison of *itpk1* and *itpk4-1*. Meanwhile, two recent studies raised compelling evidence that InsP₈ acts as a signaling molecule to regulate P_i homeostasis in Arabidopsis, as *vih1 vih2* double mutants exhibit constitutive up-regulation of PSI genes and strong P_i hyperaccumulation^{14, 15}. Here, we show that a P_i -dependent accumulation of InsP₈ and InsP₇ appears to be conserved in land plants, including dicotyledonous and monocotyledonous vascular species as well as non-vascular species (Fig. 2a-2c; Fig. 6a-e). Of note, our PAGE results from independent experiments demonstrate that InsP₈ is more sensitive to P_i than InsP₇. However, InsP₇ levels were clearly reduced when plants were exposed to more prolonged periods of P_i starvation (Fig. 6a, 6b and 6e). Furthermore, we also found that not only InsP₇ but also InsP₈ is induced by P_i in human HCT116 cells (Fig. 6f). P_i -dependent regulation of InsP₈ in human cells has likely also an impact on P_i homeostasis, as this PP-InsP was recently shown to be functionally dominant over 5-InsP₇ and 1-InsP₇ in the regulation of the cellular P_i exporter protein Xenotropic and Polytopic Retrovirus Receptor 1 (XPR1)²⁹.

In Arabidopsis, InsP₈ levels are strongly reduced while InsP₇ significantly increased in the *vih1 vih2* double mutant¹⁵, suggesting that InsP₇ itself plays only a relatively minor role in P_i signaling. However, considering the evidence for the involvement of InsP₈ in other cellular processes^{17, 30, 31}, it might well be that the severe growth defects of *vih1 vih2* plants^{14, 15} are not solely related to disturbed P_i homeostasis. Here, we analyzed the *vih2-4* single mutant,

which grows similarly to the WT (Fig. 1a), and found that whereas InsP₈ levels in *vih2-4* plants were as low as in *itpk1*, strong P_i overaccumulation was only observed for *itpk1* plants (Fig. 1b; Fig. 3a and 3b). Since InsP₈ reduction in *vih2-4* plants is accompanied by increased InsP₇ levels (Fig. 3a and 3b), it is likely that, under certain circumstances, InsP₇ may partially compensate for the loss of InsP₈. Noteworthy, also InsP₇ is involved in other cellular processes, such as auxin perception, as 5-InsP₇ can bind to the auxin receptor complex²¹. Importantly, the role of ITPK1 in auxin perception is largely independent of its function in P_i homeostasis²¹, suggesting that different tissue-specific InsP₇ pools may regulate diverse signaling pathways. This assumption is supported by the fact that the auxin-associated short-root phenotype of *itpk1* plants cannot be complemented with P_i nor attenuated by disrupting PHR1 and PHL1 (Fig. 1e and 1f; Suppl. Figs. 3 and 12). However, to directly assess the contribution of InsP₇ to different physiological processes, it would be necessary to develop a strategy to specifically disrupt InsP₇ synthesis without altering InsP₈ levels.

The genetic analysis of *phr1 itpk1* and *phr1 phl1 itpk1* multiple mutants demonstrated that, in the absence of PHRs, *ITPK1* mutation did not anymore mis-regulate the expression of P_i starvation-induced genes nor result in uncontrolled P_i accumulation under sufficient P_i (Fig. 5d and 5e). These results place ITPK1 upstream of PHRs and further support that P_i-dependent synthesis of InsP₇ by ITPK1 and of InsP₈ by VIHs is critical for undisturbed P_i signaling in plants. Our grafting experiment further demonstrated that P_i overaccumulation in *itpk1* was mainly due to missing ITPK1 activity in shoots rather than in roots (Fig. 5a), thus pointing to a major role of PP-InsPs in P_i signaling in shoots. This result was somewhat surprising, since *ITPK1*, *VIH1* and *VIH2* are expressed in shoots and roots^{13, 15, 17}. However, in line with earlier indications from [³²P]-P_i-labeling¹³, our PAGE analyses also detected much higher InsP₇ and InsP₈ levels in shoots than in roots of both Arabidopsis and rice plants (Fig. 2c and 2e; Suppl. Fig. 6). However, accumulation of these PP-InsPs in roots could be induced

by disturbing MRP5-mediated InsP₆ loading into the vacuole (Fig. 3d). Nonetheless, it remains unknown whether InsP₆ availability to ITPK1 in leaves is also regulated by P_i. Whereas ITPK1 is located both in the nucleus and in the cytoplasm¹³, VIH1 and VIH2 are reported to display cytoplasmic localizations¹⁵. Given that SPXs act as receptors for PP-InsPs^{10, 14, 32, 33, 34}, ligand availability controls the interaction between SPXs and PHRs. To affect P_i signaling, PP-InsPs most likely have to cross the nuclear envelope, especially because SPX1, SPX2 and SPX3 are localized in the nucleus^{4, 35}. Alternatively, they may also interact with proteins located in the cytoplasm, such as SPX4³⁶.

The emerging model is that in the presence of low levels of PP-InsPs ligands, as when plants suffer from P_i deficiency (Fig. 2a-2d; Fig 6a-6e), SPX receptors are unable to bind to PHRs. Consequently, these transcription factors are free to bind to the P1BS motifs present in the promoters of several P_i-responsive genes^{1, 2}. Our findings that InsP₇ and InsP₈ levels quickly increase after P_i resupply in different plant species (Fig. 2a-2d; Fig. 6a-6e) provide insights into how this mechanism can be reversed once P_i-starved plants regain access to P_i. It is likely that the increased ligand levels shortly overlap with high abundance of SPX proteins, allowing for the formation of SPX - PHR complexes and the subsequent quick inhibition of PHR-dependent transcriptional responses.

Our PAGE analyses demonstrate that InsP₇ and InsP₈ levels dynamically reflect the P_i status in different organisms, indicating that PP-InsPs synthesis and degradation (or simply turnover) must be tightly controlled. P_i-dependent accumulation of InsP₈ has been proposed to rely on the bifunctional activity of the diphosphoinositol pentakisphosphate kinases VIH1 and VIH2^{14, 15}. The relative kinase and phosphatase activities of this type of bifunctional enzymes can be shifted according to cellular ATP and P_i levels. Indeed, purified Vip1 from yeast exhibited kinase activity and, hence, increased InsP₈ synthesis at higher ATP concentrations, whereas at low ATP levels the protein functioned mainly as an InsP₈ phosphatase¹⁵. In this

context, the phosphatase activity of diphosphoinositol pentakisphosphate kinases is further inhibited by P_i itself^{15, 22}. However, unlike VIHs, ITPK1 does not harbor the bifunctional kinase domain - phosphatase domain architecture but only the atypical ‘ATP-grasp fold’ ATP-binding site. Here, we demonstrate with independent approaches that ITPK1-mediated $InsP_6$ conversion to 5- $InsP_7$ depends on ATP availability (Fig. 4). Our NMR-based kinetic assays also revealed that Arabidopsis ITPK1 has a high K_M of approximately 523 μM for ATP (Fig. 4b), which is similar to the K_M recorded for mammalian IP6Ks^{27, 28}, suggesting that ITPK1-dependent $InsP$ synthesis will be compromised at low cellular adenylate energy charge.

