Institut für Nutzpflanzenwissenschaften und Ressourcenschutz

Pflanzenernährung

The role of inositol pyrophosphates in the regulation of plant stress responses

Dissertation

zur Erlangung des Grades

Doktorin der Agrarwissenschaften (Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Esther Lange

geb. Riemer

aus

Bad Honnef

Bonn, 2023

Referent**:** Prof. Gabriel Schaaf

Korreferent**:** Prof. Frank Hochholdinger

Tag der mündlichen Prüfung: 04.07.2023

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

This dissertation is submitted as a "Cumulative Thesis" that covers two publications that are listed as following:

Published papers:

1. **Riemer, E.**, Qiu, D., Laha, D., Harmel, R. K., Gaugler, P., Gaugler, V., Frei, M., Hajirezaei, M. R., Laha, N. P., Krusenbaum, L., Schneider, R., Saiardi, A., Fiedler, D., Jessen, H. J., Schaaf, G., Giehl, R. F. H. (2021). ITPK1 is an InsP$_6$/ADP phosphotransferase that controls phosphate signaling in *Arabidopsis*. Molecular Plant 14(11):1864-1880. https://doi.org/10.1016/j.molp.2021.07.011 (Chapter II)

2. **Riemer, E.**, Pullagurla, N. J., Yadav, R., Rana, P., Jessen, H. J. J., Kamleitner, M., Schaaf, G., Laha, D. (2022). Regulation of plant biotic interactions and abiotic stress responses by inositol polyphosphates. Frontiers in Plant Science 13:944515. https://doi.org/10.3389/fpls.2022.944515 (Chapter III)

Contents

Contents

Contents

List of Abbreviations

ADP	Adenosine diphosphate
AFB	AUXIN-SIGNALING F-BOX
ARF	AUXIN RESPONSE FACTOR
ATP	Adenosine triphosphate
BGE	Background electrolyte
CE-ESI-MS	Capillary electrophoresis electrospray ionization mass spectrometry
Cd	Cadmium
COI1	Coronatine Insensitive 1
DAPI	4′,6-diamidino-2-phenylindole
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EIEs	Extracted ion electropherograms
EMS	Ethyl methanesulfonate
ET	Ethylene
ETI	Effector-triggered immunity
HR	Hypersensitive response
HR-ICP-MS	High-resolution inductively coupled plasma-mass spectrometry
HRMS	High-resolution mass spectrum
IAA	Aux/indole-3-acetic acid
ICP-OES	Inductively coupled plasma optical emission spectrometry
InsP	Inositol polyphosphate/phosphate
InsP5-ptase	Inositol polyphosphate 5-phosphatase
IPK1	INOSITOL PENTAKISPHOSPHATE 2-KINASE
ITPK1/2/3/4	INOSITOL 1,3,4-TRISPHOSPHATE 5/6 KINASE 1/2/3/4
JA	Jasmonic acid

JA-Ile	Jasmonic isoleucine
JAZ	Jasmonate ZIM
LLR	Leucine-rich repeat
Lpa	Low phytic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
n.d.	Not detected
n.s.	Not significant
NMR	Nuclear magnetic resonance spectroscopy
MBP	Maltose binding protein
Mg^{2+}	Magnesium ion
OG	Orange G
P	Phosphorus
PA	Perchloric acid
PAGE	Polyacrylamide Gel Electrophoresis
PHL1	PHR1-LIKE 1
PHR1	PHOSPHATE STARVATION RESPONSE REGULATOR 1
PHO2	PHOSPHATE2
P_i	Phosphate
pNPP	p-nitrophenyl phosphate
PP-InsP	Inositol pyrophosphate
PP-InsP$_4$	Diphosphoinositol tetrakisphosphate
PR	Pathogenesis-related
PSI	Phosphate starvation-induced
PSR	Phosphate starvation response
Pst	*Pseudomonas syringae* pv. *tomato*

PTI	PAMP-triggered immunity
R	Resistance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	Glycolsyl moiety-conjugated SA
SAR	Systemic acquired resistance
(SAX)-HPLC	Strong Anion Exchange High Performance Liquid Chromatography
SNC1	SUPPRESSOR OF nrp1-1 CONSTITUTIVE
SPX	SYG1/Pho81/XPR1-domain containing protein
T3SS	Type III secretion system
TBAS	Tetrabutylammonium bisulfate
TiO$_2$	Titanium (IV) oxide
TIR1	TRANSPORT INHIBITOR RESPONSE1
TOCSY	Total Correlation Spectroscopy
UPLC	Ultra Performance Liquid Chromatography
VIH1/2	DIPHOSPHOINOSITOL-PENTAKISPHOSPHATE KINASE 1/2
WT	Wild-type
Xops	*Xanthomonas* outer proteins
Y2H	Yeast two-hybrid

Zusammenfassung

Inositolpolyphosphate (InsPs) werden als wichtige Botenstoffe in eukaryotischen Zellen beschrieben. Besitzen sie eine oder mehrere Diphosphat-Gruppen, werden sie als Inositol-pyrophosphate (PP-InsPs) bezeichnet, denen als zelluläre Regulatoren besondere Aufmerksamkeit gilt. In Pflanzen wurde gezeigt, dass PP-InsPs in abiotische und biotische Stressantworten wie die Phosphat (P_i) Homöostase und hormonabhängige Pathogenabwehr involviert sind. Phosphor (P) ist ein essentielles Element und Schlüsselnährstoff für das Wachstum und die Entwicklung aller Organismen. In Pflanzen wird P in Form von P_i aufgenommen, dessen Verfügbarkeit stark eingeschränkt ist. Um sich an wechselnde P_i-Verfügbarkeit anzupassen, haben Pflanzen die sogenannte Phosphatmangelantwort entwickelt, welche durch die Interaktion von PHOSPHATE STARVATION RESPONSE REGULATOR (PHR) Transkriptionsfaktoren mit Proteinen, die SYG1/Pho81/XPR1 (SPX) Domänen enthalten und als PP-InsP Sensoren agieren, reguliert wird. Die hier präsentierte Studie konzentriert sich auf die P_i-abhängige Regulation des PP-InsP Metabolismus mit besonderem Fokus auf die INOSITOL 1,3,4-TRISPHOSPHATE 5/6 KINASE 1 (ITPK1). Durch die Anwendung der Kapillarelektrophorese Elektrospray Ionisations Massenspektrometrie (CE-ESI-MS) konnten unterschiedliche $InsP_7$ Isomere in *Arabidopsis* und Reis unter vollversorgten und P_i-limitierten Bedingungen getrennt und identifiziert werden. Des Weiteren wurde gezeigt, dass es im Spross von P_i-Mangel-Pflanzen nach Zugabe von Phosphat zu einer starken Akkumulation von $1/3$-$InsP_7$, 5-$InsP_7$ und $InsP_8$ kommt, was auf eine starke Abhängigkeit von zellulären P_i-Leveln hindeutet. Demnach spielt ITPK1 eine wichtige Rolle bei der P_i-abhängigen Synthese von 5-$InsP_7$, dem Vorläufer des für die P_i Signalwirkung wichtigen $InsP_8$. Darüber hinaus wird gezeigt, dass für die Generierung von 5-$InsP_7$ durch ITPK1 hohe ATP Konzentrationen benötigt werden und dass unter geringen Adenylatladungen ITPK1 den Transfer eines β-Phosphats von 5-$InsP_7$ auf ADP katalysiert um lokal ATP zu generieren. Durch die CE-ESI-MS Methode wurde pflanzliches $4/6$-$InsP_7$ identifiziert, welches bislang nur in der sozialen Amöbe *Dictyostelium discoideum* beschrieben wurde und wahrscheinlich keinen Einfluss auf die P_i Signalwirkung hat. Auch konnte eine unbekannte PP-$InsP_4$ Spezies in P_i-mangelernährten *Arabidopsis*- und Reiswurzeln detektiert werden. Diese ist nicht in *itpk1* Wurzeln nachweisbar, was darauf hindeutet, dass ITPK1 in dessen Biosynthese involviert ist. Diese Arbeit ermöglicht einen Einblick in die Beteiligung von PP-InsPs in die P_i-Homöostase und präsentiert den aktuellen Wissensstand über die Funktionen von PP-InsPs in abiotischen und biotischen Stressantworten in Pflanzen.

Summary

Inositol polyphosphates (InsPs) have been described as important messengers in eukaryotic cells. InsPs with one or more diphospho groups are also termed inositol pyrophosphates (PP-InsPs), which gained special attention as potent cellular regulators. In plants, PP-InsPs were shown to be involved in abiotic and biotic stress responses, such as phosphate (P_i) homeostasis and hormone-dependent pathogen defenses. Phosphorus is an essential element and key nutrient for growth and development in all organisms. In plants, phosphorus is taken up in form of P_i, whose availability is largely restricted. To adjust to changing P_i availabilities, plants have evolved the so-called phosphate starvation response, which is regulated by the interaction of PHOSPHATE STARVATION RESPONSE REGULATOR (PHR) transcription factors with SYG1/Pho81/XPR1-domain containing (SPX) proteins that function as sensors for PP-InsPs. The research herein presented focuses on the P_i-dependent regulation of PP-InsP metabolism, especially in view of INOSITOL 1,3,4-TRISPHOSPHATE 5/6 KINASE 1 (ITPK1). A novel tool for PP-InsP analyses, the capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS), was successfully employed to separate and identify $InsP_7$ isomers in *Arabidopsis* and rice upon P_i-limited and P_i-resupply conditions. It was shown that $1/3\text{-}InsP_7$, $5\text{-}InsP_7$ and $InsP_8$ accumulate in shoots when P_i-starved plants regained P_i, suggesting a tight link to cellular P_i levels. Notably, ITPK1 plays a crucial role in P_i-dependent synthesis of $5\text{-}InsP_7$, the precursor of $InsP_8$, whose activity is critical for undisturbed P_i signaling. In addition, this work revealed that the generation of $5\text{-}InsP_7$ by ITPK1 requires high ATP concentrations and that under conditions of low adenylate charge, ITPK1 catalyzes the transfer of the β-phosphate of $5\text{-}InsP_7$ to ADP to locally generate ATP. Using CE-ESI-MS, plant $4/6\text{-}InsP_7$ was identified, an isomer that to date was only described in social amoeba *Dictyostelium discoideum* and is unlikely to play any role in P_i signaling in plants. Further, an unknown PP-InsP species in P_i-starved *Arabidopsis* and rice roots was observed that most likely represents an unknown $PP\text{-}InsP_4$ isomer that was absent in *itpk1* roots, suggesting that ITPK1 is involved in the biosynthesis of this unknown PP-InsP.

This thesis provides an insight into the involvement of PP-InsPs in P_i homeostasis and presents the current knowledge about the functions of PP-InsPs in abiotic and biotic stress responses in plants.

Chapter I

General Introduction

Phosphorous (P) is a key determinant for growth and development of living organisms (Marschner, 1995) and functions as a component of molecules with critical cellular functions such as ATP, NADPH and nucleic acids (Marschner, 1995; White and Hammond, 2008). Plants take P up in its inorganic form phosphate (P_i) ($H_2PO_4^{1-}$), which is highly immobile, chemically fixated, as well as unevenly distributed in soils, causing its access to plants very limited (Holford, 1997). Furthermore, 30% of the world´s soils are estimated to be P-deficient or display poor plant availability of this macronutrient, which leads to a high demand of P fertilization to improve crop yields (MacDonald et al., 2011). As consequence, continuous P fertilization subsequently results in high P enrichment in soils. Further, erosion caused by wind or water can result in eutrophication of open water bodies where P represents the major limiting nutrient (Baker et al., 2015). Notably, P_i sources are limited and located in a few countries, and the major fraction of P fertilizer is acquired from heavy metal poor phosphate deposits that will be probably fully consumed in up to 50-100 years (Cordell et al., 2009; Gilbert, 2009; Cooper et al., 2011; Walan et al., 2014; Baker et al., 2015). Furthermore, the quality of P fertilizer depends on the producing country since different types of phosphate rocks harbor different levels of cadmium (Cd), which is known to cause severe irreversible damages to human health and natural ecosystems due to its toxicity (Li et al., 2021; Zheng et al., 2021). The severity of contamination with the heavy metal is related to geographic provenance or the type of ore (Suciu et al., 2022). For instance, phosphate from igneous deposits in Russia or South Africa only harbor low Cd levels about 1-4 mg kg^{-1}, whereas deposits in USA, Morocco, Togo, Senegal and Idaho contain much higher concentrations that range 1-150 mg kg^{-1} (McLaughlin et al., 2021). Before the Ukraine war, the global fertilizer market was already severely stressed causing rising fertilizer prices (World Bank, 2022). To date, the European Union restricts the import of fertilizers from Russia and Belarus following the economic sanctions (AMIS, 2022). Therefore, several aspects need to be considered in order to guarantee a sustainable use of P resources in future agriculture including adopting crops to limited P-availability. To this end, an in-depth physiological understanding of how plants take up P_i but also of how plants sense P and/or sense P-deficiency at the molecular level is critical to identify new molecular targets that can guide efforts to breed plants with increased P-use efficiency.

Plants sense cellular P_i levels and quickly react to limited P_i concentrations by activating the so-called phosphate starvation response (PSR) to maintain cellular P_i homeostasis (Vance et al., 2003; Chiou and Lin, 2011; Secco et al., 2013). As a consequence, P_i uptake is increased by the

production and secretion of acid phosphatases, organic acid exudation, root architecture modification and development of root hairs, as well as enhanced expression of P_i transporters (Marschner et al., 1986; Duff et al., 1994; Schachtman et al., 1998; Gilroy et al., 2000; Uhde-Stone et al., 2003).

Recent work has shown that the regulation of PSR relies on the involvement of phosphate-rich inositol pyrophosphates (PP-InsPs) (Wild et al., 2016, Dong et al., 2019; Zhu et al., 2019; Ried et al., 2021) by acting as ligands for SPX proteins, known negative regulators of P_i homeostasis in plants (Lv et al., 2014; Shi et al., 2014; Wang et al., 2014). PP-InsPs belong to a multifaceted family of signaling molecules that control several physiological processes in eukaryotes (Laha et al., 2015; Couso et al., 2016; Dong et al., 2019; Zhu et al., 2019; Gulabani et al., 2021). These molecules are based on a six-carbon ring structure, *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, also known as *myo*-inositol (Shears, 2015). The most abundant form of the lower phosphorylated inositol phosphates (InsPs) in eukaryotes is $InsP_6$, also referred to as *myo*-inositol 1,2,3,4,5,6 hexakisphosphate, phytic acid or phytate (Wundenberg et al., 2012; Pisani et al., 2014). $InsP_6$ is the fully phosphorylated form of the *myo*-inositol ring and serves as a P_i storage molecule in developing seeds (Raboy and Gerbasi, 1996; Secco et al., 2017). During seed germination, the storage protein bodies are degraded, leading to the rapid hydrolysis of $InsP_6$ by phytases to provide nutrients to the developing seedling (Raboy and Gerbasi, 1996; Loewus and Murthy, 2000). Due to the strong chelation of mineral cations, $InsP_6$ is known to be an antinutrient in human and animal diet. Most of the farming non-ruminant animals (e.g. pigs and poultry) lack phytases in their digestive tracts, therefore undigested P-bound phytate can be released in open water systems, eventually leading to eutrophication and environmental P pollution which is considered a major factor setting planetary health at risk (Rockström et al., 2009; Raboy, 2020).

At the molecular level, $InsP_6$ acts as precursor for higher phosphorylated PP-InsPs, which contain at least one high-energy phosphoanhydride bond. PP-InsPs are key signaling molecules for plant metabolism, development, immune responses, plant hormone perception and P_i homeostasis (Zhang et al., 2007; Mosblech et al., 2008; Jadav et al., 2013; Laha et al., 2015; Laha et al., 2016; Jung et al., 2018; Kuo et al., 2018; Dong et al., 2019; Zhu et al., 2019; Gulabani et al., 2021; Laha et al., 2022).

In this thesis, I mainly focus on the PP-InsP-mediated P_i homeostasis. Previous studies revealed that $InsP_8$ is the key player in P_i homeostasis when plants grow under sufficient P_i supply (Dong et al., 2019; Zhu et al., 2019). Under such conditions, $InsP_8$ binds to a class of stand-alone SPX proteins that negatively regulate the activity of the PHOSPHATE STARVATION RESPONSE

REGULATOR 1 (PHR1) transcription factor (Wild et al., 2016). The $InsP_8$ binding mediates the formation of the SPX-PHR complex, thereby preventing the association of PHR1 to the P1BS motif of promoters from Pi starvation-induced (PSI) genes, consequently downregulating gene expression. Under low P_i conditions, the binding affinity of SPX to the PHRs is decreased, leading to PHR-mediated expression of PSR genes (Dong et al., 2019; Zhu et al., 2019; Ried et al., 2021).

Several mutant lines disrupted in InsP and PP-InsP kinase activity were investigated in the past decade to uncover the physiological roles the phosphate-rich molecules play in regulating PSR. Those studies are discussed in details in Chapter III, where we also provide an overview of additional PP-InsP functions in regulating biotic and abiotic stress responses. For instance, mutants for the INOSITOL PENTAKISPHOSPHATE 2-KINASE (IPK1), whose levels of $InsP_6$, $InsP_7$ and $InsP_8$ are compromised, exhibit a disturbed PSR that is evident from an increased P_i overaccumulation when grown under P_i sufficient conditions (Kuo et al., 2018). This suggests that mutations in IPK1 cause the plant's inability to regulate P_i homeostasis (Stevenson-Paulik et al., 2005; Kuo et al., 2014; Kuo et al., 2018). Also mutant plants defective in INOSITOL 1,3,4-TRISPHOSPHATE 5/6 KINASE 1 (ITPK1) kinase activity show an excessive P_i overaccumulation similar to the observed in *ipk1* mutants, which suggests that IPK1 and ITPK1 attend the same pathway regulating P_i homeostasis (Kuo et al., 2018). In contrast, loss-of-function mutations in $InsP_7$ kinases DIPHOSPHOINOSITOL-PENTAKISPHOSPHATE KINASE 1/2 (VIH1 and VIH2) cause $InsP_7$ accumulation and lack of $InsP_8$, accompanied by mild developmental defects and no PSR phenotype (Dong et al., 2019; Zhu et al., 2019). Although it was shown that disrupted ITPK1 kinase activity causes a disturbed PSR (Kuo et al., 2018), the function of ITPK1 in plants was not known. Recently, *in vitro* data of recombinant ITPK1 suggests an $InsP_6$ kinase activity generating 5-$InsP_7$ (Laha et al., 2019). Interestingly, $InsP_8$ levels in *itpk1* are only slightly compromised and plants show a severe PSR phenotype, whereas in *vih2* plants, $InsP_8$ is below the limit of detection, and PSR is unaltered (Kuo et al., 2018; Dong et al., 2019). This raised the question, whether ITPK1 functions as PP-InsP synthase *in vivo* and if the lack of this activity is responsible for compromised P_i and energy homeostasis in *itpk1* mutant plants. Furthermore, it still remained unclear which $InsP_7$ isomers respond to P_i, and which isomer is the precursor for VIH2-generated $InsP_8$ linked to the plant´s P_i status. However, such studies are challenging due the lack of methods for the separation of different isomers. Addressing the abovementioned points is therefore indispensable to uncover how P_i-dependent changes in nutritional and energetic

states modulate ITPK1 activities to eventually fine-tune PP-InsP synthesis for the maintenance of P_i homeostasis.

Objectives

The objective of this work is to unveil how P_i regulates the inositol polyphosphate metabolism and how functional P_i homeostasis is dependent on the *Arabidopsis* $InsP_6$ kinase ITPK1.

In chapter II the following objectives were investigated:

1. Is the synthesis of P_i-dependent PP-InsPs conserved in multicellular organisms?
2. Is the synthesis PP-InsPs linked to cellular P_i levels?
3. In which tissue is ITPK1 critical for undisturbed P_i signaling?
4. Does ITPK1 possess an ATP-synthase activity and may the reversible reaction be important for the formation and degradation of PP-InsPs in response to P_i?
5. Is ITPK1 required for the P_i-dependent synthesis of 5-$InsP_7$ and $InsP_8$ *in planta*?
6. Does ITPK1 act in concert with VIH2 and redundantly with ITPK2 to maintain P_i homeostasis in *Arabidopsis*?
7. Is the P_i signaling controlled by ITPK1 dependent of PHR1 and PHL1 but independent of the ubiquitin conjugating enzyme PHO2 that takes part in PSR?
8. Is the subcellular $InsP_6$ compartmentalization essential for the tissue levels of $InsP_7$ and $InsP_8$?

In chapter III, I present a review of the current knowledge about the role of PP-InsPs in abiotic and biotic stress responses. Besides providing state-of-the-art information connecting PP-InsPs to hormone signaling and P_i homeostasis, as well as introducing PP-InsP-degrading bacterial effectors, the review provides an outlook of open research questions that need to be addressed to uncover the additional roles of PP-InsPs in plants in the future.

References

AMIS. Market Monitor. (2022). Available online: http://www.amis-outlook.org/fileadmin/user_upload/amis/docs/Market_monitor/AMIS_Market_Monitor_current.pdf (accessed on 8 December 2022).

Baker, A., Ceasar, S. A., Palmer, A. J., Paterson, J. B., Qi, W., Muench, S. P., Baldwin, S. A. (2015). Replace, reuse, recycle: improving the sustainable use of phosphorus by plants. J Exp Bot 66:3523-3540.

Chiou, T. J., Lin, S. I. (2011). Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol 62:185-206.

Cooper, J., Lombardi, R., Boardman, D., Carliell-Marquet, C. (2011). The future distribution and production of global phosphate rock reserves. Resour Conserv Recy 57:78-86.

Cordell, D., Drangert, J. O., White, S. (2009). The story of phosphorus: global food security and food for thought. Glob Environ Change 19:292-305.

Couso, I., Evans, B. S., Li, J., Liu, Y., Ma, F., Diamond, S., Allen, D. K., Umen, J. G. (2016). Synergism between Inositol Polyphosphates and TOR Kinase Signaling in Nutrient Sensing, Growth Control, and Lipid Metabolism in Chlamydomonas. The Plant Cell 28(9):2026-2042.

Dong, J., Ma, G., Sui, L., Wei, M., Satheesh, V., Zhang, R., Ge, S., Li, J., Zhang, T.-E., Wittwer, C., Jessen, H. J., Zhang, H., An, G.-Y., Chao, D.-Y., Liu, D., Lei, M. (2019). Inositol pyrophosphate InsP$_8$ acts as an intracellular phosphate signal in Arabidopsis. Mol Plant 12:1463-1473.

Duff, S. M. G., Sarath, G., Plaxton, W. C. (1994). The role of acid phosphatases in plant phosphorus metabolism. Phys Plantarum 90:791-800.

Gilbert, N. (2009). The disappearing nutrient. Nature 461:716-718.

Gilroy, S., Jones, D. L. (2000). Through form to function: root hair development and nutrient uptake. Trends Plant Sci 5:56-60.

Gulabani, H., Goswami, K., Walia, Y., Roy, A., Jameeta Noor, J., Ingole, K. D., Kasera, M., Laha, D., Giehl, R. F. H., Schaaf, G., Bhattacharjee, S. (2021). Arabidopsis inositol polyphosphate kinases IPK1 and ITPK1 modulate crosstalks between SA-dependent immunity and phosphate-starvation responses. Plant Cell Rep 41(2):347-363.

Hammond, J.P., White, P.J. (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. J Exp Bot 59:93-109.

Holford, I. C. R. (1997). Soil phosphorus: Its measurement, and its uptake by plants. Aust J Soil Res 35:227-239.

Jadav, R. S., Chanduri, M. V., Sengupta, S., Bhandari, R. (2013). Inositol pyrophosphate synthesis by inositol hexakisphosphate kinase 1 is required for homologous recombination repair. J Biol Chem 288:3312-3321.

Jung, J.-Y., Ried, M. K., Hothorn, M., Poirier, Y. (2018). Control of plant phosphate homeostasis by inositol pyrophosphates and the SPX domain. Curr Opin Biotech 49:156-162.

Kuo, H. F., Chang, T. Y., Chiang, S. F., Wang, W. D., Charng, Y. Y., Chiou, T. J. (2014). Arabidopsis inositol pentakisphosphate 2-kinase, AtIPK1, is required for growth and modulates phosphate homeostasis at the transcriptional level. Plant J 80:503-515.

Kuo, H.-F., Hsu, Y.-Y., Lin, W.-C., Chen, K.-Y., Munnik, T., Brearley, C. A., Chiou, T.-J. (2018). Arabidopsis inositol phosphate kinases, IPK1 and ITPK1, constitute a metabolic pathway in maintaining phosphate homeostasis. Plant J 95:613-630.

Laha, D., Johnen, P., Azevedo, C., Dynowski, M., Weiss, M., Capolicchio, S., Mao, H. B., Iven, T., Steenbergen, M., Freyer, M., Gaugler, P., de Campos, M. K. F., Zheng, N., Feussner, I., Jessen, H. J., Van Wees, S. C. M., Saiardi, A., Schaaf, G. (2015). VIH2 Regulates the Synthesis of Inositol Pyrophosphate InsP(8) and Jasmonate-Dependent Defenses in Arabidopsis. Plant Cell 27:1082-1097.

Laha, D., Parvin, N., Dynowski, M., Johnen, P., Mao, H., Bitters, S. T., Zheng, N., Schaaf,

G. (2016). Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex. Plant Phys 171:2364-2370.

Laha, D., Parvin, N., Hofer, A., Giehl, R. F. H., Fernandez-Rebollo, N., von Wiren, N., Saiardi, A., Jessen, H. J., Schaaf, G. (2019). Arabidopsis ITPK1 and ITPK2 Have an Evolutionarily Conserved Phytic Acid Kinase Activity. Acs Chem Biol 14:2127-2133.

Laha, N. P, Giehl, R. F. H., Riemer E, Qiu, D., Pullagurla, N. J., Schneider, R., Dhir, Y. W., Yadav, R., Mihiret, Y. E., Gaugler, P., Gaugler, V., Mao, H., Zheng, N., von Wirén, N., Saiardi, A., Bhattacharjee, S., Jessen, H. J., Laha, D., Schaaf, G. (2022). INOSITOL (1,3,4) TRIPHOSPHATE 5/6 KINASE1-dependent inositol polyphosphates regulate auxin responses in Arabidopsis. Plant Physiol 190(4):2722-2738.

Li, Y., Chen, W., Yang, Y., Wang, T., Dai, Y. (2021). Quantifying source-specific intake risks of wheat cadmium by associating source contributions of soil cadmium with human health risk. Ecotoxicol Environ Saf 228:112982.

Loewus, F. A., Murthy, P. P. N. (2000). *Myo*-Inositol metabolism in plants. Plant Sci 150:1-19.

Lv, Q., Zhong, Y., Wang, Y., Wang, Z., Zhang, L., Shi, J., Wu, Z., Liu, Y., Mao, C., Yi, K., Wu, P. (2014). SPX4 Negatively Regulates Phosphate Signaling and Homeostasis through Its Interaction with PHR2 in Rice. Plant Cell 26(4):1586-1597.

MacDonald, G. K., Bennett, E. M., Potter, P. A., Ramankutty, N. (2011). Agronomic phosphorus imbalances across the world's croplands. Proc Natl Acad Sci USA 108:3086-3091.

Marschner, H., Römheld, V., Horst, W.J., Martin, P. (1986). Root induced changes in the rhizosphere: importance for mineral nutrition of plants. Zeitschrift für Pflanzenernährung und Bodenkunde 149:441-456.

McLaughlin, M. J., Smolders, E., Zhao, F. J., Grant, C., Montalvo, D. (2021). Chapter One - managing cadmium in agricultural systems. Adv Agron 166:1-129.

Mosblech, A., König, S., Stenzel, I., Grzeganek, P., Feussner, I., Heilmann, I. (2008). Phosphoinositide and inositolpolyphosphate signalling in defense responses of Arabidopsis thaliana challenged by mechanical wounding. Mol Plant 1:249-261.

Pisani, F., Livermore, T., Rose, G., Chubb, J. R., Gaspari, M., Saiardi, A. (2014). Analysis of *Dictyostelium discoideum* inositol pyrophosphate metabolism by gel electrophoresis. PLoS One 9:e85533.

Raboy, V., and Gerbasi, P. (1996). Genetics of *myo*-Inositol phosphate synthesis and accumulation. Subcell Biochem 26:257-285.

Raboy, V. (2020). Low phytic acid Crops: Observations Based on Four Decades of Research. Plants 9(2):140.

Ried, M. K., Wild, R., Zhu, J., Pipercevic, J., Sturm, K., Broger, L., Harmel, R. K., Abriata, L. A., Hothorn, L. A., Fiedler, D., Hiller, S., Hothorn, M. (2021). Inositol pyrophosphates promote the interaction of SPX domains with the coiled-coil motif of PHR transcription factors to regulate plant phosphate homeostasis. Nat Commun 12(1):384.

Rockström, J., Steffen, W., Noone, K., Persson, A., Chapin, F. S., Lambin, E. F., Lenton, T. M., Scheffer, M., Folke, C., Schellnhuber, H. J., Nykvist, B., de Wit, C. A., Hughes, T., van der Leeuw, S., Rodhe, H., Sörlin, S., Snyder, P. K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R. W., Fabry, V. J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P., Foley, J. A. (2009). A safe operating space for humanity. Nature 461:472-475.

Schachtman, D. P., Reid, R. J., Ayling, S. M. (1998). Phosphorus uptake by plants: from soil to cell. Plant Physiol 116:447-453.

Secco, D., Jabnoune, M., Walker, H., Shou, H., Wu, P., Poirier, Y., Whelan J. (2013). Spatio-temporal transcript profiling of rice roots and shoots in response to phosphate starvation and recovery. Plant Cell 25(11):4285-304.

Secco, D., Bouain, N., Rouached, A., Prom-u-thai, C., Hanin, M., Pandey, A. K.,

Rouached, H. (2017). Phosphate, phytate and phytases in plants: from fundamental knowledge gained in Arabidopsis to potential biotechnological applications in wheat. Crit Rev Biotechnol 37:898-910.

Shears, S. B. (2015). Inositol pyrophosphates: why so many phosphates? Adv Biol Regul 57:203-21.

Shi, J., Hu, H., Zhang, K., Zhang, W., Yu, Y., Wu, Z., Wu, P. (2014). The paralogous SPX3 and SPX5 genes redundantly modulate Pi homeostasis in rice. J Exp Bot 65(3):859-870.

Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., York, J. D. (2005). Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc Natl Acad Sci USA 102:12612-12617.

Suciu, N.A., De Vivo, R., Rizzati R., Capri E. (2022). Cd content in phosphate fertilizer: Which potential risk for the environment and human health? Curr Opin Eviron Sc & Health 30:199392.

Uhde-Stone, C., Gilbert, G., Johnson, J. M. F., Litjens, R., Zinn, K. E., Temple, S. J., Vance, C. P., Allan, D. L. (2003). Adaptation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. Plant and Soil 248:99-116.

Vance, C. P., Uhde-Stone, C., Allan, D. L. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157(3):423-447.

Walan, P., Davidsson, S., Johansson, S., Höök, M. (2014). Phosphate rock production and depletion: regional disaggregated modeling and global implications. Resour Conserv Recycling 93:178-187.

Wang, Z. Y., Ruan, W., Shi, J., Zhang, L., Xiang, D., Yang, C., Li, C., Wu, Z., Liu, Y., Yu, Y., Shou, H., Mo, X., Mao, C., Wu, P. (2014). 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.

Wild, R., Gerasimaite, R., Jung, J.-Y., Truffault, V., Pavlovic, I., Schmidt, A., Saiardi, A., Jessen, H. J., Poirier, Y., Hothorn, M., Mayer, A. (2016). Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. Science 352:986-990.

World Bank. Commodity Markets Outlook: The Impact of the War in Ukraine on Commodity Markets. Available online: https://openknowledge.worldbank.org/bitstream/handle/10986/37223/CMO-April-2022.pdf (accessed on 8 December 2022).

Wundenberg, T., Mayr, G. W. (2012). Synthesis and biological actions of diphosphoinositol phosphates (inositol pyrophosphates), regulators of cell homeostasis. Biol Chem 393:979-998.

Zhang, Z. B., Yang, G., Arana, F., Chen, Z., Li, Y., Xia, H. J. (2007). Arabidopsis inositol polyphosphate 6-/3-kinase (AtIpk2beta) is involved in axillary shoot branching via auxin signaling. Plant Physiol 144(2):942-951.

Zheng, L., Jiang, Y. L., Fei, J., Cao, P., Zhang, C., Xie, G. F., Wang, L. X., Cao, W., Fu, L., Zhao, H. (2021). Circulatory cadmium positively correlates with epithelial-mesenchymal transition in patients with chronic obstructive pulmonary disease. Ecotoxicol Environ Saf 215:112164.

Zhu, J., Lau, K., Puschmann, R., Harmel, R. K., Zhang, Y.J., Pries, V., Gaugler, P., Broger, L., Dutta, A. K., Jessen, H. J., Schaaf, G., Fernie, A. R., Hothorn, L. A., Fiedler, D., Hothorn, M. (2019). Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. Elife 8.

Chapter II: ITPK1 is an InsP$_6$/ADP phosphotransferase that controls phosphate signaling in *Arabidopsis*

Esther Riemer, Danye Qiu, Debabrata Laha, Robert K. Harmel, Philipp Gaugler, Verena Gaugler, Michael Frei, Mohammad-Reza Hajirezaei, Nargis Parvin Laha, Lukas Krusenbaum, Robin Schneider, Adolfo Saiardi, Dorothea Fiedler, Henning J. Jessen, Gabriel Schaaf, Ricardo F.H. Giehl.

Abstract

In plants, phosphate (P$_i$) homeostasis is regulated by the interaction of PHR transcription factors with stand-alone SPX proteins, which act as sensors for inositol pyrophosphates. In this study, we combined different methods to obtain a comprehensive picture of how inositol (pyro)phosphate metabolism is regulated by P$_i$ and dependent on the inositol phosphate kinase ITPK1. We found that inositol pyrophosphates are more responsive to P$_i$ than lower inositol phosphates, a response conserved across kingdoms. Using the capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) we could separate different InsP$_7$ isomers in *Arabidopsis* and rice, and identify 4/6-InsP$_7$ and a PP-InsP$_4$ isomer hitherto not reported in plants. We found that the inositol pyrophosphates 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ increase several fold in shoots after P$_i$ resupply and that tissue-specific accumulation of inositol pyrophosphates relies on ITPK1 activities and MRP5-dependent InsP$_6$ compartmentalization. Notably, ITPK1 is critical for P$_i$-dependent 5-InsP$_7$ and InsP$_8$ synthesis *in planta* and its activity regulates P$_i$ starvation responses in a PHR-dependent manner. Furthermore, we demonstrated that ITPK1-mediated conversion of InsP$_6$ to 5-InsP$_7$ requires high ATP concentrations and that *Arabidopsis* ITPK1 has an ADP phosphotransferase activity to dephosphorylate specifically 5-InsP$_7$ under low ATP. Collectively, our study provides new insights into P$_i$-dependent changes in nutritional and energetic states with the synthesis of regulatory inositol pyrophosphates.

Introduction

To maintain cellular phosphate (P$_i$) homeostasis, plants have evolved complex sensing and signaling mechanisms that adjust whole-plant P$_i$ demand with external P$_i$ availability. Although many molecular players involved in these responses have been identified, the exact mechanism of P$_i$ sensing in complex organisms, such as plants, still remains largely unknown. In the model species *Arabidopsis thaliana*, the MYB transcription factors PHOSPHATE STARVATION

RESPONSE 1 (PHR1) and its closest paralog PHR1-LIKE1 (PHL1) control the expression of the majority of P_i starvation-induced (PSI) genes to regulate numerous metabolic and developmental adaptations induced by P_i deficiency (Rubio et al., 2001; Bustos et al., 2010). Since *PHR1* expression is only weakly responsive to P_i deficiency (Rubio et al., 2001), the existence of a post-translational control of PHR1 and PHL1 factors has been proposed. Emerging evidence indicates that a class of stand-alone SPX proteins negatively regulates the activity of PHR transcription factors in different plant species (Liu et al., 2010; Wang et al., 2014b; Lv et al., 2014; Puga et al., 2014; Qi et al., 2017; Zhong et al., 2018; Ried et al., 2021). In *Arabidopsis*, SPX proteins can interact with the plant-unique coiled-coil motif of PHR1, thereby controlling the oligomeric state and the promoter binding activity of this transcription factor (Ried et al., 2021).

The *in vivo* interaction of PHRs and SPXs is influenced by P_i (Wang et al., 2014b; Lv et al., 2014; Puga et al., 2014), suggesting that this mechanism could represent a direct link between P_i perception and downstream signaling events. However, the dissociation constants for P_i itself in an SPX–PHR complex ranged from 10 mM to 20 mM (Wang et al., 2014b; Lv et al., 2014; Puga et al., 2014). The study of Wild et al. (2016) demonstrated that SPX domains act as receptors for inositol pyrophosphates (PP-InsPs), small signaling molecules consisting of a phosphorylated *myo*-inositol ring and one or two pyrophosphate groups (Wilson et al., 2013; Shears, 2018). Isothermal titration calorimetry experiments demonstrated that 5PP-InsP$_5$ (hereafter 5-InsP$_7$) interacts more strongly with SPX domains than P_i (Wild et al., 2016). More recent studies have further shown that 1,5(PP)$_2$-InsP$_4$ (1,5-InsP$_8$ hereafter) has an even higher binding affinity toward SPX domains than 5-InsP$_7$ *in vitro* (Gerasimaite et al., 2017; Dong et al., 2019; Ried et al., 2021), and that InsP$_8$ can restore more efficiently the interaction between SPX1 and PHR1 *in vivo* (Dong et al., 2019). In line with the proposed role of InsP$_8$ as an intracellular P_i signaling molecule controlling the formation of SPX–PHR complexes, *Arabidopsis* mutants with compromised synthesis of PP-InsPs exhibit constitutive PSI gene expression and overaccumulate P_i (Stevenson-Paulik et al., 2005; Kuo et al., 2014, 2018; Dong et al., 2019; Zhu et al., 2019). Importantly, cellular pools of different PP-InsPs are significantly altered in response to P_i availability in *Arabidopsis* (Kuo et al., 2018; Dong et al., 2019), suggesting that the enzymes involved in their synthesis could act as regulators of P_i homeostasis in plants. However, the biosynthetic steps leading to dynamic changes in InsP$_8$ levels in response to P_i availability still remain unresolved.

In plants, synthesis of InsP$_8$ is mediated by VIH1 and VIH2 (Laha et al., 2015; Dong et al., 2019; Zhu et al., 2019), a class of bifunctional kinase/phosphatase enzymes (Zhu et al., 2019)

sharing homology with the yeast and animal Vip1/PPIP5Ks (Desai et al., 2014; Laha et al., 2015). However, how plants synthesize InsP$_7$ has since long remained elusive, as plant genomes do not encode homologs of the metazoan and yeast InsP$_6$ kinases IP6K/Kcs1 (Saiardi et al., 1999). We and others have recently identified the *Arabidopsis* inositol 1,3,4-trisphosphate 5/6-kinases ITPK1 and ITPK2 as putative novel plant InsP$_6$ kinases (Adepoju et al., 2019; Laha et al., 2019). We further showed that ITPK1 generates the meso InsP$_7$ isomer 5-InsP$_7$, the major form identified in seed extracts (Laha et al., 2019). Furthermore, InsP$_7$ and InsP$_8$ levels are compromised in an itpk1 mutant, showing that ITPK1 functions as an InsP$_6$ kinase *in planta* (Laha et al., 2019). Since InsP$_7$ is the precursor for InsP$_8$ synthesis, the next challenge is to determine which InsP$_7$ isomers respond to P$_i$ and how their synthesis is linked to the plant's P$_i$ status. A recent study reported that P$_i$ deficiency induces a shoot-specific increase in InsP$_7$ levels in *Arabidopsis* as determined by high-performance liquid chromatography (HPLC) analysis of [^{32}P]P$_i$-labeled extracts (Kuo et al., 2018). However, this response does not easily explain decreased InsP$_8$ levels detected in shoots of P$_i$-deficient *Arabidopsis* plants by polyacrylamide gel electrophoresis (PAGE) (Dong et al., 2019). Since ^{32}P labeling does not provide a mass assay of the inositol species, and PAGE is not suited to the analysis of lower inositol phosphates (InsPs), alternative approaches are still required to obtain a complete picture of P$_i$-dependent metabolism of InsPs and PP-InsPs in plants. The recent development of a capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) method for ultrasensitive analysis of inositol (pyro)phosphates (Qiu et al., 2020) offers a unique opportunity to perform isomer identification and quantitation in different plant tissues.

Here, we combined [^{3}H]inositol labeling, PAGE, and CE-ESI-MS to investigate in unprecedented detail P$_i$-, ITPK1-, and VIH2-dependent quantitative changes in inositol (pyro)phosphate levels. Our results reveal sensitive responses of 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ according to cellular P$_i$ levels and organ-specific accumulation of InsP$_7$ and InsP$_8$ relying on MRP5-dependent InsP$_6$ compartmentalization. We also identified two previously unreported PP-InsP isomers, including a presumptive PP-InsP$_4$ isomer that is preferentially produced in roots in an ITPK1-dependent manner. With grafting and genetic crosses, we demonstrate that ITPK1 activity in shoots is more critical for undisturbed P$_i$ signaling and relies on functional PHRs. Finally, we show that *Arabidopsis* ITPK1 mediates adenylate charge-dependent reversible reactions with high K$_M$ values for ATP and ADP, and generates 5-InsP$_7$ *in planta*, which we determined to be the main substrate for the strong InsP$_8$ synthesis induced by P$_i$ resupply to P$_i$-starved plants.

