



Intracellular phosphate sensing and regulation of phosphate transport systems in plants

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Abstract

Recent research on the regulation of cellular phosphate (Pi) homeostasis in eukaryotes has collectively made substantial advances in elucidating inositol pyrophosphates (PP-InsP) as Pi signaling molecules that are perceived by the SPX (Syg1, Pho81, and Xpr1) domains residing in multiple proteins involved in Pi transport and signaling. The PP-InsP-SPX signaling module is evolutionarily conserved across eukaryotes and has been elaborately adopted in plant Pi transport and signaling systems. In this review, we have integrated these advances with prior established knowledge of Pi and PP-InsP metabolism, intracellular Pi sensing, and transcriptional responses according to the dynamics of cellular Pi status in plants. Anticipated challenges and pending questions as well as prospects are also discussed.

Introduction

Phosphorus (P) is one of the building blocks for nucleic acids, lipid membranes, and ATP in cells and is a macronutrient essential for plant growth and development. It is also a key determinant of agricultural yield. Plants take up P in the form of orthophosphate (Pi, $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$) which is often fixed in soils, resulting in a low level of available Pi (<10 μM) in most soils (Holford, 1997). To secure the food supply, excessive application of P fertilizer is usually practiced in agriculture. Unlike nitrogen (N) fertilizer that can be chemically reproduced, P fertilizer is mainly sourced from finite and geographically restricted natural P rock reserves, which face rapid depletion due to intensive excavation to meet the agricultural demand over the past decades. Such a

situation has resulted in the increasing market price of P fertilizer, and a threat to global food security and agricultural sustainability (Obersteiner et al., 2013). A sustainable measure to circumvent this issue would be to increase the P usage efficiency of crops through breeding (van de Wiel et al., 2016; Heuer et al., 2017), which will rely on knowledge of how plants sense and respond to environmental P levels.

Plants sense the available Pi level in the soil and respond accordingly to maintain cellular Pi homeostasis through coordination of Pi acquisition, translocation, storage, metabolism, and remobilization. In response to a low Pi environment, plants initiate multifaceted adaptive mechanisms to increase external Pi acquisition and enhance internal Pi remobilization and utilization, the so-called Pi starvation response (PSR). The PSR involves a series of metabolic, morphological, and

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ADVANCES BOX

- Structural and functional analyses of the SPX domains establish their role as intracellular P sensors through the binding with PP-InsPs rather than Pi.
- Characterizations of the enzymes and their corresponding mutants involved in InsP and PP-InsP biosynthesis pathways identify PP-InsPs as metabolic messengers for intracellular Pi signaling in response to extracellular P availability.
- InsP₇ and InsP₈ are evolutionarily conserved Pi signaling molecules that are metabolically linked to cellular Pi levels.
- InsP₈ as an intracellular ligand modulates the interaction between SPX and PHR proteins, which in turn controls the transcriptional activation of PSR genes.
- SPX proteins are engaged in the regulation of the crosstalk between N and P signaling networks.

transcriptomic changes mediated through signal transduction, protein modifications, and transcriptional reprogramming, which are suppressed upon Pi replenishment (Vance et al., 2003; Chiou and Lin, 2011; Secco et al., 2013). Recently, considerable advances have been made in identifying the intracellular Pi sensing signal(s), including Pi per se and inositol phosphates (InsP). In this review, we summarize current knowledge on the dynamics of intracellular Pi metabolism and discuss how it transmits signals to coordinate the cellular Pi transport systems for cellular Pi homeostasis control.

Dynamics of Pi metabolism in response to external Pi supply

About 0.2% of plant dry mass is made up of P, which comprises inorganic forms, such as Pi and pyrophosphate (PPi), and organic forms, such as nucleic acids, phospholipids (P-lipids), and low molecular mass P-esters (Veneklaas et al., 2012). The level of Pi generally reflects P fertilizer applied to plants (White and Hammond, 2008). Depending on the external Pi supply and using different measurement methods, the cytosolic Pi concentration has been estimated to be in the micromolar range (60–80 μM) by in vivo ³¹P-NMR analyses of *Arabidopsis thaliana* suspension cells (Pratt et al., 2009) or up to the millimolar range (1–10 mM) by live imaging of a Pi biosensor (Versaw and Garcia, 2017; Sahu et al., 2020). The cytosolic Pi concentration can be maintained at the beginning of Pi fluctuation due to buffering capacity from other subcellular pools (vacuoles in particular) and adjustment of Pi acquisition but ultimately declines when the Pi from those internal pools and external resources is

exhausted after long-term Pi starvation (Mukherjee et al., 2015). On the other hand, the level of PPi, which is a byproduct in the final steps of the synthesis of macromolecules such as proteins, nucleic acids, and cellulose, etc., is relatively insensitive to Pi starvation when compared with cellular ATP pools (Heinonen, 2001; Plaxton and Podestá, 2007; Ferjani et al., 2012). At the early stage of Pi deprivation, PPi, relative to ATP, is favored as a phosphoryl donor for cytosolic glycolysis (Davies et al., 1993; Plaxton and Podestá, 2007).

When the cellular P level is in the optimal range (<4 mg/g dry weight), approximately 50% of total P is in the organic form (Veneklaas et al., 2012). In the organic P pool, ribosomal RNAs have the largest proportion (~50%), followed by P-lipids, P-esters, DNA and other RNAs, and phosphoproteins (Veneklaas et al., 2012; Busche et al., 2021). Under severe P starvation conditions, when the supply of Pi cannot meet the demand to maintain optimal growth, recycling of Pi from the organic P pools appears to be crucial. Up to 90% of RNA (Hewitt et al., 2005) and DNA from mitochondria and chloroplasts or plastids (Takami et al., 2018) were reported to be mobilized after long-term Pi deficiency. Moreover, the replacement of phospholipids by other lipids such as sulfoquinovosyl diacylglycerol and digalactosyldiacylglycerol in response to Pi starvation has been reported in many plant species (Härtel et al., 2000; Kelly and Dörmann, 2002; Nakamura, 2013). Under normal growth conditions, cytosolic ATP was measured to be 0.8–1.2 mM using a FRET-based ATP sensor (Voon et al., 2018). When the cytoplasmic Pi level declines, large reductions (up to 80%) in intracellular levels of ATP, ADP, and related nucleotides also occur (Duff et al., 1989; Plaxton and Podestá, 2007; Zhu et al., 2019).