In metazoans and yeast, PP- $InsPs$ act as energy sensors and metabolic messengers^{37, 38}, and fluctuations in ATP levels have been shown to correlate with changes in intracellular concentrations of $InsP_7$ ³⁹. In our conditions, we observed that, compared to P_i -deficient plants, ATP levels and ATP/ADP ratios are significantly higher in plants grown continuously with sufficient P_i or shortly after P_i is resupplied to P_i -deficient plants (Suppl. Fig. 10). Previous reports have indeed shown that in plants, yeast and human cells ATP levels drop in response to P_i starvation^{15, 22, 40, 41}. Interestingly, we found that ITPK1 has also a high K_M for ADP and, in the presence of this adenine nucleotide, converted 5- $InsP_7$ to $InsP_6$ generating ATP in the process (Fig. 4c, 4d and 4e; Suppl. Fig. 9a). This activity is reminiscent of the ATP synthase activity recorded for mammalian IP6K-type $InsP_6$ kinases^{27, 28} and even for the human diphosphoinositol pentakisphosphate kinase PPIP5K2⁴². Our kinetic analyses demonstrated that the enzyme has comparable K_M values for ATP and ADP and similar V_{max} values, i.e. similar efficiencies for the forward and reverse reactions (Fig. 4b and 4f), suggesting that relative adenylate energy charge determines whether ITPK1 phosphorylates $InsP_6$ or synthesizes ATP from 5- $InsP_7$. Consequently, ITPK1 activity would allow cells to, for example, rapidly remove the signaling molecule $InsP_7$ in low P_i conditions, when ATP

levels and ATP/ADP ratios decrease. This type of deactivation of InsP₇/InsP₈ signaling could bypass the requirement for dedicated PP-InsP hydrolases which are likely to slow down quick dynamic changes in PP-InsPs to induce jasmonate-related responses during wound response or insect attack¹⁷, or when P_i becomes suddenly available (Fig. 2a-2d; Fig. 6). While activation of the reverse reaction is unlikely to significantly alter global cellular ATP pools, localized ATP synthesis could quickly suppress InsP₇-mediated P_i signaling and, at the same time, might buffer the adenylate energy charge in the vicinity of ITPK1 under conditions of low energy or low P_i supply. Thus, adenylate charge-driven changes in the activities of ITPK1 and VIHs may represent one underlying mechanism by which the cellular P_i status is transduced into specific PP-InsPs levels to regulate downstream signaling events.

METHODS

Arabidopsis thaliana: plant materials and growth conditions

Seeds of *Arabidopsis thaliana* T-DNA insertion lines *itpk1* (SAIL_65_D03), *itpk2-2* (SAIL_1182_E03), *itpk3* (SALK_120653), *itpk4-1* (SAIL_33_G08), *ipk1-1* (SALK_065337C), *vih2-4* (GK-080A07), *mips1* (SALK_023626), *mips2* (SALK_031685), *mips3* (SALK_120131), *mrp5* (GK-068B10), *pho2-1* (EMS mutant described previously⁴³) and *phr1* (SALK_067629) were obtained from The European Arabidopsis Stock Centre (<http://arabidopsis.info/>). The *phr1 phl1* double mutant used in this study was described previously¹². To generate the *phr1 itpk1* double and the *phr1 phl1 itpk1* triple mutant, we crossed *itpk1* (-/-) with, respectively, *phr1* (-/-) and the homozygous *phr1 phl1* mutant. F2 and F3 plants were genotyped by PCR using the primers indicated in Suppl. Table 1 to identify homozygous lines. Transgenic lines expressing the genomic *ITPK1* fragment in the *itpk1* background were generated as described previously²¹.

For sterile culture, *Arabidopsis* seeds were surface sterilized in 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 for 15 min and washed twice with 96% (v/v) ethanol. Sterilized seeds were

sown onto modified half-strength Murashige and Skoog (MS) medium⁴⁴ containing 0.5% sucrose, 1 mM MES pH 5.5 and solidified with 1% (w/v) Difco agar (Becton Dickinson). After 7 days of preculture, seedlings were transferred to vertical plates containing fresh solid media supplemented with either sufficient P_i (625 µM P_i) or low P_i (5 µM P_i). Plants were grown under axenic conditions in a growth cabinet under the following regime: 10/14 h light/dark; light intensity 120 µmol m⁻² s⁻¹ (fluorescent lamps); temperature 22°C/18°C.

For hydroponic culture, Arabidopsis seeds were pre-cultured on rock wool moistened with tap water. After 1 week, tap water was replaced by half-strength nutrient solution containing 2 mM NH₄NO₃, 1 mM KH₂PO₄, 1 mM MgSO₄, 1 mM KCl, 250 µM K₂SO₄, 250 µM CaCl₂, 100 µM Na-Fe-EDTA, 30 µM H₃BO₃, 5 µM MnSO₄, 1 µM ZnSO₄, 1 µM CuSO₄ and 0.7 µM NaMoO₄ (pH adjusted to 5.8 by KOH). After 7 days, nutrient solution was changed to full-strength and replaced once a week (first 3 weeks), twice a week in the fourth week, and every 2 days in the following weeks. Aeration was provided to roots from the third week onwards.

To induce P_i deficiency, KH₂PO₄ was replaced by KCl and P_i resupply was performed by refeeding P_i-starved plants with 1 mM KH₂PO₄ for 12 h. Plants were grown hydroponically in a growth chamber under the above-mentioned conditions except that the light intensity was 200 µmol photons m⁻² s⁻¹ and supplied by halogen lamps.

Phenotypic characterization in soil substrate was performed by germinating seeds directly in pots filled with peat-based substrate (Klasmann-Deilmann GmbH, Germany). The pots were placed inside a conditioned growth chamber with a 22°C/18°C and 16-h/8-h light/dark regime at a light intensity of 120 µmol photons m⁻² s⁻¹ supplied by fluorescent lamps. Plants were bottom watered at regular intervals. Seedlings were thinned after 1 week to leave only two plants per pot. Whole shoots or different plant parts were harvested as indicated in the legend of figures.

Cultivation of rice in hydroponics

Rice plants (cv. Nipponbare) were cultivated in hydroponics inside a glasshouse with natural light supplemented with high pressure sodium vapor lamps to ensure a minimum light intensity of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $30^{\circ}\text{C}/25^{\circ}\text{C}$ day (16 h)/night (8 h) temperature. Seeds were germinated in darkness at 20°C for 3 days and then transferred to meshes floating on a solution containing 0.5 mM CaCl_2 and $10 \mu\text{M Na-Fe-EDTA}$, which was exchanged every third day. After 10 days, homogenous seedlings were transplanted into 60-L tanks filled with half-strength nutrient solution⁴⁵. Ten days later, the nutrient solution was changed to full-strength and exchanged every 10 days. During the whole growing period, the pH value was adjusted to 5.5 every second day. P_i starvation was imposed for 10 days before starting P_i resupply.

Cultivation of *Physcomitrella patens*

Physcomitrella patens was grown on Knop medium⁴⁶ solidified with 0.8% agar (A7921, Sigma). Light was provided by fluorescent lamps ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) under a regime of 16 h light and 8 h darkness at constant 20°C . P_i treatments were achieved by transferring pre-cultivated plants to fresh Knop solid media containing $1.8 \text{ mM KH}_2\text{PO}_4$ ($+\text{P}_i$) or 1.8 mM KCl ($-\text{P}_i$) for 30 days. At the end of P_i starvation period, part of the plants was resupplied with $1.8 \text{ mM KH}_2\text{PO}_4$ and harvested after 24 h or 96 h.

Cultivation of HCT116 cells

Mammalian cells were cultivated as described²³. Briefly, HCT116 cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and 0.45% glucose in a humidified atmosphere with 5% CO_2 . Phosphate starvation was induced with DMEM without sodium phosphate supplemented with 10% dialyzed FBS. Cells were washed twice in the phosphate-free medium before incubation with DMEM media with or without phosphate. Analysis of InsPs from HCT116 cell lines was performed as previously described²³.