Results

P_i-dependent synthesis of PP-InsPs is conserved across multicellular organisms

To assess if the synthesis of $InsP_8$ and its immediate precursor, $InsP_7$, responds to quick changes in P_i availability, we analyzed $InsP_6$, $InsP_7$, and $InsP_8$ levels with the help of titanium dioxide (TiO_2)-based pull-down followed by separation via PAGE (Losito et al., 2009; Wilson et al., 2015). In the dicotyledonous species *A. thaliana*, total phosphorus (P) concentration in shoots decreased significantly after 7 days of growth in a P_i-deficient nutrient solution (Figure 1A). When P_i was resupplied, shoot P levels were already significantly increased after 6 h and reached levels comparable to those of plants cultivated continuously on P_i-replete conditions after 12 h. In the same plants, $InsP_6$, $InsP_7$, and $InsP_8$ signals decreased significantly in response to P_i starvation (Figure 1B and 1C). However, the most dramatic changes were observed when P_i-starved plants were resupplied with P_i. In relative terms, $InsP_7$ and $InsP_8$ responded much more sensitively to P_i resupply than $InsP_6$, with $InsP_8$ signals increasing almost 100-fold and greatly surpassing the levels detected in plants grown constantly under sufficient P_i (Figure 1C). Strong recovery of $InsP_7$ and especially $InsP_8$ was also detected in shoots of the monocotyledonous species rice (Figure 1D and Supplemental Figure 1). We could also detect clear P_i-dependent accumulation of PP-InsPs in gametophores of the moss *Physcomitrium patens*, although induction by P_i resupply was less pronounced in this species (Figure 1E–1G). Together, these results suggest that P_i-dependent $InsP_7$ and $InsP_8$ synthesis is conserved in vascular and non-vascular land plants. We also used PAGE to assess P_i-dependent synthesis of PP-InsPs in the human HCT116 cell line, and found that while $InsP_6$ levels remained largely unaffected by P_i conditions, both $InsP_7$ and $InsP_8$ decreased in cells after P_i was removed from the culture and sharply increased again after P_i resupply (Figure 1H). Altogether, these results indicate that P_i-dependent synthesis of $InsP_7$ and $InsP_8$ seems to be evolutionarily conserved across a range of multicellular organisms.
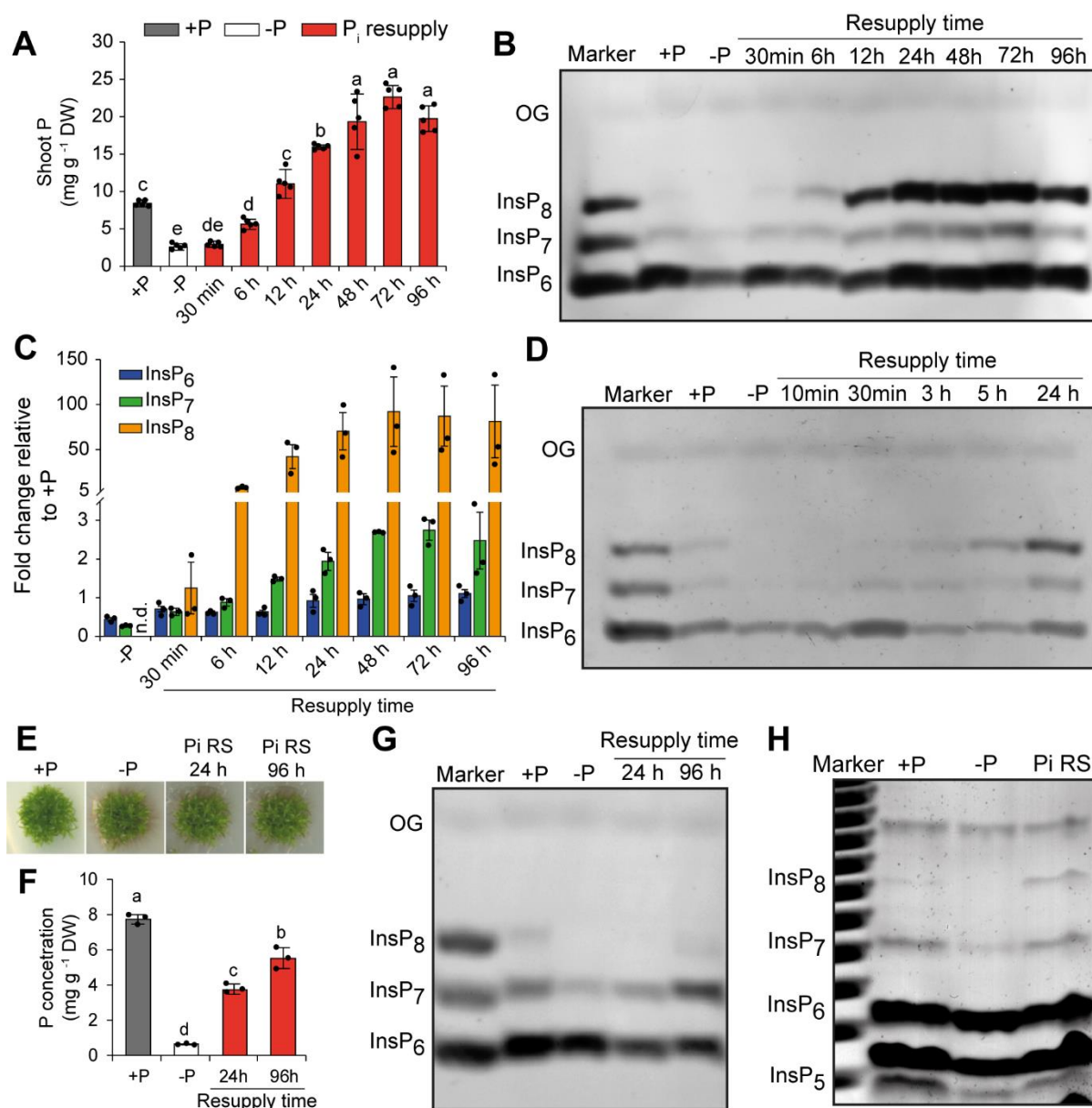
Figure 1. P_i-dependent regulation of InsP$_7$ and InsP$_8$ levels is conserved in multicellular organisms. **(A)** Shoot total P levels in *A. thaliana* in response to sufficient (+P) or deficient P_i (-P) or after P_i resupply to P_i-starved plants for the indicated time. Data are means ± SD (n = 5 plants). **(B and C)** Time-course PAGE analysis of InsP/PP-InsPs in response to P_i starvation and P_i resupply in *A. thaliana* shoots **(B)** and fold change of quantified signal intensities **(C)**. Data are means ± SE (n = 3 gels loaded with independent biological samples). OG, orange G. **(D)** Time-course PAGE analysis of rice shoots. Plants were cultivated in hydroponics under sufficient P_i (+P) or deficient P_i (−P) for 7 days (*A. thaliana* Col-0) or 10 days (rice, *Oryza sativa* cv. Nipponbare), or –P resupplied with P_i for the indicated times. Quantification of signals is shown in Supplemental Figure 1. OG, orange G. **(E-G)** Phenotype **(E)**, total P_i levels **(F)**, and PAGE analysis **(G)** of gametophores of *Physcomitrium 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 are means ± SD (*n* = 3 biological replicates). OG, orange G. **(H)** PAGE analysis of inositol (pyro)phosphates extracted from HCT116 cells cultured on sufficient P_i (+P), starved in P_i-free medium for 18 h (−P), or −P and resupplied with P_i for 3.5 h (Pi RS). Cells were harvested at the same time. The experiment was repeated twice with similar results. In **(A)** and **(F)**, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Comprehensive analysis of P_i-dependent inositol (pyro)phosphate metabolism in *Arabidopsis*

As PAGE separation and staining cannot detect lower InsPs and is unable to distinguish PP-InsP regioisomers (Losito et al., 2009), we used additional methods to investigate in more detail which InsPs and PP-InsPs respond to P_i. First, we performed strong anion-exchange chromatography–HPLC analyses of extracts from [^{3}H]inositol-labeled wild-type (WT) seedlings. Of all InsPs detected in whole seedlings, only $InsP_6$ and the PP-InsPs $InsP_7$ and $InsP_8$ decreased in response to P_i starvation and increased again after P_i resupply (Figure 2A and 2B). Two $InsP_4$ isomers of unknown isomeric nature (eluting at 41 and 46 min, respectively) also decreased under P_i deficiency, but none of the lower InsPs exhibited a comparable fast recovery after P_i resupply relative to $InsP_7$ and $InsP_8$ (Figure 2A and 2B).

Next, we used the recently developed CE-ESI-MS method, which does not rely on metabolic labeling and is therefore not blind to inositol derivatives generated *de novo* from D-glucose-6-phosphate (Qiu et al., 2020). To validate the method with plant samples of 6-week-old plants grown in hydroponics, assignment of 1-$InsP_7$, 5-$InsP_7$, and 1,5-$InsP_8$ was confirmed with fully ^{13}C-labeled internal standards (Figure 2C). Except for $InsP_3$-3 and 1/3-OH $InsP_5$, the remaining InsPs and all PP-InsPs detected with CE-ESI-MS decreased in P_i-deficient shoots (Figure 2D). Within a maximum of 12 h of P_i resupply, PP-InsP levels had recovered to P_i-sufficient levels, while at least 24 h was required to restore the levels of InsPs. In line with our PAGE analysis, $InsP_8$ showed the most dramatic relative change (up to a 40-fold increase), with levels surpassing those detected in P_i-sufficient plants already after 6 h of P_i resupply (Figure 2D). 1/3-$InsP_7$ also experienced a fast recovery during P_i resupply. We found that 5-$InsP_7$ was more abundant than 1/3-$InsP_7$ and also responded to P_i resupply, although less sensitively than 1/3-$InsP_7$ and $InsP_8$ (Figure 2D). Remarkably, we also detected a previously unreported $InsP_7$ isomer that migrated separately from [^{13}C$_6$]5-$InsP_7$ and [^{13}C$_6$]1-$InsP_7$ standards and co-migrated with synthetic 6-$InsP_7$ (Capolicchio et al., 2013), hence likely representing 4-$InsP_7$ or the 6-$InsP_7$ enantiomer (Supplemental Figure 2). Unlike 1/3-$InsP_7$ and 5-$InsP_7$, the novel $InsP_7$ isomer responded only mildly to P_i starvation and P_i resupply (Figure 2D). Together, these results demonstrate that, although most InsPs and PP-InsPs decrease during P_i deficiency, the levels of the PP-InsPs 1/3-$InsP_7$, 5-$InsP_7$, and $InsP_8$ recover faster and more strongly compared with all other InsP species when P_i-starved plants regain access to P_i.
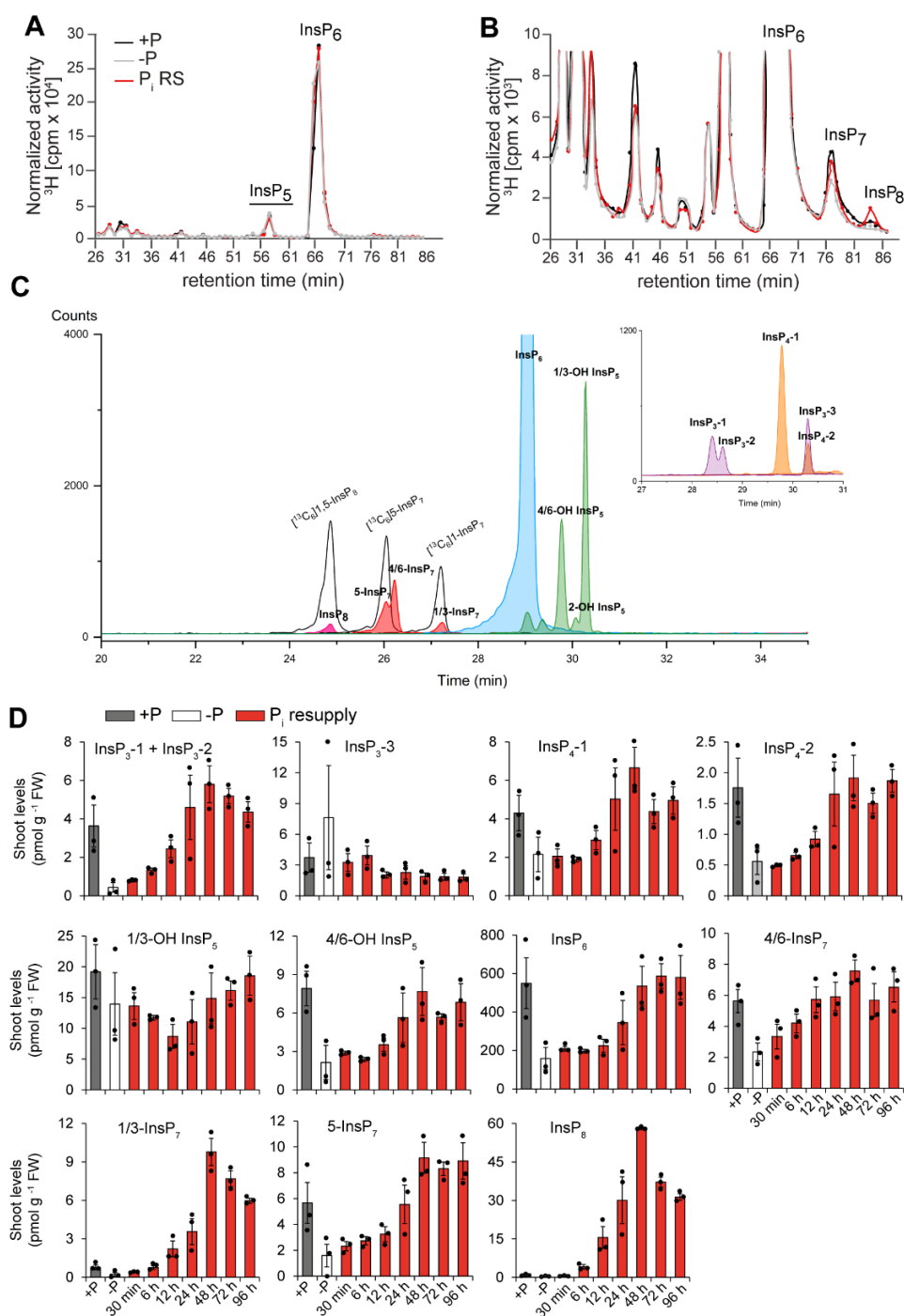
Figure 2. InsP and PP-InsP profiles in response to changes in P_i availability. (A and B) HPLC profiles of *Arabidopsis* (Col-0) seedlings radiolabeled with [^{3}H]*myo*-inositol. Seedlings were grown with P_i (+P) or without P_i (−P) or −P resupplied with P_i for 6 h (P_i RS). Full, normalized spectra **(A)** and zoom-in view of the same profile **(B)**. The experiment was repeated with similar results, and representative results from one experiment are shown. **(C)** Extracted-ion electropherograms of InsPs and PP-InsPs in *Arabidopsis* (Col-0) shoots. InsP$_8$, 5-InsP$_7$, and 1/3-InsP$_7$ were assigned by mass spectrometry and identical migration time compared with their heavy isotopic standards. A new PP-InsP isomer was assigned as 4/6-InsP$_7$, based on proofs showed in Supplemental Figure 2. Assignment of InsP$_6$ and InsP$_5$ is according to mass spectrometry and identical migration time compared with relative standards. Inset shows extracted-ion electropherograms of three InsP$_3$ and two InsP$_4$ isomers. **(D)** CE-ESI-MS analysis of inositol (pyro)phosphate levels in shoots of *Arabidopsis* (Col-0) plants exposed to variable P_i supplies. Plants were cultivated in hydroponics under sufficient P_i (+P), deficient P_i for 7 days (−P), or −P resupplied with P_i for the indicated times. Data are means ± SE (*n* = 3 biological replicates composed of two plants each).

18

The synthesis of 1/3-InsP$_7$, 5-InsP$_7$ and InsP$_8$ is tightly linked to cellular P$_i$ levels

The widespread effects of P$_i$ starvation and P$_i$ resupply on most InsPs and PP-InsPs are not unexpected, as the synthesis of these molecules relies on available P$_i$ for the phosphorylation reactions. However, the most sensitive response of 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ to P$_i$ refeeding suggested that their synthesis might be more directly associated with a signaling mechanism. To test this hypothesis, we compared P$_i$-supplied WT plants and the P-overaccumulating mutant *pho2-1* (Delhaize and Randall, 1995), defective in the E2 ubiquitin conjugase-related enzyme PHO2 (also known as UBC24) (Aung et al., 2006; Bari et al., 2006; Lin et al., 2008). Under our growth conditions, *pho2-1* plants overaccumulated P in shoots as expected (Figure 3A). PAGE revealed that InsP$_7$ and especially InsP$_8$ signals were significantly increased in *pho2-1*, while InsP$_6$ was hardly affected (Figure 3B). Subsequent CE-ESI-MS analysis showed that none of the detected lower InsP isomers was significantly increased in shoots of *pho2-1* plants (Figure 3C). In contrast, InsP$_8$ was increased approximately 50-fold in *pho2* shoots, further reinforcing that InsP$_8$ is the most P$_i$-sensitive PP-InsP in leaves. Whereas the levels of the novel presumptive 4/6-InsP$_7$ isomer did not change considerably in *pho2-1*, 5-InsP$_7$ exhibited a clear increase, albeit less dramatic than that of 1/3-InsP$_7$ (Figure 3C). Altogether, these results demonstrate that the synthesis of 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ is tightly controlled by a mechanism that relays changes in cellular P$_i$ levels specifically toward PP-InsP biosynthesis.
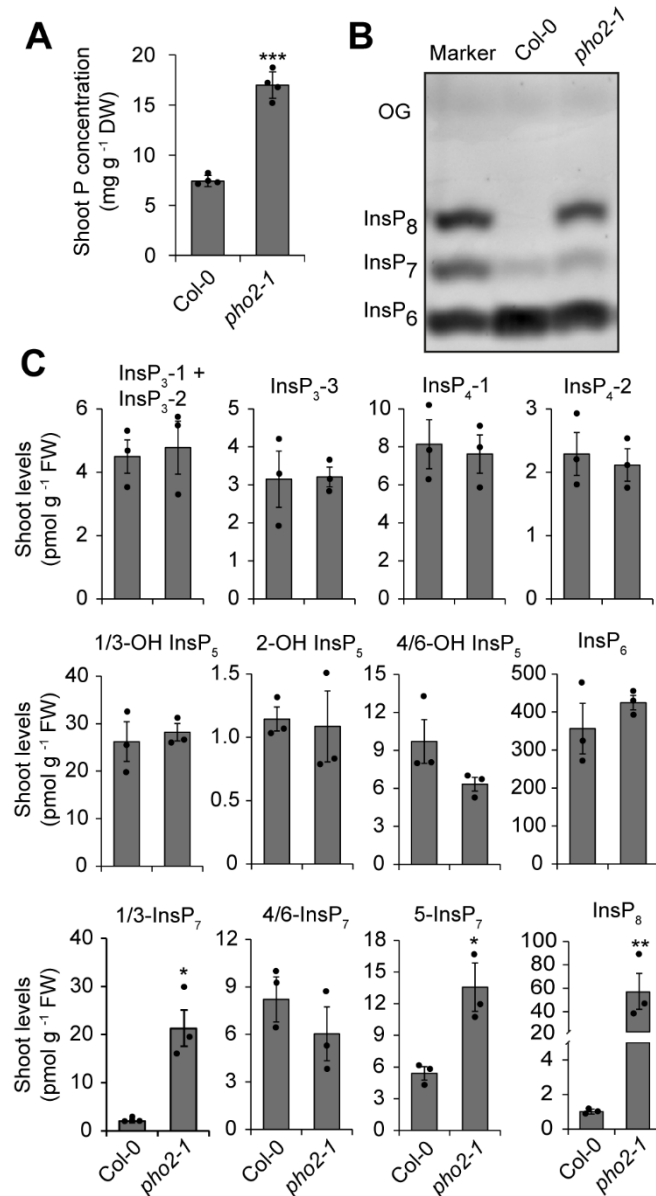
Figure 3. PP-InsPs respond more sensitively to internal P_i status than lower InsPs. (A) P-overaccumulation phenotype of *pho2-1* plants grown under sufficient P_i conditions in hydroponics. Bars show means $\pm$ SD ($n = 4$ biological replicates). **(B and C)** PAGE detection **(B)** and CE-ESI-MS analysis **(C)** of inositol (pyro)phosphates in shoots of WT (Col-0) and *pho2-1* plants. OG, orange G. Plants were cultivated in hydroponics under sufficient P_i. Data represent means $\pm$ SE ($n = 3$ biological replicates composed of two plants each). $*P < 0.05$ and $***P < 0.001$, Student's *t*-test.

ITPK1 is required for P_i-dependent synthesis of 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ *in planta*

Previously, the analysis of [^{32}P]P_i-labeled seedlings indicated that *itpk1* mutant plants display decreased levels of InsP$_6$ and InsP$_7$ and increased levels of InsP(3,4,5,6)P$_4$ and its enantiomer (Kuo et al., 2018). Analysis of [^{3}H]inositol-labeled seedlings showed that not only InsP$_7$ but also InsP$_8$ levels are decreased in the *itpk1* mutant (Laha et al., 2020). However, the function of ITPK1 in P_i-dependent synthesis of specific InsP$_7$ isomers and its link to VIH1/VIH2 remain unclear. Under our growth conditions, *itpk1* plants overaccumulated P whenever P_i was

supplied in the nutrient solution, while *vih2-4* accumulated significantly more P than WT only during short P_i resupply (Figure 4A). P_i-resupply-induced $InsP_8$ accumulation was compromised in *itpk1* and *vih2-4* single mutants, whereas $InsP_7$ levels were decreased in *itpk1* irrespective of the P_i regime (Figure 4B). Importantly, the defective synthesis of $InsP_7$ and $InsP_8$ of *itpk1* could be largely complemented by reintroducing the genomic *ITPK1* fragment into the mutant background (Supplemental Figure 3), confirming that this defect was indeed associated with the loss of *ITPK1*.

A more detailed analysis with CE-ESI-MS showed that the synthesis specifically of 5-$InsP_7$ is strongly compromised and not any more responsive to P_i in shoots of *itpk1* plants (Figure 4C). This provides the first evidence *in planta* for the ITPK1-dependent generation of 5-$InsP_7$. Loss of *ITPK1* did not significantly affect the levels of the novel 4/6-$InsP_7$ species in shoots (Figure 4C). Despite the strong reduction of 5-$InsP_7$, $InsP_8$ levels were significantly decreased in *itpk1* relative to WT plants only after P_i resupply (Figure 4C). Interestingly, 1/3-$InsP_7$ levels were compromised in *itpk1* and *vih2-4* plants resupplied with P_i. In agreement with HPLC analyses of [^{3}H]inositol-labeled seedlings (Laha et al., 2015), $InsP_8$ levels were strongly decreased in shoots of the *vih2-4* mutant (Figure 4B and 4C). When this mutant was resupplied with P_i, the lower levels of $InsP_8$ were also accompanied by a four-fold increase in 5-$InsP_7$ (Figure 4C). Accumulation of 5-$InsP_7$ increased even further when both *VIH1* and *VIH2* were knocked out (Supplemental Figure 4), providing additional evidence that 5-$InsP_7$ is the main substrate for $InsP_8$ synthesis in shoots.

Apart from an 80% decrease in 5-$InsP_7$, P_i-sufficient *itpk1* plants had also significantly decreased levels of 4/6-OH $InsP_5$ and especially of 1/3-OH $InsP_5$, but increased levels of $InsP_4$-1 and $InsP_3$-3 (Figure 4C). These results are consistent with the catalytic flexibility of inositol 1,3,4-trisphosphate 5/6-kinases (Caddick et al., 2008; Desfougeres et al., 2019; Miller et al., 2005; Whitfield et al., 2020) and provide a detailed quantitative view of the metabolic steps affected by this enzyme *in planta*. Interestingly, the synthesis of even more InsPs, including 2-OH $InsP_5$ and $InsP_6$, was dependent on ITPK1 during P_i resupply (Figure 4C). This result further indicated that a distinct set of reactions occurs in plants experiencing a sudden change in P_i availability compared with those acclimated to P_i-replete conditions. Disruption of *VIH2* resulted in little to no significant change in lower InsP forms (Figure 4C), in line with the substrate specificity of diphosphoinositol pentakisphosphate kinases (Mulugu et al., 2007; Wang et al., 2014a; An et al., 2019).

Together, our results demonstrate that the rapid synthesis of $InsP_8$ in response to P_i is largely dependent on 5-$InsP_7$ synthesized by ITPK1. However, the differences that we detected in

plants acclimated to P_i-replete conditions versus those exposed to short-term P_i resupply suggest that compensation mechanisms and metabolic rearrangements might be activated over the long run when ITPK1 or VIH2 activities are perturbed.
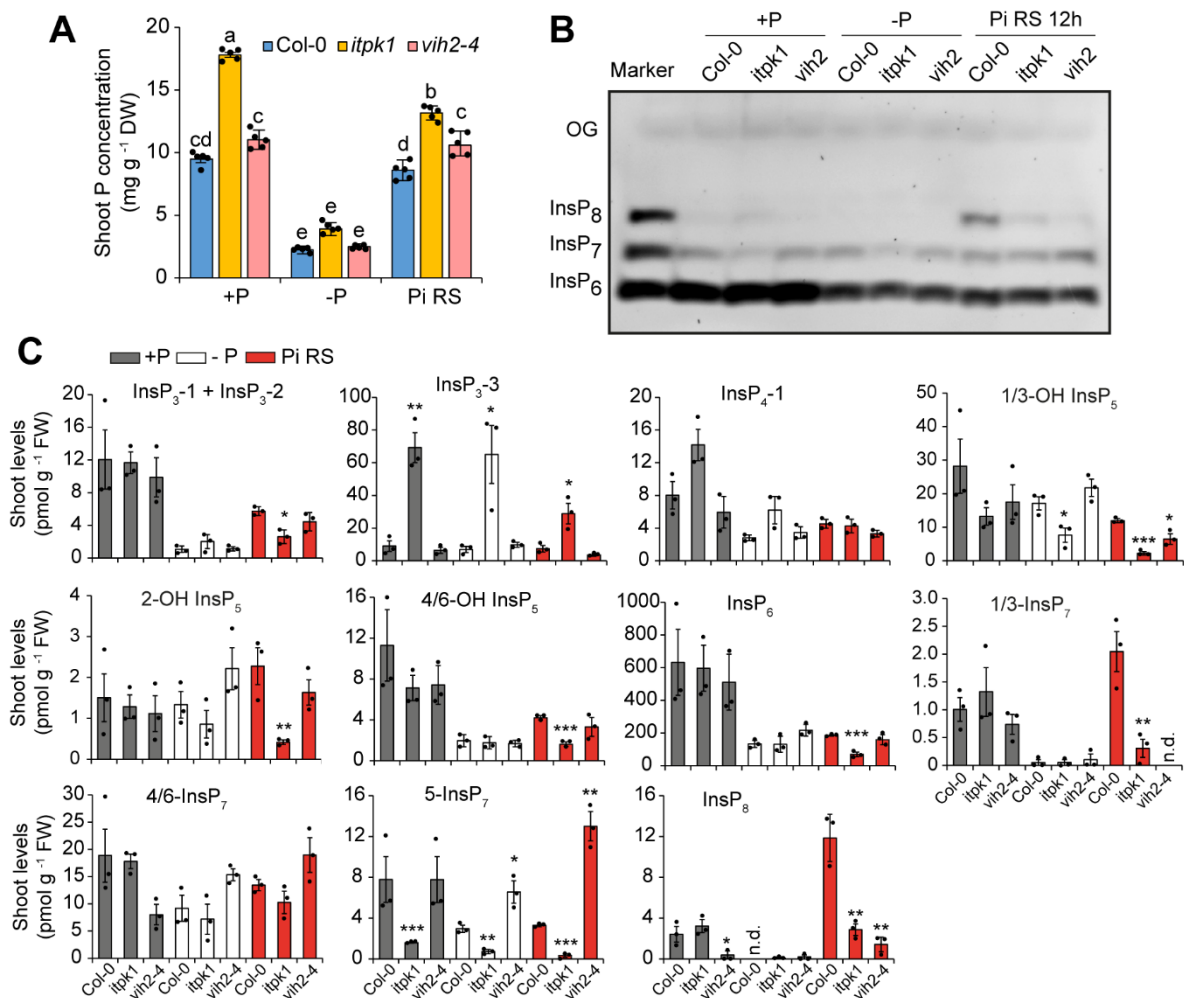
Figure 4. ITPK1 is required for 5-InsP₇ synthesis *in planta* and acts with VIH2 to generate InsP₈ in response to P_i resupply. (A) P overaccumulation of *itpk1* plants grown in hydroponics under sufficient P_i (+P), deficient P_i for 7 days (−P), or −P resupplied with P_i for 12 h (Pi RS). Bars show means ± SD (*n* = 5 biological replicates). Different letters indicate significant differences according to Tukey's test (*P* < 0.05). **(B and C)** PAGE detection **(B)** and CE-ESI-MS analysis **(C)** of inositol (pyro)phosphates in shoots of WT (Col-0), *itpk1*, and *vih2-4* plants. OG, orange. Plants were cultivated in hydroponics under sufficient P_i (+P), deficient P_i for 7 days (−P), or −P resupplied with P_i for 12 h (Pi RS). Data represent means ± SE (*n* = 3 biological replicates). ∗*P* < 0.05, ∗∗*P* < 0.01, and ∗∗∗*P* < 0.001, Student's *t*-test (mutant versus Col-0). n.d., not detected.

ITPK1 has InsP₆ kinase and ATP synthase activities

Considering that ITPK1 is required for the robust increase in InsP₈ in response to P_i resupply, we asked whether ITPK1 is also able to function as an InsP₇ kinase. However, neither 1-InsP₇ nor 5-InsP₇ appears to be a substrate for ITPK1 kinase activity *in vitro* (Supplemental

Figure 5A). Subsequently, the enzymatic properties of recombinant *Arabidopsis* ITPK1 were investigated in more detail with nuclear magnetic resonance spectroscopy (NMR). First, $InsP_6$ kinase reaction conditions were analyzed with respect to magnesium ion (Mg^{2+}) concentration and temperature dependency as well as quenching efficiency of EDTA (Supplemental Figure 5B–5D). Subsequent kinetic analysis revealed that ITPK1 exhibits a surprisingly high K_M for ATP of approximately 520 µM (Figure 5A and 5B). Unlike VIHs (Zhu et al., 2019), the kinase activity of ITPK1 was largely insensitive to P_i and not affected by the non-metabolizable P_i analog phosphite (Supplemental Figure 6). When 2-OH $InsP_5$ was presented as substrate to ITPK1, no conversion could be detected (Supplemental Figure 5E), suggesting that ITPK1 has no inositol pentakisphosphate kinase-like activity to generate $InsP_6$ from 2-OH $InsP_5$. Furthermore, in contrast to $InsP_6$ kinases of the IP6K/Kcs1 family, no activity was observed when 1-$InsP_7$ was used as a substrate (Supplemental Figure 5F and 5G), thus confirming our PAGE results from corresponding *in vitro* reactions (Supplemental Figure 5A).

The characterization of structurally and sequence-unrelated mammalian $InsP_6$ kinases of the IP6K family and of ITPK1 from potato has demonstrated that these enzymes can shift their activities from kinase to ADP phosphotransferase at low ATP-to-ADP ratios (Caddick et al., 2008; Voglmaier et al., 1996; Wundenberg et al., 2014). This prompted us to assess if *Arabidopsis* ITPK1 also possesses such activity. *In vitro* reactions with unlabeled 5-$InsP_7$ and subsequent PAGE analyses revealed that ITPK1 indeed mediates 5-$InsP_7$ dephosphorylation (Figure 5C). This activity was recently also reported by Whitfield et al. (2020) and occurred only in the presence of ADP (Supplemental Figure 7A). Interestingly, we found that the ADP phosphotransferase activity of ITPK1 was lost in stable catalytically dead ITPK1 mutants (Figure 5C), indicating that dephosphorylation is mediated by the reverse reaction of the kinase domain and not by a dedicated (albeit cryptic) phosphatase domain. Furthermore, we detected no ADP phosphotransferase activity of ITPK1 with any other $InsP_7$ isomer phosphoryl donor (Figure 5D), suggesting a high degree of substrate specificity for the dephosphorylation reaction. To determine the kinetic parameters of this reaction, we incubated ITPK1 with $^{13}C_6$-labeled 5-$InsP_7$ in the presence of ADP and detected the formation of ATP and $InsP_6$ with NMR (Supplemental Figure 7B and 7C). No ATP formation was detected when ITPK1 was incubated without 5-$InsP_7$ (Supplemental Figure 7D). Interestingly, the reverse reaction was almost two times faster than the forward, $InsP_6$ kinase, activity, whereas the K_M for ADP and ATP were relatively similar (Figure 5B, 5E, and 5F). In agreement with results obtained in agar-plate-grown seedlings (Zhu et al., 2019), 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 (Supplemental Figure 8A and 8B). Furthermore, *pho2-1* plants also had higher ATP levels and ATP/ADP ratios than WT (Supplemental Figure 8C and 8D). Thus, the P_i-dependent changes in adenylate nucleotide ratio of plants may ultimately regulate the synthesis of 5-InsP$_7$ by shifting ITPK1-mediated InsP$_6$ kinase and ADP phosphotransferase activities.
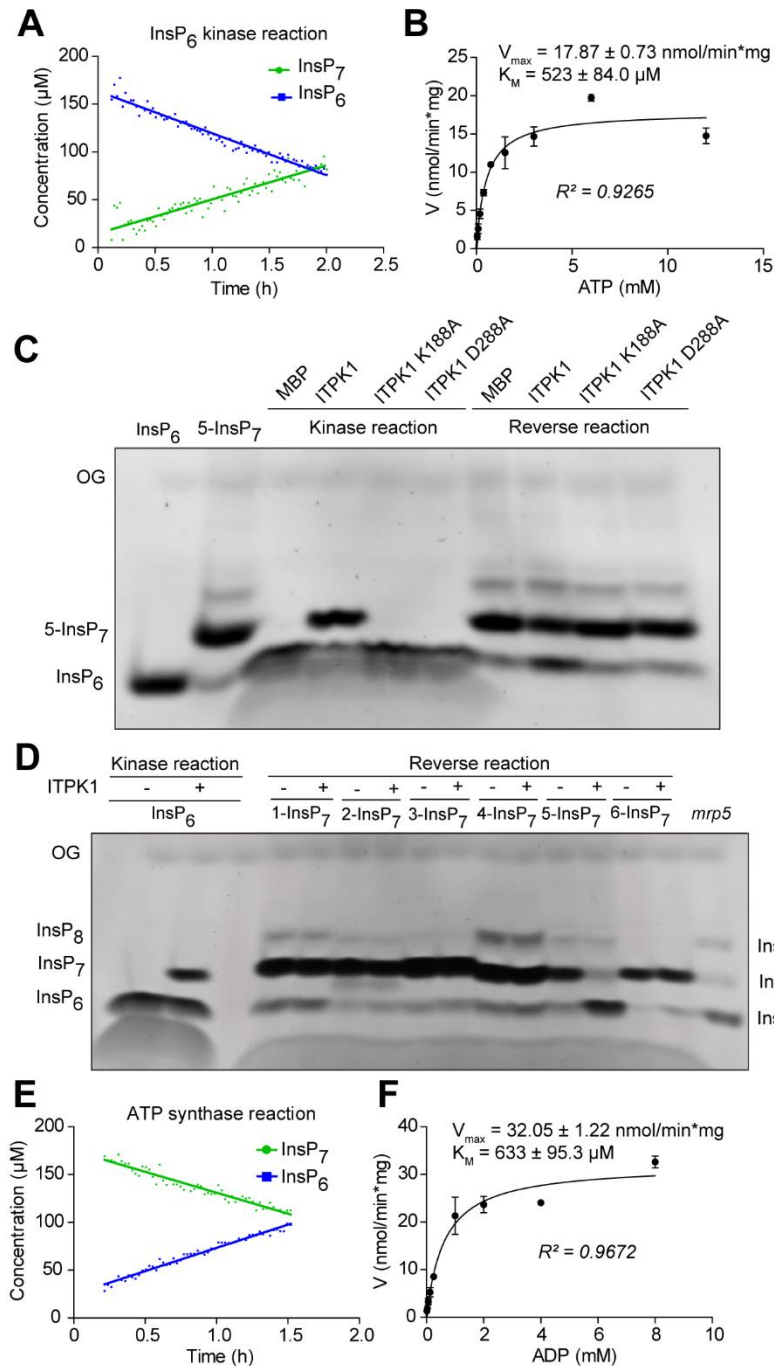
Figure 5. *In vitro* **characterization of *Arabidopsis* ITPK1 activity. (A and B)** NMR analysis of InsP$_6$ kinase activity of recombinant *Arabidopsis* ITPK1. Time-dependent conversion of InsP$_6$ to 5-InsP$_7$ **(A)** and reaction velocity determined at varying ATP concentrations **(B)**. K_M and V_{max} were obtained after fitting of the data against the Michaelis-Menten model. **(C and D)** InsP$_6$ kinase and 5-InsP$_7$ hydrolysis by recombinant *Arabidopsis* ITPK1 and designated catalytic mutants of ITPK1 **(C)** and specificity of the reverse reaction on 5-InsP$_7$ but not on other InsP$_7$ isomers **(D)**. InsPs were separated via PAGE and

visualized by toluidine blue staining. The identity of bands was determined by migration compared with InsP$_6$ and 5-InsP$_7$ standards and TiO$_2$-purified *mrp5* seed extract. InsP$_6$ kinase reaction served as positive control for the reverse reactions. Purified His$_8$-MBP tag (MBP) served as negative control for ITPK1. **(E and F)** NMR analysis of reverse reaction of recombinant *Arabidopsis* ITPK1. Accumulation of InsP$_6$ and conversion of 5-InsP$_7$**(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.

ITPK1 is genetically linked to VIH2 and acts redundantly with ITPK2 to maintain P$_i$ homeostasis in *Arabidopsis*

The P$_i$-overaccumulation phenotype of *itpk1* plants has been associated to the misregulation of PSI genes (Kuo et al., 2018; Supplemental Figure 9A). A full elemental analysis indicated that the concentrations of other nutrients were largely unaffected in shoots of *itpk1* plants (Supplemental Figure 10), demonstrating that the high P levels were not caused by a concentration effect due to the reduced shoot size. In *itpk1* plants, total P levels were also significantly increased in flowers and seeds and slightly increased in roots (Supplemental Figure 9B). A root phenotypical analysis revealed that *itpk1* plants had shorter roots than WT plants irrespective of P$_i$ supply (Supplemental Figure 9C–9E; Laha et al., 2020). This phenotype was probably not due to P$_i$ overaccumulation, as root length of *pho2-1* plants was comparable to that of WT (Supplemental Figure 9F), but likely associated with defective auxin perception (Laha et al., 2020).

To investigate the genetic link between ITPK1 and VIH2, we generated an *itpk1 vih2-4* double mutant. Compared with *itpk1* mutant plants, mutation of *VIH2* in the *itpk1* background inhibited plant growth even further and caused an approximately 27% increase specifically in shoot P levels (Figure 6A–6C). These results provide genetic evidence for the interdependence of ITPK1 and VIH2 activities to maintain undisturbed P$_i$ homeostasis in plants.

Previously, we demonstrated that ITPK2 also has InsP$_6$ kinase activity *in vitro* (Laha et al., 2019). However, at the phenotypical level, only the disruption of *ITPK1* but not of *ITPK2* results in smaller plant size and constitutive P overaccumulation (Figure 6D and 6E; Kuo et al., 2018). The levels of InsP$_8$, 5-InsP$_7$, and other (pyro)phosphates detected with CE-ESI-MS were mostly unaltered in the *itpk2-2* mutant compared with WT (Supplemental Figure 11). Despite the differential phenotypes of *itpk1* and *itpk2-2* mutants, possible functional redundancy could still explain why *itpk1* plants do not show severe growth and P-overaccumulation phenotypes like those reported for the *vih1 vih2* double mutant (Dong et al., 2019; Zhu et al., 2019). Therefore, we generated an *itpk1 itpk2-2* double mutant. When grown in P$_i$-containing substrate, *itpk1 itpk2-2* plants exhibited severe growth retardation (Figure 6D). In these plants, shoot P levels were approximately 3.5-fold and 2.1-fold higher than in WT and *itpk1* plants,

respectively (Figure 6E). These results suggested that, although ITPK2 plays a relatively minor role in P_i signaling in the presence of a functional ITPK1, it is able to partially compensate for the loss of ITPK1.
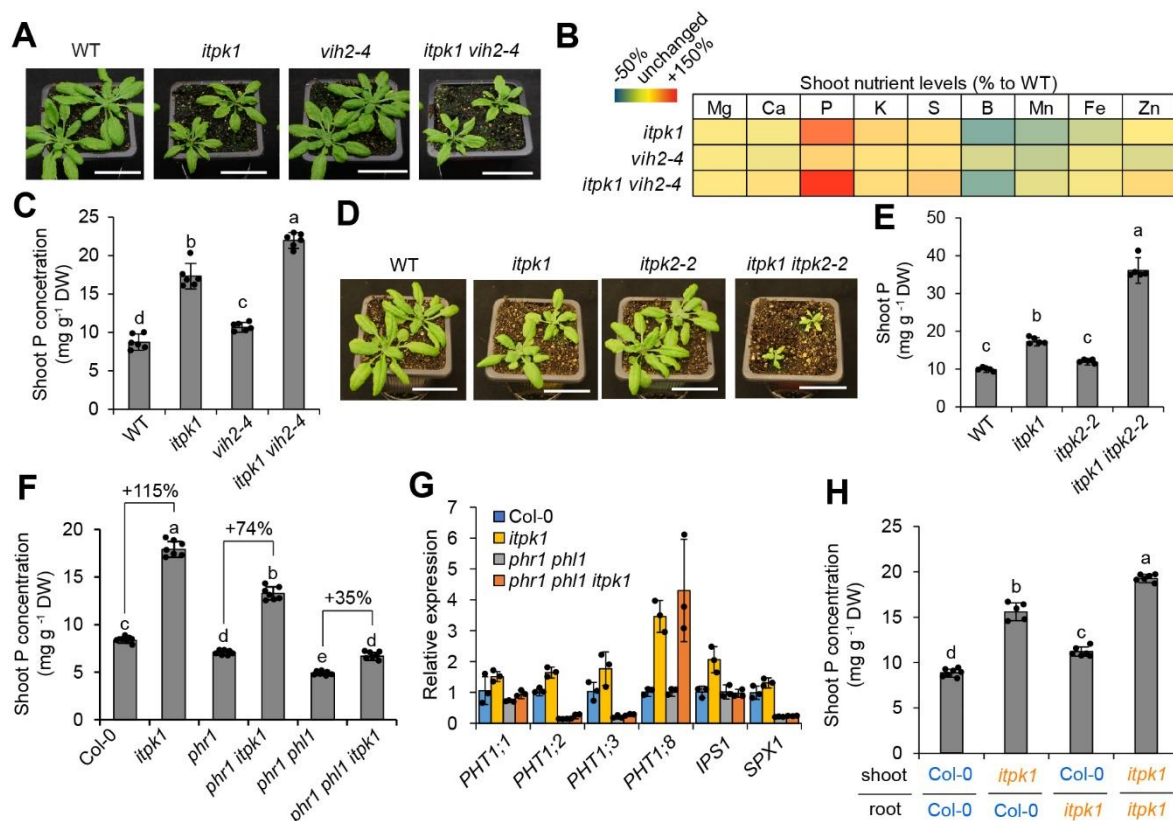
Figure 6. Genetic interaction of ITPK1 with VIH2 and ITPK2 in regulating P_i homeostasis in a PHR1- and PHL1-dependent manner. (A−C) Characterization of *itpk1 vih2-4* double mutant. Photographs of 4-week-old plants grown on peat-based substrate **(A)**, overview of relative nutrient changes in shoots **(B)**, and shoot P levels **(C)** of wild type (Col-0) and the indicated mutants. Scale bars, 3 cm. Data represent means ± SD (*n* = 6 plants). **(D and E)** Characterization of *itpk1 itpk2-2* double mutant. Photographs of 4-week-old plants grown on peat-based substrate **(D)** and shoot P levels **(E)** of wild type (Col-0) and the indicated mutants. Scale bars, 3 cm. Data represent means ± SD (*n* = 5 or 6 plants). **(F and G)** Genetic interplay between PHR1/PHL1 and ITPK1 in systemic P_i signaling. Shoot P levels **(F)** of 3-week-old wild type and indicated mutants grown on peat-based substrate. Data represent means ± SD (*n* = 6 plants). ITPK1-dependent expression of P_i deficiency-induced genes **(G)** in roots of the indicated P_i-sufficient plants. Data represent means ± SE (*n* = 3 replicates). **(H)** Total P concentration in shoots of self-grafted or reciprocally grafted wild type (Col-0) and *itpk1* grown for 2 weeks on peat-based substrate. Data represent means ± SD (*n* = 5–7 plants). In **(C)**, **(E)**, **(F)**, and **(H)**, different letters indicate significant differences according to Tukey's test (*P* < 0.05).

