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Arabidopsis inositol phosphate kinases, IPK1 and ITPK1, constitute a metabolic pathway in maintaining phosphate homeostasis

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Summary

Emerging studies have implicated a close link between inositol phosphate (InsP) metabolism and cellular phosphate (P) homeostasis in eukaryotes; however, whether a common InsP species is deployed as an evolutionarily conserved metabolic messenger to mediate P, signaling remains unknown. Here, using genetics and InsP profiling combined with P, starvation response (PSR) analysis in Arabidopsis thaliana, we showed that the kinase activity of inositol pentakisphosphate 2-kinase (IPK1), an enzyme required for phytate (inositol hexakisphosphates; InsP₆) synthesis, is indispensable for maintaining P, homeostasis under P,-replete conditions, and inositol 1,3,4-trisphosphate 5/6-kinase 1 (ITPK1) plays an equivalent role. Although both ipk1-1 and itpk1 mutants exhibited...
decreased levels of InsP₆ and diphosphoinositol pentakisphosphate (PP-InsP₆; InsP₇), disruption of another ITPK family enzyme, ITPK4, which correspondingly caused depletion of InsP₆ and InsP₇, did not display similar Pᵢ-related phenotypes, which precludes these InsP species as effectors. Notably, the level of D/L-Ins(3,4,5,6)P₄ was concurrently elevated in both ipk1-1 and itpk1 mutants, which showed a specific correlation to the misregulated Pᵢ phenotypes. However, the level of D/L-Ins(3,4,5,6)P₄ is not responsive to Pᵢ starvation that instead manifests a shoot-specific increase in InsP₇ level. This study demonstrates a more nuanced picture of the intersection of InsP metabolism and Pᵢ homeostasis and PSR than has previously been elaborated and additionally establishes intermediate steps to phytate biosynthesis in plant vegetative tissues.

**Introduction**

Elemental phosphorous (P) in its oxidized form, phosphate (PO₄³⁻; Pᵢ), is essential to all life. As a component of nucleic acids, proteins, phospholipids and numerous intermediary metabolites, Pᵢ is key to energy metabolism and signal transduction. Plants preferentially acquire P in the form of Pᵢ from the rhizosphere, where Pᵢ is often limiting owing to its sorption to soil particles and leaching (Holford, 1997). As an adaptation to fluctuating external Pᵢ concentrations, plants have evolved intricate regulatory mechanisms to maintain cellular Pᵢ homeostasis in vegetative tissue in order to coordinate growth, development, and reproduction, whereas in seeds, Pᵢ is reserved in phytate (inositol hexakisphosphate, InsP₆) that accumulates to several percentage dry weight (Raboy, 1997). In response to Pᵢ deficiency, plants initiate a systematic response, termed the Pᵢ-starvation response (PSR), which involves transcriptional, metabolic, and morphological reprogramming, to enhance Pᵢ uptake, allocation, remobilization, and conservation (Rouached et al., 2010; Yang and Finnegan, 2010). Under Pᵢ-replete or -replenishment conditions, plant cells relieve PSR and store excess Pᵢ in the vacuole to avoid cellular toxicity as a result of cytosolic Pᵢ surge (Müller et al., 2004; Lin et al.,
How plant cells perceive external and cellular Pi status to maintain Pi homeostasis remains elusive despite reports of multiple factors proposed to be signaling molecules, including sugar, phytohormones, microRNAs, InsPs and Pi, per se (Martin et al., 2000; Franco-Zorrilla et al., 2005; Liu et al., 2005; Bari et al., 2006; Chiou et al., 2006; Chiou and Lin, 2011; Puga et al., 2014; Wang et al., 2014).