520

521 **Grafting experiment**

522 Seedlings to be grafted were germinated on plates containing half-strength MS (Duchefa),
 523 0.04 mg L⁻¹ 6-benzylaminopurine (Sigma), 0.02 mg L⁻¹ indole acetic acid (Sigma), and 10 g
 524 L⁻¹ Difco agar (Becton Dickinson). Five-day-old seedlings were grafted on the plate by the
 525 90-degree blunt end technique with an ultra-fine micro knife (Fine Scientific Tools, USA)
 526 without collars. The grafted seedlings remained on the plate for 2 weeks to allow the
 527 formation of the graft union. Successfully unified seedlings were transplanted directly to peat-
 528 based soil and whole shoots harvested for elemental analysis 2 weeks later.

529

530 **RNA isolation and quantitative real-time PCR**

531 Root and shoots tissues were collected by excision and immediately frozen in liquid N₂. Total
 532 RNA was extracted with RNeasy Plant Mini Kit (Macherey-Nagel GmbH & Co KG,
 533 Germany). Quantitative reverse transcriptase PCR reactions were conducted with the
 534 CFX384TM Real-Time System (Biorad, Germany) and the Go Taq qPCR Master Mix
 535 SybrGreen I (Promega) using the primers listed in Supplementary Table 1. *UBQ2* was used as
 536 reference gene to normalize relative expression levels of all tested genes. Relative expression
 537 was calculated according to published instructions⁴⁷.

538

539 **Elemental analysis**

540 Whole shoots were dried at 65°C and digested in concentrated HNO₃ in
 541 polytetrafluoroethylene tubes under pressurized system (UltraCLAVE IV, MLS). Elemental
 542 analysis of plant samples from hydroponics or pot experiments was performed by inductively
 543 coupled plasma optical emission spectrometry (ICP-OES; iCAP 700, Thermo Fisher
 544 Scientific), whereas samples from agar plate-grown plants were analyzed by sector field high-

resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS; ELEMENT 2, Thermo Fisher Scientific). Element standards were prepared from certified reference materials from CPI International.

Titanium dioxide bead extraction and PAGE

InsPs purification and analysis was performed based on a previously established protocol^{23, 24, 25}. All steps until dilution were performed at 4°C. TiO₂ beads (Titanium (IV) oxide rutile, Sigma Aldrich) were weighted to 10 mg for each sample and washed once in water and once in 1 M perchloric acid (PA). Liquid N₂ frozen plant material was homogenized using a pestle and immediately resuspended in 800 µl ice-cold PA. Samples were kept on ice for 10 min with short intermediate vortexing, then centrifuged for 10 min at 20,000×g at 4°C using a refrigerated bench top centrifuge. The supernatants were transferred into fresh 1.5 mL tubes and centrifuged again for 10 min at 20,000×g. To absorb InsPs onto the beads the supernatants were resuspended in the pre-washed TiO₂ beads and rotated at 4°C for 30-60 min. Afterwards, beads were pelleted by centrifuging at 8000 g for 1 min and washed twice in PA. The supernatants were discarded. To elute inositol polyphosphates, beads were resuspended in 200 µl 10% ammonium hydroxide and then rotated 5 min at room temperature. After centrifuging, the supernatants were transferred into fresh 1.5-mL tubes. The elution process was repeated and the second supernatants were added to the first. Eluted samples were vacuum evaporated at 45°C to dry completely. InsPs were resuspended in 20 µL ultrapure water and separated by 33% polyacrylamide gel electrophoresis and visualized by Toluidine Blue staining, followed by 4',6-diamidino-2-phenylindole (DAPI) staining.

ITPK1 *in vitro* kinase and ATP synthase assay

Recombinant *A. thaliana* ITPK1 was purified based on the previously established protocol⁴⁸. The InsP₆ kinase assay was performed by incubating 10.17 μM enzyme in a reaction mixture containing 5 mM MgCl₂, 20 mM HEPES (pH 7.5), 1 mM DTT, 5 mM phosphocreatine, 0.33 units creatine kinase, 12.5 mM ATP and 1 mM InsP₆ (Sichem) at 25°C for 6h. The ability of the enzyme to hydrolyze 5-InsP₇ was assayed in a reaction mixture containing 3 μg enzyme, 2.5 mM MgCl₂, 50 mM NaCl, 20 mM HEPES (pH 6.8), 1 mM DTT, 1 mg/mL BSA, 8 mM ADP and 1 mM 5-InsP₇ at 25°C for 6h. Reactions were separated by 33% polyacrylamide gel electrophoresis and visualized by Toluidine Blue staining.

NMR-based enzyme assays

Full-length recombinant *A. thaliana* ITPK1 in H₂O was used in all assays. 0.2-0.8 μM of ITPK1 was incubated in reaction buffer containing 20 mM HEPES pH* 7.0, 50 mM NaCl, 1 mM DTT, 5 mM creatine phosphate, 1 U/ml creatine kinase, 2.5 mM MgCl₂ (if not indicated otherwise) and 175 μM of [¹³C₆]InsP₅ [2-OH], [¹³C₆]InsP₆, [¹³C₆]5-InsP₇ or [¹³C₆]1-InsP₇ in D₂O. If not indicated otherwise, the reaction buffer also included 2.5 mM ATP or 2.5 mM ADP.

For single time-point analysis of enzyme activity, 2.25-0.375 ng (0.2-0.3 μM) ITPK1 were used. 150 μL reactions were incubated at 25°C (except when 37°C is specified), quenched with 400 μl of 20 mM EDTA (pH* 6.0 in D₂O) and 11 μL of 5 M NaCl was added for analysis. For real-time monitoring of enzyme activity, 36 ng (0.8 μM) ITPK1 were used. 600 μL reactions were maintained at 25°C in an NMR spectrometer and measured consecutively with 85 sec spectra.

Samples were measured as previously described⁴⁹ on Bruker AV-III spectrometers (Bruker Biospin, Rheinstetten, Germany) using cryogenically cooled 5 mm TCI-triple resonance probe equipped with one-axis self-shielded gradients and operating at 600 MHz for proton

nuclei, 151 MHz for carbon nuclei, and 244 MHz for P nuclei. The software to control the spectrometer was topspin 3.5 pl6. Temperature was calibrated using d₄-methanol and the formula of Findeisen et al.⁵⁰.

InsPs extraction from seedlings and HPLC analyses

Seedlings were grown vertically on half-strength MS medium supplemented with 1 % sucrose and 7 g L⁻¹ Phytagel (P8169, Sigma), pH 5.7 for 12 days (8 h light at 22°C, 16 h darkness at 20°C). Seedlings were transferred to 3 mL half-strength MS liquid media without sucrose and with 625 μM P_i (+P) or 5 μM P_i (-P). Seedlings were labeled by adding 30 μCi mL⁻¹ of [³H]-*myo*-inositol (30 to 80 Ci mmol⁻¹ and 1 mCi mL⁻¹; American Radiolabeled Chemicals) and further cultivated for 5 days. For P_i-resupply, 620 μM KH₂PO₄ was added to the media and the plants were grown for another 6 h before harvest. Afterwards seedlings were washed two times with ultrapure water, frozen in liquid N₂ and the InsPs were extracted as described previously (Azevedo and Saiardi, 2006). Inositol polyphosphates were resolved by strong anion exchange chromatography HPLC (using a partisphere SAX 4.63 x 125 mm column; HiChrom) at a flow rate of 0.5 mL min⁻¹ with a gradient of the following buffers: buffer A (1 mM EDTA) and buffer B (1 mM EDTA and 1.3 M (NH₄)₂HPO₄, pH 3.8, with H₃PO₄). The gradient was as follows: 0-2 min, 0 % buffer B; 2-7 min, up to 10 % buffer B; 7-68 min, up to 84 % buffer B; 68-82 min, up to 100 % buffer B; 82-100 min, 100 % buffer B, 100-101 min, down to 0 % buffer B; 101-125 min, 0 % buffer B. Fractions were collected each minute, mixed with scintillation cocktail (Perkin-Elmer; ULTIMA-FLO AP), and analyzed by scintillation counting. To account for differences in fresh weight and extraction efficiencies between samples, values shown are normalized activities based on the total activity of each sample. 'Total' activities for normalization were calculated by counting fractions from 26 min (InsP₃) until the end of the run.