ITPK1 controls P_i signaling dependent of PHR1 and PHL1 but independent of PHO2

We then analyzed the genetic interaction between ITPK1 and the transcription factors PHR1 and PHL1. Although *phr1 itpk1* and *phr1 phl1 itpk1* plants still accumulated significantly more P than *phr1* and *phr1 phl1*, respectively, the relative increments were smaller than in the presence of functional PHR1 and PHL1 (Figure 6F). In contrast, the short-root phenotype caused by *ITPK1* disruption could not be restored by knocking out these transcription factors (Supplemental Figure 12A). While many PSI genes were suppressed in the triple mutant, absence of ITPK1 kept *PHT1;8* upregulated in the *phr1 phl1* background (Figure 6G), suggesting that *PHT1;8* expression was further controlled by another mechanism. To investigate whether ITPK1 is also involved in P_i starvation signaling at the level of PHO2, we then generated an *itpk1 pho2-1* double mutant. Knocking out both *ITPK1* and *PHO2* increased shoot P levels by almost two times compared with single *itpk1* and *pho2-1* mutants (Supplemental Figure 12B), hence suggesting that ITPK1 function in P_i signaling is largely independent of PHO2. Collectively, our results demonstrate that the coordination of P_i signaling by PHR1 and PHL1 is tightly linked to ITPK1-dependent PP-InsP synthesis.

We then performed grafting experiments to address whether undisturbed P_i accumulation is determined by organ-specific ITPK1 activity. As expected, shoot P overaccumulation was detected when roots and shoots of *itpk1* plants were self-grafted (Figure 6H). However, shoot P was largely reverted 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. These results suggest that ITPK1 activity in shoots is more determinant for the regulation of shoot P_i accumulation.

Root-specific synthesis of a presumptive novel PP-InsP relies on ITPK1 activity in roots

The apparent dominant ITPK1 role in shoots is puzzling, as *ITPK1* is expressed in various plant tissues, including roots (Kuo et al., 2018). CE-ESI-MS analysis of roots revealed that *itpk1* plants exhibited significantly decreased levels of 1/3-InsP$_7$, 5-InsP$_7$, and InsP$_8$ whenever P_i was available (Supplemental Figure 13). Furthermore, most InsPs that were affected in shoots by *ITPK1* disruption were also affected in roots. Interestingly, PP-InsP levels in roots were lower than those detected in shoots, and the increased accumulation of InsP$_8$ after P_i resupply was less pronounced (compare Figure 4C and Supplemental Figure 13). We therefore compared the levels of PP-InsPs and InsPs in roots and shoots of WT plants and detected clear, organ-specific differences (Figure 7A and 7B). InsP$_8$ and the detected InsP$_7$ isomers were much more abundant

in shoots than in roots (Figure 7A). For instance, $InsP_8$ levels in shoots were approximately 2-, 2.4-, and 21-fold higher than those detected in roots of P_i-sufficient, P_i-starved, and P_i-resupplied plants, respectively. Interestingly, the shoot/root ratio was reversed for most InsPs (Figure 7B). While $InsP_6$ levels were comparable in roots and shoots whenever P_i was available, most other InsPs were quantitatively less abundant in shoots than in roots. However, shoot-to-root partitioning of $InsP_4$-1, 2-OH $InsP_5$, and $InsP_6$ was increased under P_i starvation, as their synthesis was inhibited more strongly in roots than in shoots. Thus, these results demonstrate strong, organ-specific differences in InsP and PP-InsP metabolism, with higher levels of PP-InsPs produced in shoots and lower InsPs in roots.

Notably, PAGE analyses of root samples revealed a P_i-deficiency-induced accumulation of a band with an electrophoretic mobility between those of $InsP_6$ and $InsP_7$, which was absent in shoots (Figure 7C; Supplemental Figure 14A). With CE-ESI-MS we identified the presence of an isomer that, to our knowledge, has not previously been reported in plants. This isomer displayed a mobility slightly increased compared with $InsP_6$, thus likely representing a PP-$InsP_4$ isomer (Supplemental Figure 14B). We were not yet able to determine the isomeric nature of this presumptive PP-$InsP_4$ isomer, but observed that it did not co-migrate with synthetic 5PP-Ins(1,3,4,6)P_4. We also detected a band with a similar mobility in roots of rice plants (Supplemental Figure 14B). CE-ESI-MS analyses of individual and mixed samples revealed that the presumptive PP-$InsP_4$ isomer appears to be indistinguishable between *Arabidopsis* and rice roots, but clearly differed in mobility from the 5PP-Ins(1,3,4,6)P_4 standard (Supplemental Figure 14B), suggesting that its root-specific synthesis is conserved in flowering plants. Interestingly, in *Arabidopsis* roots, the levels of this PP-$InsP_4$ isomer were detected only in P_i-starved roots (Figure 7D). In roots of *itpk1* plants, a band representing the presumptive PP-$InsP_4$ was not visible, and the isomer was either not detected or present at lower levels than in WT or *itpk2-2* according to CE-ESI-MS (Figure 7C and 7D), suggesting that ITPK1 is involved in its synthesis. These results together indicate that ITPK1 is active in roots, where it is also required for the root-specific synthesis of a presumptive novel PP-$InsP_4$ isomer.
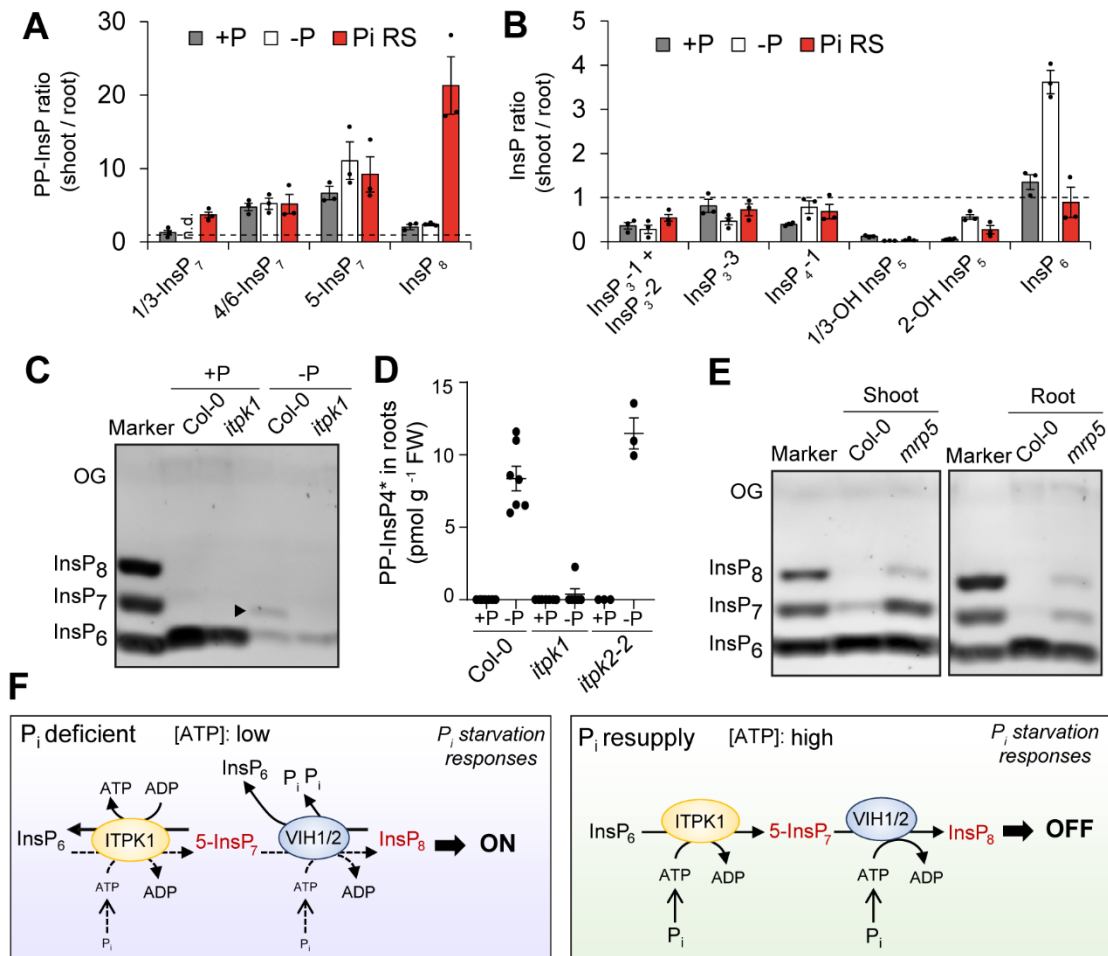
Figure 7. Amount of InsP$_7$ and InsP$_8$ synthesized in plant tissues relies on MRP5-dependent InsP$_6$ compartmentalization and ITPK1 activity. (A and B) Relative levels of PP-InsPs **(A)** and InsPs **(B)** detected by CE-ESI-MS in shoots and roots of WT (Col-0) plants exposed to variable P$_i$ supplies. Plants were cultivated in hydroponics under sufficient P$_i$ (+P), deficient P$_i$ for 7 days (−P), or −P resupplied with P$_i$ for 12 h (Pi RS). Data are means of shoot-to-root ratios ± SE (n = 3 biological replicates composed of two plants each). n.d., not detected. Dashed lines indicate a ratio of 1. **(C and D)** PAGE detection **(C)** and CE-ESI-MS quantification **(D)** of a presumptive novel PP-InsP$_4$ isomer in roots. This isomer was detected in roots of WT (Col-0) or *itpk2-2* plants but was absent or present at low levels in roots of the *itpk1* mutant. Plants were grown in hydroponics in P$_i$-sufficient solution (+P) or exposed for 7 days to P$_i$ starvation (−P). In **(D)**, data represent means ± SE (n = 6 or 7 biological replicates). Data points set to 0 indicate independent biological replicates in which the isomer was not detected. **(E)** PAGE detection of InsPs and PP-InsPs in shoots and roots of WT (Col-0) and *mrp5* mutant plants cultivated in hydroponics under sufficient P$_i$ (+P). Data represent means ± SE (n = 3 biological replicates). OG, orange G. **(F)** A proposed model for ITPK1-dependent generation and removal of 5-InsP$_7$ 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 5-InsP$_7$ to ADP, thereby generating ATP and decreasing 5-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 switch on P$_i$ starvation responses. When cells regain sufficient P$_i$, which increases ATP levels, ITPK1-mediated InsP$_6$ kinase activity is stimulated and the reverse reaction toward 5-InsP$_7$ is inhibited. 5-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. Our results also demonstrate that the amount of PP-InsPs produced in different plant tissues is further controlled by InsP$_6$ compartmentalization by MRP5, and that ITPK2 is able to partially complement ITPK1 function in P$_i$ signaling.

Subcellular InsP$_6$ compartmentalization determines tissue levels of InsP$_7$ and InsP$_8$

Since roots and shoots had comparable InsP$_6$ levels as long as P$_i$ was available to the plants (Figure 7B), we next addressed whether subcellular compartmentalization of InsP$_6$ could determine the amount of InsP$_7$ and InsP$_8$ that can be synthesized in each plant organ. To this end, we assessed these PP-InsPs in shoots and roots of *mrp5*, a mutant defective in vacuolar loading of InsP$_6$ (Nagy et al., 2009). Compared with WT, *mrp5* plants had elevated InsP$_7$ and InsP$_8$ signals in shoots and roots (Figure 7E). We then quantified these changes in shoots with CE-ESI-MS and found that InsP$_8$ especially was still responsive to P$_i$ in *mrp5* plants (Supplemental Figure 15A). Consequently, P accumulation was not significantly altered (Supplemental Figure 15B), suggesting that P$_i$ starvation responses were not misregulated in *mrp5* mutant plants. Taken together, these results indicate that the amount of PP-InsPs produced in different plant tissues is dependent on MRP5-mediated InsP$_6$ compartmentalization, while the composition may be further determined by organ-specific ITPK1 activities.

Discussion

ITPK1 reversible reactions are important for the formation and degradation of PP-InsPs in response to P_i

Regulation of cellular P_i homeostasis is critical for all living organisms. Therefore, it is not surprising that intricate P_i sensing and signaling mechanisms have evolved to dynamically adjust P_i uptake according to external and internal P_i levels. In plants, recent studies have raised compelling evidence that PP-InsPs act as signaling molecules that regulate P_i homeostasis by binding to SPX proteins (Azevedo and Saiardi, 2017; Dong et al., 2019; Ried et al., 2021; Wild et al., 2016; Zhu et al., 2019). However, it has remained challenging to establish which PP-InsP species are regulated by P_i and to link defects in P_i signaling to altered accumulation of specific InsPs in metabolic mutants. With the help of CE-ESI-MS, we show here that $1/3$-$InsP_7$, 5-$InsP_7$, and $InsP_8$ levels change dramatically not only when plants are exposed to P_i-limited conditions, but especially when P_i-starved plants are resupplied with P_i (Figures 1A–1G and 2). $InsP_8$, which co-migrated with a $[^{13}C_6]1,5$-$InsP_8$ standard (Figure 2C), responded more sensitively to P_i than any other PP-InsP or lower InsP assessed in this study, with concentrations increasing from approximately 0.32% of $InsP_6$ in shoots of P_i-starved plants to approximately 10% of $InsP_6$ 48 h after P_i resupply (Figure 2D). Due to the severe P_i signaling defects of *vih1 vih2* double mutants and the fact that $1,5$-$InsP_8$ can restore SPX1–PHR1 interaction *in vivo* more efficiently than 5-$InsP_7$ (Dong et al., 2019; Zhu et al., 2019; Ried et al., 2021), $InsP_8$ has been suggested as the preferred ligand for SPX proteins. Notably, in contrast to $InsP_8$ accumulation, the expression of *SPX1* and *SPX3* is strongly induced by P_i starvation (Duan et al., 2008). These seemingly opposing responses suggest that when P_i-deficient plants regain access to P_i, the increased accumulation of $InsP_8$ shortly overlaps with the high abundance of its receptors, allowing the quick formation of large amounts of repressive SPX–PHR complexes. As soon as 6 h after P_i resupply, when $InsP_8$ levels were elevated substantially (Figure 2D), the expression of PSI genes in roots was already strongly repressed compared with P_i-starved plants (Supplemental Table 1). Such a mechanism might thereby help plants to efficiently modulate P_i uptake according to the severity of P_i deficiency, thus preventing toxicity after P_i-starved plants regain access to P_i.

Since $InsP_8$ is generated in plants by phosphorylation of $InsP_7$ via VIH1 and VIH2 (Dong et al., 2019; Laha et al., 2015; Zhu et al., 2019), P_i-dependent $InsP_8$ synthesis relies on the availability of the substrate, $InsP_7$. Previous analyses with PAGE or $[^{32}P]P_i$ labeling indicated that $InsP_7$ levels were relatively unchanged or mildly increased in the shoots of P_i-starved plants (Dong et al., 2019; Kuo et al., 2018). In our study we observed, under slightly different conditions, a

mild global reduction in $InsP_7$ levels in response to P_i starvation and a quick recovery when P_i was resupplied to plants (Figures 1, 2, 3, and 4). However, this picture became much clearer when employing CE-ESI-MS, which enabled us to distinguish several different $InsP_7$ species. We found that, similar to $InsP_8$, both $5\text{-}InsP_7$ and $1/3\text{-}InsP_7$ were strongly reduced under P_i starvation and recovered quickly after P_i resupply (Figure 2D). Recovery of $1/3\text{-}InsP_7$ was dependent on functional ITPK1 and VIH2. Previous NMR assays showed that the recombinant kinase domain of *Arabidopsis* VIH2 catalyzes the synthesis of $InsP_8$ from $5\text{-}InsP_7$ and of $1\text{-}InsP_7$ from $InsP_6$ (Zhu et al., 2019). Thus, the lack of $1/3\text{-}InsP_7$ in P_i-resupplied *vih2-4* plants provides first support *in planta* for the $InsP_6$ kinase activity of VIH2. Nonetheless, the concomitant increase in $5\text{-}InsP_7$ in shoots of *vih2-4* plants (Figure 4C) indicated that $5\text{-}InsP_7$ is the main VIH2 substrate responsible for the robust $InsP_8$ synthesis induced by P_i resupply in WT plants. In line with the *in vitro* activity of ITPK1 (Adepoju et al., 2019; Laha et al., 2019), shoot $5\text{-}InsP_7$ levels were strongly decreased in *itpk1* mutant plants irrespective of P_i availability (Figure 4C). Since we detected only $InsP_6$ kinase and no $1\text{-}InsP_7$ or $5\text{-}InsP_7$ kinase activity with purified recombinant *Arabidopsis* ITPK1 (Supplemental Figure 5A), the decrease in $InsP_8$ in P_i-resupplied *itpk1* plants likely results from diminished $5\text{-}InsP_7$ and thus reduced availability of this $InsP_7$ isomer for the VIH1- or VIH2-catalyzed phosphorylation at the C1 phosphate. Notably, disruption of *ITPK1* or *VIH2* resulted in distinct changes in a number of inositol (pyro)phosphates in plants acclimated to sufficient P_i compared with plants exposed to short-term P_i resupply (Figure 4C), which could point to time-dependent activation of metabolic readjustments and compensatory mechanisms. Indeed, the phenotypical analysis of an *itpk1 itpk2-2* double mutant provided evidence that ITPK2 is able to partially complement the function of an absent ITPK1 (Figure 6D and 6E). However, future research will have to assess PP-InsPs at higher tissue resolution and in different cellular compartments to determine if the $InsP_8$ detected in shoots of *itpk1* plants acclimated to P_i-replete conditions is produced at the sites relevant for P_i signaling.

The dynamic changes in $1/3\text{-}InsP_7$, $5\text{-}InsP_7$, and $InsP_8$ levels according to the plant's P_i status indicate that PP-InsP synthesis and degradation must be tightly controlled. P_i-dependent accumulation of $InsP_8$ has been proposed to rely on the bifunctional activity of VIH1 and VIH2 (Dong et al., 2019; Zhu et al., 2019), whose kinase and phosphatase activities can be shifted according to cellular ATP and P_i levels (Zhu et al., 2019). However, unlike VIHs, ITPK1 harbors only the atypical "ATP-grasp fold" and no phosphatase domain. Nonetheless, we demonstrate that *Arabidopsis* ITPK1 can shift its activity and become an ADP phosphotransferase that dephosphorylates $5\text{-}InsP_7$ but no other $InsP_7$ isomer in the presence of

ADP (Figure 5C–5F and Supplemental Figure 7). Considering that 5-InsP$_7$ represents only one of at least three different InsP$_7$ isomers detected in plants, this high specificity suggests that the reverse reaction is most likely used to specifically switch off 5-InsP$_7$ signaling (and in consequence InsP$_8$ signaling) and probably makes no major contribution to global ATP synthesis under P$_i$-deficient conditions. Thus, ITPK1 can mediate reversible InsP$_6$ kinase and 5-InsP$_7$ dephosphorylation, which is reminiscent of the Ins(1,3,4,5,6)P$_5$/ADP phosphotransferase activities recorded previously for recombinant ITPK1 from potato (Caddick et al., 2008). Our kinetic analyses with NMR also demonstrate that *Arabidopsis* ITPK1 has comparable K$_M$ values for ATP and ADP (Figure 5). Thus, P$_i$-dependent (and perhaps tissue-dependent) changes in ATP levels and ATP/ADP ratios will determine whether ITPK1 phosphorylates InsP$_6$ or dephosphorylates 5-InsP$_7$ to produce or remove PP-InsPs required for undisturbed P$_i$ signaling (Figure 7F). The ADP phosphotransferase activity of ITPK1 could bypass the requirement for dedicated PP-InsP hydrolases, which are likely to slow down quick dynamic changes in InsP$_7$ and InsP$_8$ to induce, e.g., jasmonate-related responses during wound response or insect attack (Laha et al., 2015, 2016), or when P$_i$ becomes suddenly available (Figures 1B–1D and 2D). During the completion of the present study, Whitfield and colleagues (Whitfield et al., 2020) reported that, in addition to 5-InsP$_7$, *Arabidopsis* ITPK1 can also dephosphorylate Ins(1,3,4,5,6)P$_5$ at high ADP/ATP ratios. Thus, the catalytic flexibility of ITPK1 and its ability to mediate adenylate charge-dependent forward and reverse reactions at different steps along the metabolic pathway make ITPK1 a central component that transduces cellular P$_i$ status into specific inositol (pyro)phosphate changes.

ITPK1 activity in shoots is critical for undisturbed P$_i$ signaling

With genetic crossings and grafting, we demonstrated that the uncontrolled P$_i$ accumulation and misregulated expression of PSI genes in the *itpk1* mutant was strongly attenuated in the absence of PHR1 and PHL1 and was more significantly affected by missing ITPK1 activity in shoots (Figure 6F–6H). The latter result is somewhat surprising, since *ITPK1*, *VIH1*, and *VIH2* are expressed in shoots and roots (Kuo et al., 2014, 2018; Laha et al., 2015; Zhu et al., 2019) and ITPK1 activity also affects PP-InsP accumulation in roots (Supplemental Figure 13). One possibility is that the disturbed PHR-dependent P$_i$ signaling in *itpk1* shoots further amplifies PHR-dependent P$_i$ signaling defects in roots. Furthermore, in line with earlier indications from [^{32}P]P$_i$ labeling (Kuo et al., 2018), we found that the concentration of all PP-InsPs—except for a presumptive novel PP-InsP$_4$ isomer—was higher in shoots than in roots (Figure 7A–7D and Supplemental Figure 14A). Future studies are required to investigate the relevance of these differences for whole-plant P$_i$ homeostasis. Interestingly, our results indicated that subcellular

compartmentalization of $InsP_6$ seems determinant for the overall level of $InsP_6$-dependent PP-InsPs that can be produced in different plant tissues (Figure 7E and Supplemental Figure 15A), while the composition is likely defined by the predominant catalytic activity executed by different enzymes according to substrate availability and the energetic state of each tissue.

Identification of novel PP-InsPs in plants

One surprising finding from our CE-ESI-MS analysis was the identification of a previously unreported $4/6$-$InsP_7$ isomer, which, together with 5-$InsP_7$, appears to be the most abundant $InsP_7$ isomer in plants (Figure 2D and Supplemental Figure 2). The $4/6$-$InsP_7$ isomer is not misregulated in the *pho2* mutant and does not show the strong overshoot reaction observed for $1/3$-$InsP_7$ and $InsP_8$ after P_i resupply and is hence likely not involved in P_i signaling (Figures 2D and 3C). Neither 4-$InsP_7$ nor 6-$InsP_7$ has been described, to our knowledge, in other organisms, with the exception of the social amoeba *Dictyostelium discoideum*, in which 6-$InsP_7$ represents the most abundant $InsP_7$ isomer (Laussmann et al., 1997). The amounts of $InsP_7$ and $InsP_8$ are very high in *Dictyostelium*, reaching concentrations of several hundred millimolar (Wilson et al., 2015). Their synthesis is required for chemotactic responses and depends on an $InsP_6$ kinase related to mammalian IP6Ks (Luo et al., 2003). We therefore hypothesize that $4/6$-$InsP_7$ synthesis in plants and *Dictyostelium* might have evolved differently, and additional experiments will be required to determine the exact isomeric nature of this species.

We also identified a root-specific, ITPK1-dependent PP-$InsP_4$ isomer that is regulated by P_i availability but is distinct from the known $5PP$-$Ins(1,3,4,6)P_4$ isomer (Figure 7C and 7D; Supplemental Figure 14) that appears to accumulate in the yeast *ipk1* mutant (Draskovic et al., 2008). Interestingly, a recent study showed that recombinant ITPK1 is able to phosphorylate $Ins(1,2,3,4,5)P_5$, but none of the other simple $InsP_5$ isomers (Whitfield et al., 2020), possibly explaining the synthesis of this unknown PP-$InsP_4$ in roots. Future work is necessary to reveal the structure of this isomer and whether it potentially also binds to SPX domains, and to assess if the strict organ-specific and P_i-dependent accumulation of this presumptive PP-$InsP_4$ isomer is involved in P_i signaling.

Methods

Plant materials and growth conditions

Seeds of *A. thaliana* T-DNA insertion lines *itpk1* (SAIL_65_D03), *itpk2-2* (SAIL_1182_E03), *vih2-4* (GK-080A07), *mrp5* (GK-068B10), *pho2-1* (ethyl methanesulfonate mutant described in Delhaize and Randall, 1995), and *phr1* (SALK_067629) were obtained from The European *Arabidopsis* Stock Centre (http://arabidopsis.info/). The *phr1 phl1* double mutant and the *phr1 phl1 vih1 vih2* quadruple mutant used in this study were described previously (Kuo et al., 2014; Zhu et al., 2019). 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. The double mutants *itpk1 itpk2-2*, *itpk1 pho2-1*, and *itpk1 vih2-4* were generated by crossing the respective single homozygous mutants. F2 and F3 plants were genotyped by PCR using the primers indicated in Supplemental Table 2 to identify homozygous lines. The homozygous *pho2-1* allele was confirmed by sequencing. Transgenic lines expressing the genomic *ITPK1* fragment in the *itpk1* background were generated as described in Laha et al. (2020).

To investigate P_i-dependent regulation of inositol (pyro)phosphate metabolism with PAGE and CE-ESI-MS, *Arabidopsis* and rice plants were grown in hydroponics as described in detail in the supplemental methods. *P. patens* was grown on Knop medium (Reski and Abel, 1985) solidified with 0.8% agar (A7921, Sigma). Light was provided by fluorescent lamps (60 μmol m^{-2} 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 medium containing 1.8 mM KH_2PO_4 (+P) or 1.8 mM KCl (−P) for 30 days. At the end of P_i starvation period, some of the plants were resupplied with 1.8 mM KH_2PO_4 and harvested after 24 h or 96 h.

Phenotypic characterization of *Arabidopsis* WT and mutants 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 HCT116 cells

Mammalian cells were cultivated as described (Wilson et al., 2015). Briefly, HCT116 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 0.45% glucose in a humidified atmosphere with 5% CO_2. P_i starvation was induced with DMEM without

sodium phosphate supplemented with 10% dialyzed fetal bovine serum. Cells were washed twice in the phosphate-free medium before incubation with DMEM medium with or without phosphate. Analysis of InsPs from HCT116 cell lines was performed as previously described (Wilson et al., 2015).

Grafting experiment

Collar-free grafting was performed exactly as described in Rus et al. (2006). Successfully grafted seedlings were transplanted directly to peat-based soil and whole shoots harvested for elemental analysis 2 weeks later.

RNA isolation and quantitative real-time PCR

Root and shoot tissues were collected by excision and immediately frozen in liquid N_2. Total RNA was extracted with the RNeasy Plant Mini Kit (Macherey-Nagel, Germany). Quantitative reverse transcriptase PCR was conducted with the CFX384TM real-time system (Bio-Rad, Germany) and Go Taq qPCR Master Mix SybrGreen I (Promega) using the primers listed in Supplemental Table 2. *UBQ2* was used as a reference gene to normalize relative expression levels of all tested genes. Relative expression was calculated according to Pfaffl (2001).

Elemental analysis

Whole shoots were dried at 65°C and digested in concentrated HNO_3 in polytetrafluoroethylene tubes under a pressurized system (UltraCLAVE IV, MLS). Elemental analysis of plant samples from hydroponics or pot experiments was performed by inductively coupled plasma optical emission spectrometry (iCAP 700, Thermo Fisher Scientific), whereas *P. patens* samples were analyzed by sector field high-resolution inductively coupled plasma–MS (ELEMENT 2, Thermo Fisher Scientific). Element standards were prepared from certified reference materials from CPI International.

Titanium dioxide bead extraction and PAGE

All steps until dilution were performed at 4°C. TiO_2 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-N_2-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 and then centrifuged for 10 min at 20 000 *g* at 4°C using a refrigerated benchtop 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 TiO_2 beads and rotated at 4°C for 30–60 min. Afterward, the

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 for 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% PAGE and visualized by toluidine blue staining, followed by 4′,6-diamidino-2-phenylindole staining based on previously established protocols (Losito et al., 2009; Wilson et al., 2015; Wilson and Saiardi, 2018). Signal intensities of PAGE were quantified with ImageJ.

CE-ESI-MS/MS

CE-ESI-MS/MS was performed on an Agilent 7100 CE system directly interfaced with a triple-quadrupole tandem MS Agilent 6495c system, equipped with an Agilent Jet Stream ESI source. CE-MS coupling was carried out using a sheath liquid coaxial interface, with an Agilent 1200 isocratic LC pump constantly delivering the sheath liquid (via a splitter set with a ratio of 1:100). Agilent MassHunter Workstation (version 10.1) was employed to control the entire system, data acquisition, and analysis. All experiments were performed with a bare fused silica capillary with a length of 100 cm and 50 µm internal diameter. Forty millimolar ammonium acetate titrated by ammonia solution to pH 9.0 was used as background electrolyte. Before the first use, the capillary was conditioned by rinsing with 1 M sodium hydroxide (10 min), water (10 min), and background electrolyte (20 min). A constant CE current of 27 µA was established by applying +30 kV over the capillary. Five microliters of InsP extracts from TiO_2 purification were mixed with 5 µl isotopic standards mixture (Puschmann et al., 2019; 4 µM [$^{13}C_6$]1,5-InsP$_8$, 4 µM [$^{13}C_6$]5-InsP$_7$, 4 µM [$^{13}C_6$]1-InsP$_7$, 40 µM [$^{13}C_6$]InsP$_6$, and 8 µM [$^{13}C_6$]2-OH InsP$_5$). Samples were injected by applying 100 mbar pressure for 10 s (20 nl). In some cases, 35 mM ammonium acetate titrated by ammonia solution to pH 9.7 was used for a second measurement for the quantitation of 1/3-InsP$_7$ and 2-OH InsP$_5$. The sheath liquid was composed of a water–isopropanol (1:1) mixture, which was introduced at a flow rate of 10 µl/min. The MS source parameters setting with respect to the sensitivity and stability were as follows: nebulizer pressure was 8 psi, gas temperature was 150°C, and with a flow of 11 l/min, sheath gas temperature was 175°C, and with a flow at 8 l/min, the capillary voltage was −2000 V with nozzle voltage 2000 V. Negative high-pressure RF and low-pressure RF (ion funnel parameters) were 70 V and 40 V, respectively. Mass spectrometer parameters for MRM transitions are shown below, and were identified by MassHunter Optimizer software. Mass spectrometer

parameters for the analysis of InsPs are shown in Supplemental Table 3. $InsP_8$, 5-$InsP_7$, 1/3-$InsP_7$, $InsP_6$, and 2-OH $InsP_5$ were assigned by MS/MS transitions and identical migration time compared with their heavy isotopic reference. 4/6-$InsP_7$, 4/6-OH $InsP_5$, and 1/3-OH $InsP_5$ were identified by MS/MS transition and same migration time compared with relative standards. Three $InsP_3$ and two $InsP_4$ isomers were assigned by MS/MS transitions and based on a comparison with results of [³H]inositol HPLC labeling experiments. Quantification of $InsP_8$, 5-$InsP_7$, 1/3-$InsP_7$, $InsP_6$, and 2-OH $InsP_5$ was performed with known amounts of corresponding heavy isotopic references spiked into the samples. Due to the close migration, $[^{13}C_6]$5-$InsP_7$ was employed as internal standard for 4/6-$InsP_7$, $[^{13}C_6]$2-OH $InsP_5$ was taken as internal standard for 4/6-OH $InsP_5$, 1/3-OH $InsP_5$, $InsP_4$s and $InsP_3$s.

ITPK1 *in vitro* kinase and ATP synthase assay

Recombinant *A. thaliana* ITPK1 was purified based on the previously established protocol (Schaaf et al., 2006). The $InsP_6$ kinase assay was performed by incubating 10.17 µM enzyme in a reaction mixture containing 5 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 1 mM DTT, 5 mM phosphocreatine, 0.33 units creatine kinase, 12.5 mM ATP, and 1 mM $InsP_6$ (Sichem) at 25°C for 6 h. The ability of the enzyme to dephosphorylate 5-$InsP_7$ was assayed in a reaction mixture containing 3 µg enzyme, 2.5 mM $MgCl_2$, 50 mM NaCl, 20 mM HEPES (pH 6.8), 1 mM DTT, 1 mg/ml BSA, 8 mM ADP, and 1 mM 5-$InsP_7$ at 25°C for 6 h. Reactions were separated by 33% PAGE and visualized by toluidine blue staining.

NMR-based enzyme assays

Full-length recombinant *A. thaliana* ITPK1 in H_2O was used in all assays. ITPK1 (0.2–0.8 µM) was incubated in reaction buffer containing 20 mM HEPES (pH 7.0, measured in D_2O), 50 mM NaCl, 1 mM DTT, 5 mM creatine phosphate, 1 U/ml creatine kinase, 2.5 mM $MgCl_2$ (if not indicated otherwise), and 175 µM $[^{13}C_6]InsP_5$ [2-OH], $[^{13}C_6]InsP_6$, $[^{13}C_6]$5-$InsP_7$, or $[^{13}C_6]$1-$InsP_7$ in D_2O. If not indicated otherwise, the reaction buffer also included 2.5 mM ATP or 2.5 mM ADP.

For single timepoint analysis of enzyme activity, 2.25–0.375 ng (0.2–0.3 µM) ITPK1 was used. Reactions (150 µl) were incubated at 25°C (except when 37°C is specified) and quenched with 400 µl of 20 mM EDTA (pH 6.0 measured in D_2O), and then 11 µl of 5 M NaCl was added for analysis. For real-time monitoring of enzyme activity, 36 ng (0.8 µM) ITPK1 was used. Reactions (600 µl) were incubated at 25°C in an NMR instrument and measured consecutively with 85 sec spectra. Samples were measured as previously described (Harmel et al., 2019) 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 pl 6. Temperature was calibrated using d_4-methanol and the formula of Findeisen et al. (2007).

InsPs extraction from seedlings and HPLC analyses

Seedlings were grown vertically on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 7 g/l Phytagel (P8169, Sigma) (pH 5.7) for 12 days (8 h light at 22°C, 16 h darkness at 20°C). Ten to 20 seedlings were transferred to 3 ml half-strength Murashige and Skoog liquid medium 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 [^{3}H]*myo*-inositol (30–80 Ci mmol^{-1} and 1 mCi ml^{-1}; American Radiolabeled Chemicals) and further cultivated for 5 days. For P_i resupply, 620 µM KH_2PO_4 was added to the medium and the plants were grown for another 6 h before harvest. Afterward, seedlings were washed two times with ultrapure water and frozen in liquid N_2 and the InsPs were extracted as described previously (Azevedo and Saiardi, 2006) and resolved exactly as described in Gaugler et al. (2020).

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

Adepoju, O., Williams, S. P., Craige, B., Cridland, C. A., Sharpe, A. K., Brown, A. M., Land, E., Perera, I. Y., Mena, D., Sobrado, P., Gillaspy, G. E. (2019). Inositol Trisphosphate Kinase and Diphosphoinositol Pentakisphosphate Kinase Enzymes Constitute the Inositol Pyrophosphate Synthesis Pathway in Plants. bioRxiv:724914.

An, Y., Jessen, H. J., Wang, H., Shears, S. B., Kireev, D. (2019). Dynamics of Substrate Processing by PPIP5K2, a Versatile Catalytic Machine. Structure 27:1022-1028 e1022.
Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., and Chiou, T.J. (2006). pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a MicroRNA399 target gene. Plant Physiology 141:1000-1011.

Aung, K., Lin, S. I., Wu, C. C., Huang, Y. T., Su, C. L., Chiou, T. J. (2006). pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol. 141(3):1000-11.

Azevedo, C., Saiardi, A. (2006). Extraction and analysis of soluble inositol polyphosphates from yeast. Nat Protoc 1:2416-2422.

Azevedo, C., Saiardi, A. (2017). Eukaryotic Phosphate Homeostasis: The Inositol Pyrophosphate Perspective. Trends Biochem Sci 42:219-231.

Bari, R., Pant, B. D., Stitt, M., Scheible, W. R. (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiology 141:988-999.

Bustos, R., Castrillo, G., Linhares, F., Puga, M. I., Rubio, V., Perez-Perez, J., Solano, R., Leyva, A., Paz-Ares, J. (2010). A Central Regulatory System Largely Controls Transcriptional Activation and Repression Responses to Phosphate Starvation in Arabidopsis. Plos Genet 6:-.

Caddick, S. E. K., Harrison, C. J., Stavridou, I., Mitchell, J. L., Hemmings, A. M., Brearley, C.A. (2008). A Solanum tuberosum inositol phosphate kinase (StITPK1) displaying inositol phosphate-inositol phosphate and inositol phosphate-ADP phosphotransferase activities. Febs Lett 582:1731-1737.

Capolicchio, S., Thakor, D. T., Linden, A., Jessen, H. J. (2013). Synthesis of unsymmetric diphospho-inositol polyphosphates. Angew Chem Int Ed Engl 52:6912-6916.

Delhaize, E., Randall, P. J. (1995). Characterization of a Phosphate-Accumulator Mutant of Arabidopsis-Thaliana. Plant Physiology 107:207-213.

Desai, M., Rangarajan, P., Donahue, J. L., Williams, S. P., Land, E. S., Mandal, M. K., Phillippy, B. Q., Perera, I. Y., Raboy, V., Gillaspy, G. E. (2014). Two inositol hexakisphosphate kinases drive inositol pyrophosphate synthesis in plants. Plant J 80:642-653.

Desfougeres, Y., Wilson, M. S. C., Laha, D., Miller, G. J., Saiardi, A. (2019). ITPK1 mediates the lipid-independent synthesis of inositol phosphates controlled by metabolism. Proc Natl Acad Sci U S A 116:24551-24561.

Dong, J., Ma, G., Sui, L., Wei, M., Satheesh, V., Zhang, R., Ge, S., Li, J., Zhang, T. E., Wittwer, C., Jessen, H. J., Zhang, H., An, G. Y., Chao, D. Y., Liu, D., Lei, M. (2019). Inositol pyrophosphate InsP$_8$ acts as an intracellular phosphate signal in Arabidopsis. Mol Plant 12(11):1463-1473.

Draskovic, P., Saiardi, A., Bhandari, R., Burton, A., Ilc, G., Kovacevic, M., Snyder, S. H., Podobnik, M. (2008). Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. Chem Biol 15:274-286.

Duan, K., Yi, K. K., Dang, L., Huang, H. J., Wu, W., Wu, P. (2008). Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant Journal 54:965-975.

Findeisen, M., Brand, T., Berger, S. (2007). A 1H-NMR thermometer suitable for cryoprobes. Magnetic Resonance in Chemistry 45:175-178.

Gaugler, P., Gaugler, V., Kamleitner, M., Schaaf, G. (2020). Extraction and Quantification of Soluble, Radiolabeled Inositol Polyphosphates from Different Plant Species using SAX-HPLC. Jove-J Vis Exp.

Gerasimaite, R., Pavlovic, I., Capolicchio, S., Hofer, A., Schmidt, A., Jessen, H. J., and Mayer, A. (2017). Inositol Pyrophosphate Specificity of the SPX-Dependent Polyphosphate Polymerase VTC. Acs Chem Biol 12:648-653.

Harmel, R. K., Puschmann, R., Nguyen Trung, M., Saiardi, A., Schmieder, P., Fiedler, D. (2019). Harnessing (13)C-labeled myo-inositol to interrogate inositol phosphate messengers by NMR. Chem Sci 10:5267-5274.

Kuo, H. F., Chang, T. Y., Chiang, S. F., Wang, W. D., Charng, Y. Y., Chiou, T. J. (2014). Arabidopsis inositol pentakisphosphate 2-kinase, AtIPK1, is required for growth and modulates phosphate homeostasis at the transcriptional level. Plant J 80:503-515.

Kuo, H. F., Hsu, Y. Y., Lin, W. C., Chen, K. Y., Munnik, T., Brearley, C. A., Chiou, T. J. (2018). Arabidopsis inositol phosphate kinases IPK1 and ITPK1 constitute a metabolic pathway in maintaining phosphate homeostasis. Plant Journal 95:613-630.

Laha, D., Johnen, P., Azevedo, C., Dynowski, M., Weiss, M., Capolicchio, S., Mao, H. B., Iven, T., Steenbergen, M., Freyer, M., Gaugler, P., de Campos, M. K. F., Zheng, N., Feussner, I., Jessen, H. J., Van Wees, S. C. M., Saiardi, A., Schaaf, G. (2015). VIH2 Regulates the Synthesis of Inositol Pyrophosphate InsP(8) and Jasmonate-Dependent Defenses in Arabidopsis. Plant Cell 27:1082-1097.

Laha, D., Parvin, N., Dynowski, M., Johnen, P., Mao, H., Bitters, S. T., Zheng, N., Schaaf, G. (2016). Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex. Plant Physiol 171:2364-2370.

Laha, D., Parvin, N., Hofer, A., Giehl, R. F. H., Fernandez-Rebollo, N., von Wiren, N., Saiardi, A., Jessen, H. J., Schaaf, G. (2019). Arabidopsis ITPK1 and ITPK2 Have an Evolutionarily Conserved Phytic Acid Kinase Activity. Acs Chem Biol 14:2127-2133.

Laha, N. P., Dhir, Y. W., Giehl, R. F. H., Schaefer, E. M., Gaugler, P., Shishavan, Z. H., Gulabani, H., Mao, H., Zheng, N., von Wiren, N., Jessen, H. J., Saiardi, A., Bhattacharjee, S., Laha D., Schaaf, G. (2020). ITPK1-Dependent Inositol Polyphosphates Regulate Auxin Responses in Arabidopsis thaliana. bioRxiv:2020.2004.2023.058487.

Laussmann, T., Reddy, K. M., Reddy, K. K., Falck, J. R., Vogel, G. (1997). Diphospho-myo-inositol phosphates from Dictyostelium identified as D-6-diphospho-myo-inositol pentakisphosphate and D-5,6-bisdiphospho-myo-inositol tetrakisphosphate. Biochem J 322:31-33.

Lin, S. I., Chiang, S. F., Lin, W. Y., Chen, J. W., Tseng, C. Y., Wu, P. C., Chiou, T. J. (2008). Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiology 147:732-746.

Liu, F., Wang, Z. Y., Ren, H. Y., Shen, C. J., Li, Y., Ling, H. Q., Wu, C. Y., Lian, X. M., Wu, P. (2010). OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of OsPT2 and phosphate homeostasis in shoots of rice. Plant Journal 62:508-517.

Losito, O., Szijgyarto, Z., Resnick, A. C., Saiardi, A. (2009). Inositol Pyrophosphates and Their Unique Metabolic Complexity: Analysis by Gel Electrophoresis. Plos One 4.

Luo, H. B. R., Huang, Y. E., Chen, J. M. C., Saiardi, A., Iijima, M., Ye, K. Q., Huang, Y. F., Nagata, E., Devreotes, P., Snyder, S. H. (2003). Inositol pyrophosphates mediate chemotaxis in Dictyostelium via pleckstrin homology domain-PtdIns (3,4,5)P3 interactions. Cell 114:559-572.

Lv, Q. D., Zhong, Y. J., Wang, Y. G., Zhang, L., Shi, J., Wu, Z. C., Liu, Y., Mao, C. Z., Yi, K. K., Wu, P. (2014). SPX4 Negatively Regulates Phosphate Signaling and Homeostasis through Its Interaction with PHR2 in Rice. Plant Cell 26:1586-1597.

Miller, G. J., Wilson, M. P., Majerus, P. W., Hurley, J. H. (2005). Specificity determinants in inositol polyphosphate synthesis: crystal structure of inositol 1,3,4-trisphosphate 5/6-kinase. Mol Cell 18:201-212.

Mulugu, S., Bai, W. L., Fridy, P. C., Bastidas, R. J., Otto, J. C., Dollins, D. E., Haystead, T. A., Ribeiro, A. A., York, J. D. (2007). A conserved family of enzymes that phosphorylate inositol hexakisphosphate. Science 316:106-109.

Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring, J. K., Brearley, C., Martinoia, E. (2009). The Arabidopsis ATP-binding Cassette Protein AtMRP5/AtABCC5 Is a High Affinity Inositol Hexakisphosphate Transporter Involved in Guard Cell Signaling and Phytate Storage. J Biol Chem 284:33614-33622.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29.

Puga, M. I., Mateos, I., Charukesi, R., Wang, Z., Franco-Zorrilla, J. M., de Lorenzo, L., Irigoye, M. L., Masiero, S., Bustos, R., Rodriguez, J., Leyva, A., Rubio, V., Sommer, H., Paz-Ares, J. (2014). SPX1 is a phosphate-dependent inhibitor of PHOSPHATE STARVATION RESPONSE 1 in Arabidopsis. P Natl Acad Sci USA 111:14947-14952.

Puschmann, R., Harmel, R. K., Fiedler, D. (2019). Scalable Chemoenzymatic Synthesis of Inositol Pyrophosphates. Biochemistry-Us 58:3927-3932.

Qi, W. J., Manfield, I. W., Muench, S. P., Baker, A. (2017). AtSPX1 affects the AtPHR1-DNA-binding equilibrium by binding monomeric AtPHR1 in solution. Biochem J 474:3675-3687.

Qiu, D., Wilson, M. S., Eisenbeis, V. B., Harmel, R. K., Riemer, E., Haas, T. M., Wittwer, C., Jork, N., Gu, C., Shears, S. B., Schaaf, G., Kammerer, B., Fiedler, D., Saiardi, A., Jessen, H. J. (2020). Analysis of inositol phosphate metabolism by capillary electrophoresis electrospray ionization mass spectrometry. Nat Commun 11:6035.

Reski, R., Abel, W. O. (1985). Induction of budding on chloronemata and caulonemata of the moss, Physcomitrella patens, using isopentenyladenine. Planta 165:354-358.

Ried, M. K., Wild, R., Zhu, J., Pipercevic, J., Sturm, K., Broger, L., Harmel, R. K., Abriata, L. A., Hothorn, L. A., Fiedler, D., Hiller, S., Hothorn, M. (2021). Inositol pyrophosphates promote the interaction of SPX domains with the coiled-coil motif of PHR transcription factors to regulate plant phosphate homeostasis. Nat Commun. 12(1):384.

Rubio, V., Linhares, F., Solano, R., Martin, A. C., Iglesias, J., Leyva, A., Paz-Ares, J. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Gene Dev 15:2122-2133.

Rus, A., Baxter, I., Muthukumar, B., Gustin, J., Lahner, B., Yakubova, E., Salt, D. E. (2006). Natural variants of AtHKT1 enhance Na+ accumulation in two wild Populations of Arabidopsis. Plos Genet 2:1964-1973.

Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., Snyder, S. H. (1999). Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr Biol 9:1323-1326.

Schaaf, G., Betts, L., Garrett, T. A., Raetz, C. R., Bankaitis, V. A. (2006). 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.

Shears, S. B. (2018). Intimate connections: Inositol pyrophosphates at the interface of metabolic regulation and cell signaling. J Cell Physiol 233:1897-1912.

Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., York, J. D. (2005). Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. P Natl Acad Sci USA 102:12612-12617.

Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dorman, G., Olszewski, J. D., Prestwich, G. D., Snyder, S. H. (1996). Purified inositol hexakisphosphate kinase is an ATP synthase: diphosphoinositol pentakisphosphate as a high-energy phosphate donor. Proc Natl Acad Sci U S A 93:4305-4310.

Wang, H. C., Godage, H. Y., Riley, A. M., Weaver, J. D., Shears, S. B., Potter, B. V. L. (2014a). Synthetic Inositol Phosphate Analogs Reveal that PPIP5K2 Has a Surface-Mounted Substrate Capture Site that Is a Target for Drug Discovery. Chem Biol 21:689-699.

Wang, Z. Y., Ruan, W., Shi, J., Zhang, L., Xiang, D., Yang, C., Li, C., Wu, Z., Liu, Y., Yu,

Y., Shou, H., Mo, X., Mao, C., Wu, P. (2014b). Rice SPX1 and SPX2 inhibit phosphate starvation responses through interacting with PHR2 in a phosphate-dependent manner. P Natl Acad Sci USA 111:14953-14958.

Whitfield, H., White, G., Sprigg, C., Riley, A. M., Potter, B. V. L., Hemmings, A. M., Brearley, C. A. (2020). An ATP-responsive metabolic cassette comprised of inositol tris/tetrakisphosphate kinase 1 (ITPK1) and inositol pentakisphosphate 2-kinase (IPK1) buffers diphosphosphoinositol phosphate levels. Biochem J 477:2621-2638.

Wild, R., Gerasimaite, R., Jung, J. Y., Truffault, V., Pavlovic, I., Schmidt, A., Saiardi, A., Jessen, H. J., Poirier, Y., Hothorn, M., Mayer, A. (2016). Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. Science 352:986-990.

Wilson, M. S., Saiardi, A. (2018). Inositol Phosphates Purification Using Titanium Dioxide Beads. Bio Protoc 8.

Wilson, M. S. C., Bulley, S. J., Pisani, F., Irvine, R. F., Saiardi, A. (2015). A novel method for the purification of inositol phosphates from biological samples reveals that no phytate is present in human plasma or urine. Open Biol 5.

Wilson, M. S. C., Livermore, T. M., Saiardi, A. (2013). Inositol pyrophosphates: between signalling and metabolism. Biochem J 452:369-379.

Wundenberg, T., Grabinski, N., Lin, H. Y., Mayr, G. W. (2014). Discovery of InsP(6)-kinases as InsP(6)-dephosphorylating enzymes provides a new mechanism of cytosolic InsP(6) degradation driven by the cellular ATP/ADP ratio. Biochem J 462:173-184.

Zhong, Y., Wang, Y., Guo, J., Zhu, X., Shi, J., He, Q., Liu, Y., Wu, Y., Zhang, L., Lv, Q., Mao, C. (2018). Rice SPX6 negatively regulates the phosphate starvation response through suppression of the transcription factor PHR2. New Phytol 219:135-148.

Zhu, J., Lau, K., Puschmann, R., Harmel, R. K., Zhang, Y.J., Pries, V., Gaugler, P., Broger, L., Dutta, A. K., Jessen, H. J., Schaaf, G., Fernie, A. R., Hothorn, L. A., Fiedler,

D., Hothorn, M. (2019). Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. eLife 8.

Supplemental Information

Supplemental Methods

Hydroponics culture of *Arabidopsis* and rice

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 NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM KCl, 250 μM K_2SO_4, 250 μM $CaCl_2$, 100 μM Na-Fe-EDTA, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$ and 0.7 μM $NaMoO_4$ (pH adjusted to 5.8 by KOH). After 7 days, nutrient solution was changed to full-strength and replaced once a week during 2 weeks, then twice a week during 1 week, and every 2 days once the treatments were imposed. Aeration was provided to roots from the third week onwards. To induce P_i deficiency, KH_2PO_4 was replaced by KCl and P_i resupply was performed by refeeding P_i-starved plants with 1 mM KH_2PO_4 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.

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 μmol m^{-2} s^{-1}, and 30°C/25°C day (16 h)/night (8 h) temperature. Seeds were germinated in darkness at 30°C for 3 days and then transferred to meshes floating on a solution containing 0.5 mM $CaCl_2$ and 10 μM Na-Fe-EDTA, which was exchanged every third day. After 10 days, homogenous seedlings were transplanted into 60-L tanks filled with a modified Yoshida nutrient solution (Shrestha et al., 2018). 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.

Agar plate culture of *Arabidopsis*

Cultivation of *Arabidopsis* plants under sufficient P_i (625 μM P_i) or low P_i (5 μM P_i) in sterile solid medium was performed exactly as described in (Gruber et al., 2013).

Histochemical iron staining

Iron staining with Perls/DAB was performed as described previously (Muller et al., 2015) with slight modifications. Roots were washed three times with 10 mM EDTA followed by three washes with MQ water. Roots were then incubated for 5 min in a Perls solution containing

equal volumes of 0.5% (v/v) HCl and 0.5% (w/v) K-ferrocyanide. For DAB intensification, plants were washed three times with MQ water and incubated for 1 h in methanol containing 10 mM Na-azide and 0.3% (v/v) H_2O_2. After washing three times with 100 mM Na-phosphate buffer (pH 7.4), plants were incubated for up to 10 min in an intensification solution containing the same buffer containing 0.025% (w/v) DAB (Sigma-Aldrich) and 0.005% (v/v) H_2O_2. The reaction was stopped by washing three times with MQ water. Roots were mounted in a HCG solution (chloral hydrate:water:glycerol = 8:3:1) and imaged with an Axio Imager 2 microscope (Zeiss).

Analysis of ATP and ADP

Adenosine nucleotides were specifically determined according to (Haink and Deussen, 2003) with some modifications. Frozen leaf material (100 mg) was homogenized in liquid N_2 and extracted with methanol/chloroform according to (Ghaffari et al., 2016). 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 KH_2PO_4 for which pH was adjusted to 5.2 with KOH. To this mixture, 25 µL chloracetaldehyde (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 ATP and ADP standards 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 potassium KH_2PO_4, 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.

Supplemental References

Ghaffari, M. R., Shahinnia, F., Usadel, B., Junker, B., Schreiber, F., Sreenivasulu, N., Hajirezaei, M. R. (2016). The Metabolic Signature of Biomass Formation in Barley. Plant Cell Physiol 57:1943-1960.

Gruber, B. D., Giehl, R. F., Friedel, S., von Wiren, N. (2013). Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Physiol.

Haink, G., Deussen, A. (2003). Liquid chromatography method for the analysis of adenosine compounds. J Chromatogr B Analyt Technol Biomed Life Sci 784:189-193.

Müller, J., Toev, T., Heisters, M., Teller, J., Moore, K. L., Hause, G., Dinesh, D. C., Burstenbinder, K., Abel, S. (2015). Iron-dependent callose deposition adjusts root meristem maintenance to phosphate availability. Dev Cell 33:216-230.

Shrestha, A., Dziwornu, A. K., Ueda, Y., Wu, L.B., Mathew, B., Frei, M. (2018). Genome-wide association study to identify candidate loci and genes for Mn toxicity tolerance in rice. Plos One 13.

Supplemental Tables and Figures

Supplementary Table 1. Expression of P_i starvation-induced genes in response to changes in P_i availability. Wild-type plants (Col-0) were cultivated in hydroponics under sufficient P_i (+P), deficient P_i for 7 days (-P) or resupplied with P_i for 6 h. Values represent expression levels normalized to +P. Shown are means ± SE (n = 3 biological replicates).

Gene	+P	-P	Pi RS 6h
SPX1	1.0 ± 0.17	267 ± 10.53	6.93 ± 1.59
IPS1	1.0 ± 0.27	1601 ± 154	90 ± 13.8
PHR1	1.0 ± 0.06	1.04 ± 0.05	0.86 ± 0.03
PHT1;1	1.0 ± 0.22	16.4 ± 1.93	2.42 ± 0.52
PHT1;2	1.0 ± 0.19	126.67 ± 10.88	10.14 ± 0.79
PHT1;8	1.0 ± 0.23	50.39 ± 5.75	8.11 ± 0.57
PHT1;9	1.0 ± 0.07	27.06 ± 4.21	3.08 ± 0.58

Supplementary Table 2. List of primers used in this study.

Primers used for qPCR analysis		
AGI ID	**Gene name**	**Primer sequence**
AT2G36170	*UBQ2*	F: 5'-CCAAGATCCAGGACAAAGAAGGA-3' R: 5'-TGGAGACGAGCATAACACTTG-3'
AT5G43350	*PHT1;1*	F: 5'-AGGCGATCACGTTGCTTACA-3' R: 5'- TCTCTGGAGAGTTGAGGAGAGAC-3'
AT5G43370	*PHT1;2*	F: 5'-CCATTAGCGCACAACGGAAAG-3' R: 5'-GAAACCCATACCGGCGATGA-3'
AT5G43360	*PHT1;3*	F: 5'-GCTTTCATCGCGGCAGTGTT-3' R: 5'-TGAGGAGGCGTTGATAGAAACC-3'
AT2G38940	*PHT1;4*	F: 5'-AGCCTTTGTCTCTGCGGTTT-3' R: 5'-CGTGGATCCCAAGGCATCAT-3'
AT1G20860	*PHT1;8*	F: 5'-AGAGAAGTGGCGGTGGTTTG-3' R: 5'-TCTTGCGGTTTCAGGCATCA-3'
AT1G76430	*PHT1;9*	F: 5'-TTCGGAGAAGACGAACGTGG-3' R: 5'-GTATCTGGCGGTTTCAGGCA-3'
AT3G23430	*PHO1*	F: 5'-GACTTACAGCTCGTTGAATATGATAGC-3' R: 5'-CGATCTCTTTACGACTTTGAGATACG-3'
AT4G28610	*PHR1*	F: 5'-TGTGGAATTGCGACCTGTTA-3' R: 5'-GCTCTTTCACTACCGCCAAG-3'
AT5G20150	*SPX1*	F: 5'-GTTGATTTCCATGGAGAAATGG-3' R: 5'-GGTAAACGCATGAGATCACCAG-3'
AT3G09922	*IPS1*	F: 5'-TCCCTCTAGAAATTGGGCAAC-3' R: 5'-GGGAGTGGGTACAACCCAAA-3'
AT2G33770	*PHO2*	F: 5'-TTGCACCATGTGAAATTTGG-3' R: 5'-AGACCCGTTTCCTGATGGTT-3'
AT5G16760	*ITPK1*	F: 5'-ATTGGGACGTCGAAAGGGTC-3' R: 5'-CTCAGTCAACACAGGCTCGT-3'
Primers used for genotyping in F1, F2 and F3		
itpk1_LP		5'-ACCAATATTCGATTCCACACG-3'
itpk1_RP		5'-CCATGTCCCAGAAGAACTCAG-3'
itpk2-2_LP		5'- TCGCTTGTACTTTTCAAGTTGC-3'
itpk2-2_RP		5'- TAAGGACAAAAACATGGCAGG-3'
SAIL_LB3		5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'
phr1_LP		5'-GAGAGACCTCACACGCACTTC-3'

| phr1_RP	5'-CTTTCTGGCGAACCTGTAGTG-3'
phl1_LP	5'-GTGGAGACGTTTCTGCACTTC-3'
phl1_RP	5'-TCCCACAATCCAAATTCAGAG-3'
LBb1.3	5'-ATTTTGCCGATTTCGGAAC-3'
vih2-4_GK_LP	5'-AACAACAGCAATGACACAACG-3'
vih2-4_GK_RP	5'-CATTCCCATCTTTTGGACAAC-3'
GABI_LB1	5'-ATAATAACGCTGCGGACATCTACATTTT-3'

Supplementary Table 3. Mass spectrometer parameters used for the analysis of inositol (pyro)phosphates.

| Compound	MRM transitions	Type of transition	Collision Energy (V)	Cell Acc (V)
$InsP_8$	$408.9 \rightarrow 359.6^a$ $408.9 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}2H\text{-}H_3PO_4]^{2-}$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 42	1 4
$[^{13}C_6]InsP_8$	$411.9 \rightarrow 362.6^a$ $411.9 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}2H\text{-}H_3PO_4]^{2-}$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 42	1 4
$InsP_7$	$368.9 \rightarrow 319.6^a$ $368.9 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}2H\text{-}H_3PO_4]^{2-}$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 38	3 3
$[^{13}C_6]InsP_7$	$371.9 \rightarrow 322.6^a$ $371.9 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}2H\text{-}H_3PO_4]^{2-}$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 38	3 3
$InsP_6$	$328.9 \rightarrow 78.9^a$ $328.9 \rightarrow 481$	$[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$ $[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3\text{-}H_3PO_4]^-$	46 10	1 3
$[^{13}C_6]InsP_6$	$331.9 \rightarrow 78.9^a$ $331.9 \rightarrow 487$	$[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$ $[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3\text{-}H_3PO_4]^-$	46 10	1 3
$InsP_5$	$288.9 \rightarrow 498.7^a$ $288.9 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3]^-$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 14	1 1
$[^{13}C_6]InsP_5$	$291.0 \rightarrow 504.7^a$ $291.0 \rightarrow 78.9$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3]^-$ $[M\text{-}2H]^{2-} \rightarrow [PO_3]^-$	10 14	1 1
$InsP_4$	$249.0 \rightarrow 418.6^a$ $249.0 \rightarrow 320.6$	$[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3]^-$ $[M\text{-}2H]^{2-} \rightarrow [M\text{-}H\text{-}HPO_3\text{-}H_3PO_4]^-$	10 14	1 1
$InsP_3$	$419.0 \rightarrow 320.6^a$ $419.0 \rightarrow 78.9$	$[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}H_3PO_4]^-$ $[M\text{-}H]^- \rightarrow [PO_3]^-$	18 50	4 1