Inositol phosphates (InsPs) are metabolites of variable phosphorylation on a carbohydrate core, inositol, and are present in all eukaryotes. They are synthesized by evolutionarily conserved enzymes (Irvine and Schell, 2001) and play important roles in diverse cellular processes by functioning as structural and functional cofactors, regulators, and second messengers (Shears et al., 2012). According to the definition of a ‘signal’, being that of agonist-responsive change in concentration that is recognized by a defined receptor (Shears et al., 2012), only very few InsPs can be considered true signaling molecules, including Ins(1,4,5)P3 in the context of Ca2+ signaling (Berridge, 2009) and Ins(3,4,5,6)P4 as a regulator of the conductance of the Ca2+-activated chloride channels (Vajanaphanich et al., 1994; Shears et al., 2012). In plants, InsPs have been hypothesized to mediate signaling of multiple physiological processes, including stomatal closure, gravitropism, drought tolerance, and defense (Lemtiri-Chlieh et al., 2000; Lemtiri-Chlieh et al., 2003; Perera et al., 2006; Mosblech et al., 2008; Murphy et al., 2008; Perera et al., 2008; Laha et al., 2015); however, their roles as signaling messengers in most cases have not been assessed extensively.

The first elaboration of the involvement of InsPs in eukaryotic Pi homeostasis was revealed when a rabbit cDNA clone was shown to stimulate Pi uptake when ectopically expressed in Xenopus oocytes (Norbis et al., 1997). This so-called Pi uptake stimulator (PiUS) was identified to encode an InsP6 kinase (IP6K) that converts InsP6 to diphosphoinositol pentakisphosphates (PP-InsP5 or InsP7) (Norbis et al., 1997; Schell et al., 1999). In yeast, disruption of multiple enzymes responsible for biosynthesis of InsPs and diphosphoinositol phosphates (PP-InsPs) (e.g., Plc1p, Arg82p, and Kcs1p) led to constitutive activation of a Pi starvation-responsive phosphatase-coding gene, Pho5, under...
P<sub>i</sub>-replete conditions (Auesukaree et al., 2005). Subsequent work showed that the synthesis of InsP<sub>7</sub> by the other family of PP-InsP kinases (Vip1/PPIP5K), Vip1, is stimulated by P<sub>i</sub> starvation (Lee et al., 2007) and InsP<sub>7</sub> binds to Pho81, causing inhibition of the Pho80-Pho85 cyclin-cyclin–dependent kinase complex and unphosphorylation of the Pho4 transcription factor. The resulting reduction in phosphorylation of Pho4 localizes this protein to the nucleus, where it activates P<sub>i</sub> starvation-inducible genes (Lee et al., 2007; Lee et al., 2008). The synthesis of PP-InsPs is also metabolically linked to the synthesis of the main intracellular P<sub>i</sub> storage molecule, a linear chain of polyphosphate (polyP), and the yeast IP6K mutant, kcs1Δ, fails to accumulate polyP (Auesukaree et al., 2005; Lonetti et al., 2011).

Cellular adenylate energy is influenced by P<sub>i</sub> availability and PP-InsP synthesis (Boer et al., 2010; Sziójgyarto et al., 2011; Choi et al., 2017) and itself regulates the synthesis of PP-InsP (Voglmaier et al., 1996; Saiardi et al., 1999; Wundenberg et al., 2014). Together with the genetic and molecular evidence described previously, PP-InsPs have been proposed as metabolic messengers that mediate P<sub>i</sub> signaling. This hypothesis is further supported by structural and biochemical analyses demonstrating that InsPs and PP-InsPs bind to an evolutionarily conserved SYG1/PHO81/XPR1 (SPX) domain present in proteins that play key roles in P<sub>i</sub> sensing and transport, with PP-InsPs showing the highest binding affinity (at sub-micromolar concentrations for yeast and animal protein) (Secco et al., 2012; Secco et al., 2012; Wild et al., 2016). Disruption of InsP/PP-InsP binding sites in the SPX domain impaired yeast vacuolar transporter chaperone (VTC)-dependent polyP synthesis and failed to complement P<sub>i</sub>-related phenotypes of the Arabidopsis phosphate 1 (pho1) mutant (Wild et al., 2016). Despite the wealth of current investigation, the evidence for PP-InsPs as evolutionally conserved messengers in eukaryotic P<sub>i</sub> signaling is scattered, confounded by the absence of Pho80-Pho85-Pho81 homologs in other eukaryotic organisms and the contradictory responses of InsP<sub>7</sub> levels to P<sub>i</sub> starvation reported in yeast (Lee et al., 2007; Wild et al., 2016) as well as the presence of a Vip1-independent PHO signaling pathway (Choi et al., 2017).
In plants, a contemporary implication of InsP metabolism in regulation of P$_i$ homeostasis comes from a study in which genetic disruption of the kinase responsible for Ins$_P_6$ synthesis, inositol pentakisphosphate 2-kinase (IPK1), causes excessive P$_i$ accumulation (Stevenson-Paulik et al., 2005) as a result of elevated P$_i$ uptake/allocation activities and activation of a subset of P$_i$ starvation-responsive genes (PSR genes) under P$_i$-replete conditions (Kuo et al., 2014). In addition to decreased Ins$_P_6$ level, ipk1 mutation causes a significant change in Ins$_P$ composition, including accumulation of lower phosphorylated Ins$_P$ species (e.g., Ins$_P_3$, Ins$_P_4$ and Ins$_P_5$) and decreased levels of PP-Ins$_Ps$ [Ins$_P_7$ and Ins$_P_8$ (bisdiphosphoinositol tetrakisphosphate)] (Stevenson-Paulik et al., 2005; Laha et al., 2015). The mechanism of IPK1 modulating P$_i$ homeostasis and whether Ins$_Ps$ play a role in P$_i$-starvation signaling in plants is currently unknown.