Analysis of ATP and ADP

Adenosine nucleotides were specifically determined according to Haink and Deussen⁵¹ with some modifications. 100 mg frozen leaf material from Arabidopsis plants were homogenized in liquid N₂ and extracted with methanol/chloroform⁵². An aliquot of extracted samples was used for derivatization. Twenty µL of extract was added to 205 µL of a buffer containing 62 mM sodium citrate and 76 mM potassium dihydrogenphosphate for which pH was adjusted to 5.2 with potassium hydroxide. To this mixture, 25 µL chloroacetaldehyde (Sigma-Aldrich, Germany) was added and the whole solution was incubated for 40 min at 80°C followed by cooling and centrifugation for 1 min at 14000 rpm. Two blanks containing all reagents except plant extract were used as control. For quantification, external standards (ATP, ADP, AMP) were established with different concentrations. Separation of adenosine nucleotides was performed on a newly developed UPLC-based method using ultra pressure reversed phase chromatography (Acquity H-Class, Waters GmbH, Eschborn, Germany). The UPLC system consisted of a quaternary solvent manager, a sample manager-FTN, a column manager and a fluorescent detector (PDA eλ Detector). The separation was carried out on a C18 reversed phase column (YMC Triart, 1.9 µm, 2.0x100 mm ID, YMC Chromatography, Germany) with a flow rate of 0.6 ml per min and duration of 7 min. The column was heated at 37°C during the whole run. The detection wavelengths were 280 nm for excitation and 410 nm as emission. The gradient was accomplished with two solutions. Eluent A was 5.7 mM tetrabutylammonium bisulfate (TBAS) and 30.5 mM KH₂PO₄, pH adjusted to 5.8. Eluent B was a mixture of pure acetonitrile and TBAS in a ratio of 2:1. The column was equilibrated with eluent A (90%) and eluent B (10 %) for at least 30 minutes. The gradient was produced as follow: 90% A and 10% B for 2 min, changed to 40% A and 60% B and kept for 2.3 min, changed to 10% A and 90% B for 1.1 min and reversed to 90% A and 10% B for another 1.6 min.

Statistical analysis

To analyze the significant differences among multiple groups, one-way analysis of variance followed by Tukey's test at $P < 0.05$ was adopted. The statistical significance between two groups was assessed by two-tailed Student's *t*-test. All statistical tests were performed using SigmaPlot 11.0 software.

References

1. Rubio V, *et al.* A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Gene Dev* **15**, 2122-2133 (2001).
2. Bustos R, *et al.* A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. *Plos Genet* **6**, - (2010).
3. Lv QD, *et al.* SPX4 negatively regulates phosphate signaling and homeostasis through its interaction with PHR2 in rice. *Plant Cell* **26**, 1586-1597 (2014).
4. Puga MI, *et al.* SPX1 is a phosphate-dependent inhibitor of PHOSPHATE STARVATION RESPONSE 1 in Arabidopsis. *Proc Natl Acad Sci USA* **111**, 14947-14952 (2014).
5. Wang ZY, *et al.* Rice SPX1 and SPX2 inhibit phosphate starvation responses through interacting with PHR2 in a phosphate-dependent manner. *Proc Natl Acad Sci USA* **111**, 14953-14958 (2014).
6. Qi WJ, Manfield IW, Muench SP, Baker A. AtSPX1 affects the AtPHR1-DNA-binding equilibrium by binding monomeric AtPHR1 in solution. *Biochem J* **474**, 3675-3687 (2017).
7. Zhong Y, *et al.* Rice SPX6 negatively regulates the phosphate starvation response through suppression of the transcription factor PHR2. *New Phytol* **219**, 135-148 (2018).
8. Liu F, *et al.* OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of OsPT2 and phosphate homeostasis in shoots of rice. *Plant J* **62**, 508-517 (2010).
9. Pratt J, Boisson AM, Gout E, Bligny R, Douce R, Aubert S. Phosphate (Pi) starvation effect on the cytosolic pi concentration and pi exchanges across the tonoplast in plant cells: An in vivo ³¹P-nuclear magnetic resonance study using methylphosphonate as a Pi analog. *Plant Physiol* **151**, 1646-1657 (2009).
10. Wild R, *et al.* Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. *Science* **352**, 986-990 (2016).

- 682 11. Stevenson-Paulik J, Bastidas RJ, Chiou ST, Frye RA, York JD. Generation of phytate-
683 free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. *Proc*
684 *Natl Acad Sci USA* **102**, 12612-12617 (2005).
- 685 12. Kuo HF, Chang TY, Chiang SF, Wang WD, Chang YY, Chiou TJ. Arabidopsis
686 inositol pentakisphosphate 2-kinase, AtIPK1, is required for growth and modulates
687 phosphate homeostasis at the transcriptional level. *Plant J* **80**, 503-515 (2014).
- 688 13. Kuo HF, *et al.* Arabidopsis inositol phosphate kinases IPK1 and ITPK1 constitute a
689 metabolic pathway in maintaining phosphate homeostasis. *Plant J* **95**, 613-630 (2018).
- 690 14. Dong J, *et al.* Inositol pyrophosphate InsP8 acts as an intracellular phosphate signal in
691 Arabidopsis. *Mol Plant* **12**, 1463-1473 (2019).
- 692 15. Zhu J, *et al.* Two bifunctional inositol pyrophosphate kinases/phosphatases control
693 plant phosphate homeostasis. *eLife* **8**, (2019).
- 694 16. Lonetti A, Szijgyarto Z, Bosch D, Loss O, Azevedo C, Saiardi A. Identification of an
695 evolutionarily conserved family of inorganic polyphosphate endopolyphosphatases. *J*
696 *Biol Chem* **286**, 31966-31974 (2011).
- 697 17. Laha D, *et al.* VIH2 regulates the synthesis of inositol pyrophosphate InsP8 and
698 jasmonate-dependent defenses in Arabidopsis. *Plant Cell* **27**, 1082-1097 (2015).
- 699 18. Desai M, *et al.* Two inositol hexakisphosphate kinases drive inositol pyrophosphate
700 synthesis in plants. *Plant J* **80**, 642-653 (2014).
- 701 19. Dollins DE, *et al.* Vip1 is a kinase and pyrophosphatase switch that regulates inositol
702 diphosphate signaling. *Proc Natl Acad Sci USA*, 201908875 (2020).
- 703 20. Laha D, *et al.* Arabidopsis ITPK1 and ITPK2 have an evolutionarily conserved phytic
704 acid kinase activity. *ACS Chem Biol* **14**, 2127-2133 (2019).
- 705 21. Laha NP, *et al.* ITPK1-dependent inositol polyphosphates regulate auxin responses in
706 *Arabidopsis thaliana*. *bioRxiv*, 2020.2004.2023.058487 (2020).
- 707 22. Gu C, *et al.* The significance of the bifunctional kinase/phosphatase activities of
708 diphosphoinositol pentakisphosphate kinases (PPIP5Ks) for coupling inositol
709 pyrophosphate cell signaling to cellular phosphate homeostasis. *J Biol Chem* **292**,
710 4544-4555 (2017).
- 711 23. Wilson MSC, Bulley SJ, Pisani F, Irvine RF, Saiardi A. A novel method for the
712 purification of inositol phosphates from biological samples reveals that no phytate is
713 present in human plasma or urine. *Open Biol* **5**, (2015).
- 714 24. Losito O, Szijgyarto Z, Resnick AC, Saiardi A. Inositol pyrophosphates and their
715 unique metabolic complexity: analysis by gel electrophoresis. *Plos One* **4**, (2009).
- 716 25. Wilson MS, Saiardi A. Inositol phosphates purification using titanium dioxide beads.
717 *Bio Protoc* **8**, (2018).
- 718 26. Nagy R, *et al.* The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a
719 high affinity inositol hexakisphosphate transporter involved in guard cell signaling and
720 phytate storage. *J Biol Chem* **284**, 33614-33622 (2009).