[a] MRM transition with the highest response for each compound (used for quantification).
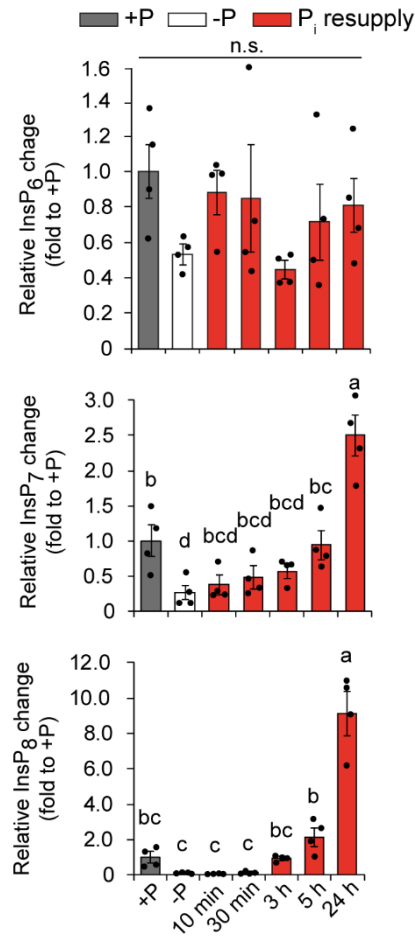
Suppl. Fig. 1. InsP$_6$ and InsP$_7$ levels respond strongly to P$_i$ deficiency and P$_i$ resupply in rice plants. Shown are fold changes of quantified signal intensities of independent PAGE gels. Data are means ± SE (n = 3 gels loaded with independent biological samples). Plants were cultivated in hydroponics under sufficient P$_i$ (+P), deficient P$_i$ (-P) for 10 days, or -P resupplied with P$_i$ for the indicated times. Different letters indicate significant differences according to Tukey's test (P < 0.05). n.s., not significant according to one-way ANOVA (P < 0.05).
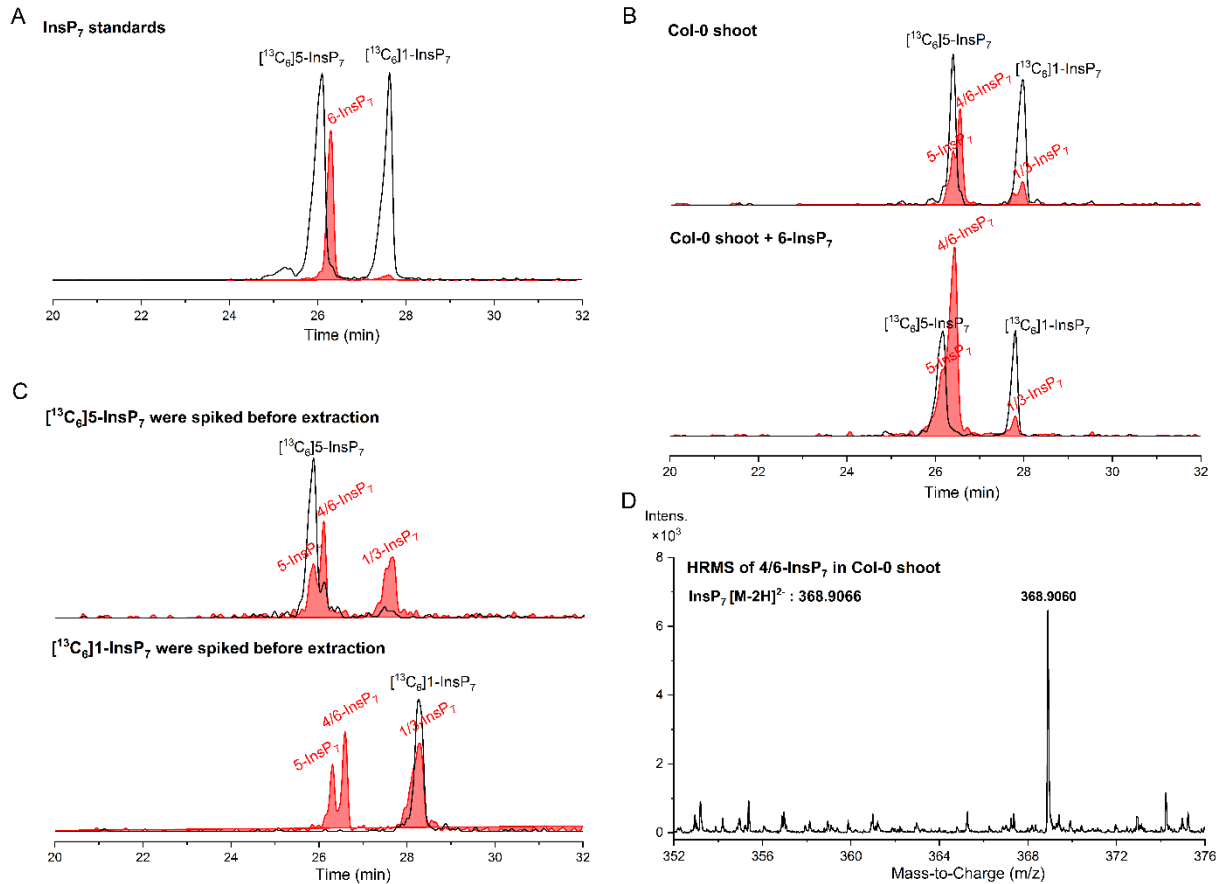
Suppl. Fig. 2. Determination of 4/6-InsP₇ as a novel PP-InsP found in plants via CE-ESI-qTOF analysis. (A) 6-InsP$_7$ could be readily distinguished with the aid of isotopic standards [^{13}C$_6$]5-InsP$_7$ and [^{13}C$_6$]1-InsP$_7$ with CE-ESI-MS. **(B)** An undefined InsP$_7$ isomer found in *Arabidopsis* (Col-0) showed same migration time as 6-InsP$_7$ standard. **(C)** [^{13}C$_6$]5-InsP$_7$ and [^{13}C$_6$]1-InsP$_7$ were spiked before InsP extractions, ruling out the possibility that 4/6-InsP$_7$ were generated during sample preparation. **(D)** A representative high-resolution mass spectrum (HRMS) of 4/6-InsP$_7$ in *Arabidopsis*.
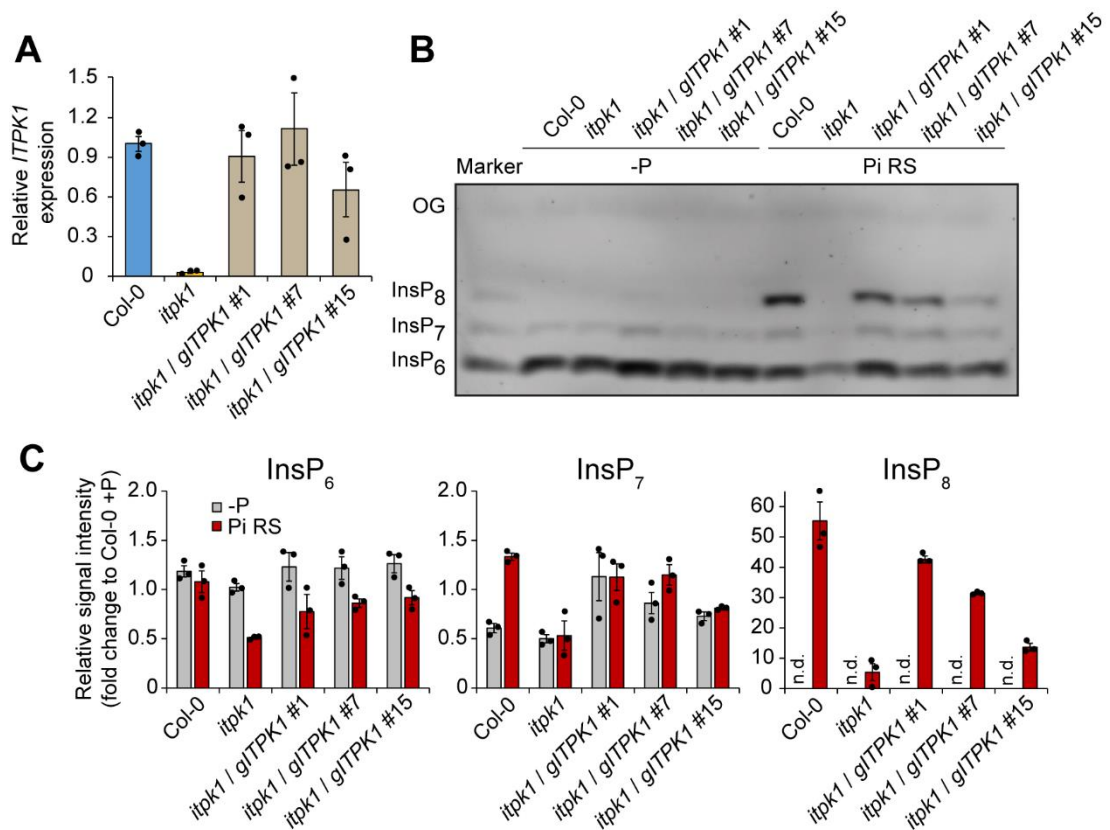
Suppl. Fig. 3. Complementation of *itpk1* mutant. (A) ITPK1 expression in 3-week-old WT (Col-0), *itpk1* and three independent *itpk1* lines transformed with *ITPK1* genomic DNA. Data represent means ± SD (n = 3 biological replicates). **(B and C)** PAGE detection **(B)** and fold change of quantified signal intensities **(C)** of inositol (pyro)phosphates in shoots of WT (Col-0), *itpk1* and three independent *itpk1* lines transformed with *ITPK1* genomic DNA. Plants were cultivated in hydroponics under deficient P_i for 7 days (-P) or -P resupplied with P_i for 12 h (Pi RS). Data represent means ± SE of values normalized to Col-0 plants continuously grown under sufficient P_i (n = 3 biological replicates). n.d., not detected. OG, orange G.
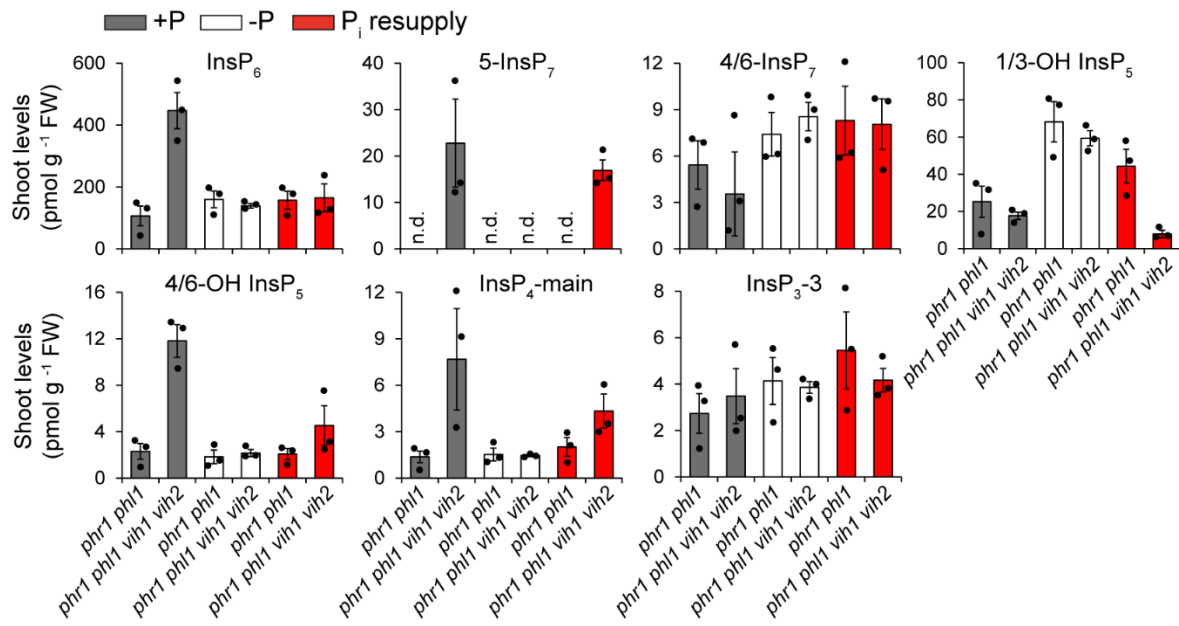
Suppl. Fig. 4. VIH1- and VIH2-dependent inositol (pyro)phosphate metabolism. CE-ESI-MS analysis of *VIH1* and *VIH2* mutations in the *phr1 phl1* background. Plants were cultivated in hydroponics under sufficient P_i (+P), deficient P_i (-P) or -P resupplied with P_i for the indicated times. Data represent means ± SE (n = 3 biological replicates). n.d., could not be detected in any of these samples. Note that $InsP_8$, $1/3-InsP_7$, $InsP_3-1$ and $2-OH\ InsP_5$ could not be detected in none of the analyzed samples.
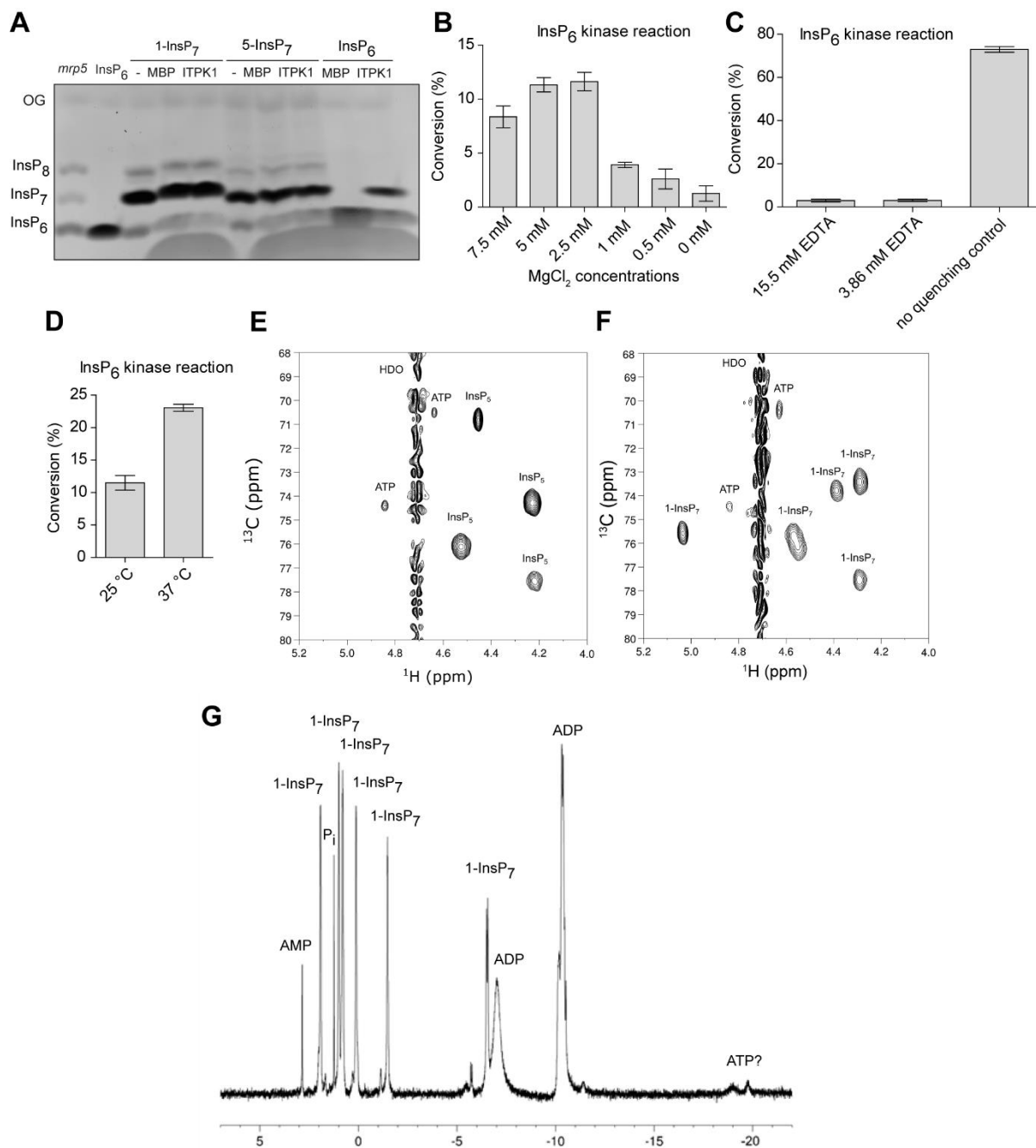
Suppl. Fig. 5. Activity of *Arabidopsis* ITPK1 on InsP$_5$ [2-OH] and InsP$_7$ isomers and control experiments for kinase assays. (A) ITPK1 has no kinase activity on 1-InsP$_7$ and 5-InsP$_7$. InsP$_7$ isomers or InsP$_6$ were incubated with recombinant *Arabidopsis* ITPK1 as indicated in presence of 12.5 mM ATP. Inositol (pyro)phosphates 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 (-) and TiO$_2$-purified *mrp5* seed extract. Purified His$_8$-MBP tag (MBP) served as negative control for ITPK1. OG, orange G. **(B-D)** Control experiments for NMR analyses. InsP$_6$ 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 Mg^{2+} concentrations **(C)** and temperature **(D)**. The conversion was determined by NMR spectroscopy after quenching with an excess of EDTA. **(E-G)** 2D ^{1}H-^{13}C-HMBC spectra. Recombinant *Arabidopsis* ITPK1 was incubated with InsP$_5$ **(E)** or 1-InsP$_7$ **(F)** at 25°C in the presence of an ATP recycling system for 24 h. The reaction mixture was analyzed by HSQC NMR spectroscopy. **(G)** Overview of the reaction shown in **(F)** as analyzed by ^{31}P NMR spectroscopy after 24 h. A small, unidentified signal potentially reflecting ATP is marked with a question mark.
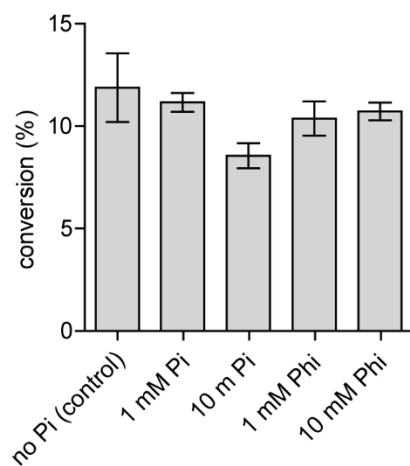
**Suppl. Fig. 6. Dependency of *Arabidopsis* ITPK1 kinase activity on P_i.** $InsP_6$ was incubated with recombinant *Arabidopsis* ITPK1 at 25°C in the presence of 2.5 mM ATP and the indicated concentrations of P_i or its non-metabolizable analog phosphite (Phi). The conversion was determined by NMR spectroscopy after quenching with an excess of EDTA. The experiment was repeated three times.
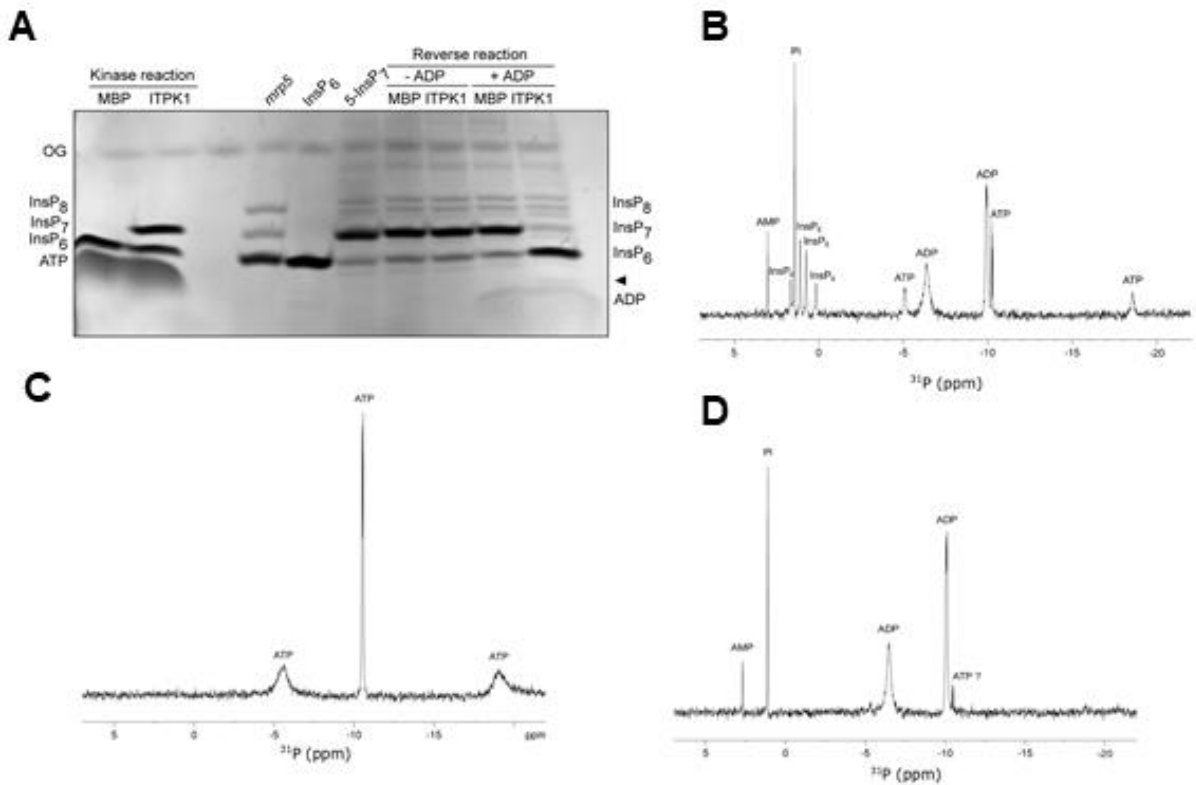
Suppl. Fig. 7. Recombinant *Arabidopsis* ITPK1 can dephosphorylate 5-InsP$_7$ in the presence of ADP. (A) ADP-dependent dephosphorylation of 5-InsP$_7$ by recombinant *Arabidopsis* ITPK1. InsPs were separated via PAGE and visualized by toluidine blue staining. The identity of bands was determined by migration compared to InsP$_6$ and 5-InsP$_7$ standards and TiO$_2$-purified *mrp5* seed extract. InsP$_6$ kinase reaction served as positive control for the reverse reactions. Purified His$_8$-MBP tag (MBP) served as negative control for ITPK1. Arrowhead indicates the presence of a small ATP band just above ADP. OG, orange G. **(B)** ^{31}P NMR spectroscopy analysis of recombinant *Arabidopsis* ITPK1 incubated with [^{13}C$_6$]-labelled 5-InsP$_7$ at 25°C in the presence of ADP. After 24 h the mixture was analyzed by NMR. **(C)** ^{31}P NMR analysis of ATP in ATP synthase reaction buffer. **(D)** ^{31}P NMR spectroscopy analysis of recombinant *Arabidopsis* ITPK1 incubated with ADP without 5-InsP$_7$ at 25°C and analyzed after 24 h. A small, unidentified signal potentially reflecting ATP is marked with a question mark.
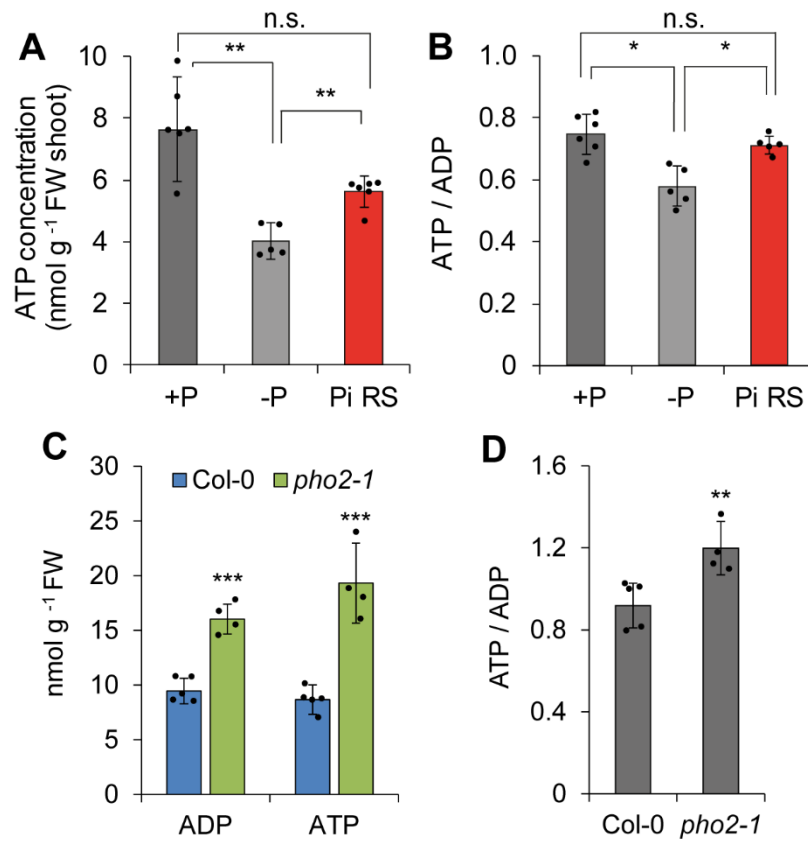
Suppl. Fig. 8. Effect of P$_i$ availability and resupply on shoot ATP levels. (A and B) Concentration of ATP **(A)** and ATP/ADP ratios **(B)** in shoots of Col-0 plants grown in hydroponics with P$_i$-sufficient solution (+P), exposed to 4 days of P$_i$ starvation (-P) or resupplied with P$_i$ for 12 h (Pi RS). Data represent means ± SD (n = 5-6 biological replicates). n.s., not significant. **(C and D)** Concentration of ATP and ADP **(C)** and ATP/ADP ratios **(D)** in shoots of Col-0 and the P-overaccumulating mutant *pho2-1* grown in hydroponics with sufficient P$_i$. Data represent means ± SD (n = 4-5 biological replicates). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ denote significant difference to wild type (Col-0) according to Student's *t*-test.
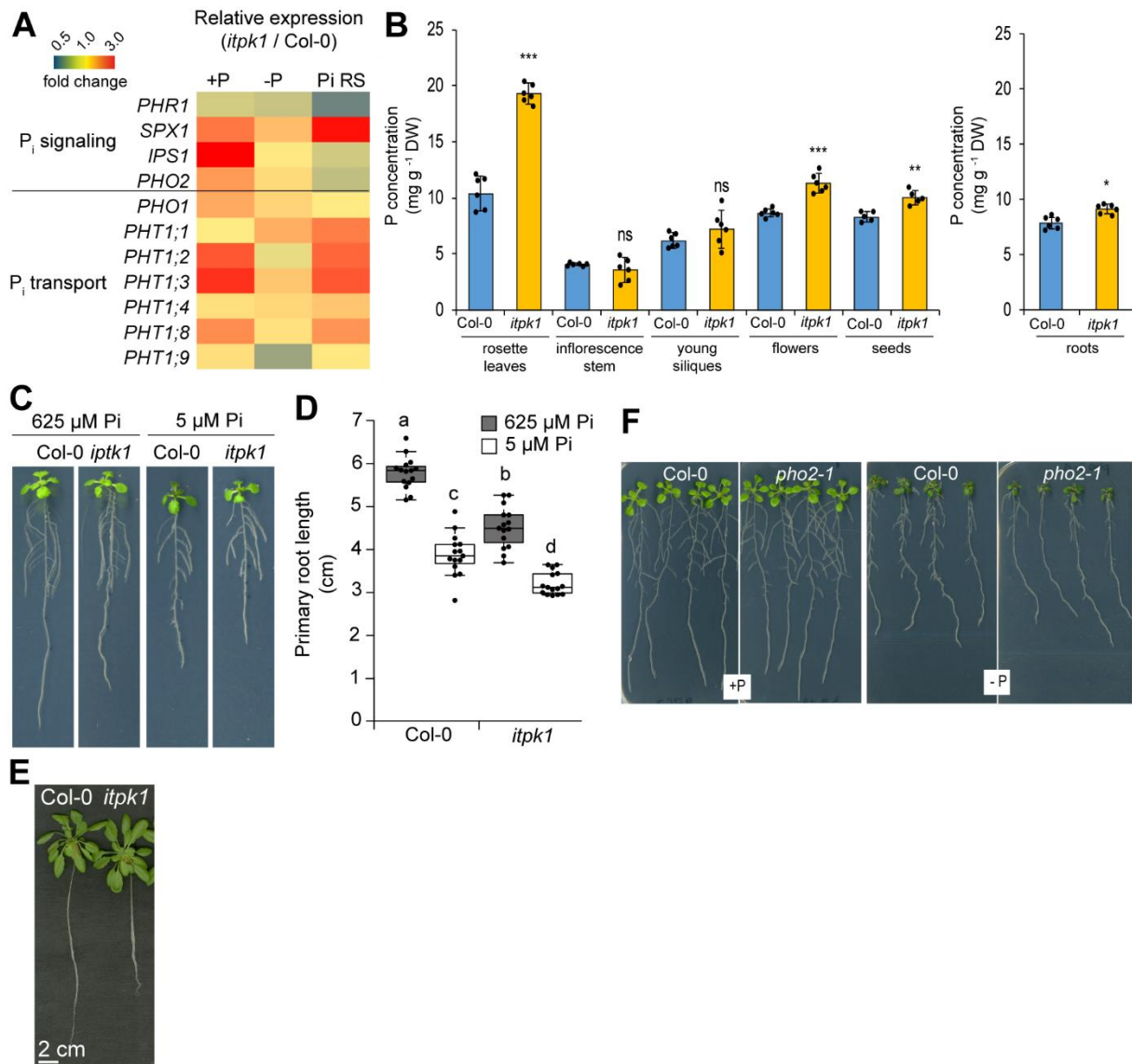
Suppl. Fig. 9. ITPK1-dependent P overaccumulation in different plant organs and root phenotypes. (**A**) Disruption of *ITPK1* results in mis-regulated expression of P_i starvation-induced genes in roots. (**B**) Total P levels in different parts of wild-type (Col-0) and *itpk1* plants. Data represent means ± SD (n = samples from 5 independent plants). Samples of above-ground tissues were collected from 5-week-old plants grown on peat-based substrate. Roots were collected from plants grown under sufficient P_i in hydroponics. *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference to wild type (Col-0) according to Student's *t*-test. n.s., not significant. Young siliques = green siliques with a length of 0.8 cm to 1.5 cm. (**C and D**) Pi-independent root growth repression in *itpk1* plants. Plant phenotype (**C**) and primary root length (**D**) after 7 days of growth under sufficient (625 μM P_i) or deficient P_i supply (5 μM P_i). Horizontal lines show medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 1.5 times the interquartile range from the 25th and 75th percentiles (n = 15 independent plants). Different letters indicate significant differences according to Tukey's test (P < 0.05). (**E**) Short-root phenotype of 5-week-old *itpk1* plants grown in hydroponics with sufficient P_i. Representative plants are shown. (**F**) Phenotype of wild-type (Col-0) and *pho2-1* plants grown in agar plates. Seven-day-old seedlings germinated on half-strength solid Murashige and Skoog agar media containing 625 μM P_i were transferred to +P (625 μM P_i) or -P (5 μM P_i) and grown for additional 7 days until imaging.
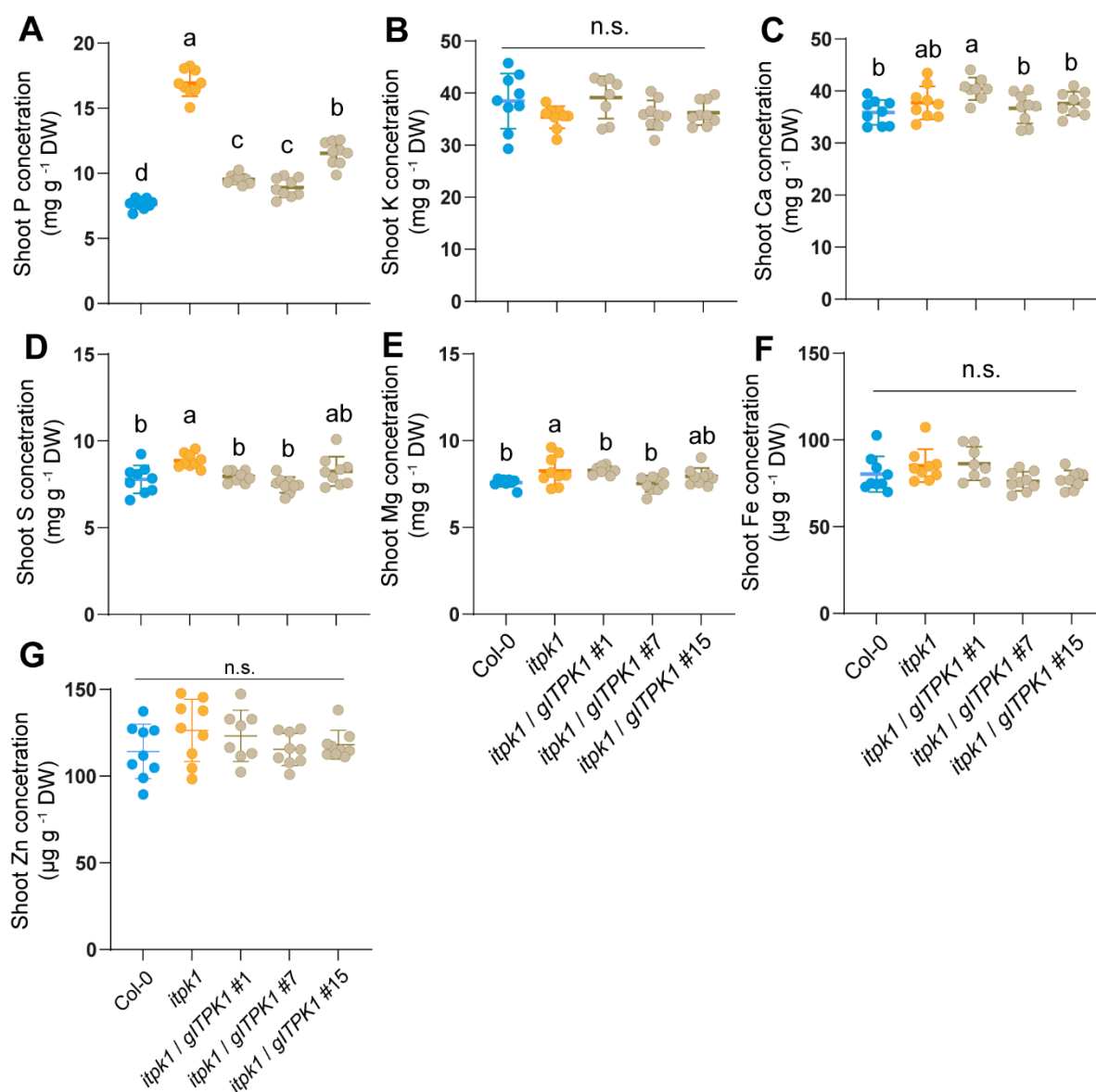
Suppl. Fig. 10. The *itpk1* mutant shows a specific overaccumulation of P that can be complemented with genomic *ITPK1*. Shoot concentrations of the macronutrients phosphorus **(A)**, potassium **(B)**, calcium **(C)**, sulfur **(D)** and magnesium **(E)**, and the micronutrients iron **(F)** and zinc **(G)** of 3-week-old plants grown on peat-based substrate. Graphs depict the means ± SD (n = 8-9 plants) and raw data points. Different letters indicate significant differences according to Tukey's test (P < 0.05). n.s., not significant according to one-way ANOVA.
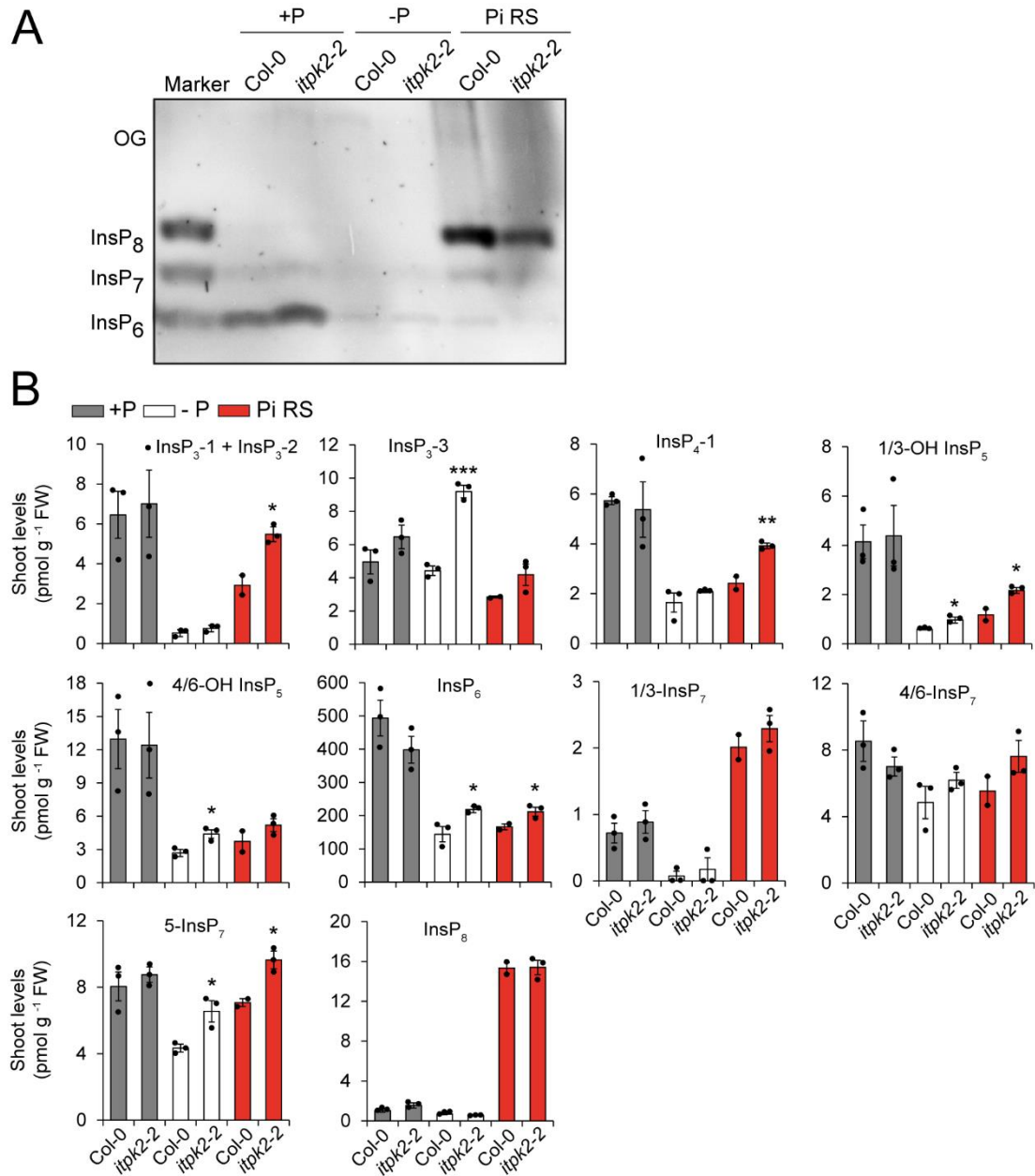
Suppl. Fig. 11. P$_i$-dependent InsP$_7$ and InsP$_8$ synthesis is not substantially altered in shoots of the *itpk2-2* **mutant. (A and B)** PAGE detection **(A)** and CE-ESI-MS analysis **(B)** of inositol (pyro)phosphates in shoots of WT and *itpk2-2* plants. Plants were grown in hydroponics under sufficient P$_i$ (+P), after 7 days of P$_i$ deficiency (-P) or after resupply of P$_i$ to P$_i$-deficient plants for 12 h (Pi RS). Data represent means ± SE (n = 2-3 biological replicates). OG, orange G. *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference to wild type (Col-0) according to Student's *t*-test.
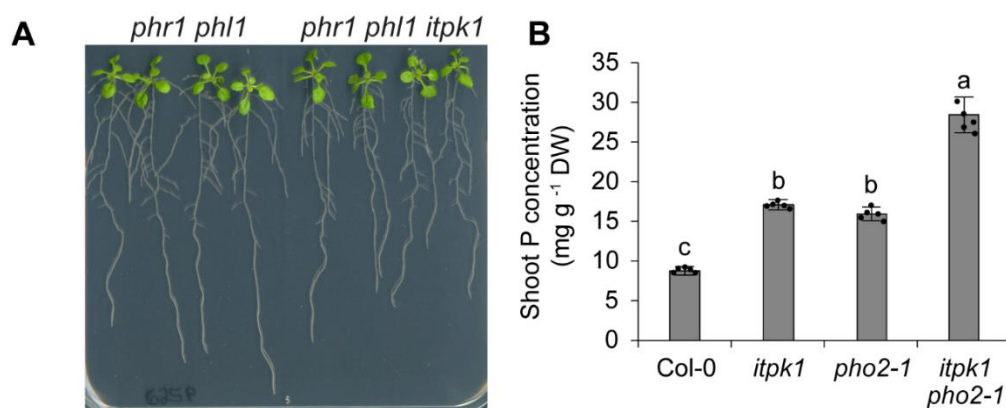
Suppl. Fig. 12. The short-root phenotype of *itpk1* plants is independent of PHR1/PHL1 and evidence for the additive role of ITPK1 and PHO2 in the regulation of P$_i$ homeostasis. (A) Root phenotypes of *phr1 phl1* double mutant and *phr1 phl1 itpk1* triple mutant. Seven-day-old seedlings germinated on half-strength solid Murashige and Skoog agar media containing 625 μM P$_i$ were transferred to +P (625 μM P$_i$) and grown for additional 7 days. Shown are representative images of the indicated mutants grown side-by-side on the same agar plate. **(B)** Total P levels were assessed in shoots of 4-week-old wild-type (Col-0) and the indicated single and double mutant. Samples of above-ground tissues were collected from 5-week-old plants grown on peat-based substrate. Data represent means ± SD (n = 5 plants). Different letters indicate significant differences according to Tukey's test (P < 0.05).
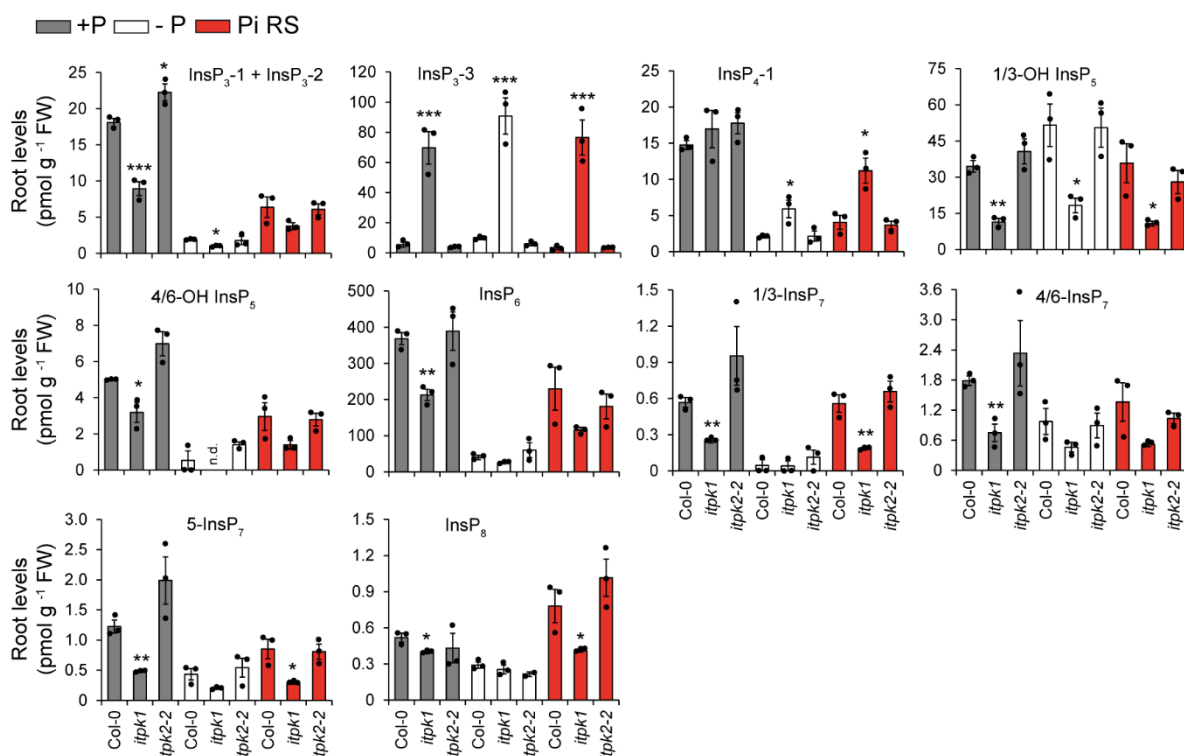
Suppl. Fig. 13. ITPK1- and ITPK2-dependent inositol (pyro)phosphate metabolism in roots. CE-ESI-MS analysis of inositol (pyro)phosphates in roots of WT (Col-0) and *itpk1* plants. Plants were cultivated in hydroponics under sufficient P_i (+P), deficient P_i for 7 days (-P) or -P resupplied with P_i for 12 h (Pi RS). Data represent means ± SE (n = 3 biological replicates). n.d., not detected. *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference to wild type (Col-0) according to Student's *t*-test.
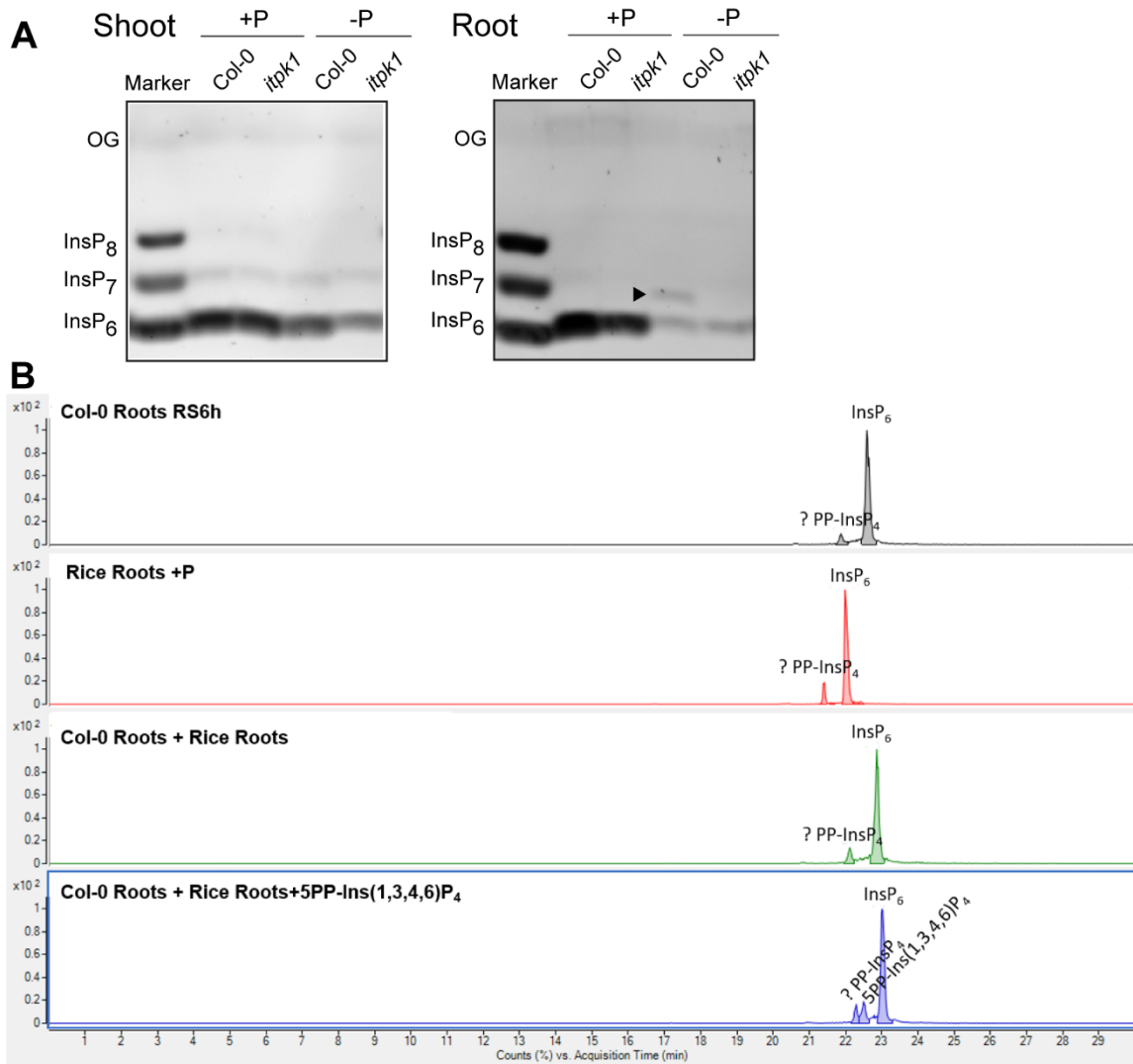
Suppl. Fig. 14. Roots of *Arabidopsis* and rice plants produce a PP-InsP$_4$ isomer not previously identified in plants. (A) PAGE detection of InsPs and PP-InsPs in shoots and roots of WT (Col-0) and *itpk1* mutant plants grown in hydroponics in P$_i$ -sufficient solution (+P) or exposed for 7 days to P$_i$ starvation (-P). Shown are PAGE results of shoots and roots of the same plants. The PAGE of roots is also shown in Fig. 7C and is displayed here for a direct comparison with shoot. The arrowhead indicates the appearance of a band between InsP$_6$ and InsP$_7$ specifically in WT root samples. OG, orange G. **(B)** CE-ESI-MS identification of a new PP-InsP$_4$ isomer in roots of *Arabidopsis* and rice plants. The identified isomer generated in the roots of both species was indistinguishable and did not co-migrate with a 5PP-Ins(1,3,4,6)P$_4$ standard.
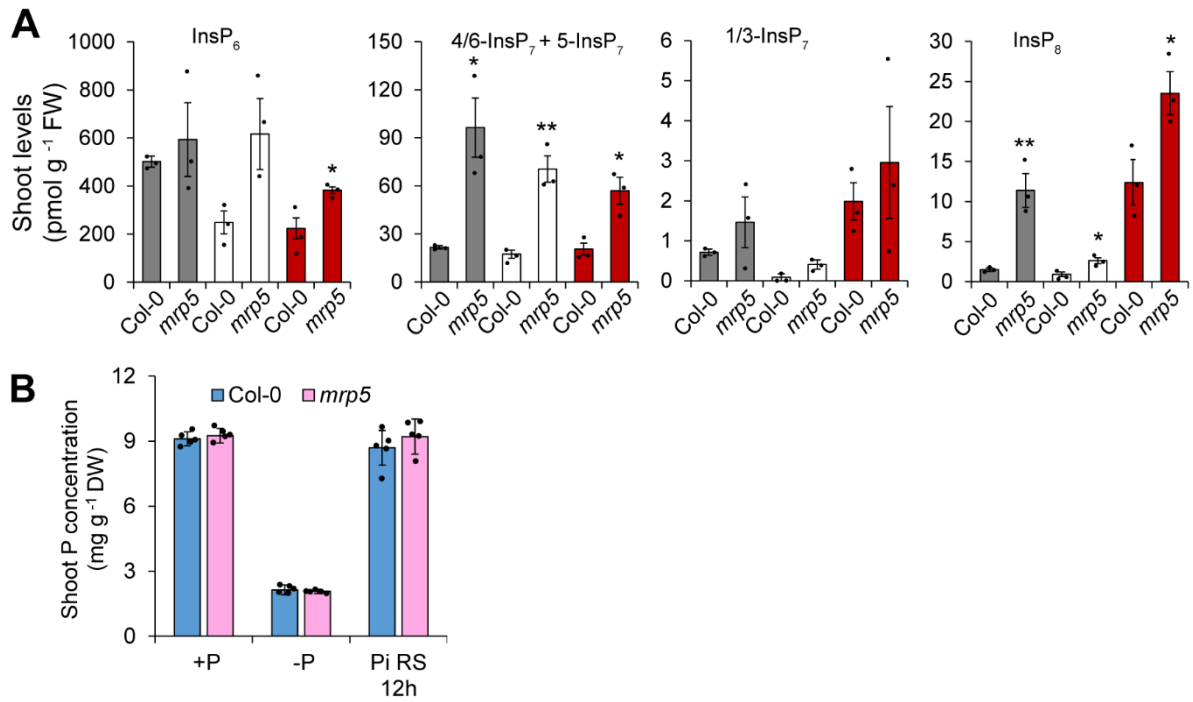
Suppl. Fig. 15. Disruption of *MRP5* increases PP-InsP synthesis in shoots but does not alter P$_i$-dependent regulation of InsP$_8$ synthesis.
(A) CE-ESI-MS analysis of InsP$_6$ and PP-InsPs in shoots of wild-type (Col-0) and *mrp5* plants. Plants were grown in hydroponics in P$_i$-sufficient solution (+P), exposed for 7 days to P$_i$ starvation (-P) or -P resupplied with P$_i$ for 12 h (Pi RS). In this run, a baseline separation of 5-InsP$_7$ and 4/6-InsP$_7$ was not possible and combined results are shown. Data represent means ± SE (n = 3 biological replicates). *P < 0.05 and **P < 0.01 denote significant difference to wild type (Col-0) according to Student's *t*-test. **(B)** Total P concentration in shoots of WT (Col-0) and *mrp5* mutant plants grown as described in **(A)**. Data represent means ± SD (n = 5 plants).

Chapter III: Regulation of plant biotic interactions and abiotic stress responses by inositol polyphosphates

Esther Riemer, Naga Jyothi Pullagurla, Ranjana Yadav, Priyanshi Rana, Henning J. Jessen, Marília Kamleitner, Gabriel Schaaf, Debabrata Laha.

Abstract

Inositol pyrophosphates (PP-InsPs), derivatives of inositol hexakisphosphate (phytic acid, $InsP_6$) or lower inositol polyphosphates, are energy-rich signaling molecules that have critical regulatory functions in eukaryotes. In plants, the biosynthesis and the cellular targets of these messengers are not fully understood. This is because, in part, plants do not possess canonical $InsP_6$ kinases and are able to synthesize PP-InsP isomers that appear to be absent in yeast or mammalian cells. This review will shed light on recent discoveries in the biosynthesis of these enigmatic messengers and on how they regulate important physiological processes in response to abiotic and biotic stresses in plants.

Introduction

Inositol phosphates (InsPs) belong to the multifaceted family of signaling molecules that control a plethora of physiological processes across the eukaryote landscape (Shears, 2015; Thota and Bhandari, 2015; Laha et al., 2021a). These molecules are based on a six-carbon ring structure, *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, commonly referred to as *myo*-inositol (Shears, 2015). Combinatorial phosphorylation of the *myo*-inositol ring could generate a large array of InsP species, of which only a few were identified in cell extracts (Shears, 2015). The physiological functions of most of these InsP species are largely unexplored. Almost 40 years ago, inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] was shown for the first time to act as a second messenger, by acting as a calcium release factor that stimulates its specific receptor/Ca^{2+}-permeable ion channel on endomembranes in pancreatic acinar cells (Streb et al., 1983; Irvine, 2003). In land plants, changes in intracellular $InsP_3$ levels were shown to be responsive to various factors, such as root gravitropism, heat shock signal transduction, mechanical wounding, osmotic stress, pollen dormancy and blue light perception (Knight et al., 1997; Liu et al., 2006; Chen et al., 2008; Mosblech et al., 2008; Wang et al., 2009, 2012). These responses to $InsP_3$ were assumed to be mediated by cytosolic Ca^{2+}, as several studies showed that either treatment with caged photoactivatable compounds to release cytosolic $InsP_3$ or direct $InsP_3$ microinjection resulted

in a transient increase in cytosolic Ca^{2+} (Blatt et al., 1990; Gilroy et al., 1990; Allen and Sanders, 1994; Tucker and Boss, 1996; Monteiro et al., 2005).

Additionally, it was shown that tomato plants expressing the human type I inositol polyphosphate 5-phosphatase (InsP5-ptase), a key enzyme in the phosphoinositide pathway, were more tolerant to drought and light stress, a phenotype that was suggested to be caused by the decrease of $InsP_3$ detected in those plants (Khodakovskaya et al., 2010; Alimohammadi et al., 2012). Notably, even though $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$ levels were shown to be decreased in InsP5-ptase-expressing plants, the role of other InsP species including PP-InsPs were not considered in these studies.

Importantly, genomes of flowering plants do not encode homologs of mammalian $InsP_3$ receptors, which appear to have been lost during the course of evolution (Krinke et al., 2007; Wheeler and Brownlee, 2008; Munnik and Testerink, 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Zhang et al., 2018). Therefore, the role of $Ins(1,4,5)P_3$ in plants remains unresolved. $InsP_6$, also known as *myo*-inositol 1,2,3,4,5,6 hexakisphosphate, phytic acid or phytate, is the most abundant form of InsPs in eukaryotes, with concentrations in the range of 10–100 μM in animal and yeast cells, and up to 500 μM in slime molds (Wundenberg and Mayr, 2012; Pisani et al., 2014). $InsP_6$ is the fully phosphorylated version of *myo*-inositol and serves as a phosphate (P_i) storage molecule during seed development. In this process, $InsP_6$ accumulates in storage microbodies in the form of mixed salts with cations, such as zinc, calcium, iron, potassium, magnesium and manganese (Raboy and Gerbasi, 1996; Otegui et al., 2002; Secco et al., 2017). The storage protein bodies are then degraded during seed germination, leading to the rapid hydrolysis of $InsP_6$ by phytases to provide nutrients to the developing seedling (Raboy and Gerbasi, 1996; Loewus and Murthy, 2000). Due to its strong affinity toward different mineral cations, $InsP_6$ is considered an antinutrient for humans and non-ruminant animals (McCance and Widdowson, 1942; Halsted et al., 1972). Since non-ruminant animals (e.g., pigs and poultry) lack phytases in their digestive tracts, excrements derived from phytate-rich diet contain phytate-bound phosphorus, which is often released in open water bodies, leading to eutrophication and environmental pollution (Rockström et al., 2009; Raboy, 2020).

$InsP_6$ also serves as an important signaling molecule, directly or indirectly as a precursor of "di/pyro-phosphate"-containing inositol polyphosphates, commonly referred to as inositol pyrophosphates (PP-InsPs). These energy-rich InsP species are ubiquitous in eukaryotes, with $InsP_7$ and $InsP_8$ representing the most well-characterized species (Wilson et al., 2013; Shears, 2015). In plants, PP-InsPs control a range of important biological functions, including immune

responses, hormone perception, and phosphate homeostasis (Zhang et al., 2007; Jadav et al., 2013; Laha et al., 2015, 2016, 2020; Jung et al., 2018; Kuo et al., 2018; Dong et al., 2019; Zhu et al., 2019; Gulabani et al., 2021; Land et al., 2021; Riemer et al., 2021).

The metabolic pathways leading to the production of PP-InsPs are well-established in metazoan and yeast. In these organisms, mammalian IP6K/yeast Kcs1-type kinases catalyze the phosphorylation of $InsP_6$ or $1\text{-}InsP_7$ at the 5 position, resulting in the generation of $5\text{-}InsP_7$ or $1,5\text{-}InsP_8$, respectively (Saiardi et al., 1999; Draskovic et al., 2008). Furthermore, mammalian PPIP5K/yeast Vip1 kinases phosphorylate the 1 position of $InsP_6$ and $5\text{-}InsP_7$ to generate $1\text{-}InsP_7$ and $1,5\text{-}InsP_8$, respectively (Mulugu et al., 2007; Lin et al., 2009; Wang et al., 2011; Zhu et al., 2019). The PP-InsP biosynthetic pathway is partially conserved in plants. For instance, while the Arabidopsis genome encodes Vip1 isoforms, genes encoding Kcs1-type kinase(s) could not be identified yet. However, recent studies have demonstrated that the *Arabidopsis thaliana* kinases ITPK1 and ITPK2 phosphorylate $InsP_6$, which is first generated by the phosphorylation of $InsP_5$ [2-OH] by IPK1, to synthesize $5\text{-}InsP_7$ *in vitro* (Adepoju et al., 2019; Laha et al., 2019; Whitfield et al., 2020) and *in planta* (Parvin Laha et al., 2020; Riemer et al., 2021). These proteins belong to the family of ATP-grasp fold proteins with the capability to bind ATP in a cleft between the β sheet toward the central and C-terminal domain (Miller et al., 2005; Josefsen et al., 2007). Notably, their homologs ITPK3 and ITPK4 do not appear to phosphorylate $InsP_6$ *in vitro* or *in vivo* (Laha et al., 2019). The Arabidopsis Vip1 isoforms VIH1 and VIH2 harbor both an N-terminal ATP-grasp kinase domain, as well as a C-terminal phosphatase-like domain and are responsible for $InsP_8$ production *in planta* (Figure 1A; Laha et al., 2015; Zhu et al., 2019).

The recent establishment of novel methods for InsP analyses led to the emergence of several plant PP-InsP studies, which have been instrumental to establish PP-InsP as novel signaling molecules in plants. Remarkably, to date it is still challenging to separate different PP-InsP isomers. This leads to the open question, whether the enantiomers $1\text{-}InsP_7$ or $3\text{-}InsP_7$ and $1,5\text{-}InsP_8$ or $3,5\text{-}InsP_8$ are the main isomers in plants or, if both exist, which of them is the most abundant.

Inositol pyrophosphates play a crucial role in the adaption to several stress responses in plants. Previous work has demonstrated the relevance of $InsP_7$ and $InsP_8$ in responses to hormones such as auxin, salicylic acid or jasmonate (Laha et al., 2015, 2016; Parvin Laha et al., 2020; Gulabani et al., 2021).

In addition, P_i homeostasis in plants was shown to be regulated by kinases involved in InsP synthesis (Kuo et al., 2014, 2018), most likely due to their contribution to the synthesis of $InsP_8$,

which serves as a proxy for P_i (Dong et al., 2019; Zhu et al., 2019; Riemer et al., 2021). Interestingly, certain bacterial plant pathogens interfere with plant $InsP_6$- and potentially PP-InsP-dependent hormone signaling by injecting XopH-like type III effectors that function as 1-phytases (Blüher et al., 2017). However, it is still unclear how this modulation of the host's InsP and PP-InsP status benefits the pathogen (Blüher et al., 2017). Beyond that, recent studies demonstrated a link between pathogen defense and P_i starvation, displaying InsPs and PP-InsPs as crosstalk mediators of abiotic and biotic stresses (Gulabani et al., 2021).

In this review, we present in detail the latest findings of the roles of these phosphate-rich molecules in regulating different biotic and abiotic responses in plants.
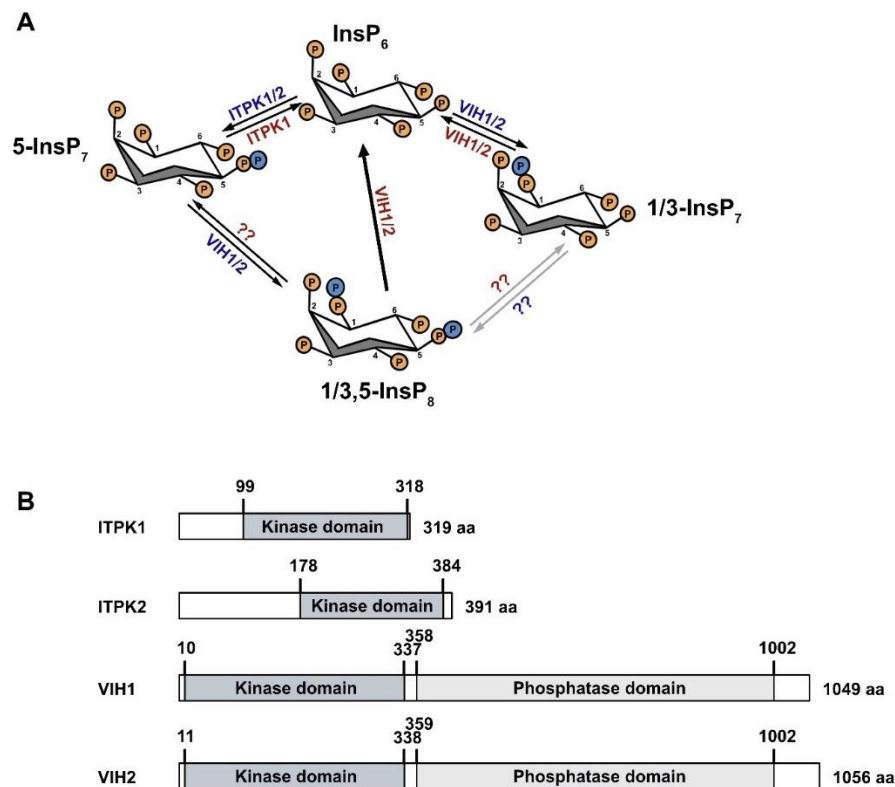
Figure 1. Inositol pyrophosphate biosynthesis pathway in plants and protein architecture of kinases. (A) ITPK1/2 and VIH1/2 phosphorylate $InsP_6$ to generate $5\text{-}InsP_7$ and $1\text{-}InsP_7$, respectively. Further, VIH1/2 use $5\text{-}InsP_7$ as substrate to generate $InsP_8$. The isomer identity of $InsP_8$ remains unresolved but presumably represents the 1,5 and/or the $3,5\text{-}InsP_8$ isomer. VIH1/2 are also able to dephosphorylate $1/3,5\text{-}InsP_8$ and $1/3\text{-}InsP_7$ to $InsP_6$. At low adenylate charge, the ITPK1 kinase domain also catalyzes the reverse reaction from $5\text{-}InsP_7$ to $InsP_6$ in the presence of ADP to locally generate ATP. The gray arrows and question marks denote alternative routes of $1/3\text{-}InsP_7$ and $1/3,5\text{-}InsP_8$ synthesis, and the responsible enzymes, respectively. (B) Schematic representation of ITPK1, ITPK2, VIH1 and VIH2 architectures. Kinase domains are shown in dark gray, phosphatase domains in light gray.

Enzymatic activity of PP-InsP kinases

The function of InsP and PP-InsP kinases is not only limited to the generation of higher inositol pyrophosphates, as they can also shift their activity from PP-InsP synthases to ATP synthases in response to different ATP ratios (Voglmaier et al., 1996; Gu et al., 2017; Zhu et al., 2019; Riemer et al., 2021). It was already shown that mammalian IP6K kinases can transfer a phosphate group from InsP$_7$ to ADP to generate ATP (Voglmaier et al., 1996). Furthermore, both mammalian and yeast IP6K/Kcs1 activities react to changes in cellular ATP levels with respect to the generation of 5-InsP$_7$ (Saiardi et al., 1999; Gu et al., 2017).

In contrast to IP6K/Kcs1, which harbor only a kinase domain, mammalian and yeast PPIP5K/Vip1 harbor both an N-terminal kinase domain and a C-terminal phosphatase domain in the same protein, enabling them to act as bifunctional enzymes (Fridy et al., 2007; Mulugu et al., 2007; Wang et al., 2015; Zhu et al., 2019). As mentioned above, Arabidopsis VIH1 and VIH2 also possess an N-terminal kinase and a C-terminal phosphatase domain (Figure 1B). Similarly to the mammalian IP6K (Voglmaier et al., 1996), Arabidopsis ITPK1 does not only transfer phosphates to inositol polyphosphates but also acts as ATP synthase under varying ATP/ADP ratios or P$_i$ concentrations (Figure 1B). The enzyme has a high K$_M$ of 520 μM for ATP and shifts its activity from kinase to ATP synthase at low adenylate energy charges by transferring the β-phosphate from 5-InsP$_7$ to generate ATP from ADP (Riemer et al., 2021). In addition, ITPK1 exclusively uses 5-InsP$_7$ and no other InsP$_7$ isomer as a substrate for this ADP phosphotransferase activity *in vitro*, in agreement with a high substrate specificity (Riemer et al., 2021). Besides, ITPK1 was shown to act as an InsP(3,4,5,6)$_4$ 1-kinase/InsP$_5$ [2-OH] 1-phosphotranferase to generate ATP from ADP *in vitro* (Whitfield et al., 2020).

Taken together, plant PP-InsP kinases catalyze both the generation and the removal of PP-InsPs. This raises the hypothesis that the enzymes might modulate energy reserves by shifting their activities, for instance, in response to environmental changes, such as phosphorus limitation or sufficiency (Saiardi et al., 1999; Riemer et al., 2021).

Discovery of new PP-InsP$_4$ and InsP$_7$ isomers in plants

The detection and quantification of plant PP-InsP species is challenging due to their low abundance, as well as the presence of high amounts of acid phosphatases in plant extracts, which leads to rapid degradation of PP-InsPs (Laha et al., 2021b). Until recently, Strong Anion Exchange High Performance Liquid Chromatography (SAX-HPLC) and Polyacrylamide Gel Electrophoresis (PAGE) were the most common methods used to analyze InsPs and PP-InsPs (Azevedo and Saiardi, 2006; Pisani et al., 2014). Owing to its easy set-up and low costs, PAGE is still widely employed to resolve higher inositol polyphosphates. The drawback of both of the

above-mentioned methods is the inability to separate PP-InsP isomers. The first clarification of isomer identity of a particular PP-InsP species in plant tissue was possible *via* two-dimensional nuclear magnetic resonance spectroscopy (NMR), by taking advantage of an Arabidopsis *mrp5* mutant (Laha et al., 2019). This mutant is defective in vacuolar loading of $InsP_6$, leading to elevated PP-InsP cyto/nucleoplasmic levels (Nagy et al., 2009; Desai et al., 2014; Laha et al., 2019; Riemer et al., 2021). NMR analyses of *mrp5* seed extracts and comparison with synthetic references demonstrated that $5\text{-}InsP_7$ is the major PP-InsP species present in *mrp5* seeds (Laha et al., 2019).