In seeds, P is predominantly stored as organic P in the form of InsP, which is a group of metabolites with various numbers of Pi covalently bound to a *myo*-inositol molecule (Irvine, 2005). Inositol hexakisphosphate (InsP₆; also known as phytic acid, PA) interacts with cations, such as Ca²⁺, Fe³⁺, K⁺, Mg²⁺, Mn²⁺ and Zn²⁺, to form phytates that commonly represent up to 75% of total P in seeds (Raboy, 1997). InsPs are present in the plant vegetative tissues at a much lower concentration (<200 μM depending on tissues or species analyzed; Phillippy et al., 2015). While the level of InsP₆ is irresponsive to changes of cellular Pi (Kuo et al., 2018; Dong et al., 2019), its diphosphorylated products, that is 5-diphosphoinositol pentakisphosphate 5PP-InsP₅ (InsP₇) and bis-diphosphoinositol tetrakisphosphate 1,5(PP)₂-InsP₄ (InsP₈) (collectively termed PP-InsP), are subjected to change (Dong et al., 2019), a phenomenon also observed in yeast and human cells (Lee et al., 2007; Lonetti et al., 2011; Wild et al., 2016; Gu et al., 2017). As discussed in the following section, PP-InsPs are considered as energy-dependent metabolic messengers that reflect the cellular Pi status and are perceived by the SPX (named after the *Saccharomyces cerevisiae* Syg1 and Pho81 proteins and the mammalian Xpr1) sensor domain for cellular Pi homeostasis control (Wild et al., 2016; Azevedo and Saiardi, 2017; Gerasimaite et al., 2017;

Jung et al., 2018; Shears, 2018; Lorenzo-Orts et al., 2020; Ried et al., 2021).

Pi transport systems and their regulation in response to external Pi supply

The initial acquisition of Pi is mainly achieved by PHOSPHATE TRANSPORTER 1 (PHT1) located in the plasma membranes of root cells (Shin et al., 2004). The activity of PHT1 is modulated by external Pi availability. Under Pi-limited conditions, transcription of *PHT1s* is upregulated by PHOSPHATE STARVATION RESPONSE 1 (PHR1) and its close homolog PHR1-like 1 (PHL1), a class of MYB transcription factors that play a major role in controlling the PSR in Arabidopsis (Rubio et al., 2001; Bustos et al., 2010). Other than PHR1, NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR1.2 (NIGT1.2), and OsWRKY21 or OsWRKY108 transcription factors were shown to directly regulate the transcription of *PHT1s* (Wang et al., 2020b; Zhang et al., 2021a). Moreover, phyB-mediated activation of *PHT1s* via phytochrome interacting factors PIF4/PIF5 and HY5 transcription factors in response to Pi deficiency was reported, which highlights the importance of optimizing plant growth under different light and nutrient conditions (Sakuraba et al., 2018).

The exit of PHT1 proteins from the endoplasmic reticulum (ER) is facilitated by PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1 (PHF1), which regulates the targeting of PHT1 proteins to plasma membranes (Gonzalez et al., 2005). In response to Pi replenishment, transcription of *PHT1s* and exiting of PHT1 proteins from the ER are suppressed (Bayle et al., 2011; Chen et al., 2015). The abundance of PHT1 proteins is controlled through ubiquitin-mediated degradation by NITROGEN LIMITATION ADAPTATION (NLA), a ubiquitin E3 ligase, in the plasma membrane, and PHOSPHATE 2 (PHO2)/UBC24, a ubiquitin E2 conjugase in the ER (Huang et al., 2013; Lin et al., 2013; Park et al., 2014). Ubiquitinated PHT1 proteins are delivered to vacuoles for degradation via ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT (ESCRT)-III-associated protein ALG2-interacting protein X (ALIX) (Cardona-López et al., 2015). Arabidopsis *AtNLA* and *AtPHO2* are regulated post-transcriptionally, cleaved by miR827 and miR399, respectively, particularly under low P (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Hsieh et al., 2009; Lin et al., 2013). PHT1 proteins can be phosphorylated depending on the external P supply. A high level of Pi promotes phosphorylation of Arabidopsis *AtPHT1;1* at Ser-514 (Bayle et al., 2011), or of rice (*Oryza sativa*) *OsPT8* at Ser-517 mediated by casein kinase 2 (Chen et al., 2015), resulting in retention of Pi transporters in the ER to prevent excess Pi accumulation. Conversely, low Pi leads to dephosphorylation of *OsPT8* at Ser-517 via *OsPP95*, a type 2C protein phosphatase, to facilitate its ER exit (Yang et al., 2020b). In addition, phosphorylation at Ser-520 of *AtPHT1;1* was reported to enhance its transport activity (Wang et al., 2020a).

Cellular Pi homeostasis is also controlled by Pi allocation between the cytosol and organelles. About 70%–95% of the

intracellular Pi is stored in vacuoles at micromolar to millimolar concentrations according to external Pi supply (Yang et al., 2017) as a result of Pi influx and efflux activities operated by Arabidopsis PHOSPHATE TRANSPORTER 5 (*AtPHT5*)/VACUOLAR PHOSPHATE TRANSPORTER (*AtVPT*) and rice VACUOLAR PHOSPHATE EFFLUX TRANSPORTER 1/2 (*OsVPE1/2*), respectively (Liu et al., 2015, 2016; Xu et al., 2019). When cytosolic Pi is high, excess Pi is stored in the vacuole by *AtPHT5*. Loss-of-function *atpht5* mutants have a significant reduction of vacuolar Pi and increased cytosolic Pi levels as revealed by ³¹P-NMR studies or a genetically encoded FRET-based Pi sensor (Liu et al., 2016; Luan et al., 2019; Sahu et al., 2020). When the cytosolic Pi level is low, vacuolar Pi is released by the Pi efflux transporter *OsVPE1/2* (Xu et al., 2019) to support the cytosolic demand of Pi. It is important to note that the change in *AtPHT5* activity can affect the expression of *AtPHT1s* (Liu et al., 2016), suggesting intracellular coordination to maintain an optimal cytosolic Pi concentration.