- 721 27. Voglmaier SM, *et al.* Purified inositol hexakisphosphate kinase is an ATP synthase:
722 diphosphoinositol pentakisphosphate as a high-energy phosphate donor. *Proc Natl*
723 *Acad Sci U S A* **93**, 4305-4310 (1996).
- 724 28. Wundenberg T, Grabinski N, Lin HY, Mayr GW. Discovery of InsP₆-kinases as InsP₆-
725 dephosphorylating enzymes provides a new mechanism of cytosolic InsP₆ degradation
726 driven by the cellular ATP/ADP ratio. *Biochem J* **462**, 173-184 (2014).
- 727 29. Li X, *et al.* Control of XPR1-dependent cellular phosphate efflux by InsP₈ is an
728 exemplar for functionally-exclusive inositol pyrophosphate signaling. *Proc Natl Acad*
729 *Sci U S A* **117**, 3568-3574 (2020).
- 730 30. Couso I, *et al.* Synergism between inositol polyphosphates and TOR kinase signaling
731 in nutrient sensing, growth control, and lipid metabolism in *Chlamydomonas*. *Plant*
732 *Cell* **28**, 2026-2042 (2016).
- 733 31. Laha D, *et al.* Inositol polyphosphate binding specificity of the jasmonate receptor
734 complex. *Plant Physiol* **171**, 2364-2370 (2016).
- 735 32. Wilson MS, Jessen HJ, Saiardi A. The inositol hexakisphosphate kinases IP6K1 and -
736 2 regulate human cellular phosphate homeostasis, including XPR1-mediated
737 phosphate export. *J Biol Chem* **294**, 11597-11608 (2019).
- 738 33. Ried MK, *et al.* Inositol pyrophosphates promote the interaction of SPX domains with
739 the coiled-coil motif of PHR transcription factors to regulate plant phosphate
740 homeostasis. *bioRxiv*, 2019.2012.2013.875393 (2019).
- 741 34. Azevedo C, Saiardi A. Eukaryotic phosphate homeostasis: The inositol pyrophosphate
742 perspective. *Trends Biochem Sci* **42**, 219-231 (2017).
- 743 35. Duan K, Yi KK, Dang L, Huang HJ, Wu W, Wu P. Characterization of a sub-family
744 of Arabidopsis genes with the SPX domain reveals their diverse functions in plant
745 tolerance to phosphorus starvation. *Plant J* **54**, 965-975 (2008).
- 746 36. Osorio MB, *et al.* SPX4 acts on PHR1-dependent and -independent regulation of shoot
747 phosphorus status in Arabidopsis. *Plant Physiol* **181**, 332-352 (2019).
- 748 37. Shears SB. Diphosphoinositol polyphosphates: metabolic messengers? *Mol*
749 *Pharmacol* **76**, 236-252 (2009).
- 750 38. Wilson MSC, Livermore TM, Saiardi A. Inositol pyrophosphates: between signalling
751 and metabolism. *Biochem J* **452**, 369-379 (2013).
- 752 39. Szijgyarto Z, Garedew A, Azevedo C, Saiardi A. Influence of inositol pyrophosphates
753 on cellular energy dynamics. *Science* **334**, 802-805 (2011).
- 754 40. Choi J, Rajagopal A, Xu YF, Rabinowitz JD, O'Shea EK. A systematic genetic screen
755 for genes involved in sensing inorganic phosphate availability in *Saccharomyces*
756 *cerevisiae*. *Plos One* **12**, (2017).
- 757 41. Desfougeres Y, Wilson MSC, Laha D, Miller GJ, Saiardi A. ITPK1 mediates the lipid-
758 independent synthesis of inositol phosphates controlled by metabolism. *Proc Natl*
759 *Acad Sci U S A* **116**, 24551-24561 (2019).

42. An Y, Jessen HJ, Wang H, Shears SB, Kireev D. Dynamics of substrate processing by PPIP5K2, a versatile catalytic machine. *Structure* **27**, 1022-1028 e1022 (2019).
43. Delhaize E, Randall PJ. Characterization of a phosphate-accumulator mutant of *Arabidopsis thaliana*. *Plant Physiol* **107**, 207-213 (1995).
44. Gruber BD, Giehl RFH, Friedel S, von Wirén N. Plasticity of the *Arabidopsis* root system under nutrient deficiencies. *Plant Physiol* **163**, 161-179 (2013).
45. Yoshida S. *Laboratory manual for physiological studies of rice*. IRRI (1976).
46. Reski R, Abel WO. Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. *Planta* **165**, 354-358 (1985).
47. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45 (2001).
48. Schaaf G, Betts L, Garrett TA, Raetz CR, Bankaitis VA. Crystallization and preliminary X-ray diffraction analysis of phospholipid-bound Sfh1p, a member of the *Saccharomyces cerevisiae* Sec14p-like phosphatidylinositol transfer protein family. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **62**, 1156-1160 (2006).
49. Harmel RK, Puschmann R, Nguyen Trung M, Saiardi A, Schmieder P, Fiedler D. Harnessing ¹³C-labeled myo-inositol to interrogate inositol phosphate messengers by NMR. *Chem Sci* **10**, 5267-5274 (2019).
50. Findeisen M, Brand T, Berger S. A ¹H-NMR thermometer suitable for cryoprobes. *Magn Reson Chem* **45**, 175-178 (2007).
51. Haink G, Deussen A. Liquid chromatography method for the analysis of adenosine compounds. *J Chromatogr B Analyt Technol Biomed Life Sci* **784**, 189-193 (2003).
52. Ghaffari MR, *et al.* The metabolic signature of biomass formation in barley. *Plant Cell Physiol* **57**, 1943-1960 (2016).

Acknowledgments

This work was funded by grants from the Deutsche Forschungsgemeinschaft (DFG, German Research Council) (HE 8362/1-1 to R.F.H.G.; SCHA 1274/4-1, SCHA 1274/5-1, Research Training Group GRK 2064 and under Germany's Excellence Strategy, EXC-2070-390732324, PhenoRob to G.S.; CIBSS – EXC 2189 – 390939984 as well as JE 572/4-1 to H.J.J.; and LA 4541/1-1, Postdoctoral Research Fellowship, to D.L.) and from the Medical Research Council (MRC) MC_UU_00012/4 to A.S. We thank Annett Bieber, Jacqueline Fuge, Nicole Schäfer and Yudelsy A. Tandron Moya (Leibniz Institute of Plant Genetics and

Crop Plant Research) for excellent technical assistance, and Nicolaus von Wirén for critically reading the article. We thank Saikat Bhattacharjee (RCB, India) for providing seeds of published *Arabidopsis thaliana* mutants.

Author contributions

G.S. and R.F.H.G. conceived the study. D.L., R.K.H., M.F., G.S., and R.F.H.G. designed experiments. E.R., D.L., R.K.H., P.G., V.P., M.F., N.P.L., L.K., R.S., and R.F.H.G. performed experiments. M.-R.H. performed UPLC analysis of ATP and ADP. H.J.J. synthesized various InsP isomers. A.S., D.F., G.S., and R.F.H.G. supervised experimental work. R.F.H.G. and G.S. wrote the manuscript with critical inputs from all authors.

Conflict of interest

Conflicts of interest: No conflicts of interest declared.