As compared with the situation in other eukaryotic organisms, the investigation of biosynthesis of Ins$_Ps$ and their composition in the vegetative tissues of plants is necessarily more complicated than in other eukaryotes due to the presence of complex gene families of Ins$_P$ biosynthesis enzymes. Mammalian Ins$_P$ metabolism is dominated by receptor-coupled activation of phospholipase C (PLC) and subsequent metabolic conversion of Ins(1,4,5)$P_3$ to multiple higher and lower Ins$_Ps$ (Irvine and Schell, 2001), but few plant studies offer detailed identification of Ins$_P$ species in vegetative tissues due to the limited levels of labeling achieved with myo-[³H]inositol. Nevertheless, specific short-term non-equilibrium labeling with [³²P]P$_i$ has afforded a metabolic test capable of distinguishing the order in which phosphates are added to the inositol core (Stephens and Downes, 1990; Stephens and Irvine, 1990; Whiteford et al., 1997) and applied to vegetative tissues of plants that revealed a ‘lipid-independent’ pathway of Ins$_P_6$ synthesis (Brearley and Hanke, 1993; Brearley et al., 1997).

Here, using reverse genetics and Ins$_P$ profiling by [³H]inositol and [³²P]P$_i$ labelling, we show that maintenance of P$_i$ homeostasis in plants under P$_i$-replete conditions depends on the kinase activity of IPK1 and an additional inositol 1,3,4-trisphosphate 5/6-kinase ITPK1. Profile comparison of Ins$_Ps$ between ipk1-1, itpk1, and another mutant defective in Ins$_P_6$ synthesis, itpk4, reveals a correlation...
between elevated D/L-Ins(3,4,5,6)P₄ [Ins(1,4,5,6)P₄ and/or Ins(3,4,5,6)P₄] level and activation of Pᵢ uptake and PSR gene expression. However, the InsP profile in response to Pᵢ starvation is distinct from that of the ipk1-1 and itpk1 mutants and marked a shoot-specific increase in InsP₇ level accompanied by ATP increase. Our study reveals a complex relationship between InsP metabolism and Pᵢ homeostasis in plants and identifies ITPK4 as a key enzyme in generating InsP₄ precursors for phytate biosynthesis.

Results

Kinase activity of IPK1 is required for maintenance of Pᵢ homeostasis

We previously demonstrated Pᵢ overaccumulation in ipk1-1 mutants associated with activation of PSR genes involved in Pᵢ uptake, allocation, remobilization, and signaling (Kuo et al., 2014). Because InsP kinases have been implicated in transcriptional regulation independent of their catalytic activities (Bosch and Saiardi, 2012; Xu et al., 2013; Xu et al., 2013), we examined whether regulation of Pᵢ homeostasis by IPK1 is kinase-dependent. We constructed two forms of IPK1 bearing mutations in conserved kinase motifs (Stevenson-Paulik et al., 2005) (Figure S1a) at Lys168 (IPK1K168A) or Asp368 (IPK1D368A), both shown to cause loss of kinase activity in vitro (Gonzalez et al., 2010). The expression of wild-type (WT) IPK1 complemented low InsP₆ content in ipk1-1 seeds, whereas InsP₆ levels in seeds of transgenic lines expressing either of the two point-mutated forms of IPK1 remained as low as that in ipk1-1 seeds (Figure 1a). These point-mutated IPK1 forms were expressed both at the transcriptional and translational levels (Figures 1b and S1b), with subcellular protein localization in the cytosol and nucleus, similar to the WT IPK1 (Figure S1c). These results indicated that Lys168 and Asp368 are required for kinase activity of IPK1 in vivo.