Pi is also transported across the membranes of other organelles. PHT2 (Daram et al., 1999; Versaw and Harrison, 2002; Rausch et al., 2004; Guo et al., 2013; Liu et al., 2020) and some members of PHT4 (Ferro et al., 2002; Roth et al., 2004; Guo et al., 2008) mediate Pi transport across the envelope membranes of the plastid/chloroplast. They contribute to ATP synthesis or starch accumulation in chloroplasts or participate in stress responses, which affects plant growth (Irigoyen et al., 2011; Wang et al., 2011; Karlsson et al., 2015). Additionally, Pi can be exchanged with phosphorylated C3, C5, or C6 carbon compounds via plastidic phosphate translocators (pPTs) across the plastid envelope to ensure Pi balance between the stroma and cytosol (Weber and Linka, 2011; Poirier and Jung, 2015).

PHT3 is involved in Pi transport across mitochondrion inner membranes to support the generation of ATP (Hamel et al., 2004; Zhu et al., 2012) and PHT4;6 is responsible for Pi export from Golgi essential for sugar-nucleotide metabolism (Guo et al., 2008; Cubero et al., 2009). Nevertheless, very little is known about the regulation of these organellar Pi transporters.

The Pi allocation between organs in plants relies on the Pi efflux activities of the PHOSPHATE1 (PHO1) family of proteins (Poirier et al., 1991). PHO1 is located in the Arabidopsis root stellar cells and mediates unloading of Pi into the xylem apoplastic space for subsequent translocation up to the shoots (Poirier et al., 1991; Hamburger et al., 2002; Arpat et al., 2012; Liu et al., 2012). Its physiological role for root-to-shoot Pi allocation was demonstrated by the reduced Pi in the shoots and higher Pi accumulation in the roots of *pho1* mutants in Arabidopsis and rice (Poirier et al., 1991; Secco et al., 2010; Che et al., 2020). *AtPHO1* is also expressed in leaf guard cells where it is involved in the stomatal response to ABA (Zimmerli et al., 2012), and in the chalazal seed coat to transfer Pi from maternal tissues to embryos in developing seeds (Vogiatzaki et al., 2017). In rice, PHO1s are also expressed at the vasculature of the

uppermost node connecting to the panicle and in developing seed tissues, where they are involved in the allocation of Pi during grain filling (Che et al., 2020; Ma et al., 2021). In *Medicago* (*Medicago truncatula*), PHO1 mediates the transfer of Pi from nodule infected cells to bacteroids (Nguyen et al., 2021). Negative regulations of Arabidopsis PHO1 either by WRKY6 at the transcriptional level (Chen et al., 2009) or by its upstream open-reading frame at the translational level (Reis et al., 2020) were reported. Conversely, the interaction between rice PHO1.2 mRNA and its cis-natural antisense transcript can enhance its translation (Jabnour et al., 2013; Reis et al., 2021).

Taken together, the activities of cellular Pi transport systems in plant cells are modulated by the external Pi levels as well as internal demands at the cellular and organismal levels via multiple mechanisms, including transcriptional, post-transcriptional, and post-translational regulation. It is worth noting that several of these transporters or their regulators (i.e. NLA, PHT5, and PHO1) possess a common SPX domain, a protein domain that perceives cellular Pi levels by binding to InsPs/PP-InsPs as discussed below.

Intracellular P sensors: SPX domains and their roles in Pi signaling and transport

Plant genomes encode four groups of SPX domain-containing proteins: SPX single-domain proteins and three other types of proteins comprising an N-terminal SPX domain in conjunction with an additional domain including PHO1 (SPX-EXS (*S. cerevisiae* Erd1, mammalian Xpr1, and *S. cerevisiae* Syg1)) and NLA (SPX-RING (Really Interesting New Gene)) and PHT5/VPT (SPX-MFS (Major Facilitator Superfamily)) (Secco et al., 2012a). In yeast, SPX domain-containing proteins include a cyclin-dependent kinase inhibitor Phosphatase 81 (Pho81); Pi transporters of PHO87, PHO90, and PHO91; glycerophosphocholine phosphodiesterase GDE1; polyphosphate (polyP) synthesis complex vacuolar transporter chaperone 2–5 (VTC2-VTC5); and a plasma membrane protein with unknown function SYG1 (Secco et al., 2012b; Desfougères et al., 2016). In contrast, the human genome encodes only one SPX domain-containing protein Xenotropic and Polytopic Retrovirus Receptor 1 (XPR1), a homolog of PHO1, which is involved in cellular Pi efflux (Giovannini et al., 2013; Legati et al., 2015). Diversification of SPX domain protein-coding genes in yeast and plant genomes may imply the requirement of more elaborate regulation of P sensing in sessile organisms.

Several lines of evidence suggest that SPX domains have a Pi sensing function. First, mutations of the SPX domain impair Pi transport activities of the transporters that bear it (Wege et al., 2016; Wild et al., 2016). Second, SPX domains mediate protein–protein interactions to regulate Pi transport (Wykoff et al., 2007; Hürlimann et al., 2009; Lin et al., 2013) or transcription of PSR genes (Lv et al., 2014; Puga et al., 2014; Wang et al., 2014; Desmarini et al., 2020) in various organisms from yeast to plants. Importantly, structural studies of the SPX domain and identification of Pi signaling

molecules, InsP/PP-InsP as its binding ligands provide direct evidence for its role as an intracellular Pi sensor (Wild et al., 2016). Crystal structures demonstrated that the SPX domain comprising 135–380 amino acids is arranged in six alpha-helices with highly conserved and positively charged amino acid residues located in the alpha 2 and alpha 4 helices that are denoted by the Pi binding cluster (PBC) and lysine surface cluster (KSC). The PBC and KSC constitute the basic binding surface for negatively charged InsP/PP-InsP and are essential for the SPX protein functions (Wild et al., 2016). The SPX domains have a higher binding affinity to InsP/PP-InsP (at the sub-micromolar level for InsP_{6–7}) than to Pi and PPI (at the millimolar and sub-millimolar level, respectively; Wild et al., 2016). The presence of SPX domains in a variety of proteins involved in Pi transport and transcriptional regulation of PSR accentuates the need for multifaceted sensing mechanisms for cellular Pi homeostasis coordination in plants. Current knowledge regarding the roles of SPX domains for the function of different groups of SPX-domain-containing proteins is discussed below.