Figure legends

Fig. 1. Role of distinct InsP kinases in plant P_i accumulation.

a-b Photographs of 3-week-old *Arabidopsis* plants grown on peat-substrate (**a**), and total P_i levels in shoots (**b**) of WT (Col-0) and the indicated mutants. Data represent means $\pm$ SD ($n = 6-9$ plants). Scale bars = 2 cm. **c-d** *ITPK1* expression (**c**) and shoot P_i levels (**d**) in 3-week-old WT (Col-0), *itpk1* and three independent *itpk1* lines transformed with *ITPK1* genomic DNA. Data represent means $\pm$ SD ($n = 3$ biological replicates in **c** and $n = 8-9$ plants in **d**). **e-h** Loss of ITPK1 results in P_i overaccumulation only in shoots but P_i-independent root growth repression. Phenotypes (**e**), primary root length (**f**) and total P_i concentrations in shoots (**g**) or roots (**h**) of plants grown in agar plates with sufficient (625 μ M P_i) or deficient P_i supply (5

$\mu\text{M P}_i$) for 7 days. Bars show means $\pm$ SD ($n = 6$ replicates with 3 plants each). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Fig. 2. InsP₇ and InsP₈ levels in shoots respond to P_i availability in an ITPK1-dependent manner.

a-b HPLC profiles of 17-day-old *Arabidopsis* WT (Col-0) seedlings radiolabeled with [³H]-*myo*-inositol. Seedlings were grown either with P_i (+P) or without P_i (-P) or resupplied with P_i for 6 h prior to harvest (P_i RS 6h). Full, normalized spectra (**a**) and zoom-in view of the same profile (**b**). The experiment was repeated two times with similar results, and representative results from one experiment are shown. **c-e** PAGE of InsP levels in shoots (**c**), quantification of PAGE signals from shoots (**d**) and PAGE of roots (**e**) of WT (Col-0) and *itpk1* plants. Plants were grown in hydroponics in P_i-sufficient solution (+P), exposed for 4 days to P_i starvation (-P) or resupplied with P_i for 12 hours (P_i RS 12h). InsPs were eluted from TiO₂ beads, separated by PAGE and visualized by Toluidine blue and DAPI. Data represent means $\pm$ SE ($n = 3-4$ biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's *t*-test. **f-g** InsP₇ and InsP₈ levels are strongly increased in the shoots of the P_i-overaccumulating mutant *pho2-1*. PAGE of shoots (**f**) and shoot P_i levels in response to P_i deficiency and P_i resupply (**g**). Plants were cultivated as described in **c-e**. Data represent means $\pm$ SE ($n = 4$). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Fig. 3. ITPK1- and VIH2-dependent synthesis of PP-InsPs and dependency on InsP₆ compartmentation.

a-b Loss of VIH2 increases InsP₇ levels in shoots. InsP detection in shoots of *Arabidopsis* WT, *itpk1* and *vih2-4* plants (**a**) and quantification of PAGE signals (**b**) 12 h after P_i resupply to P_i-starved plants in hydroponics. Data represent means $\pm$ SE ($n = 4$ biological replicates).

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's t -test. **c-d** Impaired InsP₆ transport into the vacuole increases InsP₇ and InsP₈ levels in shoots and roots. InsP determination in shoots (**c**) and roots (**d**) of WT (Col-0) and *mrp5*. Plants were grown in hydroponics in P_i-sufficient solution (+P), exposed for 4 days to P_i starvation (-P) or resupplied with P_i for 12 hours (Pi RS 12h).

Fig. 4. *In vitro* characterization of ITPK1 activity.

a-b NMR analysis of InsP₆ kinase activity of recombinant *Arabidopsis* ITPK1. Time-dependent conversion of InsP₆ to 5-InsP₇ (**a**) and reaction velocity determined at varying ATP concentrations (**b**). K_M and V_{max} were obtained after fitting of the data against the Michaelis-Menten model. **c-d** In the presence of ADP, recombinant *Arabidopsis* ITPK1 mediates 5-InsP₇ hydrolysis (**c**) but not the hydrolysis of other InsP₇ isomers (**d**). InsPs were separated via PAGE and visualized by Toluidine Blue staining. The identity of bands was determined by migration compared to InsP₆ and 5-InsP₇ standards and TiO₂-purified *mrp5* seed extract. InsP₆ kinase reaction served as positive control for the reverse reactions. Purified His₈-MBP tag (MBP) served as negative control for ITPK1. Arrowhead in **c**, indicates the presence of a small ATP band just above ADP. **e-f** NMR analysis of reverse reaction of recombinant *Arabidopsis* ITPK1. Accumulation of InsP₆ and conversion of 5-InsP₇ (**e**) and reaction velocity determined at varying ADP concentrations (**f**). K_M and V_{max} were obtained after fitting of the data against the Michaelis-Menten model.

Fig. 5. P_i starvation responses are mainly regulated by ITPK1 activity in shoots.

a Total P_i concentration in shoots of self-grafted or reciprocally grafted WT (Col-0) and *itpk1*. Plants were grafted on agar plates and left recovering for 2 weeks. Positive grafts were transferred to peat-based substrate for another 2 weeks. Data represent means $\pm$ SD ($n = 5-7$ plants). **b-d** Genetic interplay between PHR1/PHL1 and ITPK1 in P_i sensing. Phenotype (**b**),

shoot dry weight (**c**) and shoot P_i levels (**d**) of 3-week-old WT and indicated mutants grown on peat-based substrate. Data represent means $\pm$ SD ($n = 6$ plants). In **a**, **c** and **d**, letters indicate significant differences according to Tukey's test ($P < 0.05$). **e** ITPK1-dependent expression of P_i deficiency-induced genes in roots of the indicated P_i -sufficient plants. Data represent means $\pm$ SE ($n = 4$ replicates). **f-g** PHR1- and PHL1-dependent synthesis of InsPs as revealed by PAGE (**f**) and relative quantification of signals (**g**) in indicated double and triple mutants grown in hydroponics with P_i -sufficient solution (+P), exposed to 4 days of P_i starvation (-P) or resupplied with P_i for 12 hours (P_i RS 12h). Data represent means $\pm$ SE ($n = 4$ biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's *t*-test.

Fig. 6. P_i -dependent regulation of InsP₈ levels is conserved in multicellular organisms.

a-b Time-course analysis of InsPs in response to P_i starvation and P_i resupply in rice shoots. PAGE (**a**) and relative quantification of bands (**b**) of rice plants cv. Nipponbare grown in hydroponics. Data represent means $\pm$ SE ($n = 4$ biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$). **c-e** Phenotype (**c**), total P_i levels (**d**) and PAGE of P_i -dependent synthesis of InsP₇ and InsP₈ (**e**) in gametophores of *Physcomitrella patens*. Plants were cultivated on sufficient P_i (+P), starved of P_i for 30 days (-P) or resupplied with P_i for the indicated time. Data represent means $\pm$ SD ($n = 3$ biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$). **f** PAGE analysis of wild-type HCT116 cell extracts during P_i starvation and resupply. Cells were starved in P_i -free media for 18 h and resupplied with P_i for 3.5 h. Cells were harvested at the same time. The experiment was repeated twice with similar results.

Fig. 7. Model for ITPK1-dependent generation and removal of InsP₇ and its link with VIHs and P_i signaling.

In P_i -deficient cells, low ATP levels stimulate ITPK1 to catalyze P_i transfer from $InsP_7$ to ADP, thereby generating ATP and decreasing $InsP_7$. Decreased ATP and P_i levels also activate the pyrophosphatase activity of VIHs to break down $InsP_8$. The removal of PP- $InsPs$ destabilizes the association between PHRs and SPXs, allowing PHRs to induce P_i starvation responses. When cells regain sufficient P_i , which increase ATP levels, ITPK1-mediated $InsP_6$ kinase activity is stimulated and the reverse reaction towards $InsP_7$ is inhibited. $InsP_7$ generated by ITPK1 serves then as substrate for $InsP_8$ production via the kinase domain of VIHs. As a consequence of increased PP- $InsPs$, SPX proteins recruit PHRs to repress P_i starvation responses.