Coupling of the two powerful tools "capillary electrophoresis" and "electrospray ionization mass spectrometry" (CE-ESI-MS) has enabled new insights into the abundance of InsP and PP-InsP isomers in mammalian cells, yeast, amoeba and plants (Qiu et al., 2020). Due to its high tolerance of complex sample matrices, the combined CE-ESI-MS enables separation of highly charged metabolites with compelling sensitivity. By employing this technique, the generation of $1/3\text{-}InsP_7$ and $5\text{-}InsP_7$ by VIH2 and ITPK1, respectively, was finally confirmed *in planta* (Riemer et al., 2021). Notably, this work also revealed for the first time the presence of $4/6\text{-}InsP_7$ in plants. In Arabidopsis, this $InsP_7$ isomer was found to be more prominent than $5\text{-}InsP_7$ and $1/3\text{-}InsP_7$. However, in contrast to $1/3\text{-}InsP_7$ and $5\text{-}InsP_7$, the new isomer is less responsive to P_i deplete and replete conditions (Riemer et al., 2021) and the function(s) of $4/6\text{-}InsP_7$ and the potential kinase(s) that generate this new isomer in plants are still unknown.

Notably, not only $InsP_6$ is converted to higher PP-InsPs, but also isomers of pentakisphosphates ($InsP_5$) can serve as precursors for the generation of 5-diphosphoinositol tetrakisphosphate ($5PP\text{-}InsP_4$) in yeast and mammalian cells (Wang et al., 2018). For instance, Saiardi et al. (2000) demonstrated that the yeast $InsP_6$ kinase Kcs1 can generate $PP\text{-}InsP_4$ from $InsP_5$ [2-OH] *in vitro*. Interestingly, the affinity of Kcs1 for $InsP_5$ was shown to be threefold higher (mean $K_M = 1.2$ μM) than for $InsP_6$ (mean $K_M = 3.3$ μM). The mammalian IP6K1 also phosphorylates $InsP_5$ [2-OH] to $PP\text{-}InsP_4$, but in this case with similar affinities for $InsP_5$ and $InsP_6$ phosphorylation (Saiardi et al., 2000). While $InsP_5$ levels in yeast are low and therefore probably do not represent the main Kcs1 substrate *in vivo*, this might be different in mammalian cells, where $InsP_5$ [2-OH] and $InsP_6$ levels are similar and represent physiologically relevant substrates of IP6K1 (Saiardi et al., 2000).

A recent study reported the identification of a novel $PP\text{-}InsP_4$ isomer that does not co-migrate with a synthetic $5PP\text{-}InsP_4$ standard, suggesting a distinct structural identity as compared to $PP\text{-}InsP_4$ isomers identified in yeast and mammalian cells (Riemer et al., 2021). CE-MS and PAGE data show that this plant $PP\text{-}InsP_4$ isomer increases under P_i-starvation, as well as under P_i-

resupply conditions, and is not detectable in nutrient-repleted plant roots. Interestingly, in roots of *itpk1* loss-of-function mutants, this novel PP-InsP isomer seems to be less abundant, suggesting that ITPK1 might catalyze the generation of PP-InsP$_4$ *in planta* (Riemer et al., 2021). Interestingly, ITPK1 catalyzes the generation of PP-InsP$_4$ from InsP$_5$ [6-OH] *in vitro* (Whitfield et al., 2020) but it remains to be shown whether plants possess the InsP$_5$ [6-OH] isomer and whether the ITPK1-dependent PP-InsP$_4$ derives from it.

The finding that other PP-InsP species than 1/3-InsP$_7$ or 5-InsP$_7$ were detected in plant extracts unveil an unexplored diversity of inositol pyrophosphates in plants. Also the involvement of putative unknown kinases responsible for the production of additional isomers in environmental responses still have to be investigated.

Inositol pyrophosphate kinases and their role in the adaption of plants to biotic and abiotic stress responses

Inositol pyrophosphate kinases are involved in salicylic acid-dependent immunity

The plant hormone salicylic acid (SA) regulates several processes like flower induction, stomatal closure and heat production mediated by alternative respiration in flowers (Rai et al., 1986; Raskin, 1992). Besides, SA is known to play a crucial role in defense mechanisms against bacteria, fungi, viruses and insects (Raskin et al., 1989; Chaerle et al., 1999; Martínez et al., 2004; Zarate et al., 2007; Kim and Hwang, 2014; Hao et al., 2018). Plant immune responses include the so-called PAMP-triggered immunity (PTI), characterized by the recognition of pathogen-associated molecular patterns (PAMPs, e.g., the bacterial peptide flagellin 22, or flg22), which triggers ion fluxes, ROS production and a series of signaling cascades that ultimately lead to local or systemic responses to restrict pathogen invasion (Seybold et al., 2014). Besides PTI, plants count on a second layer of protection, the effector triggered immunity (ETI), in which plants recognize effector proteins secreted by the pathogen. The ETI usually triggers fast defense reactions, such as hypersensitive response (HR), to promptly restrict pathogen colonization (Dodds and Rathjen, 2010).

Both PTI and ETI are modulated by SA, which is also key for the establishment of systemic acquired resistance (SAR), an additional layer of defense that protects plants from subsequent pathogen attacks (Hõrak, 2020). For instance, SA activates, *via* the regulatory protein NPR1, expression of several pathogenesis-related (PR) genes, which encode different types of proteins with antimicrobial properties (Van Loon et al., 2006; Hõrak, 2020).

A defined role of PP-InsPs in SA-signaling is still unclear. This is because studies showing an involvement of InsPs and PP-InsPs in SA-dependent immunity have in part contradictory

outcomes. Arabidopsis mutants disrupted in InsP$_6$ biosynthesis, for instance, showed increased susceptibility to bacterial, fungal and viral infections (Murphy et al., 2008; Poon et al., 2020), as well as to cyst nematode infestation (Jain, 2015). In fact, the Arabidopsis *ips2* and *ipk1* mutants defective in the activities of enzymes for the first and last steps in InsP$_6$ biosynthesis, respectively, were similarly susceptible to microbial pathogens than *NahG*-transgenic lines and to *sid2* mutants, both of which are unable to accumulate normal levels of SA (Murphy et al., 2008). The SA contents in *ips2* and *ipk1*, however, did not differ from those of wild-type plants, and also increased, similarly to wild-type, after challenge with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 *AvrB* (Murphy et al., 2008). These results indicate that the enhanced susceptibility of *ips2* and *ipk1* is not related to low SA levels, but could be caused by the disruption of InsP$_6$ biosynthesis (Murphy et al., 2008). Further studies of the *ipk1* mutant and of loss-of-function mutants of another *IPS* isoform (*IPS3*) confirmed an involvement of InsP$_6$ in basal pathogen responses (Poon et al., 2020). While displaying a higher susceptibility to *Pst* than wild-type plants, when the *ips2*, *ips3*, and *ipk1* mutants were assessed for SAR acquirement, no impairment was detected. Besides, all mutants except *ipk1* presented flg22-induced resistance to *Pst*, indicating that PTI was inhibited in *ipk1* only (Poon et al., 2020). In this case, however, disruption of InsP$_6$ synthesis in *ipk1* did not affect typical responses to flg22, such as Ca^{2+} influx, oxidative burst, root growth inhibition and activation of PAMP-triggered genes. Taken together, these data suggest that InsP$_6$ biosynthesis is important for maintaining basal resistance against various pathogens, contributing to defense mechanisms different from canonical PTI (Murphy et al., 2008; Poon et al., 2020).

In contrast to findings presented by those previous studies, a recent analysis of *ipk1*, *itpk1*, and *vih2* mutants revealed that these enzymes act as negative regulators of SA-dependent immunity (Gulabani et al., 2021). Mutant plants, in which either InsP$_7$ or InsP$_8$ levels are impaired (Stevenson-Paulik et al., 2005; Sweetman et al., 2007; Desai et al., 2014; Laha et al., 2015; Kuo et al., 2018; Riemer et al., 2021), were significantly more resistant to bacterial infection by *Pst* in comparison to wild-type (Gulabani et al., 2021). Such a response was associated with an apparent constitutive activation of defenses observed in these plants. For instance, they showed a strong upregulation of SA biosynthesis genes, such as *SID2/ICS1*, and higher levels of free or glycolsyl moiety-conjugated SA (SAG) than wild-type plants. Along with these findings, an increase in the expression of *PR1* and *PR2*, together with an accumulation of the respective proteins was observed. Also protein levels of ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and SUPPRESSOR OF nrp1-1 CONSTITUTIVE 1 (SNC1), both of which are required for basal defenses, were higher in these mutants than in wild-type plants, probably due to their

elevated SA levels (Gulabani et al., 2021).

Although previous studies highlighted the importance of $InsP_6$ in maintaining basal defenses against bacteria (Murphy et al., 2008; Ma et al., 2017; Poon et al., 2020), a set of mutants reduced in phytic acid levels, such as *mik-1*, *ipk2β*, or *itpk4*, displayed comparable PTI to wild-type (Gulabani et al., 2021). These findings suggest that $InsP_6$ is not directly involved in triggering plant defenses but point toward a role of higher inositol pyrophosphates in regulating basal immunity (Gulabani et al., 2021).

Currently, it remains unclear whether $InsP_7$ or $InsP_8$ is the main player in SA-mediated defense. Both molecules might act indirectly by the regulation of an antagonistic crosstalk between auxin-SA and jasmonic-acid (JA)-SA, respectively. As described in details in section "As described in the section 5.5," of this review, $5\text{-}InsP_7$ was proposed to regulate auxin signaling by acting as a co-ligand of the ASK1-TIR1-Aux/IAA auxin receptor complex (Parvin Laha et al., 2020), and exogenous application of auxin enhances the *Pst* infestation by interfering with SA-defenses (Navarro et al., 2006; Wang et al., 2007). Therefore, disruption of auxin signaling in *itpk1* mutants might enhance basal immunity (Gulabani et al., 2021). On the other hand, the antagonism between JA-SA crosstalk in plants is well-described and even pathogens have the capability to secrete hormone-mimicking effectors to hijack host defense mechanisms (Zheng et al., 2012; Caarls et al., 2015). For instance, coronatine, a *Pst*-produced phytotoxin that mimics JA, triggers virulence by downregulating SA-dependent defenses in plants (Brooks et al., 2005; Zheng et al., 2012). Furthermore, several studies indicate that endogenous SA is antagonistic to JA-dependent defense mechanisms in plants, leading to a prioritized SA-driven resistance over JA-regulated defense (reviewed in Pieterse et al., 2012). Along these lines, *ipk1* plants primed with injection of air or water to mimic wounding were less susceptible to *Pst* than corresponding wild-type plants that were primed in the same way (Poon et al., 2020). It remains unclear which *ipk1*-dependent inositol phosphate species might be responsible for this phenotype, as *ipk1* mutants are defective in $InsP_6$, $InsP_7$, and $InsP_8$ synthesis (Laha et al., 2015; Gulabani et al., 2021).

Taken together, elevated SA levels and expression of PTI-responsive genes in *ipk1*, *itpk1* and *vih2* might be related to disrupted JA signaling by low PP-InsP levels, causing enhanced SA-defense mechanisms (Gulabani et al., 2021). Further research is needed to unveil the involvement of specific PP-InsPs and other InsP species in regulating plant SA-dependent immunity.

The role of inositol pyrophosphates in jasmonate perception

Jasmonic acid and its derivates, collectively known as jasmonates (JA), play a crucial role in regulating plant development and defense against several necrotrophs and herbivores (Wasternack and Hause, 2013). In response to wounding or herbivory insects, the level of the bioactive JA conjugate jasmonic isoleucine (JA-Ile) is elevated (Fonseca et al., 2009; Koo et al., 2009), which in turn binds to the Coronatine Insensitive 1 (COI1) protein (Feys et al., 1994; Xie et al., 1998; Xu et al., 2002; Katsir et al., 2008), the F-box component of the SCF ubiquitin E3 ligase complex (Devoto et al., 2002). Binding of JA-Ile to COI1 recruits the Jasmonate ZIM Domain (JAZ) transcriptional repressor, which subsequently undergoes polyubiquitylation and SCF^{COI1}-mediated proteasome degradation (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). JAZ degradation then de-represses MYC2 and other transcription factors and consequently triggers the expression of JA-dependent genes (Boter et al., 2004; Browse, 2009). Crystallization of the insect-purified auxin receptor TIR1/IAA complex that contained insect-derived InsP$_6$ provided important information to better understand phytohormone-mediated signaling in plants (Tan et al., 2007). Nano-electrospray mass spectroscopy of the ASK1-COI1 complex that was purified from an insect cell line ectopically expressing the Arabidopsis ASK1-COI1 complex indeed revealed the existence of a molecule whose molar mass corresponded to InsP$_5$ (Sheard et al., 2010). A multistep purification strategy followed by ^{1}H NMR analysis and Total Correlation Spectroscopy (TOCSY) allowed to identify this ligand as either InsP$_5$ [1-OH] or InsP$_5$ [3-OH] (Sheard et al., 2010). In the crystal structure of the ASK1-COI1 complex, the presence of strong electron densities congregating in the core of the solenoid structure likely represents individual phosphates that replaced the insect-derived InsP5 ligand, probably due to high concentrations of ammonium phosphate, used as a precipitant during crystallization (Sheard et al., 2010). To further evaluate the functional role of InsPs in ASK1-COI1-JAZ1 co-receptor complex, a ligand-binding reconstitution assay revealed that both Ins(1,4,5,6)P$_4$ and InsP$_5$ [3-OH] can strongly induce ASK1-COI1-JAZ co-receptor complex formation *in vitro*, whereas InsP$_6$ appeared to be less effective (Sheard et al., 2010). However, it is still unclear whether plants contain InsP$_5$ [3-OH], its enantiomer InsP$_5$ [1-OH] or both isomers.

Several studies pointed to an involvement of InsPs in wound response, as well as disease resistance in plants (Mosblech et al., 2008; Murphy et al., 2008). Arabidopsis plants heterologously expressing human inositol phosphate 5-phosphatase exhibit reduced levels of InsP$_3$ (Perera et al., 2006; Hung et al., 2014) and are found to be susceptible to the cabbage moth *Plutella xylostella* (Mosblech et al., 2008). As previously mentioned, Murphy et al. (2008)

also showed that Arabidopsis *ipk1* and *ics2* mutant plants with defects the in production of $InsP_6$ are compromised in plant defense against bacterial (*Pseudomonas syringae*), viral (*Tobacco Mosaic Virus*), and necrotrophic fungal (*Botrytis cinerea*) pathogens.

To gain insights into the functional role of InsPs in JA signaling, mutant lines defective in putative inositol phosphate binding residues of COI1 were analyzed (Mosblech et al., 2011). Yeast two-hybrid (Y2H) analysis of the COI1 mutant variants revealed reduced interaction with JAZ proteins in presence of the JA analog coronatine, which suggests that InsP binding to the receptor complex might be important (Mosblech et al., 2011). Supporting this statement, Y2H studies using mutant lines defective in InsP biosynthesis revealed an enhanced COI1/JAZ interaction in the *ipk1Δ* yeast strain, which has high levels of $InsP_5$ [2-OH] (Mosblech et al., 2011).

Although one cannot simply compare the yeast *ipk1Δ* mutant with Arabidopsis *ipk1-1* lines, as yeast *ipk1* strains show elevated levels of a specific $PP-InsP_4$ isomer that cannot be detected in Arabidopsis (Saiardi et al., 2002; Laha et al., 2015; Riemer et al., 2021), these studies suggest a potential role of InsPs in regulating JA responses in plants. The enhanced interaction of COI1 and JAZ in *ipk1Δ* yeast strain could also be explained by the high levels of $PP-InsP_4$. Additionally, Arabidopsis *ipk1-1* plants are not only defective in $InsP_6$ but are also severely compromised in $InsP_7$ and $InsP_8$ (Laha et al., 2015). To further evaluate the potential role of $PP-InsPs$ in JA-dependent responses, VIH2-deficient plants defective in $InsP_8$ synthesis were investigated (Laha et al., 2015). The mutant plants had unchanged levels of $InsP_5$ [2-OH], but were shown to be severely susceptible to the generalist herbivore *Mamestra brassicae* and the Brassicaceae specialist *Pieris rapae*. This suggests that VIH2-dependent $InsP_8$ but not $InsP_5$ [2-OH] is critical for defense against these insects (Laha et al., 2015). In addition, *vih2* mutants showed reduced expression of JA-dependent genes, despite an increase in JA. Therefore, the compromised resistance of Arabidopsis *vih2* mutants against herbivory insects might be explained by a defect in JA perception, and not by compromised JA production (Laha et al., 2015). Additionally, *in vitro* binding assays of ASK1-COI1-JAZ1-coronatine with different radiolabeled InsPs indicated that higher inositol polyphosphates, such as $InsP_6$ and $InsP_7$, are capable to bind to the JA-receptor complex with higher efficiency than lower InsPs (Laha et al., 2015, 2016). Unfortunately, plant-purified $InsP_8$ was not included in these binding assays due to its low amounts in plants and its high susceptibility to acid phosphatases present in plant extracts (Laha et al., 2015).

Taken together, it has been proposed that coincidence detection of VIH2-dependent $InsP_8$ and JA is important for plant defense against necrotrophic and herbivorous pathogens (Laha et al.,

2015, 2016). While these studies provide some mechanistic insights into the role of $InsP_8$ in JA responses, future work is needed to clarify the molecular basis of VIH2 functions. For instance, it has not been established whether the catalytic activity of VIH2 solely contributes to JA responses, or whether VIH2 regulates JA responses through a yet unidentified mechanism. It might be also interesting to learn whether the phosphatase domain of VIH2 contributes to JA-related defense responses.

The studies presented here led to the assumption that plants are able to use different InsPs to cope against several pathogens and herbivores (Mosblech et al., 2011; Laha et al., 2015). However, whether InsPs allow plants to differentially respond against pathogens and how this process takes place remains an interesting question. The precise mechanism by which the JA co-receptor complex discriminates and specifically binds to a particular InsP isomer is still unclear. Furthermore, the physiological relevance of various InsP isomers in the context of the JA signaling pathway is still unsolved. It would be interesting to explore the possibility that different InsPs could form a series of distinctive JA-co-receptor complexes, which would help plants to induce specific immune responses against distinct pathogens.

The 1-phytase activity of *Xanthomonas* type III effector XopH

Several Gram-negative bacteria of the genus *Xanthomonas* cause diseases in different plant hosts, such as pepper, rice, wheat, tomato, citrus, cabbage, and banana, leading to substantial crop yield losses (Ryan et al., 2011; Jacques et al., 2016). A broad range of factors influence host specificity and pathogenicity. These include bacterial lipopolysaccharides, adhesins, transcription factors and TonB-dependent receptors, as well as the type III secretion system (T3SS) (Raetz and Whitfield, 2002; Ghosh, 2004; Blanvillain et al., 2007; Das et al., 2009; Büttner, 2016). The latter is responsible for the translocation of effector proteins into the plant cell cytosol (Büttner, 2016; Constantin et al., 2017; Newberry et al., 2019). The tomato and pepper pathogen *Xanthomonas campestris* pv. *vesicatoria* (Xcv) encodes more than 30 T3S effector proteins, which are generally designated as Xops (*Xanthomonas* outer proteins) and are known to cause characteristic bacterial spot disease symptoms (Thieme et al., 2005; Teper et al., 2015). In resistant plants, the effectors are recognized by immune receptors, often leading to HR at the infected area to suppress spreading of biotrophic pathogens from the site of infection (Goodman and Novacky, 1994; Mur et al., 2008). One member of the Xops effector family, XopH, depicts typical features of dual-specific protein phosphatases and can dephosphorylate the generic substrate p-nitrophenyl phosphate (pNPP) (Potnis et al., 2012). Blüher et al. (2017) reported a novel phytate-degrading activity of XopH *in vitro* and *in planta*, which is assumed to account for the activation of HR in resistant plants. Using a novel NMR

method coupled with spiking experiments, as well as biochemical studies with recombinant XopH, the authors identified XopH as a 1-phytase that cleaves the phosphate from the C1 hydroxy group of $InsP_6$, resulting in the generation of $InsP_5$ [1-OH] (Blüher et al., 2017). HPLC data of *S. cerevisiae* and *N. benthamiana* ectopically expressing XopH revealed a reduction of $InsP_6$ and a strong accumulation of $InsP_5$ [1/3-OH] also *in vivo* (Blüher et al., 2017). To confirm whether XopH executes 1-phytase activity *in planta*, the authors performed XopH digestion of $InsP_5$ [1/3-OH] species purified from [3H]-*myo*-inositol-labeled transgenic *N. benthamiana* overexpressing *xopH*. The plant-purified $InsP_5$ [1/3-OH] was resistant to XopH degradation and was not phosphorylated by plant enzymes, supporting the idea that this PP-InsP isomer is absent in plants and is more likely a product of XopH phytase activity (Blüher et al., 2017). Strikingly, the XopH-induced HR in pepper plants harboring the *Bs7* resistance (R) gene seems to be dependent on the effector's phytase activity. This led to the assumption that Bs7 more likely recognizes the result of XopH activity, such as changes in inositol polyphosphate levels, but not the protein itself (Blüher et al., 2017). It was also observed that heterologous expression of XopH in *N. benthamiana* resulted in a strong reduction of $InsP_7$ and $InsP_8$, presumably interfering with $InsP_7$- and $InsP_8$-dependent hormone signaling. In agreement with this, qRT-PCR analysis of *N. benthamiana* leaves constitutively expressing *xopH* showed an induction of the JA marker genes *PR1b*, *PR4*, and *PI-II* after wounding, strengthening the involvement of the effector protein in JA signaling (Blüher et al., 2017). Since those genes are also responsive to ethylene (ET), a hormone acting synergistically to JA, it cannot be excluded that XopH also affects the ET pathway. Indeed, virus-induced gene silencing of *EIN2* and *EBF1*, which are the positive and negative regulators of the ET pathway, respectively, caused the suppression of *xopH*-induced upregulation of *PR4* and *PI-II* in *N. benthamiana* (Donnell et al., 1996; Adie et al., 2007; Zhu and Lee, 2015; Blüher et al., 2017).

It still remains unclear for what purpose Xanthomonas secretes XopH into the host cells. One possibility is that the XopH phytase activity might release phosphate from the plant tissue, which could enhance the nutritional status of the pathogen. A similar activity was observed for the phytase PhyA, which is secreted by the rice pathogen *X. oryzae* pv. *Oryzae*. It was suggested that this bacterial pathogen uses phytate as the sole phosphate source and that this activity contributes to its virulence (Chatterjee et al., 2003). In addition, XopH might also degrade higher inositol pyrophosphates and thereby influence hormone signaling pathways of the host, leading to manipulation of JA- or ET-mediated defense responses to the pathogen's benefit (Blüher et al., 2017).

Inositol pyrophosphates are involved in phosphate homeostasis

Phosphorus is an essential element and a key determinant for growth and development of all living organisms, as it composes essential molecules such as ATP, nucleic acids and phospholipids (Marschner, 1995). Plants take up phosphorus in the form of P_i, which is highly immobile, chemically fixated, as well as unevenly distributed in soils, causing a very limited access of available P_i (Holford, 1997; Seidel et al., 2021). Plants respond to low P_i levels by metabolic changes such as an increase of sulfo- and galactolipids at the expense of phospholipids (Essigmann et al., 1998; Härtel et al., 2000) and by increasing RNA degradation to release phosphate for other cellular processes (Taylor et al., 1993; Bariola et al., 1994). Furthermore, P_i-starved plants increase P_i acquisition *via* the production and secretion of phosphatases, exudation of organic acids, modification of root architecture and development of root hairs, as well as enhanced expression of P_i transporters (Karthikeyan et al., 2002; Mudge et al., 2002; Rausch and Bucher, 2002; Vance et al., 2003; Shin et al., 2004; Plaxton and Tran, 2011; Péret et al., 2011). These metabolic, morphological and transcriptional mechanisms belong to the so called phosphate starvation response (PSR), which is interrupted upon P_i replenishment (Vance et al., 2003; Chiou and Lin, 2011; Secco et al., 2013).

The majority of P_i starvation-induced (PSI) genes in plants is regulated by the MYB-CC transcription factor PHOSPHATE STARVATION RESPONSE REGULATOR 1 (PHR1) and its homolog PHR1-LIKE 1 (PHL1) (Rubio et al., 2001). PHR1 is expressed under P_i-sufficient conditions and controls P_i signaling and homeostasis through binding as a dimer to an imperfect palindromic sequence (PHR1-binding sequence, or P1BS) present in the promoters of P_i starvation-induced genes (Rubio et al., 2001).

Recent studies showed that a class of stand-alone SPX (SYG1/Pho81/XPR1-domain containing protein 1) proteins negatively regulates the activity of PHR transcription factors by high affinity binding to PHRs under sufficient P_i supply (Puga et al., 2014). The formation of the SPX-PHR complex in turn prevents the binding of the transcription factors to the P1BS motifs, thereby repressing the expression of PSI genes. Under low P_i conditions, the binding affinity of SPX to the PHRs is decreased, leading to the activation of their transcriptional targets (Puga et al., 2014; Qi et al., 2017).

Structural studies of SPX domains from proteins of different organisms indicate that PP-InsPs bind to SPX domains on a conserved cluster of basic residues and regulate the activity of such proteins, as shown for an SPX-containing component of the Vacuolar Transporter Chaperone (VTC) complex that mediates polyphosphate synthesis in baker's yeast (Wild et al., 2016). Similar conserved clusters of basic residues at the surface of the SPX N-terminus were also

identified in plant SPX proteins (Wild et al., 2016).

Recently, Dong et al. (2019) demonstrated that $InsP_8$ binds to the rice OsSPX1 domain with a Kd of approximately 5.7 μM *in vitro*. In addition, Co-IP results revealed that SPX1 is not able to interact with PHR1 under P_i starvation conditions but can be restored by adding 1 μM $InsP_8$ (Dong et al., 2019). On the other hand, the SPX-PHR interaction cannot be restored by the addition of $InsP_7$, corroborating the idea that $InsP_8$ but not $InsP_7$ acts as the key regulator of P_i starvation responses in plants (Dong et al., 2019).

Several *in vivo* studies confirmed the involvement of PP-InsPs in PSR in plants. Arabidopsis mutants defective in IPK1 activity exhibit a disturbed phosphate starvation phenotype (Kuo et al., 2014). This results in an increased P_i overaccumulation when grown under P_i sufficient conditions and P_i accumulation in response to increasing external P_i concentrations (Stevenson-Paulik et al., 2005; Kuo et al., 2014, 2018). In addition, the mutants displayed reduced levels of $InsP_6$, $InsP_7$, and $InsP_8$ (Laha et al., 2015; Kuo et al., 2018; Land et al., 2021).

Under P_i-replete conditions, the loss of *ITPK1* but not of *ITPK2* causes a robust overaccumulation of P_i similar to what was observed in *ipk1* plants, even though only a decrease in 5-$InsP_7$ and not in $InsP_8$ was observed in *itpk1* plants under such conditions (Riemer et al., 2021). In contrast, P_i-starved *itpk1* plants that were resupplied with P_i displayed strong defects in both 5-$InsP_7$ and $InsP_8$ synthesis, again coinciding with a robust PSR phenotype (Riemer et al., 2021). An earlier study reported reduced $InsP_8$ levels of *itpk1* plants, as revealed by PAGE analyses also under P_i-replete conditions (Wang et al., 2021). The difference between the works of Riemer et al. (2021) and Wang et al. (2021) might be explained by different growth conditions employed by these two independent studies, including different P_i-availabilities at the P_i-replete condition.

While disruption of VIH1 and VIH2 did not cause any PSR phenotype, such as PSR gene expression and P_i-accumulation under P_i-replete conditions (Kuo et al., 2018; Land et al., 2021; Riemer et al., 2021), loss of VIH2 caused a mild PSR phenotype upon P_i-resupply to P_i-starved plants (Riemer et al., 2021). Importantly, *vih1 vih2* double mutants (in which the respective kinase domains are defective) are seedling lethal (Zhu et al., 2019).

This is explained by the severe PSR phenotype of the double mutant seedlings caused by the strong overaccumulation of P_i, confirmed by the high expression of P_i starvation marker genes (Dong et al., 2019; Zhu et al., 2019). On the PP-InsP level, an increase in 5-$InsP_7$ was observed in the double mutant, while $InsP_8$ was below the limit of detection (Zhu et al., 2019). In contrast, HPLC profiles of a *vih1 vih2* double mutant shown in Land et al. (2021) displayed reduced $InsP_7$ and $InsP_8$ levels. It is worth mentioning that the T-DNA insertion in this particular *vih2*

allele (*vip1-2*) is positioned outside the core *VIH2* kinase domain. Taken together, the disruption of both *VIH1* and *VIH2* appears to result in the loss of the plant's ability to maintain intracellular P_i levels due to defective $InsP_8$ synthesis (Dong et al., 2019; Zhu et al., 2019). Notably, the *itpk1 vih2* double mutant displays inhibited plant growth and an increase of approximately 27% in shoot P levels (Riemer et al., 2021). This strongly suggests that the combined activities of ITPK1 and VIH2 are critical for maintaining P_i homeostasis in plants, by concomitantly generating both the precursor (5-$InsP_7$) as well as the main substrate ($InsP_8$) of P_i sensing (Figure 2; Riemer et al., 2021). Lack of a PSR phenotype of ITPK4-deficient plants that display reduced levels of $InsP_6$, $InsP_7$, and $InsP_8$ (Kuo et al., 2018; Wang et al., 2021) suggests that regulation of phosphate homeostasis by InsP and PP-InsPs might be even more complex. Future work should try to clarify the identities of $InsP_7$ and $InsP_8$ isomers by CE-ESI-MS analyses and, by taking advantage of chiral selectors, to address the question which PP-InsP species are altered. Besides, whether certain $InsP_7$ or $InsP_8$ isomers, or even enantiomers, play antagonistic roles in regulating the interaction of free standing SPX proteins with PHR1/PHL1 still needs clarification.
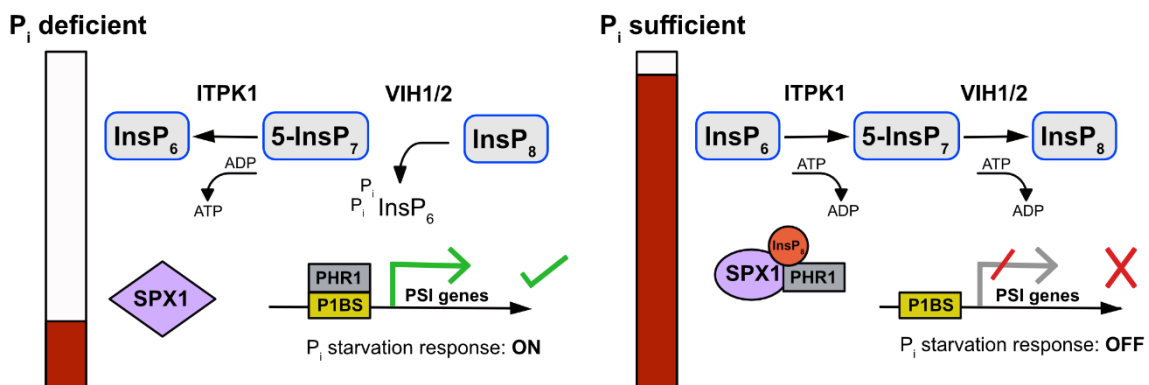

Figure 2. Model for the ITPK1-dependent phosphorylation of $InsP_6$ and 5-$InsP_7$ removal and possible link of ITPK1 with VIHs and phosphate homeostasis. Upon P_i-deficiency, ATP levels drop and stimulate ITPK1 to transfer the P-phosphate from 5-$InsP_7$ to ADP, leading to the local generation of ATP and decreased 5-$InsP_7$ levels. Additionally, low ATP/ADP ratios (i.e., low adenylate charge) and low P_i levels cause the switch from kinase to phosphatase activity of VIH proteins to hydrolyze $InsP_8$. Lacking PP-InsPs, the interaction between PHR1 and SPX1 is destabilized, which promotes the binding of PHR1 to the P1BS motif in the promoter region of PSI genes. As a result, the P_i starvation response is activated. When plant cells regain sufficient P_i, ATP levels increase and the kinase activity of ITPK1 is activated, leading to the generation of 5-$InsP_7$, which further serves as substrate for the kinase-activated VIH proteins to produce $InsP_8$. Consequently, the accumulation of PP-InsPs facilitates the binding of SPX proteins to PHR1 to suppress Pi starvation responses.

Recent studies have pointed to a connection between plant's P_i status and immune responses (Campos-Soriano et al., 2020; Val-Torregrosa et al., 2022). These findings are based on the involvement of a miRNA species (miR399) in the regulation of P_i homeostasis in Arabidopsis (Chiou et al., 2006; Paul et al., 2015). Upon P_i starvation, miR399 accumulates and represses its target gene *PHOSPHATE2* (*PHO2*, encoding an E2 ubiquitin-conjugating enzyme) that is responsible for phosphate transporter degradation, leading to an enhanced P_i uptake in plants (Fujii et al., 2005; Kraft et al., 2005; Chiou et al., 2006; Liu et al., 2012; Huang et al., 2016). In rice, miR399 overexpression resulted in P_i accumulation in leaves and higher susceptibility to the fungal pathogen *Magnaporthe oryzae*, which was also observed upon high P_i fertilization (Campos-Soriano et al., 2020). In contrast, Val-Torregrosa et al. (2022) demonstrated an enhanced resistance to necrotrophic and hemibiotrophic fungal pathogens in Arabidopsis lines overexpressing miR399, as well as in *pho2* loss-of-function lines. The high P_i accumulation in Arabidopsis leaves caused by *miR399* overexpression and lack of functional PHO2 was linked to an elevated ROS production. This was assumed to be related to an increased HR in these plants during pathogen infection. Besides the changes in ROS levels, the mutant lines showed elevated SA and JA levels, combined with the upregulation of SA- and JA-dependent defense genes (Val-Torregrosa et al., 2022). Intriguingly, *pho2* mutants were also shown to accumulate high levels of $InsP_8$ (Riemer et al., 2021). As previously mentioned, $InsP_8$ is a key player in JA- and P_i signaling (Laha et al., 2015, 2016; Dong et al., 2019; Riemer et al., 2021), raising the hypothesis that P_i homeostasis and pathogen defense mechanisms might be linked by the plant's PP-InsP status.

Recently, Gulabani et al. (2021) demonstrated that the products of IPK1, ITPK1 and VIH2 kinase activities also function as crosstalk mediators between pathogen defense and P_i homeostasis, and that these enzymes act as suppressors of SA-dependent defense mechanisms. Strikingly, previous studies indicated the suppression of SA-responsive genes by PHR1 and as a consequence, *phr1 phl1* double mutants appear to be more resistant to infections with *PstDC3000* (Castrillo et al., 2017).

While *PR1* and *PR2* transcripts are upregulated in *ipk1*, *itpk1*, and *vih2* lines, which are compromised in $InsP_8$ levels or disrupted in functional PSR, the opposite was observed with the introduction of *ipk1* and *itpk1* into the *phr1 phl1* mutant background (Gulabani et al., 2021). In this case, a reduced expression of both SA marker genes in comparison to Col-0 and *phr1 phl1* was observed (Gulabani et al., 2021). The authors assumed that the downregulation of SA-associated defense genes in PP-InsP-compromised mutants is stimulated by a PHR1/PHL1-dependent increase in PSR. Furthermore, it is known that PSI genes might harbor SA-inducible

elements in their promoters (Baek et al., 2017). Double mutants of the SA-biosynthesis gene *SID2* in the *ipk1* and *itpk1* backgrounds, respectively, indeed resulted in decreased P_i overaccumulation phenotypes. Exogenous application of SA to wild-type plants led to increased transcripts of the PSI gene *SPX1* or the PAMP-responsive gene *WRKY38*, both shown to be reduced in the *ipk1 sid2* and *itpk1 sid2* mutants, strengthening the hypothesis that SA may directly activate the transcription of PSI-genes (Gulabani et al., 2021). The phenotypes observed in *ipk1*, *itpk1* and *vih2* mutants are assumed to be related to the low $InsP_8$ concentration that was observed at least in *ipk1* and *vih2* lines (Gulabani et al., 2021; Riemer et al., 2021), supporting a further putative link between PP-InsP-driven PSR and the capability to defend against pathogens.

To summarize, the connection of PSR and pathogen defense might give another perspective of how P_i is managed in crops. By having a deeper understanding of the factors affecting P_i homeostasis in plants, a more precise adjustment of fertilizer conditions may be employed in the field. This might help, for instance, to avoid strong pathogen infestation caused by excessive application of P_i, as well as to reduce environmental pollution and the depletion of global P_i-deposits, all of which will improve sustainability in crop production.

The role of inositol phosphates in auxin signaling

Auxin regulates a multitude of plant functions, including cell division, elongation, differentiation, embryonic development, root and stem tropisms, apical dominance, and flower formation (Young et al., 1990; Woodward and Bartel, 2005; Tanaka et al., 2006; Möller and Weijers, 2009; Leyser, 2010; Müller and Leyser, 2011; Christie and Murphy, 2013; Gallavotti, 2013; Geisler et al., 2014). This phytohormone coordinates those physiological processes by modulating the transcription of auxin-responsive genes through the action of the three protein families: TRANSPORT INHIBITOR RESPONSE1 (TIR1) and AUXIN- SIGNALING F-BOX proteins (AFB1-5), Aux/indole-3-acetic acid (IAA) transcriptional repressors, and the AUXIN RESPONSE FACTORS (ARFs) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Auxin mediates their functions by binding to TIR1/AFB F-box proteins, enhancing the interaction of TIR1/AFB with Aux/IAAs repressors, which are in turn degraded by the Skp, Cullin, F-box-containing complex (SCF) ubiquitin ligase to activate ARF transcription factors (Wang and Estelle, 2014; Salehin et al., 2015). The Arabidopsis genome encodes 6 TIR1/AFBs, 29 Aux/IAA proteins, and 23 ARFs, which act combinatorically to regulate a wide range of auxin-dependent processes (Calderón Villalobos et al., 2012; Shimizu-Mitao and Kakimoto, 2014; Dinesh et al., 2016). The auxin co-receptors TIR1/AFB proteins comprise an F-box domain in the N terminus and 18 Leucine-rich repeat (LRR) domains at the C terminus (Tan et

al., 2007). Structural analyses of the auxin co-receptor complexes purified from an insect cell line ectopically expressing Arabidopsis ASK1-TIR1 were instrumental in unveiling the molecular basis of auxin perception (Tan et al., 2007). The TIR1 crystal structure contained insect-derived $InsP_6$ as a cofactor (Tan et al., 2007). $InsP_6$ interacts with a highly basic surface area formed by 10 positively charged residues of TIR1, supporting the formation of the auxin binding pocket. These residues are also conserved in Arabidopsis AFBs, suggesting its binding importance in this subfamily of F-box proteins. When TIR1 is mutated in three residues that are involved in the coordination with $InsP_6$, it fails to interact with either IAA7 or ASK1, implying a key role of $InsP_6$ in the structural architecture of TIR1 (Calderón Villalobos et al., 2012). $InsP_6$ interacts primarily with halves of the TIR1-LRR solenoids, loop-2, and the Arg403 residue. The Arg403 residue also binds to the carboxy group of auxin and is essential for the structural function of TIR1 (Calderón Villalobos et al., 2012). The authors demonstrated further that the mutation in His78, Arg403, and Ser438 residues of TIR1, which are involved in both auxin and $InsP_6$ binding, failed to reconstitute the interaction between TIR1 and IAA7 in the presence of auxin (Calderón Villalobos et al., 2012). While these findings suggest an important function of InsP binding to TIR1, it needs to be investigated whether the designated $InsP_6$ binding pocket of TIR1 can accommodate also other InsPs or is specific to $InsP_6$. Even before $InsP_6$ was identified as cofactor for the auxin receptor complex, inositol polyphosphates have been linked with several auxin-dependent physiological processes (Xu et al., 2005; Zhang et al., 2007). In Arabidopsis, two Inositol 1,4,5-Trisphosphate 3-Kinases (IPK2α and IPK2β) were found to harbor 6-/3-kinase activities and sequentially phosphorylate $Ins(1,4,5)P_3$ to generate $InsP_5$ [2-OH] *via* an $Ins(1,3,4,6)P_4$ intermediate *in vitro* (Stevenson-Paulik et al., 2002; Xia et al., 2003). Expression analyses of *IPK2α* and *IPK2β* in different tissues of Arabidopsis plants pointed to a role of those kinases in plant growth and development (Xia et al., 2003; Xu et al., 2005). Silencing of *IPK2α* through antisense gene expression led to enhanced root growth and pollen germination in transgenic Arabidopsis plants (Xu et al., 2005), both of which are auxin-regulated processes (Fu and Harberd, 2003; Wu et al., 2008). Subsequent work investigating the physiological functions of IPK2β kinase uncovered that *IPK2β* is an early responsive gene that regulates axillary branching by an auxin signaling pathway. Furthermore, the application of exogenous IAA induced *IPK2β* expression and overexpressing of *IPK2β* results in altered auxin responses such as lateral root formation and primary root development (Zhang et al., 2007). Reverse Transcription-Polymerase chain reaction analysis (RT-PCR) of *IPK2β* overexpression lines revealed decreased expression of *CYP83B1*, a regulator of auxin production (Bartel et al., 2001; Woodward and Bartel, 2005; Zhang et al., 2007), and enhanced

expression of *PIN4*, which mediates auxin transport (Friml, 2003; Zhang et al., 2007). Moreover, the expression levels of *MAX4* and *SPS*, which are required for auxin-mediated shoot branching, was downregulated in *IPK2β* overexpression lines (Tantikanjana et al., 2001; Sorefan et al., 2003; Bainbridge et al., 2005; Zhang et al., 2007). Future work on auxin responses using *ipk2β* knockout lines will provide more insight into the IPK2β functions in auxin signaling. *IPK2α* and *IPK2β* are homologous genes with high sequence similarities (Stevenson-Paulik et al., 2002), and deletion of a single gene might not reveal its biological function due to redundancy. To date, the generation of *ipk2α ipk2β* double mutants was not successful because the homozygous double knockout appears to be lethal probably due to defects in pollen development, pollen tube guidance, and embryogenesis (Zhan et al., 2015). As such, the catalytic dead variants of *IPK2β* could not complement *ipk2α ipk2β*–associated lethality, suggesting an essential role of inositol polyphosphate signaling in plant reproduction (Zhan et al., 2015).