SPX single-domain proteins

The function of SPX single-domain proteins has been studied extensively with regard to their interactions with AtPHR1 in Arabidopsis or OsPHR2 in rice (Lv et al., 2014; Puga et al., 2014; Wang et al., 2014). The Arabidopsis genome encodes four (AtSPX1–4) whereas rice contains six SPX (OsSPX1–6) single-domain proteins. The expression of all SPX genes except AtSPX4 and OsSPX4 is upregulated in response to Pi starvation (Duan et al., 2008; Wang et al., 2009). Under Pi-replete conditions, interactions between SPX1/2 and AtPHR1/OsPHR2 in the nucleus prevent AtPHR1/OsPHR2 from binding to the P1BS cis-element (GNATATNC) in the promoter of many PSR genes (Puga et al., 2014; Wang et al., 2014). Recently, it was reported that the KHR motif (AtPHR1^{K325}, AtPHR1^{H328}, and AtPHR1^{R335}) at the surface of the coiled-coil domain of AtPHR1 is required for its interaction with AtSPX1 (Ried et al., 2021). Alternatively, cytosolic OsSPX4 interacts with OsPHR2 to sequester OsPHR2 from targeting to the nucleus (Lv et al., 2014). Under Pi starvation, OsSPX4 undergoes rapid turnover mediated by RING-finger SPX4 degradation E3 ligases (OsSDEL1/2) (Ruan et al., 2019). The interaction between AtSPX1/OsSPX1 and AtPHR1/OsPHR2 was originally shown to be facilitated by Pi and its analog phosphite (Phi) at an optimal concentration of 15 mM in vitro (Puga et al., 2014; Wang et al., 2014). While the dynamics of cytosolic Pi levels in response to external Pi changes remains debatable, InsP₇ that is metabolically dependent on cytosolic Pi levels was later shown to trigger the OsSPX4–OsPHR2 interaction at sub-micromolar concentrations (Wild et al., 2016), suggesting a role for SPX as a sensor to transducing cellular Pi levels.

Interestingly, OsSPX4 also interacts with a master transcription regulator of the nitrate response NIN-LIKE PROTEIN 3 (OsNLP3), a rice homolog of AtNLP7 (Hu et al., 2019). Under low nitrate conditions, regardless of Pi level, OsSPX4 interacts with OsPHR2 and OsNLP3 and retains

them in the cytosol to prevent their activation on PSR genes and nitrate responsive genes (Hu et al., 2019; Hu and Chu, 2020). Under high nitrate and low Pi conditions, the nitrate sensor NITRATE TRANSPORTER 1.1 (NRT1.1) (Ho et al., 2009) recruits a plasma membrane-localized E3 ubiquitin ligase NRT1.1B INTERACTING PROTEIN 1 (OsNBIP1), together with OsSDEL1/2, to ubiquitinate OsSPX4 (Hu et al., 2019). This leads to a low level of OsSPX4 in the cytosol and an increase in the nucleus translocation of OsPHR2 and OsNLP3 for gene activation (Hu et al., 2019), suggesting that OsSPX4 has a pivotal role in the N–P interactive regulation network. Further study showed that REGULATOR OF LEAF INCLINATION 1 (RLI1)/HIGHLY INDUCED BY NITRATE GENE 1 (HINGE1), a MYB-transcription factor closely related to PHR2, could compete with OsPHR2 for binding to OsSPX1/2/4 to enhance PSR (Zhang et al., 2021b). Moreover, the nitrate-inducible GARP-type transcriptional repressor 1 (NIGT1) was reported to act as a transcriptional repressor of SPX genes by directly binding to the SPX promoters in Arabidopsis (Ueda et al., 2020). AtSPX4 can also interact with AtPAP1 to regulate anthocyanin biosynthesis in an InsP-dependent manner (He et al., 2021b). In rice, a basic helix–loop–helix protein OsbHLH6 can competitively inhibit the interaction between OsSPX4 and OsPHR2 and thus regulate PSRs (He et al., 2021a). Therefore, sequestration of AtPHR1/OsPHR2 by SPX proteins can be modulated by repression of SPX transcription, degradation of SPX proteins, or competitive binding with the SPX domain by other proteins.

PHO1 (SPX-EXS)

PHO1 contains a cytosolic SPX domain at the N terminus, followed by four transmembrane motifs, and an EXS domain at the C-terminus (Wege et al., 2016). It has been shown that the SPX-spanning region of PHO1 interacts with PHO2 that mediates its degradation (Liu et al., 2012), and the truncation of the SPX domain did not affect the Pi efflux activity of PHO1 in a transient expression assay (Wege et al., 2016). However, a truncated version lacking the SPX domain or mutations at the PBC or KSC residues of PHO1 was unable to complement the impaired activity of root-to-shoot Pi translocation in *pho1* mutants, suggesting that the SPX domain is essential and InsP/PP-InsP binding regulates its activity (Wege et al., 2016; Wild et al., 2016). In comparison, depletion of the cytosolic SPX domains of yeast PHO87 and PHO90 Pi transporters resulted in unrestricted Pi uptake and accumulation of cytosolic Pi to a toxic level, suggesting a repressive function of the SPX domain on these plasma-membrane Pi transporters (Hürliemann et al., 2009). While the Pi transport mechanism of PHO1 and its membrane localization remain enigmatic (Chiou, 2020), these confounding observations emphasize the necessity of structural analysis of PHO1 to dissect the role of SPX for its regulation. PHO1 has 10 other SPX-domain bearing homologs in Arabidopsis that have overlapping or non-overlapping expression and functions (Hamburger et al., 2002). It is

therefore intriguing whether or how their SPX domains may differentially regulate their activity.

PHT5 (SPX-MFS)

As a major vacuolar Pi import transporter, PHT5 bears an N-terminal SPX domain predicted to face the cytosol and a MFS domain at the C-terminus. Unlike OsVPE1/2, the transcription level of *AtPHT5* is irresponsive to Pi status (Liu et al., 2016) and therefore its activity may be regulated at the protein or posttranslational level, likely through its SPX domain. While the role of the SPX domain and its interaction with InsP/PP-InsP for AtPHT5 activity remains to be investigated, studies of the vacuolar P transport system in yeast demonstrated that PP-InsP and SPX control its activity. In yeast, Pi was stored in the vacuole in the form of inorganic polyP synthesized and transported by the VTC complex located on the vacuolar membranes, in which four of the five subunits (VTC2–VTC5) bear SPX domains. The polymerase activity of the VTC complex is dependent on the PP-InsP (InsP₇) synthesis (Lonetti et al., 2011). Noticeably, while mutations of most PBC or KSC residues disrupt polyP polymerase activity of the VTC complex, substitutions at a particular residue (K126/129 in VTC3/4) result in constitutive activation of polyP synthesis in the absence of InsP₇ (Wild et al., 2016), suggesting that PP-InsP binding may affect a structural change of the complex and hence its activity. InsP/PP-InsP can also stimulate the activity of the Pi exporter PHO91 in yeast vacuoles and *Trypanosoma brucei* acidocalcisomes (Potapenko et al., 2018), suggesting conservation in adoption of the SPX domain for vacuolar P-storage control in eukaryotes.