Supplementary Figure 1. Shoot elemental analysis of WT, *itpk1* and recombined lines.

Dry weight of whole shoots (a) and shoot concentrations of the macronutrients calcium (b), potassium (c), magnesium (d) and sulfur (e), and the micronutrients iron (f), and zinc (g) of 3-week-old plants grown on peat-substrate. Data represent the mean $\pm$ SD ($n = 8-9$ plants). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Supplementary Figure 2. ITPK1-dependent P overaccumulation in different plant organs.

Total P_i levels in different parts of WT (Col-0) and *itpk1* plants grown on peat-based. Data represent means $\pm$ SD ($n =$ samples from 5 independent plants). Letters indicate significant differences according to Tukey's test ($P < 0.05$). Young siliques = green siliques with a length of 0.8 cm to 1.5 cm.

Supplementary Figure 3. Root phenotype of *itpk1* plants grown in hydroponics and of *pho2-1* grown in agar.

a Phenotype of 5-week-old WT and *itpk1* plants grown in hydroponics with sufficient P_i . Representative plants are shown. **b** Phenotype of WT and *pho2-1* plants grown in agar plates. Seven-day-old seedlings germinated on half-strength solid MS agar media containing 625 μ M P_i were transferred to +P (625 μ M P_i) or -P (5 μ M P_i) and grown for additional 7 days.

Supplementary Figure 4. Expression of PSI genes in *itpk1* plants under different P_i conditions.

Expression analysis of representative P_i starvation-induced genes in *itpk1* relative to WT (Col-0). In **a** and **b**, the expression of P_i uptake- and signaling-related genes is shown, respectively. Seven-day-old seedlings germinated on half-strength solid MS agar media containing 625 μ M P_i were transferred to same agar media containing either sufficient (625 μ M) or deficient (5 μ M) P_i levels for 4 days. For P_i refeeding, P_i -deficient plants were transferred back to P_i -containing media for 6 hours. Data represents means $\pm$ SE ($n = 4$ biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's *t*-test (*itpk1* versus Col-0).

Supplementary Figure 5. P_i -dependent InsP₇ and InsP₈ synthesis is not altered in *itpk2-2* and *itpk4-1* mutant.

InsP detection in shoots of WT and *itpk2-2* (**a**) and *itpk4-1* plants (**b**) and relative quantification of PAGE signals for *itpk4-1* (**c**). Plants were grown in hydroponics under sufficient P_i (+P), after 4 days of P_i deficiency (-P) or after resupply of P_i to P_i -deficient plants for 12 h (RS 12h). Data represent means $\pm$ SE ($n = 3$ biological replicates).

Supplementary Figure 6. P_i -dependent regulation of InsP levels in roots and shoots of rice plants.

PAGE analysis of rice plants cv. Nipponbare grown in hydroponics under the indicated P_i conditions. Shown are representative gels from root and shoot samples.

Supplementary Figure 7. ITPK1 activity on InsP₅ [2-OH] and InsP₇ isomers and control experiments for the kinase activity.

a ITPK1 has no kinase activity on InsP₇ isomers. 1-InsP₇, 5-InsP₇ or InsP₆ were incubated with recombinant *Arabidopsis* ITPK1 as indicated in presence of 12.5 mM ATP. InsPs were separated via PAGE and visualized by Toluidine Blue staining. The identity of bands was determined by migration compared to the substrates in absence of enzyme (-). Purified His₈-MBP tag (MBP) served as negative control for ITPK1. **b-d** Control experiments for NMR analyses. InsP₆ was incubated with recombinant *Arabidopsis* ITPK1 at 25 °C in the presence of 2.5 mM ATP. Enzymatic activity was determined after 24 h in the presence of varying EDTA concentrations (**b**), after 1.5 h at changing Mg²⁺ concentrations (**c**) and temperature (**d**). The conversion was determined by NMR spectroscopy after quenching with an excess of EDTA. **e-f** 2D ¹H-¹³C-HMBC spectra. Recombinant *Arabidopsis* ITPK1 was incubated with InsP₅ (**e**) or 1-InsP₇ (**f**) at 25°C in the presence of an ATP recycling system for 24 h. The reaction mixture analyzed by HSQC NMR spectroscopy. **g** Overview of the reaction shown in (**f**) as analyzed by ³¹P NMR spectroscopy after 24 h. A small, unidentified signal potentially reflecting ATP is marked with a question mark.

Supplementary Figure 8. Dependency of ITPK1 kinase activity on P_i .

InsP₆ was incubated with recombinant *Arabidopsis* ITPK1 at 25°C in the presence of 2.5 mM ATP and the indicated concentrations of P_i or its non-metabolizable analog phosphite (Phi).

The conversion was determined by NMR spectroscopy after quenching with an excess of EDTA. The experiment was repeated three times.

Supplementary Figure 9. Recombinant *Arabidopsis* ITPK1 can hydrolyze 5-InsP₇ in the presence of ADP.

a ³¹P NMR spectroscopy analysis of recombinant *Arabidopsis* ITPK1 incubated with 5-InsP₇ at 25°C in the presence of ADP. After 24 h the mixture was analyzed by. **b** ³¹P NMR analysis of ATP in ATP synthase reaction buffer. **c** ³¹P NMR spectroscopy analysis of recombinant *Arabidopsis* ITPK1 incubated with ADP without 5-InsP₇ at 25°C and analyzed after 24 h. A small, unidentified signal potentially reflecting ATP is marked with a question mark.

Supplementary Figure 10. Effect of P_i availability and resupply on shoot ATP levels.

Concentration of ATP (**a**) and ATP/ADP ratios (**b**) in shoots of Col-0 plants grown in hydroponics with P_i-sufficient solution (+P), exposed to 4 days of P_i starvation (-P) or resupplied with P_i for 12 hours (Pi RS 12h). Data represent means ± SE (*n* = 6–7 biological replicates). * *P* < 0.05 and ***P* < 0.01 for the indicated pairwise comparisons with Student's *t*-test.

Supplementary Figure 11. Shoot ITPK1 function is more determinant for P_i accumulation in plants.

Shoot dry weight and shoot concentrations of the macronutrients potassium (K), calcium (Ca), magnesium (Mg) and the micronutrient iron (Fe) of self-grafted or reciprocally grafted WT (Col-0) and *itpk1*. Plants were grafted on agar plates and left recovering for 2 weeks. Positive grafts were transferred to peat-based substrate for another 2 weeks. Data represent means ± SD (*n* = 5–7 plants). Different letters indicate significant differences according to Tukey's test (*P* < 0.05). n.s., not significant.

994

995 **Supplementary Figure 12. ITPK1-dependent root phenotype in the absence of PHR1**
 996 **and PHL1.**

997 Seven-day-old seedlings germinated on half-strength solid MS agar media containing 625 μ M
 998 P_i were transferred to +P (625 μ M P_i) and grown for additional 7 days. Shown are
 999 representative images of the indicated mutants grown side-by-side on the same agar plate.

1000

1001 **Supplementary Figure 13. P_i -dependent transcriptional regulation of InsP-related genes**
 1002 **in Col-0 roots.**

1003 Seven-day-old Col-0 seedlings germinated on half-strength solid MS agar media were
 1004 transferred to the indicated treatments for 4 days. +P, 625 μ M P_i ; -P, 5 μ M P_i ; P_i RS, P_i -
 1005 starved plants were transferred back to P_i -containing media for 6 hours. Data represent mean
 1006 $\pm$ SE ($n = 3$ biological replicates).