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In contrast to WT IPK1, which was able to restore the P_i content of the ipk1-1 mutant to the WT level, both kinase-inactive IPK1 forms failed to complement excessive P_i accumulation and PSR gene activation in ipk1-1 (Figure 1b, c). Therefore, the kinase activity of IPK1 is required for regulation of P_i homeostasis. In addition to regulating P_i content, the kinase activity of IPK1 is also required for root system architecture (RSA), because neither of the kinase-inactive IPK1 proteins complemented the PSR-like RSA phenotypes (i.e., reduced primary root and enhanced lateral root growth) of ipk1-1 (Figure S1d).

Misregulation of P_i homeostasis in ipk1-1 is not caused by defective InsP_6-mediated mRNA export

In yeast, InsP_6 is required for mRNA export by activating the RNA-dependent ATPase activity of DEAD-box protein 5 (Dbp5p) in conjunction with GLFG lethal 1 (Gle1p), and mutations in ipk1 and gle1 resulted in mRNA retention in the nucleus and temperature-sensitive growth defects (York et al., 1999; Alcazar-Roman et al., 2006). A conserved mechanism was recently reported in Arabidopsis, and part of the growth defect of ipk1-1 is attributed to compromised mRNA export due to reduced level of InsP_6 (Lee et al., 2015). To address whether defective mRNA export in the ipk1-1 mutant is a cause of the misregulation of P_i homeostasis, we examined P_i-related phenotypes of the mRNA export mutants reported (Lee et al., 2015). As shown in Figure S2, the loss-of-function mutation in the Dbp5 homologous gene LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4 (LOS4), and inducible GLE1 RNAi lines exhibited WT P_i content (Figure S2a,b) and PSR gene expression (Figure S2c). Furthermore, expression of variants of Gle1 (IS1 and IS2), which exhibit increased InsP_6 sensitivity to LOS4 stimulation and improved growth defects of ipk1-1 (Lee et al., 2015), did not reduce P_i content or suppress PSR gene activation of the ipk1-1 mutant (Figure S2c, d). These results suggest that misregulation of P_i homeostasis in ipk1-1 is not caused by defective mRNA export due to reduced InsP_6 level.
Genetic dissection of the roles for InsP and PP-InsP biosynthesis enzymes in P\textsubscript{i} homeostasis regulation

The dependence of P\textsubscript{i} homeostasis on the kinase activity of IPK1 suggested that the PSR activation signal is derived from InsP biosynthesis. To dissect which step(s) of InsP and PP-InsP biosynthesis controls this signal, we examined P\textsubscript{i}-related phenotypes of mutants defective in several InsP and PP-InsP biosynthesis enzymes previously characterized in Arabidopsis, including *myo*-inositol-3-phosphate synthases (MIPS1-3) (Torabinejad and Gillaspy, 2006), Ins(1,4,5)P\textsubscript{3} 6/-3-kinases (inositol phosphate multikinases; IPK2\textalpha{} and IPK2\textbeta{}) (Stevenson-Paulik *et al.*, 2002), Ins(1,3,4)P\textsubscript{3} 5/-6-kinase enzymes (inositol phosphate tris/tetrakisphosphate kinases; ITPK1-4) (Wilson and Majerus, 1997; Sweetman *et al.*, 2007), PP-InsP synthesizing enzyme PPIP5K (VIP1/VIH2 and VIP2/VIH1) (Desai *et al.*, 2014; Laha *et al.*, 2015), and a mutant of an InsP\textsubscript{6} transporter, multidrug resistance-associated protein 5 (MRP5) (Nagy *et al.*, 2009). T-DNA insertional mutants were obtained and confirmed by RT-PCR to be null mutants (Table S1 and Figure S3a, b).