NLA (SPX-RING)

AtNLA controls the protein abundance of AtPHT1 Pi transporters at plasma membranes according to the availability of Pi (Lin et al., 2013; Park et al., 2014). The N-terminal SPX domain of AtNLA interacts with AtPHT1 and directs it for ubiquitination mediated by its C-terminal RING domain E3 ligase (Lin et al., 2013; Yang et al., 2020a). Under Pi-deficient conditions, AtNLA-mediated ubiquitination of AtPHT1 is attenuated by Pi starvation-induced miR827 (Hsieh et al., 2009; Lin et al., 2013). AtNLA also modulates the degradation of the nitrate transporter NRT1.7 through the binding of its SPX domain (Liu et al., 2017), providing a potential argument for N–P crosstalk. Moreover, AtNLA was shown to interact with ORESARA1 (ORE1) through its SPX domain in the nucleus to control leaf senescence (Park et al., 2018). Whether InsP/PP-InsP also mediates the interaction of AtNLA–AtPHT1, AtNLA–AtNRT1.7, or AtNLA–AtORE1 in response to cellular Pi levels awaits further studies.

Collectively, it is evident that the SPX domain is relevant to the functions of Pi transport proteins, yet its mechanistic effects on the transport activities in association with their InsP/PP-InsP ligands remain largely unknown, which emphasizes the need for structural dissection with functional studies in the future.

Metabolic messengers for intracellular Pi signaling: PP-InsPs and their metabolism

Prior to the discovery of InsP and PP-InsP as the SPX-domain binding ligands, genetic and physiological studies suggested a role for InsP biosynthesis in the regulation of Pi homeostasis in yeast, animals, and plants (Norbis et al., 1997; Schell et al., 1999; Stevenson-Paulik et al., 2005; Lee et al., 2007; Lonetti et al., 2011; Kuo et al., 2014, 2018). InsP and PP-InsP are synthesized through step-wise phosphorylation catalyzed by evolutionarily conserved kinases (Figure 1). In plants, mutations in the inositol-tetrakisphosphate 1-kinase (ITPK1) or inositol pentakisphosphate 2-kinase (IPK1) cause excessive Pi accumulation and activation of PSR genes under Pi-replete conditions (Stevenson-Paulik et al., 2005; Kuo et al., 2018). Mutations of *ITPK1* and *IPK1* disturb the stoichiometry of InsP intermediates, including accumulation of InsP₄ or InsP₅ and decreases in InsP₆₋₇₋₈ levels (Stevenson-Paulik et al., 2005; Laha et al., 2015; Kuo et al., 2018; Riemer et al., 2020), confounding differentiation of the causal InsP species that control Pi homeostasis. Two recent studies collectively provided evidence that InsP₈ is a key molecule in mediating Pi signaling. They showed that kinase-dead mutations of the DIPHOSPHOINOSITOL PENTAKISPHOSPHATE KINASES (PPIP5K) in Arabidopsis, Vip Homologs (VIH1 and VIH2) resulted in no detectable InsP₈ and the *vih1 vih2* mutant exhibited severe growth defects with constitutive activation of PSR genes and excessive cellular Pi accumulation (Dong et al., 2019; Zhu et al., 2019). These phenotypes were significantly suppressed by loss-of-function mutations of PHR1 and PHL1, indicating that VIH1/2 control Pi homeostasis predominantly through PHR1/PHL1 (Zhu et al., 2019). Also, InsP₈ levels were positively corresponded to cellular Pi levels (Dong et al., 2019). Together with the observation that PP-InsPs facilitate the SPX–PHR1 interaction at a near sub-micromolar concentration (Wild et al., 2016), it is hypothesized that high concentrations of cellular Pi drive VIH to synthesize InsP₈ that promotes the SPX–PHR1 interaction and thus sequesters PHR1 from transcriptional activation of PSR genes, whereas under low cellular Pi, InsP₈ decreases and releases PHR1 from interacting with SPX to activate PSR gene expression (Dong et al., 2019; Zhu et al., 2019).

Emerging evidence suggests that InsP₇ and InsP₈ are evolutionarily conserved Pi signaling molecules that are metabolically linked to cellular Pi levels. In yeast and human cells, the levels of cellular InsP₇ and InsP₈ are also positively associated with cellular Pi levels (Lonetti et al., 2011; Wild et al., 2016; Gu et al., 2017). Disruption of the yeast pKc1 suppressor (Kcs1; an inositol hexakisphosphate and inositol heptakisphosphate kinase) inhibited vacuolar polyP synthesis (Lonetti et al., 2011). Human inositol hexakisphosphate kinases IP6K (InsP₇ synthesis kinase) and PPIP5K knockout lines or pharmacological inhibition of IP6K enzymatic activity blocked XPR1-mediated Pi efflux (Wilson et al., 2019; Li et al., 2020). It is therefore relevant to understand how cellular Pi status relays InsP₇ and InsP₈ synthesis and their turnover.

Recent studies demonstrated that ITPK1 and VIH are bifunctional enzymes that are capable of catalyzing reversible