Other InsP kinases were found to be also involved in auxin-dependent physiological processes. Notably, transcriptome analysis of *ipk1-1* plants showed that genes involved in root hair differentiation and root system development were misregulated in the mutant line. Moreover, *ipk1-1* plants display a phenotype similar to the *mrp5* mutant (Kuo et al., 2014). *MRP5* encodes an ABC-type transporter mediating $InsP_6$ loading into the vacuole (Nagy et al., 2009). In consequence, *mrp5* mutant plants display reduced levels of $InsP_6$, as well as elevated cytoplasmic levels of $InsP_7$ and $InsP_8$ (Desai et al., 2014; Laha et al., 2019), and exhibit a root system architecture (RSA) phenotype in response to elevated auxin (Gaedeke et al., 2001). Further, *ipk1-1* plants having reduced levels of $InsP_6$ also exhibit an altered RSA, which might be caused by compromised auxin signaling (Kuo et al., 2014). In line with this, the *ipk1-1* mutant exhibited defects in gravitropic responses. Both *ipk1-1* and *mrp5* mutant plants were also insensitive to exogenous auxin supply, as evidenced by an increase in relative primary root length (Gaedeke et al., 2001; Laha et al., 2020). Taken together, these findings put forward the importance of IPK1 function in auxin signaling. The fact that the *mrp5* mutant has elevated levels of $InsP_7$ and $InsP_8$ (Desai et al., 2014; Laha et al., 2019; Riemer et al., 2021), whereas *ipk1-1* is severely compromised in those PP-InsP species (Laha et al., 2015), raise the possibility that the decreased levels of $InsP_6$ or PP-InsP might contribute to auxin signaling. To further corroborate the role of PP-InsPs in auxin responses, the *itpk1* and *vih2* mutant lines were investigated. The *itpk1* plants were shown to be defective in primary root elongation, leaf venation and compromised gravitropic root curvature, as well as thermomorphogenic adaptation, all of which are reminiscent of auxin deficient phenotypes (Laha et al., 2020). In

auxin sensitivity assays, *itpk1* plants displayed resistance to exogenous auxin, which could be fully rescued by *itpk1* lines carrying a genomic *ITPK1* fragment. This reinforces the idea that phenotypic defects of *itpk1* mutant lines might be related to impaired auxin perception (Laha et al., 2020). ITPK1-deficient plants are defective not only in 5-InsP$_7$ synthesis but are also perturbed in lower inositol phosphates homeostasis (Laha et al., 2019, 2020; Riemer et al., 2021), and their role in building auxin receptor complexes cannot be ignored (Laha et al., 2020). Specifically, HPLC profiles of both *itpk1* and *ipk1* mutants show reduced levels of InsP$_5$ [1/3-OH], InsP$_7$, and InsP$_8$ and an increase in InsP$_{4a}$, an unknown InsP$_4$ isomer (Stevenson-Paulik et al., 2005; Laha et al., 2015).

Taken together, these results suggest that one or several inositol polyphosphate isomers might be important for auxin signaling. Future work is needed to clarify whether the control of auxin responses depends on the catalytic activity of ITPK1. Furthermore, the *vih2* mutant lacking detectable InsP$_8$ levels, as revealed by SAX-HPLC, did not exhibit auxin-related phenotypes, suggesting that InsP$_8$ might not be critical for auxin responses (Laha et al., 2020). To further clarify the role of InsP$_8$ in auxin signaling, future work on *vih1 vih2* double knockout lines is necessary to account for a potential redundancy of the two VIH homologs.

Notably, competitive binding assays revealed that InsP$_6$ and 5-InsP$_7$ bind with similar affinities to the TIR1-ASK1-Aux/IAA7 auxin receptor complex (Laha et al., 2020). Considering the large amount of InsP$_6$ present in plant cell extracts, an obvious question is how InsP$_7$ (which comprises around 3% of global InsP$_6$) could specifically control auxin perception. As mentioned earlier, several studies established that the major pool of InsP$_6$ is stored in the vacuole (Nagy et al., 2009; Desai et al., 2014; Laha et al., 2019; Riemer et al., 2021), suggesting that the cyto/nucleo-plasmic concentration of InsP$_6$ and InsP$_7$ is distinct from the global cellular pool of InsP$_6$ and InsP$_7$. Investigating the localization of InsP$_6$ and InsP$_7$ at different compartments with the development of InsP$_6$- and InsP$_7$-specific sensors might clarify many of these open questions. Interestingly, a previous study reported that an InsP$_6$ kinase interacts with certain protein complexes to generate InsP$_7$ in close proximity to dedicated effector proteins (Rao et al., 2014). Similarly, recent work in Arabidopsis suggests that ITPK1 physically interacts with TIR1, presumably to channel 5-InsP$_7$ to the auxin receptor complex (Laha et al., 2020). In addition, the potential of InsP molecules to induce a conformational change in TIR1 and promote the degradation of AUX/IAA is another conjecture to be solved. Knowing that different inositol phosphates have different affinities toward the auxin receptor complex is intriguing and raises the question whether these InsP molecules act differentially by forming distinct sets of auxin receptor complexes to regulate diverse auxin-related physiological

processes. Altogether, many unsolved puzzles demand further research to identify the mechanism behind these phosphate-rich molecules playing a pivotal role in auxin-mediated plant growth and development.

In addition to the role of auxin in plant developmental and growth processes, several studies have also implicated a role of auxin in abiotic and biotic stresses (Cheong et al., 2002; Dowd et al., 2004; Hannah et al., 2005; Navarro et al., 2006; Wang et al., 2007; Jain and Khurana, 2009). The expression profiles of auxin-responsive genes of plants subjected to different biotic and abiotic stresses have pointed to a potential role of auxin in regulating plant defense responses, suggesting a possible crosstalk between auxin, abiotic and biotic stress signaling pathways (Ghanashyam and Jain, 2009). Recent findings revealed a potential role of auxin in regulating host-pathogen interaction. Auxin produced by different plant-associated microbes promotes disease susceptibility and antagonizes plant defense responses (Kunkel and Harper, 2018). Furthermore, *Arabidopsis thaliana* mutant lines defective in auxin signaling and perception showed increased levels of bacterial growth and suppressed host defenses, highlighting the role of auxin in biotic stress modulation (Djami-Tchatchou et al., 2020). Future work is needed to clarify whether inositol polyphosphates are involved in auxin-mediated pathogen defense responses.

Outlook

Here we pointed out the several roles InsPs and PP-InsPs play in regulating biotic and abiotic stress responses, and highlight these molecules as supporting modulators of plant metabolism to adapt to several environmental conditions (Figure 3). The recent development of more sensitive tools for the detection and quantification of low abundant PP-InsPs like CE-ESI-MS provides new insights into the large network of these molecules in eukaryotic systems (Qiu et al., 2020). However, the separation of enantiomeric PP-InsPs such as $1/3$-InsP$_7$ and $4/6$-InsP$_7$ still remains challenging with current chromatographic and electrophoretic methods. Future research needs to develop methods to distinguish between the mirror images to delineate which isomers are relevant in living plants. Besides the identification of the enantiomeric identity of these PP-InsP species, it will be a milestone to determine the responsible kinase for the newly identified $4/6$-InsP$_7$ and to determine the physiological processes this isomer regulates. We speculate that this might involve also responses to biotic stresses. Furthermore, the involvement of PP-InsPs in hormone signaling still remains enigmatic. Besides the role of these small molecules in auxin-, JA- and SA-dependent functions (Laha et al., 2015, 2020; Gulabani et al., 2021), a direct involvement in ethylene or brassinosteroid responses should be addressed, since a role of *myo*-inositol phosphate synthase in regulating plant growth and stress responses *via* ethylene- mediated signaling has been observed in Arabidopsis and wheat (Sharma et al., 2020a,b).

Finally, the identification of PP-InsPs and their different isomers will help to understand plant-pathogen interactions, which will be useful for improving crop growth and yield under abiotic and biotic stresses.
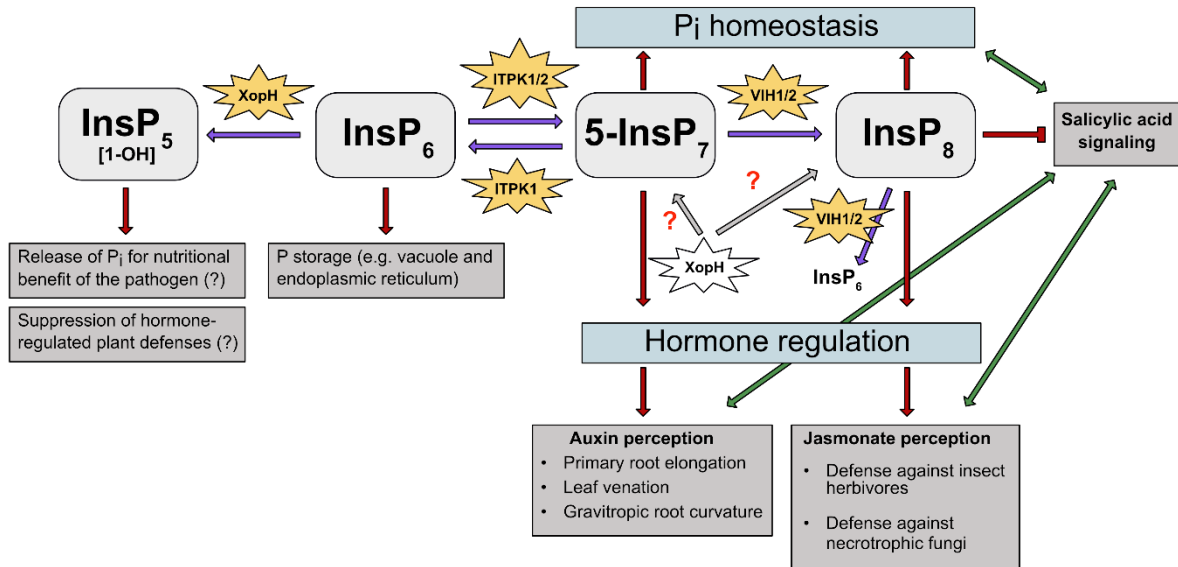
Figure 3. PP-InsPs and their kinases are involved in different abiotic and biotic stress responses in plants. PP-InsPs' involvement in P_i homeostasis, hormone perception and regulation is depicted. Purple arrows indicate the kinase/phosphatase activity of the respective enzymes on InsPs and PP-InsPs. Gray arrows and red question marks depict a putative effect of XopH on PP-InsPs. Red arrows and T-shaped line indicate promotion and suppression of specific InsPs and PP-InsPs in regulating stress responses, respectively. Green arrows depict the interplay between plant hormones auxin, JA and SA, respectively. ITPK1 phosphorylates $InsP_6$ to $5\text{-}InsP_7$. The latter serves as precursor for $InsP_8$, which plays a crucial role in adaption to changing P_i levels. Additionally, $InsP_6$ is degraded by XopH to potentially release P_i for the pathogen's nutritional benefit. ITPK1 and VIH2 interaction is needed to maintain P_i homeostasis. Higher PP-InsPs are also involved in hormone perception and regulation. The ITPK1-generated $5\text{-}InsP_7$ is speculated to be involved in auxin and SA signaling. The VIH2- generated $InsP_8$ has been proposed to represent a critical co-ligand of the JA receptor complex and is also assumed to regulate SA signaling. The bacterial type III effector XopH displays 1- phytase activity but may also have hydrolytic activities against PP-InsPs that might disrupt hormone-regulated defense mechanisms.

References

Adepoju, O., Williams, S. P., Craige, B., Cridland, C. A., Sharpe, A. K., Brown, A. M., Land, E., Perera, I. Y., Mena, D., Sobrado, P., Gillaspy, G. E. (2019). Inositol Trisphosphate Kinase and Diphosphoinositol Pentakisphosphate Kinase Enzymes Constitute the Inositol Pyrophosphate Synthesis Pathway in Plants. bioRxiv:724914.

Adie, B., Rubio-Somoza, I., Solano, R. (2007). Modulation of plant defenses by ethylene. J. Plant Growth Regul 26(2):160-177.

Alimohammadi, M., de Silva, K., Ballu, C., Ali, N., Khodakovskaya, M. V. (2012). Reduction of inositol (1,4,5)-trisphosphate affects the overall phosphoinositol pathway and leads to modifications in light signalling and secondary metabolism in tomato plants. J Exp Bot 63(2):825-35.

Allen, G. J., Sanders, D. (1994). Osmotic stress enhances the competence of Beta vulgaris vacuoles to respond to inositol 1,4,5-trisphosphate. Plant J. 6(5):687-695.

Azevedo, C., Saiardi, A. (2006). Extraction and analysis of soluble inositol polyphosphates from yeast. Nat Protoc 1(5):2416–2422.

Baek, D., Chun, H. J., Yun, D. J., Kim, M. C. (2017). Cross-talk between phosphate starvation and other environmental stress signaling pathways in plants. Mol Cells 40(10):697-705.

Bainbridge, K., Sorefan, K., Ward, S., Leyser, O. (2005). Hormonally controlled expression of the Arabidopsis MAX4 shoot branching regulatory gene. Plant J 44(4):569–580.

Bariola, P. A., Howard, C. J., Taylor, C. B., Verburg, M. T., Jaglan, V. D. Green, P. J. (1994) The *Arabidopsis* ribonuclease gene *RNS1* is tightly controlled in response to phosphate limitation. Plant J 6:673-685.

Bartel, B., LeClere, S., Magidin, M., Zolman, B. K. (2001). Inputs to the active indole-3-acetic acid pool: de novo synthesis, conjugate hydrolysis, and indole-3-butyric acid β-oxidation. J Plant Growth Regul 20(3):198-216.

Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denancé, N., Vasse, J., Lauber, E., Arlat, M. (2007). Plant carbohydrate scavenging through tonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. PLoS One 2(2):e224.

Blatt, M., Thiel, G., Trentham, D. (1990). Reversible inactivation of K^+ channels of *Vcia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. Nature 346:766-769.

Blüher, D., Laha, D., Thieme, S. Hofer, A., Eschen-Lippold, L., Masch, A., Balcke, G., Pavlovic, I., Nagel, O., Schonsky, A., Hinkelmann, R., Wörner, J., Parvin, N., Greiner, R., Weber, S., Tissier, A., Schutkowski, M., Lee, J., Jessen, H. J., Schaaf, G., Bonas, U. (2017). A 1-phytase type III effector interferes with plant hormone signaling. Nat Commun 8:2159.

Boter, M., Ruı́z-Rivero, O., Abdeen, A., Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18(13):1577-1591.

Brooks, D. M., Bender, C. L., Kunkel, B. N. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. Mol. Plant Pathol 6(6):629-639.

Browse, J. (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. Annu Rev Plant Biol 60:183-205.

Büttner, D. (2016). Behind the lines-actions of bacterial type III effector proteins in plant cells. FEMS Microbiol Rev 40(6):894-937.

Caarls, L., Pieterse, C. M., Van Wees, S. C. (2015). How salicylic acid takes transcriptional control over jasmonic signaling. Front Plant Sci 6:170.

Calderón Villalobos, L. I., Lee, S., De Oliveira, C., Ivetac, A., Brandt, W., Armitage, L., Sheard, L. B., Tan, X., Parry, G., Mao, H., Zheng, N., Napier, R., Kepinski, S., Estelle, M. (2012). A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of

auxin. Nat Chem Biol 8(5):477-485.

Campos-Soriano, L., Bundó, M., Bach-Pages, M., Chiang, S. F., Chiou, T. J., San Segundo, B. (2020). Phosphate excess increases susceptibility to pathogen infection in rice. Mol Plant Pathol 21(4):555–570.

Castrillo, G., Teixeira, P. J., Paredes, S. H., Law, T. F., de Lorenzo, L., Feltcher, M. E., Finkel, O. M., Breakfield, N. W., Mieczkowski, P., Jones, C. D., Paz-Ares, J., Dangl, J. L. (2017). Root microbiota drive direct integration of phosphate stress and immunity. Nature 543:513-518.

Chaerle, L., Van Caeneghem, W., Messens, E., Lambers, H., Van Montagu, M., Van Der Straeten, D. (1999). Presymptomatic visualization of plant-virus interactions by thermography. Nat Biotechnol 17(8):813-816.

Chatterjee, S., Sankaranarayanan, R., Sonti, R. V. (2003). PhyA, a secreted protein of Xanthomonas oryzae pv. oryzae, is required for optimum virulence and growth on phytic acid as a sole phosphate source. Mol Plant Microbe Interact 16(11):973–982.

Chen, X., Lin, W., Wang, Y., Luan, S., Xue, H. W. (2008). An inositol polyphosphate 5-phosphatase functions in PHOTOTROPIN1 signaling in Arabidopis by altering cytosolic Ca2+. Plant Cell 20(2):353-366.

Cheong, Y. H., Chang, H. S., Gupta, R., Wang, X., Zhu, T., Luan, S. (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol 129:661-677.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J., Lorenzo, O., García-Casado, G., Lopéz-Vidriero, I., Lozano, F. M., Ponce, M. R., Micol, J. L., Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448:666-671.

Chiou, T. J., Aung, K., Lin, S. I., Wu, C. C., Chiang, S. F., Su, C. L. (2006). Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell 18(2):412–421.

Chiou, T. J., Lin, S. I. (2011). Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol 62:185-206.

Christie, J. M., Murphy, A. S. (2013). Shoot phototropism in higher plants: new light through old concepts. Am J Bot 100(1):35-46.

Constantin, E. C., Haegeman, A., Van Vaerenbergh, J., Baeyen, S., Van Malderghem, C., Maes, M., Cottyn, B. (2017). Pathogenicity and virulence gene content of *Xanthomonas* strains infecting Araceae, formerly known as *Xanthomonas axonopodis* pv. *dieffenbachiae*. Plant Pathol 66(9):1539-1554.

Das, A., Rangaraj, N., Sonti, R. V. (2009). Multiple adhesin-like functions of Xanthomonas oryzae pv. oryzae are involved in promoting leaf attachment, entry, and virulence on rice. Mol Plant Microbe Interact 22(1):73-85.

Desai, M., Rangarajan, P., Donahue, J. L., Williams, S. P., Land, E. S., Mandal, M. K., Phillippy, B. Q., Perera, I. Y., Raboy, V., Gillaspy, G. E. (2014). Two inositol hexakisphosphate kinases drive inositol pyrophosphate synthesis in plants. Plant J. 80(4):642–653.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., Turner, J. G. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin–ligase complex in Arabidopsis. Plant J 32(4):457-466.

Dharmasiri, N., Dharmasiri, S., Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature 435:441-445.

Dinesh, D. C., Villalobos, L. I. A. C., Abel, S. (2016). Structural Biology of Nuclear Auxin Action. Trends Plant Sci 21(4):302-316.   .

Djami-Tchatchou, A. T., Harrison, G. A., Harper, C. P., Wang, R., Prigge, M. J., Estelle, M., Kunkel, B. N. (2020). Dual Role of Auxin in Regulating Plant Defense and Bacterial Virulence Gene Expression During *Pseudomonas syringae* PtoDC3000 Pathogenesis. Mol Plant Microbe Interact 33(8):1059-1071.

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet 11(8):539-548.

Donnell, P., Calvert, C., Atzorn, R., Wasternack, C. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science 274(5294):1914-1917.

Dong, J., Ma, G., Sui, L., Wei, M., Satheesh, V., Zhang, R., Ge, S., Li, J., Zhang, T. E., Wittwer, C., Jessen, H. J., Zhang, H., An, G. Y., Chao, D. Y., Liu, D., Lei, M. (2019). Inositol pyrophosphate InsP$_8$ acts as an intracellular phosphate signal in Arabidopsis. Mol Plant 12(11):1463-1473.

O'Donnell, P. J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H. M. O., Bowles, D. J. (1996). Ethylene as a Signal Mediating the Wound Response of Tomato Plants. Science 274(5294):1914-1917.

Dowd, C., Wilson, I. W., McFadden, H. (2004). Gene expression profile changes in cotton root and hypocotyl tissues in response to infection with *Fusarium oxysporum* f. sp. *vasinfectum*. Mol Plant Microbe Interact (6):654-67.

Draskovic, P., Saiardi, A., Bhandari, R., Burton, A., Ilc, G., Kovacevic M. (2008). Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. Chem Biol 15(3):274-286.

Essigmann, B., Gueler, S., Narang, R.A., Linke, D. Benning, C. (1998) Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. Proc. Natl Acad. Sci. USA 95:1950-1955.

Feys, B., Benedetti, C. E., Penfold, C. N., Turner, J. G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6(5):751-759.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., Solano, R. (2009). (+)-7-iso-Jasmonoyl-Lisoleucine is the endogenous

bioactive jasmonate. Nat Chem Biol 5(5):344-350.

Fridy, P. C., Otto, J. C., Dollins, D. E., York, J. D. (2007). Cloning and Characterization of Two Human VIP1-like Inositol Hexakisphosphate and Diphosphoinositol Pentakisphosphate Kinases. J Biol Chem 292(42):30754-30762.

Friml, J. (2003). Auxin transport—shaping the plant. Curr Opin Plant Biol 6(1):7-12.

Fu, X., and Harberd, N. P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. Nature 421(6924):740-743.

Fujii, H., Chiou, T.-J., Lin, S.-I., Aung, K., Zhu, J.-K. (2005). A miRNA involved in phosphate-starvation response in Arabidopsis. Curr Biol. 15(22):2038-2043.

Gaedeke, N., Klein, M., Kolukisaoglu, U., Forestier, C., Müller, A., Ansorge, M., Becker, D., Mamnun, Y., Kuchler, K., Schulz, B., Mueller-Roeber, B., Martinoia, E. (2001) The Arabidopsis thaliana ABC transporter AtMRP5 controls root development and stomata movement. EMBO J 20(8):1875-1887.

Gallavotti, A. (2013). The role of auxin in shaping shoot architecture. J Exp Bot 64(9):2593-2608.

Geisler, M., Wang, B., Zhu, J. (2014). Auxin transport during root gravitropism: transporters and techniques. Plant Biol 16:50-57.

Ghanashyam, C., Jain, M. (2009). Role of auxin-responsive genes in biotic stress responses. Plant Signal Behav 4(9):846-848.

Ghosh P. (2004). Process of protein transport by the type III secretion system. Microbiol Mol Biol Rev 68(4):771-795.

Gilroy, S., Read, N. D., Trewavas, A. J. (1990). Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature 346:769–771.

Goodman, R. N., Novacky, A. J. (1994). The hypersensitive reaction in plants to pathogens: a resistance phenomenon. Chicago: American Phytopathological Society.

Gu, C., Nguyen, H.-N., Hofer, A., Jessen, H. J., Dai, X., Wang, H., Shears, S. B. (2017). The Significance of the Bifunctional Kinase/Phosphatase Activities of Diphosphoinositol Pentakisphosphate Kinases (PPIP5Ks) for Coupling Inositol Pyrophosphate Cell Signaling to Cellular Phosphate Homeostasis. J Biol Chem 292:4544-4555.

Gulabani, H., Goswami, K., Walia, Y., Roy, A., Jameeta Noor, J., Ingole, K. D., Kasera, M., Laha, D., Giehl, R. F. H., Schaaf, G., Bhattacharjee, S. (2021). Arabidopsis inositol polyphosphate kinases IPK1 and ITPK1 modulate crosstalks between SA-dependent immunity and phosphate-starvation responses. Plant Cell Rep 41(2):347-363.

Halsted, J. A., Ronaghy, H. A., Abadi, P., Haghshenass, M., Amirhakemi, G. H., Barakat, R. M., Reinhold, J. G. (1972). Zinc deficiency in man. The Shiraz experiment. Am J Med 53(3):277-284.

Hannah, M. A., Heyer, A. G., Hincha, D. K. (2005). A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. PLoS Genet 1(2):e26.

Hao, Q., Wang, W., Han, Q., Wu, J., Lyo, B., Chen, F., Caplan, A., Li, C., Wu, J., Wang, W., Xu, Q., Fu, D. (2018). Isochorismate-based salicylic acid biosynthesis confers basal resistance to *Fusarium graminearum* in barley. Mol Plant Pathol 19(8):1995-2010.

Härtel, H., Dormann, P., Benning, C. (2000). DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in Arabidopsis. Proc Natl Acad Sci U S A 97(19):10649-10654.

Holford, I. C. R. (1997). Soil phosphorus: Its measurement, and its uptake by plants. Aust J Soil Res 35(2):227-239

Hõrak, H. (2020). Zones of Defense? SA Receptors Have It Under Control. Plant Cell 32(12):3658-3659.

Huang, J., Yang, M., Lu, L., Zhang, X. (2016). Diverse functions of smallRNAs in different plant-pathogen communications. Front Microbiol 4(7):1552.

Hung, C. Y., Aspesi, P. Jr., Hunter, M. R., Lomax, A. W., Perera, I. Y. (2014). Phosphoinositide-signaling is one component of a robust plant defense response. Front Plant Sci 5:267.

Irvine, R. F. (2003). 20 years of Ins(1,4,5)P3 and 40 years before. Nat Rev Mol Cell Bio 4(7):586-590.

Jacques, M.-A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., Chen, N. W., Cociancich, S., Darrasse, A., Denancé, N., Fischer-Le Saux, M., Gagnevin, L., Koebnik, R., Lauber, E., Noël, L. D., Pieretti, I., Portier, P., Pruvost, O., Rieux, A., Robène, I., Royer, M., Szurek, B., Verdier, V., Vernière, C. (2016). Using Ecology, Physiology, and Genomics to Understand Host Specificity in *Xanthomonas*. Ann Rev Phytopathol 54:163-187.

Jadav, R. S., Chanduri, M. V., Sengupta, S., Bhandari, R. (2013). Inositol pyrophosphate synthesis by inositol hexakisphosphate kinase 1 is required for homologous recombination repair. J Biol Chem 288(5):3312-3321.

Jain, R. (2015). Reduction of phytate by down-regulation of *Arabidopsis thaliana MIPS* and *IPK1* genes alters susceptibility to beet cyst nematodes. Nematology 17(1):401-407.

Jain, M., and Khurana, J. P. (2009). Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive development and abiotic stress in rice. FEBS J 276(11):3148-3162.

Josefsen, L., Bohn, L., Sørensen, M. B., Rasmussen, S. K. (2007). Characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfamily. Gene 397(1-2):114–125.

Jung, J.-Y., Ried, M. K., Hothorn, M., Poirier, Y. (2018). Control of plant phosphate homeostasis by inositol pyrophosphates and the SPX domain. Curr Opin Biotechnol 49:156-

162.

Karthikeyan, A. S., Varadarajan, D. K., Mukatira, U. T., D'Urzo, M. P., Damsz, B., Raghothama, K. G. (2002). Regulated expression of Arabidopsis phosphate transporters. Plant Physiol 130(1):221-233.

Katsir, L., Schilmiller, A. L., Staswick, P. E., He, S. Y., Howe, G. A. (2008). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA 105(19):7100-7105.

Kepinski, S., Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435:446-451.

Khodakovskaya, M., Sword, C., Wu, Q., Perera, I. Y., Boss, W. F., Brown, C. S., Winter Sederoff, H. (2010). Increasing inositol (1,4,5)-trisphosphate metabolism affects drought tolerance, carbohydrate metabolism and phosphate-sensitive biomass increases in tomato. Plant Biotechnol J 8(2):170-83.

Kim, D. S., Hwang, B. K. (2014). An important role of pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J Exp Bot 65(9):2295-2306. ^

Knight, H., Trewavas, A. J., Knight, M. R. (1997). Calcium signalling in Arabidopsis thaliana responding to drought and salinity. Plant J 12(5):1067-1078.

Koo, A. J., Gao, X., Jones, A. D., Howe, G. A. (2009). A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J 59(6):974-986.

Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O. S., Deng, X. W., Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol 139(4):1597-1611.

Krinke, O., Novotná, Z., Valentová, O., Martinec, J. (2007). Inositol trisphosphate receptor in higher plants: is it real? J Exp Bot 58(3):361-76.

Kunkel, B. N., and Harper, C. P. (2018). The roles of auxin during interactions between bacterial plant pathogens and their hosts. J Exp Bot 69(2):245-254.

Kuo, H. F., Chang, T. Y., Chiang, S. F., Wang, W. D., Charng, Y. Y., Chiou, T. J. (2014). Arabidopsis inositol pentakisphosphate 2-kinase, AtIPK1, is required for growth and modulates phosphate homeostasis at the transcriptional level. Plant J 80(3):503-515.

Kuo, H.-F., Hsu, Y.-Y., Lin, W.-C., Chen, K.-Y., Munnik, T., Brearley, C. A., Chiou, T.-J. (2018). Arabidopsis inositol phosphate kinases, IPK1 and ITPK1, constitute a metabolic pathway in maintaining phosphate homeostasis. Plant J 95:613-630.

Laha, D., Johnen, P., Azevedo, C., Dynowski, M., Weiss, M., Capolicchio, S., Mao, H. B., Iven, T., Steenbergen, M., Freyer, M., Gaugler, P., de Campos, M. K. F., Zheng, N., Feussner, I., Jessen, H. J., Van Wees, S. C. M., Saiardi, A., Schaaf, G. (2015). VIH2 Regulates the Synthesis of Inositol Pyrophosphate InsP(8) and Jasmonate-Dependent Defenses in Arabidopsis. Plant Cell 27:1082-1097.

Laha, D., Parvin, N., Dynowski, M., Johnen, P., Mao, H., Bitters, S. T., Zheng, N., Schaaf, G. (2016). Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex. Plant Phys171:2364–2370.

Laha, D., Parvin, N., Hofer, A., Giehl, R. F. H., Fernandez-Rebollo, N., von Wiren, N., Saiardi, A., Jessen, H. J., Schaaf, G. (2019). Arabidopsis ITPK1 and ITPK2 Have an Evolutionarily Conserved Phytic Acid Kinase Activity. Acs Chem Biol 14:2127-2133.

Laha, N. P., Dhir, Y. W., Giehl, R. F. H., Schaefer, E. M., Gaugler, P., Shishavan, Z. H., Gulabani, H., Mao, H., Zheng, N., von Wiren, N., Jessen, H. J., Saiardi, A., Bhattacharjee, S., Laha D., Schaaf, G. (2020). ITPK1-Dependent Inositol Polyphosphates Regulate Auxin Responses in Arabidopsis thaliana. bioRxiv:2020.2004.2023.058487.

Laha, D., Portela-Torres, P., Desfougères, Y., Saiardi, A. (2021). Inositol phosphate kinases in the eukaryote landscape. Adv Biol Regul 79(3):100782.

Laha, D., Kamleitner, M., Johnen, P., Schaaf, G. (2021). Analyses of Inositol Phosphates and Phosphoinositides by Strong Anion Exchange (SAX)-HPLC. In: Bartels, D., Dörmann, P. (eds) Plant Lipids. Methods in Molecular Biology, vol 2295. Humana, New York, NY.

Land, E. S., Cridland, C. A., Craige, B., Dye, A., Hildreth, S. B., Helm, R. F., Gillaspy, G. E., Perera, I. Y. (2021). A Role for Inositol Pyrophosphates in the Metabolic Adaptations to Low Phosphate in Arabidopsis. Metabolites 11(9), 601.

Leyser O. (2010). The power of auxin in plants. Plant Physiol 154(2):501-505.

Lin, H, Fridy, P. C., Ribeiro, A. A., Choi, J. H., Barma, D. K., Vogel, G., Falck, J. R., Shears, S. B., York, J. D., Mayr, G. W. (2009). Structural analysis and detection of biological inositol pyrophosphates reveal that the family of VIP/diphosphoinositol pentakisphosphate kinases are 1/3-kinases. J Biol Chem 284(3):1863-1872.

Liu, H. T., Gao, F., Cui, S. J., Han, J. L., Sun, D. Y., Zhou, R. G. (2006). Primary evidence for involvement of IP3 in heat-shock signal transduction in Arabidopsis. Cell Res 16(4):394-400.

Liu, T. Y., Huang, T. K., Tseng, C. Y., Lai, Y. S., Lin, S. I., Lin, W. Y., Chen, J. W., Chiou, T. J. (2012). PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in Arabidopsis. Plant Cell 24(5):2168-2183.

Loewus, F. A., Murthy, P. P. N. (2000). *Myo*-Inositol metabolism in plants. Plant Sci 150(1):1-19.

Ma, Y., Zhao, Y., Berkowitz, G. A. (2017). Intracellular Ca^{2+} is important for flagellin-triggered defense in Arabidopsis and involves inositol polyphosphate signaling. J Exp Bot 68(13):3617-3628.

Marschner, H. (1995). Mineral nutrition of higher plants. Academic press, San Diego, CA.

Martínez, C., Pons, E., Prats, G., León, J. (2004). Salicylic acid regulates flowering time and links defence responses and reproductive development. Plant J 37(2):209-217.

McCance, R. A., Widdowson, E. M. (1942). Mineral metabolism of healthy adults on white and brown bread dietaries. J Physiol 101(1):44-85.

Miller, G. J., Wilson, M. P., Majerus, P. W., Hurley, J. H. (2005). Specificity determinants in inositol polyphosphate synthesis: crystal structure of inositol 1,3,4-trisphosphate 5/6-kinase. Mol Cell 15;18(2):201-212.

Möller, B., Weijers, D. (2009). Auxin control of embryo patterning. Cold Spring Harb Perspect Biol 1:a001545.

Monteiro, D., Liu, Q., Lisboa, S., Scherer, G. E. F., Quader, H., Malhó, R. (2005). Phosphoinositides and phosphatidic acid regulate pollen tube growth and reorientation through modulation of $[Ca^{2+}]c$ and membrane secretion. J Exp Bot 56(416):1665-1674.

Mosblech, A., König, S., Stenzel, I., Grzeganek, P., Feussner, I., Heilmann, I. (2008). Phosphoinositide and inositolpolyphosphate signalling in defense responses of Arabidopsis thaliana challenged by mechanical wounding. Mol Plant 1(2):249-261.

Mosblech, A., Thurow, C., Gatz, C., Feussner, I., Heilmann, I. (2011). Jasmonic acid perception by COI1 involves inositol polyphosphates in *Arabidopsis thaliana*. Plant J 65(6):949-957.

Mudge, S. R., Rae, A. L., Diatloff, E., Smith, F. W. (2002). Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in Arabidopsis. Plant J 31(3):341-353.

Müller, D., Leyser, O. (2011). Auxin, cytokinin and the control of shoot branching. Ann Bot 107(7):1203-1212.

Mulugu, S., Bai, W., Fridy, P. C., Bastidas, R. J., Otto, J. C., Dollins, D. E., Haystead, T. A., Ribeiro, A. A., York, J. D. (2007). A conserved family of enzymes that phosphorylate inositol hexakisphosphate. Science 316:106-109.

Munnik, T., Testerink, C. (2009). Plant phospholipid signaling: "in a nutshell". J Lipid Res 50 Suppl(Suppl):260-265.

Munnik, T., and Vermeer, J. E. (2010). Osmotic stress-induced phosphoinositide and inositol phosphate signalling in plants. Plant Cell Environ 33(4):655-669.

Munnik, T., Nielsen, E. (2011). Green light for polyphosphoinositide signals in plants. Curr Opin Plant Biol 14(5):489-497.

Mur, L. A., Kenton, P., Lloyd, A. J., Ougham, H., Prats, E. (2008). The hypersensitive response; the centenary is upon us but how much do we know? J Exp Bot 59(3):501-520.

Murphy, A. M., Otto, B., Brearley, C. A., Carr, J. P., Hanke, D. E. (2008). A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. Plant J 56(4):638-652.

Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring, J. K., Brearley, C., Martinoia, E. (2009). The Arabidopsis ATP-binding Cassette Protein AtMRP5/AtABCC5 Is a High Affinity Inositol Hexakisphosphate Transporter Involved in Guard Cell Signaling and Phytate Storage. J Biol Chem 284:33614-33622.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., Jones, J. D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312(5772):436-439.

Newberry, E. A., Bhandari, R., Minsavage, G. V., Timilsina, S., Jibrin, M. O., Kemble, J., Sikora, E. J., Jones, J. B., Potnis, N. (2019). Independent Evolution with the Gene Flux Originating from Multiple *Xanthomonas* Species Explains Genomic Heterogeneity in *Xanthomonas* performance. Appl Environ Microbiol 85(20):e00885-19.

Otegui, M. S., Capp, R., Staehelin, L. A. (2002). Developing seeds of Arabidopsis store different minerals in two types of vacuoles and in the endoplasmic reticulum. The Plant Cell 14(6):1311-1327.

Parvin Laha, N., Dhir, Y. W., Giehl, R. F. H., Schäfer, E. M., Gaugler, P., Haghighat Shishavan, Z., Gulabani, H., Mao, H., Zheng, N., von Wirén, N., Jessen, H. J., Saiardi, A., Bhattacharjee, S., Laha, D., Schaaf, G. (2020). ITPK1-dependentinositol polyphosphates regulate auxin responses in *Arabidopsis thaliana*. bioRxiv [Preprint].

Paul, S., Datta, S., Datta, K. (2015). miRNA regulation of nutrient homeostasis in plants. Front Plant Sci 6:232.

Perera, I. Y., Hung, C. Y., Brady, S., Muday, G. K., Boss, W. F. (2006). A universal role for inositol 1,4,5-trisphosphate-mediated signaling in plant gravitropism. Plant Physiol 140(2):746-60.

Péret, B., Clément, M., Nussaume, L., Desnos, T. (2011). Root developmental adaptation to phosphate starvation: better safe than sorry. Trends Plant Sci 16(8):442-450.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. Annu Rev Cell Dev Biol 28:489-521.

Pisani, F., Livermore, T., Rose, G., Chubb, J.R., Gaspari, M., Saiardi, A. (2014). Analysis of *Dictyostelium discoideum* inositol pyrophosphate metabolism by gel electrophoresis. PLoS One 9:e85533.

Plaxton, W. C., and Tran, H. T. (2011). Metabolic adaptations of phosphate-starved plants. Plant Physiol 156(3):1006-1015.

Poon, J. S. Y., Le Fevre, R. E., Carr, J. P., Hanke, D. E., Murphy, A. M. (2020). Inositol hexakisphosphate biosynthesis underpins PAMP-triggered immunity to Pseudomonas syringae pv. tomato in Arabidopsis thaliana but is dispensable for establishment of systemic acquired resistance. Mol Plant Pathol 21(3):376-387.

Potnis, N., Minsavage, G., Smith, J. K., Hurlbert, J. C., Norman, D., Rodrigues, R., Stall, R. E., Jones, J. B. (2012). Avirulence proteins AvrBs7 from *Xanthomonas gardneri* andAvrBs1.1 from *Xanthomonas euvesicatoria* contribute to a novel gene-for-geneinteraction in pepper. Mol. Plant Microbe Interact 25(3):307-320.

Puga, M. I., Mateos, I., Charukesi, R., Wang, Z., Franco-Zorrilla, J. M., de Lorenzo, L., Irigoye, M. L., Masiero, S., Bustos, R., Rodriguez, J., Leyva, A., Rubio, V., Sommer, H., Paz-Ares, J. (2014). SPX1 is a phosphate-dependent inhibitor of PHOSPHATE STARVATION RESPONSE 1 in Arabidopsis. P Natl Acad Sci USA 111:14947-14952.

Qi, W., Manfield, I. W., Muench, S. P, Baker, A. (2017). AtSPX1 affects the AtPHR1-DNA-binding equilibrium by binding monomeric AtPHR1 in solution. Biochem J 474(21):3675-3687.

Qiu, D., Wilson, M. S., Eisenbeis, V. B., Harmel, R. K., Riemer, E., Haas, T. M., Wittwer, C., Jork, N., Gu, C., Shears, S. B., Schaaf, G., Kammerer, B., Fiedler, D., Saiardi, A., Jessen, H. J. (2020). Analysis of inositol phosphate metabolism by capillary electrophoresis electrospray ionization mass spectrometry. Nat Commun 11:6035.

Raboy, V., Gerbasi, P. (1996). Genetics of *myo*-Inositol phosphate synthesis and accumulation. Subcell Biochem 26:257-285.

Raboy, V. (2020). Low phytic acid Crops: Observations Based on Four Decades of Research. Plants 9(2)*:*140.

Raetz, C. R. H., Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu Rev Biochem. 71:635-700.

Rai V.K., Sharma S.S., Sharma S. (1986). Reversal of ABA-Induced Stomatal Closure by Phenolic Compounds. J Exp Bot 37(174):129-134

Raskin, I., Turner, I. M., Melander, W. R. (1989). Regulation of heat production in the inflorescences of an Arum lily by endogenous salicylic acid. Proc Natl Acad Sci U S A 86(7):2214-2218.

Raskin, I. (1992). Salicylate, a new plant hormone. Plant Physiol 99(3):799–803.

Rausch, C., Bucher, M. (2002). Molecular mechanisms of phosphate transport in plants. Planta

216(1):23-37.

Rao, F., Cha, J., Xu, J., Xu, R., Vandiver, M. S., Tyagi, R., Tokhunts, R., Koldobskiy, M. A., Fu, C., Barrow, R., Wu, M., Fiedler, D., Barrow, J. C., Snyder, S. H. (2014). Inositol pyrophosphates mediate the DNA-PK/ATM-p53 cell death pathway by regulating CK2 phosphorylation of Tti1/Tel2. Mol Cell 54(1):119-132.

Riemer, E., Qiu, D., Laha, D., Harmel, R. K., Gaugler, P., Gaugler, V., Frei, M., Hajirezaei, M.-R., P. Laha, N., Krusenbaum, L., Schneider, R., Saiardi, A., Fiedler, D., Jessen, H. J., Schaaf, G., Giehl, R. F. H. (2021). ITPK1 is an InsP6/ADP phosphotransferase that controls phosphate signaling in Arabidopsis. Mol Plant 14(11):1864-1880.

Rockström, J., Steffen, W., Noone, K., Persson, A., Chapin, F. S., Lambin, E. F., Lenton, T. M., Scheffer, M., Folke, C., Schellnhuber, H. J., Nykvist, B., de Wit, C. A., Hughes, T., van der Leeuw, S., Rodhe, H., Sörlin, S., Snyder, P. K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R. W., Fabry, V. J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P., Foley, J. A. (2009). A safe operating space for humanity. Nature 461:472-475.

Rubio, V., Linhares, F., Solano, R., Martín, A. C., Iglesias, J., Leyva, A., Paz-Ares, J. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Gene Dev 15:2122-2133.

Ryan, R. P., Vorhölter, F.-J., Potnis, N., Jones, J. B., Van Sluys, M.-A., Bogdanove, A. J., Dow, J. M. (2011). Pathogenomics of *Xathomonas*: understanding bacterium-plant interactions. Nat Rev Microbiol 9(5):344-355.

Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., Snyder, S. H. (1999). Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr Biol 9(22):1323-1326.

Saiardi, A., Caffrey, J. J., Snyder, S. H., Shears, S. B. (2000). The inositol hexakisphosphate kinase family: Catalytic flexibility and function in yeast vacuole biogenesis. J Biol Chem 275(32):24686-24692.

Saiardi, A., Sciambi, C., McCaffery, J. M., Wendland, B., Snyder, S. H. (2002). Inositol pyrophosphates regulate endocytic trafficking. Proc Natl Acad Sci U S A 99(22):14206-14211.

Salehin, M., Bagchi, R., Estelle, M. (2015). SCFTIR1/AFB-based auxin perception: mechanism and role in plant growth and development. Plant Cell 27(1):9-19.

Secco, D., Jabnoune, M., Walker, H., Shou, H., Wu, P., Poirier, Y., Whelan J. (2013). Spatio-temporal transcript profiling of rice roots and shoots in response to phosphate starvation and recovery. Plant Cell 25(11):4285-304 (2013).

Secco, D., Bouain, N., Rouached, A., Prom-u-thai, C., Hanin, M., Pandey, A. K., Rouached, H. (2017). Phosphate, phytate and phytases in plants: from fundamental knowledge gained in Arabidopsis to potential biotechnological applications in wheat. Crit Rev Biotechnol 37:898-910.

Seidel, S. J., Gaiser, T., Ahrends, H. E., Hueging, H., Siebert, S., Bauke, S. L., Glocke,, M. I., Koch, M., Schweitzer, K., Schaaf, G., Ewert, F. (2021). Crop response to P fertilizer omission under a changing climate-experimental and modeling results over 115 years of a long-term fertilizer experiment. Field Crops Res 268:108174.

Seybold, H., Trempel, F., Ranf, S., Scheel, D., Romeis, T., Lee, J. (2014). Ca_{2+} signaling in plant immune response: from pattern recognition receptors to Ca_{2+} decoding mechanisms. New Phytol 204(4):782-790.

Sharma, N., Chaudhary, C., Khurana, P. (2020a). Role of *myo*-inositol during skotomorphogenesis in *Arabidopsis*. Sci Rep 10:17329.