1007

1008

1009

Fig. 1. Role of distinct InsP kinases in plant P_i accumulation.

a-b Photographs of 3-week-old *Arabidopsis* plants grown on peat-substrate (**a**), and total P_i levels in shoots (**b**) of WT (Col-0) and the indicated mutants. Data represent means $\pm$ SD ($n = 6-9$ plants). Scale bars = 2 cm. **c-d** *ITPK1* expression (**c**) and shoot P_i levels (**d**) in 3-week-old WT (Col-0), *itpk1* and three independent *itpk1* lines transformed with *ITPK1* genomic DNA. Data represent means $\pm$ SD ($n = 3$ biological replicates in **c** and $n = 8-9$ plants in **d**). **e-h** Loss of ITPK1 results in P_i overaccumulation only in shoots but P_i-independent root growth repression. Phenotypes (**e**), primary root length (**f**) and total P_i concentrations in shoots (**g**) or roots (**h**) of plants grown in agar plates with sufficient (625 μ M P_i) or deficient P_i supply (5 μ M P_i) for 7 days. Bars show means $\pm$ SD ($n = 6$ replicates with 3 plants each). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Fig. 2. InsP₇ and InsP₈ levels in shoots respond to P_i availability in an ITPK1-dependent manner.

a-b HPLC profiles of 17-day-old *Arabidopsis* WT (Col-0) seedlings radiolabeled with [3 H]-myo-inositol. Seedlings were grown either with P_i (+P) or without P_i (-P) or resupplied with P_i for 6 h prior to harvest (Pi RS 6h). Full, normalized spectra (**a**) and zoom-in view of the same profile (**b**). The experiment was repeated two times with similar results, and representative results from one experiment are shown. **c-e** PAGE of InsP levels in shoots (**c**), quantification of PAGE signals from shoots (**d**) and PAGE of roots (**e**) of WT (Col-0) and *itpk1* plants. Plants were grown in hydroponics in P_i-sufficient solution (+P), exposed for 4 days to P_i starvation (-P) or resupplied with P_i for 12 hours (Pi RS 12h). InsPs were eluted from TiO₂ beads, separated by PAGE and visualized by Toluidine blue and DAPI. Data represent means $\pm$ SE ($n = 3-4$ biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's *t*-test. **f-g** InsP₇ and InsP₈ levels are strongly increased in the shoots of the P_i-overaccumulating mutant *pho2-1*. PAGE of shoots (**f**) and shoot P_i levels in response to P_i deficiency and P_i resupply (**g**). Plants were cultivated as described in **c-e**. Data represent means $\pm$ SE ($n = 4$). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Fig. 3. ITPK1- and VIH2-dependent synthesis of PP-InsPs and dependency on InsP₆ compartmentation.

a-b Loss of VIH2 increases InsP₇ levels in shoots. InsP detection in shoots of *Arabidopsis* WT, *itpk1* and *vih2-4* plants (**a**) and quantification of PAGE signals (**b**) 12 h after P_i resupply to P_i-starved plants in hydroponics. Data represent means $\pm$ SE ($n = 4$ biological replicates). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ according to pairwise comparison with Student's *t*-test. **c-d** Impaired InsP₆ transport into the vacuole increases InsP₇ and InsP₈ levels in shoots and roots. InsP determination in shoots (**c**) and roots (**d**) of WT (Col-0) and *mrp5*. Plants were grown in hydroponics in P_i -sufficient solution (+P), exposed for 4 days to P_i starvation (-P) or resupplied with P_i for 12 hours (Pi RS 12h).

Fig. 4. In vitro characterization of ITPK1 activity.

a-b NMR analysis of InsP₆ kinase activity of recombinant *Arabidopsis* ITPK1. Time-dependent conversion of InsP₆ to 5-InsP₇ (**a**) and reaction velocity determined at varying ATP concentrations (**b**). K_M and $V_{\max}$ were obtained after fitting of the data against the Michaelis-Menten model. **c-d** In the presence of ADP, recombinant *Arabidopsis* ITPK1 mediates 5-InsP₇ hydrolysis (**c**) but not the hydrolysis of other InsP₇ isomers (**d**). InsPs were separated via PAGE and visualized by Toluidine Blue staining. The identity of bands was determined by migration compared to InsP₆ and 5-InsP₇ standards and TiO₂-purified *mrp5* seed extract. InsP₆ kinase reaction served as positive control for the reverse reactions. Purified His₈-MBP tag (MBP) served as negative control for ITPK1. Arrowhead in **c**, indicates the presence of a small ATP band just above ADP. **e-f** NMR analysis of reverse reaction of recombinant *Arabidopsis* ITPK1. Accumulation of InsP₆ and conversion of 5-InsP₇ (**e**) and reaction velocity determined at varying ADP concentrations (**f**). K_M and $V_{\max}$ were obtained after fitting of the data against the Michaelis-Menten model.

Fig. 5. P_i starvation responses are mainly regulated by ITPK1 activity in shoots.

a Total P_i concentration in shoots of self-grafted or reciprocally grafted WT (Col-0) and *itpk1*. Plants were grafted on agar plates and left recovering for 2 weeks. Positive grafts were transferred to peat-based substrate for another 2 weeks. Data represent means ± SD (*n* = 5-7 plants). **b-d** Genetic interplay between PHR1/PHL1 and ITPK1 in P_i sensing. Phenotype (**b**), shoot dry weight (**c**) and shoot P_i levels (**d**) of 3-week-old WT and indicated mutants grown on peat-based substrate. Data represent means ± SD (*n* = 6 plants). In **a**, **c** and **d**, letters indicate significant differences according to Tukey's test (*P* < 0.05). **e** ITPK1-dependent expression of P_i deficiency-induced genes in roots of the indicated P_i-sufficient plants. Data represent means ± SE (*n* = 4 replicates). **f-g** PHR1- and PHL1-dependent synthesis of InsPs as revealed by PAGE (**f**) and relative quantification of signals (**g**) in indicated double and triple mutants grown in hydroponics with P_i-sufficient solution (+P), exposed to 4 days of P_i starvation (-P) or resupplied with P_i for 12 hours (Pi RS 12h). Data represent means ± SE (*n* = 4 biological replicates). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 according to pairwise comparison with Student's *t*-test.

Fig. 6. P_i-dependent regulation of InsP₈ levels is conserved in multicellular organisms.

a-b Time-course analysis of InsPs in response to P_i starvation and P_i resupply in rice shoots. PAGE (**a**) and relative quantification of bands (**b**) of rice plants cv. Nipponbare grown in hydroponics. Data represent means $\pm$ SE ($n = 4$ biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$). **c-e** Phenotype (**c**), total P_i levels (**d**) and PAGE of P_i-dependent synthesis of InsP₇ and InsP₈ (**e**) in gametophores of *Physcomitrella patens*. Plants were cultivated on sufficient P_i (+P), starved of P_i for 30 days (-P) or resupplied with P_i for the indicated time. Data represent means $\pm$ SD ($n = 3$ biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$). **f** PAGE analysis of wild-type HCT116 cell extracts during P_i starvation and resupply. Cells were starved in P_i-free media for 18 h and resupplied with P_i for 3.5 h. Cells were harvested at the same time. The experiment was repeated twice with similar results.

Fig. 7. Model for ITPK1-dependent generation and removal of InsP₇ and its link with VIHs and P_i signaling. In P_i-deficient cells, low ATP levels stimulate ITPK1 to catalyze P_i transfer from InsP₇ to ADP, thereby generating ATP and decreasing InsP₇. Decreased ATP and P_i levels also activate the pyrophosphatase activity of VIHs to break down InsP₈. The removal of PP-InsPs destabilizes the association between PHRs and SPXs, allowing PHRs to induce P_i starvation responses. When cells regain sufficient P_i, which increase ATP levels, ITPK1-mediated InsP₆ kinase activity is stimulated and the reverse reaction towards InsP₇ is inhibited. InsP₇ generated by ITPK1 serves then as substrate for InsP₈ production via the kinase domain of VIHs. As a consequence of increased PP-InsPs, SPX proteins recruit PHRs to repress P_i starvation responses.