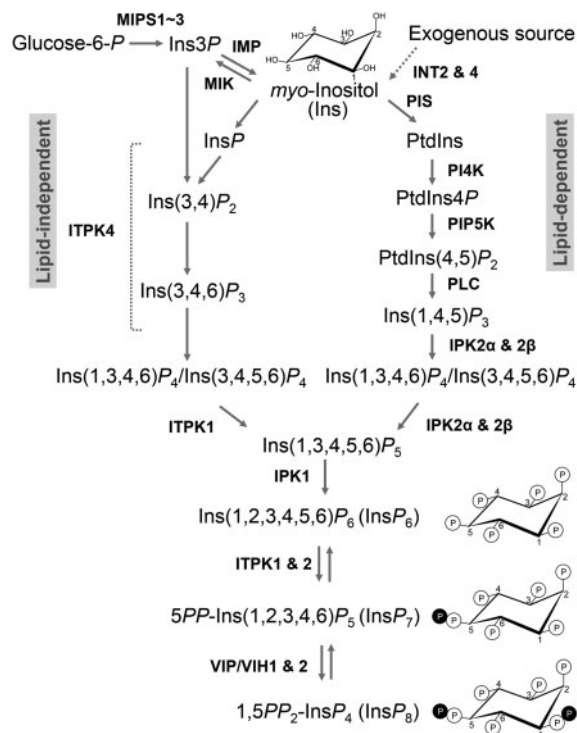


Figure 1 InsP and PP-InsP biosynthesis pathways in plants. Schematic pathways for the synthesis of InsP₆ and PP-InsP in plants. The biosynthesis of InsP consists of a “lipid-dependent pathway” based on biochemical activities of homologous enzymes in Arabidopsis and a “lipid-independent pathway” identified in duckweed (*Spirodela polyrrhiza*) (Brearley and Hanke, 1996; Stevenson-Paulik et al., 2005; Kuo et al., 2018). There are two classes of kinases that synthesize the PP-InsP in eukaryotes: IP6K that phosphorylates InsP₆ to generate 5PP-InsP₅ (InsP₇) and PPIP5K that can phosphorylate 5PP-InsP₅ to produce 1,5(PP)2-InsP₄ (InsP₈) (Shears, 2018). Plant genomes do not encode IP6K homologs and the InsP₆ kinase activities of ITPK1 (and ITPK2) have been postulated to generate 5PP-InsP₅ (Adepoju et al., 2019; Laha et al., 2019; Whitfield et al., 2020). The plant PPIP5K homologs, VIP1/VIH2 and VIP2/VIH1 coordinately catalyze 5PP-InsP₅ into 1,5(PP)2-InsP₄ (Desai et al., 2014; Laha et al., 2015; Zhu et al., 2019). MIPS, inositol-3-phosphate synthases; IMP, inositol monophosphate; MIK, myo-inositol kinase; INT2 and 4, inositol transporters; PtdIns, phosphatidylinositol; PIS, phosphatidylinositol synthase; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PLC, phospholipase C; IPK2α and 2β, inositol polyphosphate 6-/3-kinases; ITPK1 and 4, inositol 1,3,4-trisphosphate 5-/6-kinase; IPK1, inositol pentakisphosphate 2-kinase; and VIP/VIH1 and 2, Vip1 homologs. The dashed bracket, unresolved catalytic steps mediated by ITPK4. The dashed arrow, an uptake process; reversible arrows, kinase/ADP phosphotransferase, and kinase/pyrophosphatase activities of ITPK1 and VIP/VIH1 and 2, respectively.

steps to synthesize and hydrolyze InsP₇ and InsP₈, respectively, depending on the cellular ATP levels (Zhu et al., 2019; Riemer et al., 2020; Whitfield et al., 2020). The cellular ATP/ADP ratio changes in accordance with the cellular Pi level, being high when cells are supplied with sufficient Pi, and decreasing upon Pi starvation (Gu et al., 2017; Zhu et al., 2019). At a high cellular ATP/ADP ratio, the ITPK1 kinase phosphorylates InsP₆ to generate InsP₇, which is in turn

phosphorylated into InsP_8 by VIH. On the other hand, as the cellular Pi level decreases in response to Pi starvation, a decreased ATP/ADP ratio stimulates ADP-transferase activity of ITPK1 and the VIH phosphatase activity (possessed by the C-terminal domain of VIH) for InsP_7 and InsP_8 hydrolysis (Zhu et al., 2019; Riemer et al., 2020; Whitfield et al., 2020).

Because the human IP6K displays a K_m of 1 mM for ATP that is comparable to the cellular ATP concentration (Wundenberg and Mayr, 2012; Voon et al., 2018), InsP_7 has been considered to be a metabolic messenger, acting as a cellular energy sensor (Shears, 2009; Wundenberg and Mayr, 2012; Wilson et al., 2013). However, genetic studies in plants and human cell lines, in which loss-of-function mutations of VIH1/2 and PPIP5Ks exerted the strongest effects, respectively, on the PSR activation and attenuation of XPR1-mediated Pi efflux (phenocopied XPR1 knockout lines), argued that InsP_8 is the cellular Pi-sensing signal (Zhu et al., 2019; Li et al., 2020). The fact that levels of InsP_8 are more responsive to cellular ATP and Pi changes (Gu et al., 2017; Dong et al., 2019), and that Pi and Phi are both able to inhibit PPIP5K phosphatase activity or suppress PSR in planta (Gu et al., 2017; Zhu et al., 2019), further substantiate the claim that InsP_8 is the cellular Pi signal despite that in vivo impacts of Phi on the cellular InsP_8 level has yet to be demonstrated.

The earlier observations that the non-metabolizable Pi analog Phi could mimic Pi to suppress the PSR when Pi was depleted implied that Pi per se is a signal (Ticconi et al., 2001; Varadarajan et al., 2002). A more direct demonstration of Pi as a possible signal in triggering the PSR was that Pi or Phi was able to promote the interaction between PHR1 and SPX1 in vitro (Puga et al., 2014; Wang et al., 2014). The binding affinity of Pi to the SPX domain according to isothermal titration calorimetry was measured to be in the millimolar range, which despite being lower than $\text{InsP}/\text{PP-InsP}$ by two–three orders, is compatible with the physiological concentrations (Wild et al., 2016; Versaw and Garcia, 2017; Sahu et al., 2020). In contrast, no binding between OsSPX4 and OsPHR2 was detected in the presence of Pi (Wild et al., 2016) arguing against Pi as a signaling molecule. Moreover, replenishment of Pi, but not Phi, to P-starved rice was able to stabilize OsSPX4 (Osorio et al., 2019), hinting that P metabolites rather than Pi per se mediate the OsSPX4–OsPHR2 interaction.

Unresolved issues regarding intracellular Pi signaling mediated by PP-InsPs and SPX domains

An overview based on our current knowledge of intracellular Pi signaling and the activities of Pi transport systems under different cellular Pi levels is shown in Figure 2. Considering the different roles of SPX-bearing proteins in modulating cellular Pi homeostasis, how they perceive Pi-signals to function in accordance will be the focus of future research. Whether $\text{InsP}/\text{PP-InsP}$ act as ligands in mediating protein–protein interactions as a general mechanism (as exemplified by the PHR1– InsP_8 –SPX1 interaction) that applies to the regulation of other SPX-proteins awaits further investigation. To

investigate this scenario, identifying the interacting components of other SPX proteins becomes a prerequisite. Nevertheless, it is possible that the binding of PP- InsP to the SPX domain per se may regulate the function of the protein it bears independent of interacting partners as a result of structural changes (Furkert et al., 2020; Lorenzo-Orts et al., 2020).