Sharma, N., Chaudhary, C., Khurana, P. (2020b). Wheat *Myo*-inositol phosphate synthase influences plant growth and stress responses via ethylene mediated signaling. Sci Rep 10**:**10766.

Sheard, L. B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T. R., Kobayashi, Y., Hsu, F. F., Sharon, M., Browse, J., He, S. Y., Rizo, J., Howe, G. A., Zheng, N. (2010).

Jasmonate perception by inositol phosphate-potentiated COI1-JAZ co-receptor. Nature 468(7322):400-405.

Shears, S. B. (2015). Inositol pyrophosphates: why so many phosphates? Adv Biol Regul 57:203-221.

Shimizu-Mitao, and Y., Kakimoto, T. (2014). Auxin sensitivities of all Arabidopsis Aux/IAAs for degradation in the presence of every TIR1/AFB. Plant Cell Physiol 55(8):1450-1459.

Shin, H., Shin, H. S., Dewbre, G. R., Harrison, M. J. (2004). Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 39(4):629–642.

Sorefan, K., Booker, J., Haurogne, K., Goussot, M., Bainbridge, K., Foo, E., Chatfield, S., Ward, S., Beveridge, C., Rameau, C., Leyser, O. (2003). MAX4 and RMS1 are orthologous dioxygenase-like genes that regulate shoot branching in Arabidopsis and pea. Genes Dev 17(12):1469-1474.

Stevenson-Paulik, J., Odom, A. R., York, J. D. (2002). Molecular and biochemical characterization of two plant inositol polyphosphate 6-/3-/5-kinases. J Biol Chem 277(45):42711-42718.

Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., York, J. D. (2005). Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc Natl Acad Sci USA 102(35):12612-12617.

Streb, H., Irvine, R. F., Berridge, M. J., Schulz, I. (1983). Release of Ca^{2+} from a non mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5- trisphosphate. Nature 306(5938):67-69.

Sweetman, D., Stavridou, I., Johnson, S., Green, P., Caddick, S. E. K., Brearley, C. A. (2007). Arabidopsis thaliana inositol 1,3,4-trisphosphate 5/6-kinase 4 (AtITPK4) is an outlier to a family of ATP-grasp fold proteins from Arabidopsis. FEBS Letters 581(22):4165-4171.

Tan, X., Calderon-Villalobos, L. I. A., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M., Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446(7136):640–645.

Tanaka, H., Dhonukshe, P., Brewer, P. B., Friml, J. (2006). Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci 63(23):2738-2754.

Tantikanjana, T., Yong, J. W., Letham, D. S., Griffith, M., Hussain, M., Ljung, K., Sandberg, G., Sundaresan, V. (2001). Control of axillary bud initiation and shoot architecture in Arabidopsis through the SUPERSHOOT gene. Genes Dev 15(12):1577-1588.

Taylor, C.B., Bariola, P.A., DelCardyre, S.B., Raines, R.T. and Green, P.J. (1993) RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. Proc. Natl Acad. Sci. USA 90, 5118-5122.

Teper, D., Sunitha, S., Martin, G. B., Sessa, G. (2015). Five Xanthomonas type III effectors suppress cell death induced by components of immunity-associated MAP kinase cascades. Plant Signal Behav 10(10):e1064573.

Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Büttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A. C., Meyer, F., Mittenhuber, G., Nies, D. H., Niesbach-Klösgen, U., Patschkowski, T., Rückert, C., Rupp, O., Schneiker, S., Schuster, S. C., Vorhölter, F. J., Weber, E., Pühler, A., Bonas, U., Bartels, D., Kaiser O. (2005). Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. J Bacteriol 187(21):7254-7266.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S. Y., Howe, G. A., Browse, J. (2007). JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signaling. Nature 448(7154):661-665.

Thota, S.G., Bhandari, R. (2015). The emerging roles of inositol pyrophosphates in eukaryotic

cell physiology. J Biosci 40(3):593-605.

Tucker, E. B., Boss, W. F. (1996). Mastoparan-induced intracellular Ca^{2+} fluxes may regulate cell-to-cell communication in plants. Plant Physiol 111(2):459-467.

Val-Torregrosa, B., Bundó, M., Martín-Cardoso, H., Bach-Pages, M., Chiou, T.-J., Flors, Segundo B.S. (2022). Phosphate-induced resistance to pathogen infection in Arabidopsis. Plant J 110(2):452-469.

Van Loon, L. C., Rep, M., Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135-162.

Vance, C. P., Uhde-Stone, C., Allan, D. L. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157(3):423-447.

Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dormán, G., Olszewski, J. D., Prestwich, G. D., Snyder, S. H. (1996). Purified inositol hexakisphosphate kinase is an ATP synthase: diphosphoinositol pentakisphosphate as a high-energy phosphate donor. Proc Natl Acad Sci U S A.

Wang, D., Pajerowska-Mukhtar, K., Culler, A. H., Dong, X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr Biol 17(20):1784-1790.

Wang, Y., Lin, W. H., Chen, X., Xue, H. W. (2009). The role of Arabidopsis 5PTase13 in root gravitropism through modulation of vesicle trafficking. Cell Res 19(10):1191-1204.

Wang, H., Falck, J. R., Hall, T. M., Shears, S. B. (2011). Structural basis for an inositol pyrophosphate kinase surmounting phosphate crowding. Nat Chem Biol 8(1):111-116.

Wang, Y., Chu, Y. J., Xue, H. W. (2012). Inositol polyphosphate 5-phosphatase-controlled Ins(1,4,5)P3/Ca2+ is crucial for maintaining pollen dormancy and regulating early germination of pollen. Development 139(12):2221-2233.

Wang, R., Estelle, M. (2014) Diversity and specificity: auxin perception and signaling through the TIR1/AFB pathway. Curr Opin Plant Biol 21:51-58.

Wang, H., Nair, V. S., Holland, A. A., Capolicchio, S., Jessen, H. J., Johnson, M. K., Shears, S. B. (2015). Asp1 from Schizosaccharomyces pombe binds a [2Fe-2S](2+) cluster which inhibits inositol pyrophosphate 1-phosphatase activity. Biochemistry 54:6462-6474.

Wang, H., Gu, C., Rolfes, R.J., Jessen, H. J, Shears, S. B. (2018). Structural and biochemical characterization of Siw14: A protein-tyrosine phosphatase fold that metabolizes inositol pyrophosphates. J Biol Chem 293(18):6905-6910.

Wang, Z., Kuo, H.-F., Chiou, T.-J. (2021). Intracellular phosphate sensing and regulation of phosphate transport systems in plants. Plant Physiol. 187(4):2043-2055.

Wasternack, C., Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. an update to the 2007 review in *Annals of Botany.* Ann Bot 111(6):1021-1058.

Wheeler, G. L., Brownlee, C. (2008). Ca^{2+} signalling in plants and green algae - changing channels. Trends Plant Sci 13(9):506-514.

Whitfield, H., White, G., Sprigg, C., Riley, A. M., Potter, B. V. L, Hemmings, A. M., Brearley, C. A. (2020). An ATP-responsive metabolic cassette comprised of inositol tris/tetrakisphosphate kinase 1 (ITPK1) and inositol pentakisphosphate 2-kinase (IPK1) buffers diphosphosphoinositol phosphate levels. Biochem 31:477 (14), 2621-2638.

Wild, R., Gerasimaite, R., Jung, J.-Y., Truffault, V., Pavlovic, I., Schmidt, A., Saiardi, A., Jessen, H. J., Poirier, Y., Hothorn, M., Mayer, A. (2016). Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. Science 352:986-990.

Wilson, M. S. C., Livermore, T. M., Saiardi, A. (2013). Inositol pyrophosphates: between signalling and metabolism. Biochem J 452(3):369-379.

Woodward, A. W., Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann Bot

95(5):707-735.

Wu, J., Qin, Y., Zhao, J. (2008). Pollen tube growth is affected by exogenous hormones and correlated with hormone changes in styles in *Torenia fournieri* L. Plant Growth Regul 55(2):137-148.

Wundenberg, T., Mayr, G. W. (2012). Synthesis and biological actions of diphosphoinositol phosphates (inositol pyrophosphates), regulators of cell homeostasis. Biol Chem 393(9):979-998.

Xia, H. J., Brearley, C., Elge, S., Kaplan, B., Fromm, H., Mueller-Roeber, B. (2003). Arabidopsis inositol polyphosphate 6-/3-kinase is a nuclear protein that complements a yeast mutant lacking a functional ArgR-Mcm1 transcription complex. Plant Cell 15(2):449-463.

Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M., Turner, J. G. (1998). COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280(5366):1091-1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W. L., Ma, H., Peng, W., Huang, D., Xie, D. (2002). The SCF(COI1) ubiquitin–ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 14(8):1919-1935.

Xu, J., Brearley, C. A., Lin, W. H., Wang, Y., Ye, R., Mueller-Roeber, B., Xu, Z. H., Xue, H. W. (2005). A role of Arabidopsis inositol polyphosphate kinase AtIpk2a, in pollen germination and root growth. Plant Physiol 137(1):94-103.

Yan, Y., Stolz, S., Chételat, A., Reymond, P., Pagni, M., Dubugnon, L., Farmer, E. E. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. The Plant Cell 19(8). 2470-2483.

Young, L. M., Evans, M. L., Hertel, R. (1990). Correlations between gravitropic curvature and auxin movement across gravistimulated roots of Zea mays. Plant Physiol 92(3):792-796.

Zarate, S. I., Kempema, L. A., Walling, L. (2007). Silverleaf whitefly induces salicylic acid

defenses and suppresses effectual jasmonic acid defenses. Plant Physiol 143(2):866-875.

Zhan, H., Zhong, Y., Yang, Z., Xia, H. (2015). Enzyme activities of Arabidopsis inositol polyphosphate kinases AtIPK2α and AtIPK2β are involved in pollen development, pollen tube guidance and embryogenesis. Plant J 82(5):758-771.

Zhang, Z. B., Yang, G., Arana, F., Chen, Z., Li, Y., Xia, H. J. (2007). Arabidopsis inositol polyphosphate 6-/3-kinase (AtIpk2beta) is involved in axillary shoot branching via auxin signaling. Plant Physiol 144(2):942-951.

Zhang, Q., van Wijk, R., Shahbaz, M., Roels, W., Schooten, B.V., Vermeer, J. E. M., Zarza, X., Guardia, A., Scuffi, D., García-Mata, C., Laha, D., Williams, P., Willems, L. A. J., Ligterink, W., Hoffmann-Benning, S., Gillaspy, G., Schaaf, G., Haring, M. A., Laxalt, A. M., Munnik, T. (2018). Arabidopsis Phospholipase C3 is Involved in Lateral Root Initiation and ABA Responses in Seed Germination and Stomatal Closure. Plant Cell Physiol 59(3):469-486.

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F., He, S. Y., Dong, X. (2012). Coronatine promotes Pseudomonas syringae virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. Cell Host Microbe 11(6):587-596.

Zhu, Z., Lee, B. (2015). Friends or foes: new insights in jasmonate and ethylene co-actions. Plant Cell Physiol 56(3):414-420.

Zhu, J., Lau, K., Puschmann, R., Harmel, R. K., Zhang, Y.J., Pries, V., Gaugler, P., Broger, L., Dutta, A. K., Jessen, H. J., Schaaf, G., Fernie, A. R., Hothorn, L. A., Fiedler, D., Hothorn, M. (2019). Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. Elife 8.

Chapter IV: General discussion and outlook

The world´s growing population causes an increased demand for food, fiber and fuel which is in large part dependent on crop production (OECD/FAO, 2022), which could either be satisfied by increased land use or increased yield (Edgerton, 2009). Increased land use causes loss of natural ecosystems and can cause negative effects on the environment and biodiversity, leading to a high demand to modify crop production systems (Rockström et al, 2009; Roberts and Mattoo, 2018).

In order to guarantee stable crop yields that would fulfill the higher demand for food, breeding of variants adapted to various abiotic and biotic stresses (Razzaq et al., 2021) and crop management such as the application of fertilizers and pesticides is required (Cooper and Dobson, 2007; Popp et al., 2013; Ahrends et al., 2021). But the excessive use of P fertilizer and pesticides is known to be resource intensive and negatively impact the environment, either via pollution of open water bodies and other natural ecosystems (Vos et al., 2000; Hussain et al., 2009; Rockström et al., 2009; Raboy, 2020). The fundamental understanding of how plants sense nutritional changes in the environment, as well as how they recognize and defend against pests and pathogens might help to improve future farming systems, in a way to maintain or even increase yield without compromising the environment.

Here we contribute to this knowledge by focusing on inositol pyrophosphates, and their roles in the regulation of P_i homeostasis and hormone signaling, which ultimately leads to the regulation of abiotic and biotic stress responses. Taking advantage of the novel CE-ESI-MS method that was developed with the Jessen lab at the University of Freiburg (Qiu et al., 2020), we were able to show for the first time that the $InsP_6$ kinase ITPK1 mediates the generation of 5-$InsP_7$ *in vivo*, importantly providing the precursor for $InsP_8$, which functions as ligand for SPX protein to regulate P_i homeostasis in plants (Riemer et al., 2021). Previous studies addressing PP-InsPs in plants were limited to techniques such as SAX-HPLC, PAGE or ion chromatography and subsequently failed to discriminate different $InsP_7$ isomers (Desai et al., 2014; Couso et al., 2016; Laha et al., 2015; Laha et al.,2016; Kuo et al., 2016; Blüher et al., 2017; Dong et al., 2019; Zhu et al., 2019). In previous studies focusing on P_i homeostasis, only pools of $InsP_7$ could be analyzed (Kuo et al., 2016; Dong et al., 2019; Zhu et al., 2019), while with the help of CE-ESI-MS we were able to distinguish between different PP-InsP isomers. We showed that 1/3-$InsP_7$, 5-$InsP_7$ and 1/3,5-$InsP_8$ levels were changed upon P_i-starvation and resupply conditions, suggesting that ITPK1 and VIH2 activities together control the PSR. The data also revealed decreased 5-$InsP_7$ levels in mutants lacking functional ITPK1 upon sufficient P_i conditions. Additionally, the mutant exhibit reduced 1/3,5-$InsP_8$ level after P_i replete,

assuming that ITPK1-generated 5-InsP$_7$ serves as substrate for P$_i$-responsive 1/3,5-InsP$_8$ production via VIH2. Notably, we detected similar 1/3-InsP$_7$ concentrations as observed for 5-InsP$_7$ when plants regain P$_i$. Previous NMR data showed that the recombinant kinase domain of *Arabidopsis* VIH2 catalyzes the generation of 1/3-InsP$_7$ from InsP$_6$ (Zhu et al., 2019), which we could confirm *in planta* by analyzing *vih2* mutants via CE-ESI-MS. However, the increase in 1/3-InsP$_7$ under P$_i$-resupply conditions might not be directly involved in P$_i$ homeostasis since we observed overaccumulation of 5-InsP$_7$, and not 1/3-InsP$_7$, in *vih2* shoots regaining P$_i$. Additionally, we identified a previously undetected InsP$_7$ species corresponding to either 4-InsP$_7$ or 6-InsP$_7$ (4/6-InsP$_7$), which was found to be the most abundant InsP$_7$ isomer in plants. We could not observe a strong overaccumulation of the 4/6-InsP$_7$ species under P$_i$-resupply, differently than what has been shown for 1/3-InsP$_7$ and InsP$_8$, whose levels under such conditions were significantly increased when compared to P$_i$ sufficient status. Therefore, we hypothesize that the isomer might not play a physiological relevant role in regulating P$_i$ starvation responses. Future research has to determine the kinase(s) that generate 4/6-InsP$_7$ *in planta*, to provide the basis to study the function of these novel InsP$_7$ isomer(s). To date, both 4-InsP$_7$ and 6-InsP$_7$ were not reported in other organisms, with the exception of 6-InsP$_7$, which was found in the social amoeba *Dictyostelium discoideum* (Laussmann et al., 1997) and is there responsible for chemotactic responses (Luo et al., 2003). Since 4/6-InsP$_7$ represents the major fraction of InsP$_7$ in *Arabidopsis*, whether this isomer is involved in abiotic and biotic stress responses, such as drought/heat/water stress or pathogen and herbivory attack is a fundamental question to be answered. Previous research demonstrated the key role of InsP$_8$ in JA perception and of 5-InsP$_7$ in auxin signaling, and both were shown to be putative candidates regulating salicylic acid-mediated immunity (Laha et al., 2015; Laha et al., 2022; Gulabani et al., 2021). It is possible that also 4-InsP$_7$ or 6-InsP$_7$ may be phosphorylated to a novel InsP$_8$ isomer with functions other than the previously reported regulation of P$_i$ homeostasis (Dong et al., 2019; Riemer et al., 2021) or JA perception (Laha et al., 2015).

Since our study identified other InsP$_7$ isomers in plants, it is important to gain a broader picture of the function of both kinases, as well as of a potential occurrence of other InsP$_7$ isomers in nature. One interesting way to approach those questions would be to investigate whether recombinant *Arabidopsis* ITPK and VIH isoforms possess kinase activity on 1-InsP$_7$, 3-InsP$_7$, 4-InsP$_7$, 5-InsP$_7$ or 6-InsP$_7$ isomers, respectively. Notably, to date it is still assumed that 1-InsP$_7$ and 1,5-InsP$_8$ are the major species found at least in yeast and mammals (Shears, 2018), but since it is technically challenging to separate the chiral isomers, it still remains unclear whether 1-InsP$_7$ or 3-InsP$_7$ and 1,5-InsP$_8$ or 3,5-InsP$_8$, respectively, are present in plants. Therefore,

there is a demand to develop and improve methods to distinguish between mirrored isomers (so called enantiomers) and determine the identity of detected PP-InsPs. This, for instance, might be achieved by taking advance of chiral separation via spiking L-arginine amide to 4/6-InsP$_7$ extracted from plant tissue followed by NMR analyses (Blüher et al., 2017).

Interestingly, we also detected an unknown PP-InsP$_4$ species in P$_i$-starved *Arabidopsis* and rice roots that migrates between InsP$_6$ and InsP$_7$ on PAGE, and we suggest that this isomer is regulated by P$_i$ availability in plants. However, the species does not co-migrate with the available standard 5PP-Ins(1,3,4,6)P$_4$ when analyzed via CE-ESI-MS. Notably, the species was less abundant in *itpk1* roots (Riemer et al., 2021), in agreement to a recent study that revealed the phosphorylation of Ins(1,2,3,4,5)P$_5$ to PP-InsP$_4$ by recombinant *Arabidopsis* ITPK1 (Whitfield et al., 2020), which supports our *in vivo* observations suggesting that ITPK1 is the putative kinase generating the unknown PP-InsP$_4$ species in roots (Riemer et al., 2021). Further research is needed to clarify the nature of this species, for instance, via spiking synthetic PP-InsP$_4$ standards based on Ins(1,2,3,4,5)P$_5$ pyrophosphorylation to plant extracts for CE-ESI-MS analyses. Moreover, another question to be addressed is whether the species is involved in P$_i$ homeostasis, especially in roots. Previous studies revealed that the auxin receptor TIR1 is a PHR1 target (Castrillo et al., 2017), which may suggest a link between auxin signaling, P$_i$ starvation and lateral root formation, a typical plant response to P$_i$-limited conditions. Since ITPK1 is known to physically interact with TIR1, assuming to generate 5-InsP$_7$ to activate the auxin receptor complex (Laha et al., 2022), an involvement of PP-InsP$_4$ isomers may be a point of interest to investigate a putative role of PP-InsP$_4$ in auxin-mediated root architecture-adaption to P$_i$-stress. Strikingly, *itpk1* mutants show defective primary root elongation, which is known to be an auxin defective phenotype (Laha et al., 2022), pointing more towards a putative connection of PP-InsP$_4$ and auxin signaling in roots.

Besides the function of ITPK1 as an InsP$_6$ kinase, we also demonstrated that ITPK1 directly controls the plant´s P$_i$ status via PP-InsP synthesis and degradation. The enzyme can shift its activity from kinase to an ADP phosphotransferase that exclusively dephosphorylates its product 5-InsP$_7$ in the presence of ADP *in vitro*. This observation is in accordance with previously demonstrated Ins(1,3,4,5,6)P$_5$ phosphotransferase activities of recombinant potato and *Arabidopsis* ITPK1 (Caddick et al., 2008; Whitfield et al., 2020). These findings suggest that the reverse activity of ITPK1 might be used to switch off InsP$_8$ signaling by turning down the levels of its precursor 5-InsP$_7$ upon P$_i$-starvation.

Interestingly, most of the PP-InsP level were unaltered in mutants lacking functional *ITPK2*, the close homolog of *ITPK1*. Since *itpk1* does not exhibit the severe P-overaccumulation and

growth phenotype as observed for *vih1 vih2* double mutants (Dong et al., 2019; Zhu et al., 2019), a functional redundancy might cause the weak phenotype of *itpk1*. Generating *itpk1 itpk2* double mutants resulted in lethality and only the progeny of homozygous-heterozygous parents was capable to survive despite severe growth retardation (Riemer et al., 2021). In these plants, the shoot P level were higher compared to Col-0 and *itpk1*, assuming that, despite ITPK2 is not a key player in P_i signaling, it may partially compensate the loss of ITPK1 (Riemer et al., 2021). Moreover, at least one of the candidates needs to be functional to circumvent lethality, suggesting a crucial role of ITPK1 and ITPK2 for plant growth.

To summarize, our observations highlight ITPK1 as a crucial component connecting the cellular P_i status with changes in PP-InsP levels. Furthermore, ITPK1 acts in concert with VIH2 to generate or withhold $InsP_8$ to maintain proper P_i homeostasis in plants and either functional ITPK1 or ITPK2 are necessitated to maintain plants survival.

Our findings on how plants sense their P_i status lay the basis for future research aiming at generating plants that are more efficient in P_i uptake, without facing the drawback of phytate accumulation. The generation of crop plants containing less phytate in their seeds is crucial to circumvent issues such as water pollution and malnutrition, or the so called "hidden hunger", which affects two billion people worldwide (von Grebmer et al., 2014) and is a result of consuming an energy-dense but nutrient-poor diet (Black et al., 2013; World Health Organization, 2006). In the human diet, zinc, iron, iodine and vitamin A belong to the most limiting micronutrients (World Health Organization, 2006). Especially zinc and iron deficiency are often found in low income countries (Gibson and Ferguson, 1998; Bohn et al., 2008; World Health Organization, 2009), while in addition, iron deficiency is also a problem of children, women of childbearing age, and older people (McLean et al., 2009; Stevens et al., 2013; Petry et al., 2016; World Health Organization, 2017). These issues mostly rely on the high consumption of grains and legumes with high contents of phytate, that binds to essential minerals such as zinc, iron, magnesium and calcium in the digestive tract and therefore inhibits the absorption by the body (Erdman, 1981; Brown and Solomons, 1991; Raboy, 2002). At first glance, the generation of low-phytate crops may be an important aim to prevent people from being exposed to iron and zinc deficiency. Previous studies focused on *low phytic acid* (*lpa*) crops. Unfortunately, these mutants displayed several issues, such as the reduced germination rate, accompanied with less yield and negative pleiotropic effects (Raboy et al., 2000; Pilu et al., 2005; Zhao et al., 2008; Cerino Badone et al., 2012). Some *lpa* plants showed reduced level of $InsP_7$ and $InsP_8$ that may result in a disturbed P_i homeostasis, combined with higher susceptibility to pathogens and pests, since both species are involved in SA and JA signaling,

respectively (Laha et al., 2015; Gulabani et al., 2021). On the other hand, higher levels of $InsP_7$ and $InsP_8$ combined with lower $InsP_6$ levels were measured in *Arabidopsis* seeds, shoots and roots of mutants disrupted in MRP5-mediated transport of $InsP_6$ into the vacuole (Riemer et al., 2021). Future research has to unveil whether these mutants result in more resistant plants against pathogen and pest attacks. In some cases, the disruption of MRP-transporters results in lethal phenotypes in the homozygous stage of maize and rice. This might be caused by the strong reduction of phytate in crop seeds (80-90%) (Colombo et al., 2020), an impaired embryo development due to the displacement of the root primordium (Cerino Badone et al., 2012) or the higher content of free iron associated with more free radicals in the kernels and higher production of hydrogen peroxide in the embryo (Doria et al., 2009). Downregulating the vacuolar transporter, e.g., by RNA interference technology, may be a helpful approach to accumulate less $InsP_6$ in the seed storage tissue without facing the above mentioned issues. This may be accompanied with increased $InsP_7$ and $InsP_8$ levels in roots and shoots. The isomer identity of both species still needs to be uncovered by using the novel CE-ESI-MS tool or NMR, if sufficient quantities can be purified, in combination with a chiral selector as successfully applied to distinguish $InsP_5$ entaniomers (Blüher et al., 2017). Moreover, it may be an interesting finding whether the increase of specific PP-InsP isomers caused by disrupted MRP5 transporter activity would probably lead to an enhanced resistance of crops against pathogens and pests, resulting in less pesticide and fertilizer application, as well as less yield losses. Taken together, this work has uncovered the role of the *Arabidopsis* $InsP_6$ kinase ITPK1 and its product 5-$InsP_7$ as critical regulators of P_i homeostasis in plants, providing an insight in how plants sense their P_i status. Furthermore, the finding of new $InsP_7$ and PP-$InsP_4$ isomers in plants will contribute to further knowledge on the PP-InsP pathway in plants. Further research on the new candidates may enable us to generate plants that are more efficient in nutrient acquisition, and more tolerant to abiotic and biotic stresses, which will contribute to future agricultural sustainability. Moreover, the understanding of how plants sense P_i with help of PP-InsPs will lay the basis for using those molecular targets in future plant breeding research and, in addition, may answer the question why specific genotypes in crop diversity panels show an enhanced P-use efficiency, that could be used for conventional breeding methods in future.

References

Ahrends, H. E., Siebert, S. Rezaei, E. E., Seidel, S. J., Hüging, H., Ewert, F., Döring, T., Rueda-Ayala, V., Eugster, W., Gaiser, T. (2021). Nutrient supply affects the yield stability of major European crops – a 50 year study. Environ Res Lett 16 014003.

Black, R. E., Victora, C. G., Walker, S. P. Bhutta, Z. A., Christian, P., de Onis, M., Ezzati, M., Grantham-McGregor, S., Katz, J., Martorell, R., Uauy, R. (2013). Maternal and child undernutrition and overweight in low-income and middle-income countries. Lancet 382:427-451.

Blüher, D., Laha, D., Thieme, S. Hofer, A., Eschen-Lippold, L., Masch, A., Balcke, G., Pavlovic, I., Nagel, O., Schonsky, A., Hinkelmann, R., Wörner, J., Parvin, N., Greiner, R., Weber, S., Tissier, A., Schutkowski, M., Lee, J., Jessen, H. J., Schaaf, G., Bonas, U. (2017). A 1-phytase type III effector interferes with plant hormone signaling. Nat Commun 8:2159.

Bohn, L., Meyer, A. S., Rasmussen, S. K. (2008). Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B 9(3):165-191.

Brown, K. H., Solomons, N. W. (1991). Nutritional problems of developing countries. Infect Dis Clin North Am 5(2):297-317.

Caddick, S. E. K., Harrison, C. J., Stavridou, I., Mitchell, J. L., Hemmings, A. M., Brearley, C. A. (2008). A Solanum tuberosum inositol phosphate kinase (StITPK1) displaying inositol phosphate-inositol phosphate and inositol phosphate-ADP phosphotransferase activities. Febs Lett 582:1731-1737.

Castrillo, G., Teixeira, P. J., Paredes, S. H., Law, T. F., de Lorenzo, L., Feltcher, M. E., Finkel, O. M., Breakfield, N. W., Mieczkowski, P., Jones, C. D., Paz-Ares, J., Dangl, J. L. (2017). Root microbiota drive direct integration of phosphate stress and immunity. Nature 543:513-518.

Cerino Badone, F., Amelotti, M., Cassani, E., Pilu, R. (2012). Study of low phytic acid1-7 (lpa1-7), a new ZmMRP4 mutation in maize. J Hered 103(4):598-605.

Colombo, F., Paolo, D., Cominelli, E., Sparvoli, F., Nielsen, E., Pilu, R. (2020). MRP Transporters and Low Phytic Acid Mutants in Major Crops: Main Pleiotropic Effects and Future Perspectives. Front Plant Sci 27(11):1301.

Cooper, J., Dobson, H. *(2007).* The benefits of pesticides to mankind and the environment. Crop Prot 26*:*1337-1348

Desai, M., Rangarajan, P., Donahue, J. L., Williams, S. P., Land, E. S., Mandal, M. K., Phillippy, B. Q., Perera, I. Y., Raboy, V., Gillaspy, G. E. (2014). Two inositol hexakisphosphate kinases drive inositol pyrophosphate synthesis in plants. Plant J 80(4):642-653.

Dong, J., Ma, G., Sui, L., Wei, M., Satheesh, V., Zhang, R., Ge, S., Li, J., Zhang, T. E., Wittwer, C., Jessen, H. J., Zhang, H., An, G. Y., Chao, D. Y., Liu, D., Lei, M. (2019). Inositol pyrophosphate InsP$_8$ acts as an intracellular phosphate signal in Arabidopsis. Mol Plant 12(11):1463-1473.

Doria, E., Galleschi, L., Calucci, L., Pinzino, C., Pilu, R., Cassani, E., Nielsen, E. (2009). Phytic acid prevents oxidative stress in seeds: Evidence from a maize (Zea mays L.) low phytic acid mutant. J Exp Bot 60:967-978.

Edgerton, M. D. (2009). Increasing crop productivity to meet global needs for feed, food, and fuel. Plant Physiol 149(1):7-13.

Erdman, J. W. (1981). Bioavailability of trace minerals from cereals and legumes. Cereal Chem 58:21-26.

Gibson, R. S., Ferguson, E. L. (1998). Assessment of dietary zinc in a population. Am J Clin Nutr 68(2 Suppl):430-434.

Gulabani, H., Goswami, K., Walia, Y., Roy, A., Jameeta Noor, J., Ingole, K. D., Kasera, M., Laha, D., Giehl, R. F. H., Schaaf, G., Bhattacharjee, S. (2021). Arabidopsis inositol polyphosphate kinases IPK1 and ITPK1 modulate crosstalks between SA-dependent immunity and phosphate-starvation responses. Plant Cell Reports 41(2):347-363.

Hussain, S., Siddique, T., Saleem, M., Arshad, M., Khalid, A. (2009). Impact of pesticides on soil microbial diversity, enzymes, and biochemical reactions. Adv Agron 102:159-200.

Kuo, H.-F., Hsu, Y.-Y., Lin, W.-C., Chen, K.-Y., Munnik, T., Brearley, C. A., Chiou, T.-J. (2018). Arabidopsis inositol phosphate kinases, IPK1 and ITPK1, constitute a metabolic pathway in maintaining phosphate homeostasis. Plant J 95:613-630.

Laha, D., Johnen, P., Azevedo, C., Dynowski, M., Weiss, M., Capolicchio, S., Mao, H. B., Iven, T., Steenbergen, M., Freyer, M., Gaugler, P., de Campos, M. K. F., Zheng, N., Feussner, I., Jessen, H. J., Van Wees, S. C. M., Saiardi, A., Schaaf, G. (2015). VIH2 Regulates the Synthesis of Inositol Pyrophosphate InsP(8) and Jasmonate-Dependent Defenses in Arabidopsis. Plant Cell 27:1082-1097.

Laha, D., Parvin, N., Dynowski, M., Johnen, P., Mao, H., Bitters, S. T., Zheng, N., Schaaf, G. (2016). Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex. Plant Phys 171:2364–2370.

Laha, N. P, Giehl, R. F. H., Riemer E, Qiu, D., Pullagurla, N. J., Schneider, R., Dhir, Y. W., Yadav, R., Mihiret, Y. E., Gaugler, P., Gaugler, V., Mao, H., Zheng, N., von Wirén, N., Saiardi, A., Bhattacharjee, S., Jessen, H. J., Laha, D., Schaaf, G. (2022). INOSITOL (1,3,4) TRIPHOSPHATE 5/6 KINASE1-dependent inositol polyphosphates regulate auxin responses in Arabidopsis. Plant Physiol 190(4):2722-2738.

Laussmann, T., Reddy, K. M., Reddy, K. K., Falck, J. R., Vogel, G. (1997). Diphospho-myo-inositol phosphates from Dictyostelium identified as D-6-diphospho-myo-inositol pentakisphosphate and D-5,6-bisdiphospho-myo-inositol tetrakisphosphate. Biochem J 322:31-33.

Luo, H. B. R., Huang, Y. E., Chen, J. M. C., Saiardi, A., Iijima, M., Ye, K. Q., Huang, Y. F., Nagata, E., Devreotes, P., Snyder, S. H. (2003). Inositol pyrophosphates mediate chemotaxis in Dictyostelium via pleckstrin homology domain-PtdIns (3,4,5)P3 interactions. Cell 114:559-572.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D., de Benoist, B. (2009). Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. Public Health Nutr 12:444-454.

OECD/FAO (2022), OECD-FAO Agricultural Outlook 2022-2031, OECD Publishing, Paris. Available online:
https://www.oecd-ilibrary.org/sites/f1b0b29c-en/index.html?itemId=/content/publication/f1b0b29c-en   (accessed on 04.01.2023).

Petry, N., Olofin, I., Hurrell, R.F., Boy, E., Wirth, J.P., Moursi, M., Donahue Angel, M., Rohner, F. (2016). The Proportion of Anemia Associated with Iron Deficiency in Low, Medium, and High Human Development Index Countries: A Systematic Analysis of National Surveys. Nutrients 8:693.

Pilu, R., Landoni, M., Cassani, E., Doria, E., Nielsen, E. (2005). The maize lpa241 mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci 45:2096-2105.

Popp, J., Petö, K., Nagy, J. *(2013).* Pesticide productivity and food security. A review. Agron Sustain Dev 33*:*243-255.

Qiu, D., Wilson, M. S., Eisenbeis, V. B., Harmel, R. K., Riemer, E., Haas, T. M., Wittwer, C., Jork, N., Gu, C., Shears, S. B., Schaaf, G., Kammerer, B., Fiedler, D., Saiardi, A., Jessen, H. J. (2020). Analysis of inositol phosphate metabolism by capillary electrophoresis electrospray ionization mass spectrometry. Nat Commun 11:6035.

Raboy, V., Gerbasi, P. F., Young, K. A., Stoneberg, S. D., Pickett, S. G., Bauman, A. T., Murthy, P. P., Sheridan, W. F., Ertl, D.S. (2000). Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol 124(1):355-68.

Raboy, V. (2002). Progress in Breeding Low Phytate Crops. J Nutr 132(3):503–505.

Raboy, V. (2020). *Low phytic acid* Crops: Observations Based on Four Decades of Research. Plants 9(2)*:*140.

Razzaq, A., Kaur, P., Akhter, N., Wani, S. H., Saleem, F. (2021). Next-Generation Breeding Strategies for Climate-Ready Crops. Front Plant Sci 12:620420.

Riemer, E., Qiu, D., Laha, D., Harmel, R. K., Gaugler, P., Gaugler, V., Frei, M., Hajirezaei, M.-R., P. Laha, N., Krusenbaum, L., Schneider, R., Saiardi, A., Fiedler, D., Jessen, H. J., Schaaf, G., Giehl, R. F. H. (2021). ITPK1 is an InsP6/ADP phosphotransferase that controls phosphate signaling in Arabidopsis. Mol Plant 14(11):1864-1880.

Roberts, D. P., and Mattoo, A. K. (2018). Sustainable agriculture – enhancing environmental benefits, food nutritional quality and building crop resilience to abiotic and biotic stresses. Agriculture 8:8.

Rockström, J., Steffen, W., Noone, K., Persson, A., Chapin, F. S., Lambin, E. F., Lenton, T. M., Scheffer, M., Folke, C., Schellnhuber, H. J., Nykvist, B., de Wit, C. A., Hughes, T., van der Leeuw, S., Rodhe, H., Sörlin, S., Snyder, P. K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R. W., Fabry, V. J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P., Foley, J. A. (2009). A safe operating space for humanity. Nature 461:472-475.

Shears, S. B. (2018). Intimate connections: Inositol pyrophosphates at the interface of metabolic regulation and cell signaling. J Cell Physiol 233(3):1897-1912.

Stevens, G. A., Finucane, M. M., De-Regil, L. M., Paciorek, C. J., Flaxman, S. R., Branca, F., Peña-Rosas, J. P., Bhutta, Z. A., Ezzati, M. (2013). Nutrition Impact Model Study Group (Anaemia). Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995-2011: a systematic analysis of population-representative data. Lancet Glob Health 1(1):e16-25.

von Grebmer, A. S. K., Saltzman, A., Birol, E., Wiesmann, D. Prasai, N., Yin, S., Yohannes Y., Menon, P., Thompson, J., Sonntag, A. (2014). 2014 Global Hunger Index: the challenge of hidden hunger. In [IFPRI Welthungerhilfe, and Concern Worldwide, editor]. Bonn, Washington, D.C., and Dublin.

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambré, C., Tarazona, J. V., Brandt, I., Vethaak, A. D. (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol 30(1):71-133.

World Health Organization (2006). Guidelines on Food Fortification with Micronutrients. World Health Organization, Geneva. Available online: https://www.who.int/publications/i/item/9241594012 (accessed on 19.12.2022).

Word Health Organization (2009). Global Health Risks: Mortality and Burden of Disease Attributable to Selected Major Risks, World Health Organization. Available online: https://apps.who.int/iris/bitstream/handle/10665/44203/9789241563871_eng.pdf?sequence=1 &isAllowed=y (accessed on 21.20.2022).

World Health Organization (2017). Nutritional Anaemias: Tools for Effective Prevention and Control; WHO: Geneva, Switzerland. Available online: https://apps.who.int/iris/bitstream/handle/10665/259425/?sequence=1 (accessed on 21.12.2022).

Whitfield, H., White, G., Sprigg, C., Riley, A. M., Potter, B. V. L., Hemmings, A. M., Brearley, C. A. (2020). An ATP-responsive metabolic cassette comprised of inositol tris/tetrakisphosphate kinase 1 (ITPK1) and inositol pentakisphosphate 2-kinase (IPK1) buffers diphosphosphoinositol phosphate levels. Biochem J 477:2621-2638.

Zhao, H. J., Liu, Q. L., Fu, H. W., Xu, X. H., Wu, D. X., Shu, Q. Y. (2008). Effect of non-lethal low phytic acid mutations on grain yield and seed viability in rice. F Crop Res 108:206-211.

Zhu, J., Lau, K., Puschmann, R., Harmel, R. K., Zhang, Y.J., Pries, V., Gaugler, P., Broger, L., Dutta, A. K., Jessen, H. J., Schaaf, G., Fernie, A. R., Hothorn, L. A., Fiedler, D., Hothorn, M. (2019). Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. Elife 8.

Other Publications

Qiu, D., Wilson, M. S., Eisenbeis, V. B., Harmel, R. K., <u>Riemer, E.</u>, Haas, T. M., Wittwer, C., Jork, N., Gu, C., Shears, S. B., Schaaf, G., Kammerer, B., Fiedler, D., Saiardi, A., Jessen, H. J. (2020). Analysis of inositol phosphate metabolism by capillary electrophoresis electrospray ionization mass spectrometry. Nat Commun 11:6035.
https://doi.org/10.1038/s41467-020-19928-x

Laha, N. P., Giehl, R. F. H., <u>Riemer, E.</u>, Qiu, D., Pullagurla, N. J., Schneider, R., Dhir, Y. W., Yadav, R., Mihiret, Y. E., Gaugler, P., Gaugler, V., Mao, H., Zheng, N., von Wirén, N., Saiardi, A., Bhattacharjee, S., Jessen, H. J., Laha, D., Schaaf, G. (2022). INOSITOL (1,3,4) TRIPHOSPHATE 5/6 KINASE1-dependent inositol polyphosphates regulate auxin responses in *Arabidopsis*. Plant Physis 190(4):2722–2738.
https://doi.org/10.1093/plphys/kiac425

Siddique, S., Radakovic, Z.S., Hiltl, C. Pellegrin, C., Baum, T. J., Beasley, H., Bent, A. F., Chitambo, O., Chopra, D., Danchin, E. G. J., Grenier, E., Habash, S. S., Hasan, M. S., Helder, J., Hewezi, T., Holbein, J., Holterman, M., Janakowski, S., Koutsovoulos, G. D., Kranse, O. P., Lozano-Torres, J. L., Maier, T. R., Masonbrink, R. E., Mendy, B., <u>Riemer, E.</u>, Sobczak, M., Sonawala, U., Sterken, M. G., Thorpe, P., van Steenbrugge, J. J. M., Zahid, N., Grundler, F., Eves-van den Akker, S. (2022). The genome and lifestage-specific transcriptomes of a plant-parasitic nematode and its host reveal susceptibility genes involved in trans-kingdom synthesis of vitamin B5. Nat Commun 13:6190.
https://doi.org/10.1038/s41467-022-33769-w

Liu, G., <u>Riemer, E.</u>, Schneider, R., Cabuzu, D., Bonny, O., Wagner, C. A., Qiu, D., Saiardi, A., Strauss, A., Lahaye, T., Knoll, T., Jessen, J. P., Jessen, H. J. (2023). The phytase RipBL1 enables the assignment of a specific inositol phosphate isomer as a structural component of human kidney stones. RSC Chemical Biology.
https://doi.org/10.1039/D2CB00235C

Danksagung

An erster Stelle möchte ich mich bei meinem Doktorvater Gabriel bedanken. Du hast mir den Raum gegeben, eigene Ideen und Projekte zu entwickeln und mich dabei immer unterstützt. Somit konnte ich in meiner Zeit als Doktorandin bei dir eine Menge lernen. Vielen Dank!

Auch möchte ich meinen Kollegen im Schaaf Lab danken. Zuerst meinem Studienfreund und Büromitbewohner Robin für das gemeinsame Lachen, das Grübeln über unsere Projekte und natürlich die Kaffeepausen! Danke auch an Philipp und Verena, durch euch bin ich an meine Doktorandenstelle gelangt und ihr standet mir bei vielen immer Fragen mit Rat und Tat zur Seite. Großer Dank gilt Brigitte für die tatkräftige Unterstützung bei meinen Experimente, ohne diese Hilfe hätte ich nur halb so viel schaffen können! Ich möchte mich auch herzlich bei Marília für den immer willkommenen und sehr lehrreichen Input und Feinschliff an meinen schriftlichen Arbeiten bedanken. Natürlich möchte ich mich auch bei alle anderen Schaaf Lab Mitgliedern für die gute Zeit bedanken.

Ebenso möchte ich mich bei allen Kollaboratoren, besonders bei Henning und Danye aus dem Jessen Lab der Universität Freiburg. Durch diese Zusammenarbeit konnte ich den Grundstein für mein eigenes kleines Projekt legen. Ein riesisges Dankeschön geht auch an Ricardo vom IPK Gatersleben für die tolle Zusammenarbeit und die Möglichkeit, an dem Projekt mitzuwirken, welches nun Teil meiner Doktorarbeit ist!

Und das Beste kommt zum Schluss: Ein ganz besonderer Dank geht an Richard! Du hast mich immer wieder motiviert, Input gegeben, mich aufgebaut und gut zugeredet aber auch geholfen, manche Dinge aus einem anderen Blickwinkel zu betrachten. Ohne deine Unterstützung wäre meine Doktorarbeit nur halb so gut gelungen. Nun ist das Abenteuer Promotion geschafft und ich freue mich darauf, die nächsten Herausforderungen gemeinsam mit dir zu meistern!