Another glaring question regards how $\text{InsP}/\text{PP-InsP}$ as Pi-signaling molecules coordinate the activities of different Pi transport systems temporally and spatially to modulate cellular Pi homeostasis in response to changes in cellular Pi levels. Comparisons between the affinities of different InsPs and PP- InsPs to SPX domains and their relative cellular levels suggest the possibility of competitive bindings. For example, the ratio of cellular concentration of InsP_6 to InsP_7 (more than three orders) is greater than that of their binding affinity to SPX (two orders; Laha et al., 2015; Wild et al., 2016; Kuo et al., 2018), and InsP_6 has been demonstrated to compete with InsP_7 in protein pyrophosphorylation (Saiardi et al., 2004; Wu et al., 2016). Therefore, the competitive outcomes of binding of $\text{InsP}/\text{PP-InsP}$ to SPX-domain proteins need to be assessed in the physiological context. Similarly, the discrepancy in the binding affinity of the SPX domains from different proteins for a given $\text{InsP}/\text{PP-InsP}$ species (such as InsP_8) has to be considered equally. SPX domains from different proteins (SPX, PHO1, NLA, and PHT5) may also sense the change in InsP or PP- InsP at different affinities, which allows them to function differently at particular cellular $\text{InsP}/\text{PP-InsP}$ levels. Recently, the development of capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE–ESI–MS) allowed differentiating PP- InsP isomers and has identified isomers of InsP_7 in planta, that is 5-PP- InsP_5 and 1,3-PP- InsP_5 (Qiu et al., 2020). Implementation of this analytical approach should facilitate dissecting the biological relevance of PP- InsP isomers and their interacting proteins.

Furthermore, the subcellular compartmentalization of the $\text{InsP}/\text{PP-InsP}$ should be considered. We and others have observed that changes in overall cellular InsP_7 and InsP_8 levels did not necessarily alter Pi homeostasis. The Arabidopsis *itpk4* mutation resulted in substantial decreases of InsP_{6-7-8} as *ipk1* and *itpk1* without affecting cellular Pi content nor constitutively activating PSR genes under Pi-replete conditions (Figure 3; Kuo et al., 2018). On the other hand, the multidrug resistance-associated protein 5 (*mrp5*) mutants defective in storage of InsP_6 into vacuoles exhibited overall elevated InsP_7 and InsP_8 in vegetative tissues (Figure 3; Riemer et al., 2020), similar to the *pho2* mutants (Dong et al., 2019), yet they displayed wild-type levels of cellular Pi (Figure 3; Kuo et al., 2018). One conceivable explanation is that the PP- InsPs have to be generated and/or present at a definite subcellular location to serve as “effective” Pi-signals. The development of subcellular PP- InsP tracing markers will facilitate addressing this question.

By using synthetic InsP_7 as bait to search for its interacting proteins, a recent study identified a myriad of proteins

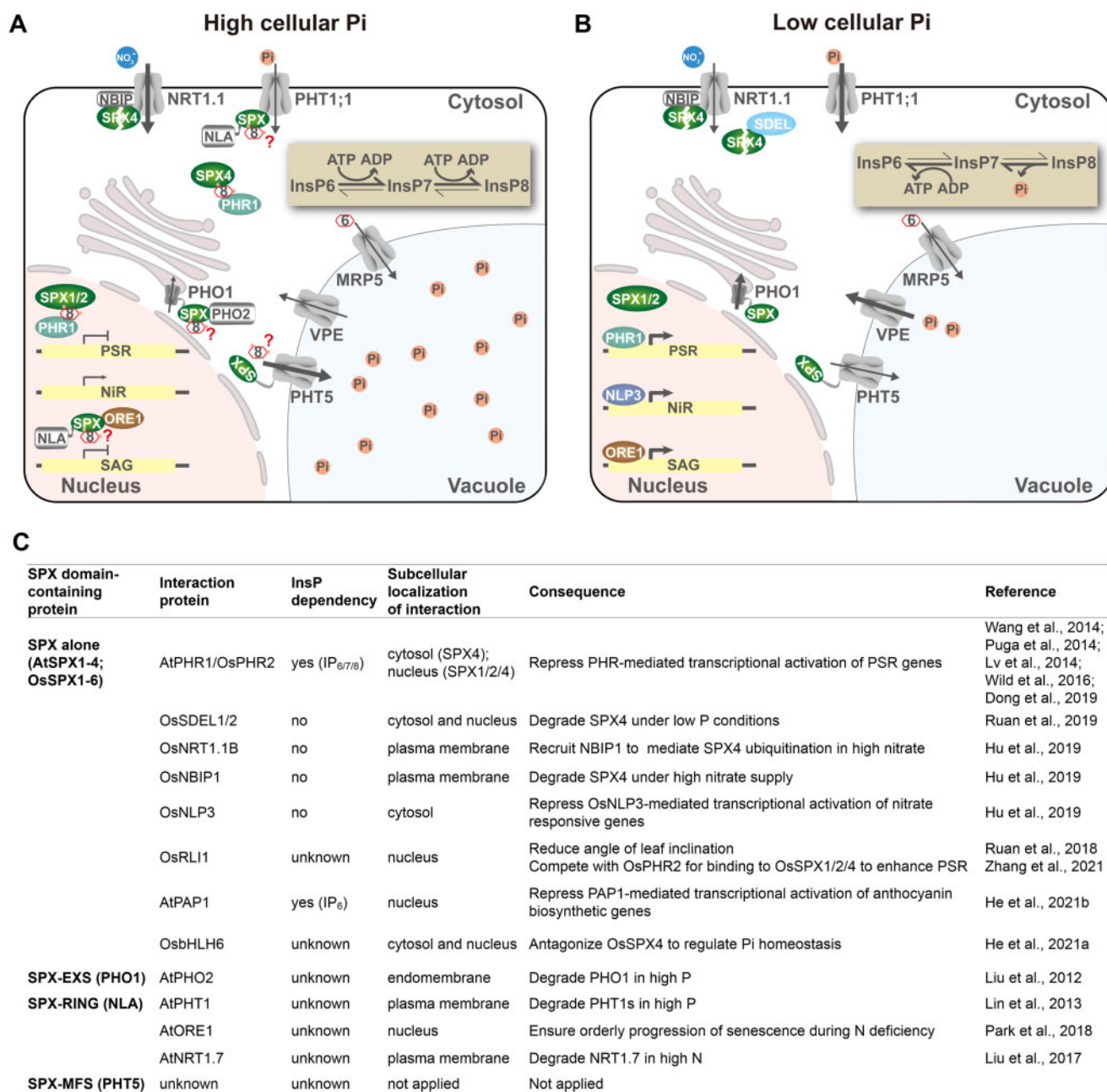


Figure 2 A model for PP-InsP sensing by SPX domain proteins in plants. Under Pi-replete conditions (A), high cellular Pi and ATP levels drive InsP₈ (8) synthesis, which promotes the PHR–SPX interaction and thus suppresses the activation of PSR genes. Under Pi-depleted conditions (B), the decrease of cellular Pi and ATP results in a decline of InsP₈ level. Accordingly, PHR1 is released to activate PSR genes. Expression of nitrate-responsive genes (NiR) and senescence-associated genes (SAG) is also activated by NLP3 and ORE1, respectively. It is likely that changes in the InsP₈ level also regulates the activity of other SPX-domain proteins, including NLA, PHO1, and PHT5 involved in Pi acquisition, translocation, and storage, respectively. MRP5 is a vacuolar InsP₆ (6) transporter. Thickness of arrows indicates the strength of activities. C, A summary of proteins interacting with SPX domain-containing proteins and their mediated physiological responses (see text for details).

absent of SPX domains, among which are the ribose-phosphate pyrophosphokinases 1 and 2 (PRPS1 and PRPS2) and their associated proteins PRPSAP1 and PRPSAP2 that are involved in nucleotide biosynthesis (Furkert et al., 2020). This finding suggests a potential correlation between the Pi-signaling and nucleotide biosynthesis and inspires speculation about whether SPX-independent proteins can perceive

PP-InsPs to modulate cellular Pi homeostasis. On the other hand, InsP₇ has been shown to mediate enzyme-independent pyrophosphorylation of proteins involved in biological processes that are tightly linked to the cellular energy status such as glycolysis and ribosome biogenesis (Bhandari et al., 2007; Szijgyarto et al., 2011; Thota et al., 2015; Wu et al., 2016). Protein pyrophosphorylation has not

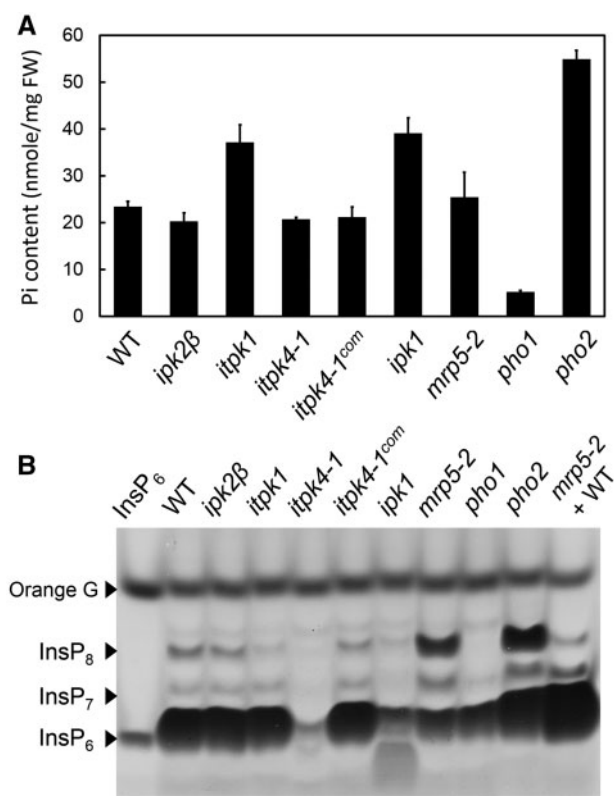


Figure 3 Cellular InsP₇₋₈ levels are not necessarily positively associated with cellular Pi levels. A, The shoot Pi contents of various mutants (14-d-old seedlings grown on half-strength Murashige and Skoog medium) involved in InsP/PP-InsP and Pi metabolism as described by Kuo *et al.* (2018). *itpk4-1^{com}* denotes the *itpk4-1* complementary line expressing the YFP-tagged *ITPK4* CDS (Kuo *et al.*, 2018). Error bars, SE of three independent experiments. B, InsP/PP-InsP profiles in the shoots of the corresponding genotypes shown in (A). Commercially purchased InsP₆ (Sigma) and the mixture of *mrp5-2* and Col-0 wild-type seed extract (*mrp5-2* +WT) were used as markers for InsP₆₋₇₋₈ (Desai *et al.*, 2014). InsP/PP-InsP were extracted from equal mass of 14-d-old seedlings (1 g) and enriched by TiO₂ beads followed by polyacrylamide gel electrophoresis analysis as described (Wilson *et al.*, 2015).

been reported in plants and whether PP-InsPs can modulate the activity of plant Pi transport systems via this mechanism remains to be explored.

Conclusions

Over recent years, active research into InsP metabolism driven by the study of Pi signaling in plants has not only elucidated key kinases responsible for InsP/PP-InsP biosynthesis but also extended our knowledge of InsP metabolism in plants. This advance is also expected to foster the investigation of previously unsolved biological processes that intersect with InsPs/PP-InsPs metabolism. With the identification of SPX domains as intracellular sensors that perceive InsP/PP-InsP as cellular Pi signaling molecules, we expect accelerated progress toward a comprehensive understanding of the regulation of Pi transport systems at the organismal level.

OUTSTANDING QUESTIONS BOX

- What is the molecular basis underlying PP-InsP-mediated regulation of Pi transport proteins? Do PP-InsPs generally act as ligands in mediating protein–protein interactions and/or directly modulate the activities of their receptor proteins through structural or chemical modifications?
- How are the subcellular levels of InsP/PP-InsP controlled in accordance with the dynamics of cellular Pi levels?
- Are the activities of SPX-bearing Pi transporters modulated by competitive bindings of different InsP/PP-InsP species according to their differential affinities and physiological levels?

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