Morphologically, none of the mutants displayed growth defects as severe as *ipk1-1* (stunted growth and leaf necrosis), although *mips1*, *itpk1* and *mrp5-2* mutants were smaller than the WT (Figure 2a). The leaf epinasty and PSR-like RSA phenotypic characteristics of *ipk1-1* mutants (Stevenson-Paulik *et al.*, 2005; Kuo *et al.*, 2014) were observed in *itpk1* and *mrp5-2* mutants (Figures 2a and S3c, d) (Kuo *et al.*, 2014). Analysis of P\textsubscript{i} content in the shoot tissues revealed that only *itpk1* accumulated excessive P\textsubscript{i} comparable to *ipk1-1* (Figure 2b), and this phenotype persisted to the mature stage (Figure S3e). Mild but significantly elevated P\textsubscript{i} content was observed in *mrp5-2* seedlings but was no longer seen at the mature stage (Figures 2b and S3e). Consistent with the elevated P\textsubscript{i} content, *itpk1* exhibited elevated uptake of P\textsubscript{i} activity comparable with that of *ipk1-1*, whereas all other mutants showed WT activities (Figure 2c-f). The excessive P\textsubscript{i} accumulation in *itpk1* mutants could be restored to the WT level by ectopic expression of a genomic construct of the *ITPK1* sequence (Figure S4a), which confirms a role for ITPK1 in regulating P\textsubscript{i} homeostasis.

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In addition to decreased InsP₆ level, levels of InsP₇ and InsP₈ are also reduced in ipk1-1 mutants (Laha et al., 2015). We therefore examined whether PP-InsPs also play a role in the regulation of Pᵢ homeostasis or PSR in plants. Two families of kinases, IP6K and Vip/PPIP5K, are involved in PP-InsP synthesis in eukaryotes (Wundenberg et al., 2014); however, only Vip1/PPIP5K homologs are identified in plants and shown to be responsible for InsP₈ but not InsP₇ synthesis in Arabidopsis (Mulugu et al., 2007; Desai et al., 2014; Laha et al., 2015). We analyzed mutants defective in each of the two Arabidopsis Vip1/PPIP5K homologs, AtVIP1/VIH2 and AtVIP2/VIH1, and observed slightly decreased Pᵢ content in two alleles of atvip1 mutants (abbreviated as vip1-1 and vip1-2) with T-DNA disrupting the phosphatase-like domain but not in the alleles disrupted in the ATP-grasp kinase domain (vih2-3 and vih2-4) (Figure S3b) (Laha et al., 2015). Three atvip2 mutants (abbreviated as vip2-1, vip2-2 and vih1) did not show Pᵢ-content phenotype, but vip1-2 vip2-1 double mutants exhibited lower Pᵢ content comparable to the vip1-2 single mutant (Figures 2b and S3e), which suggests a dominant role for vip1 mutation in determining this phenotype. Despite the lower Pᵢ content, Pᵢ uptake and root-to-shoot allocation activity did not change in the vip1-1 or vip1-2 mutants (Figures 2f and S5a). Furthermore, the expression of PSR genes under Pᵢ-replete conditions and the magnitude of PSR gene activation in response to Pᵢ starvation in the vip1/vih2 and vip2/vih1 mutants were similar to that in the WT (Figure S5b, c). The cause of reduced Pᵢ content observed in vip1 alleles defective in the phosphatase-like domain is unclear, but the contrasting Pᵢ-related phenotypes between these vip1 alleles and ipk1-1 indicates that the decreased level of InsP₈ in ipk1 mutants is not responsible for Pᵢ homeostasis misregulation.

**ITPK1 and IPK1 constitute a pathway involved in the maintenance of Pᵢ homeostasis**

The common phenotypes observed in itpk1 and ipk1-1 mutants (i.e., excessive Pᵢ accumulation and elevated Pᵢ uptake under Pᵢ-replete growth conditions) suggest that ITPK1 and IPK1 are involved in the same pathway that regulates Pᵢ homeostasis. Consistently, a common set of representative PSR genes was upregulated in itpk1 and ipk1-1 mutants (Figure 3a), and overexpression of **ITPK1** or **IPK1**
reduced shoot P\textsubscript{i} content (Figure 3b). Correspondingly, ITPK1 overexpression significantly decreased P\textsubscript{i} uptake activity, in contrast to the elevated uptake activity shown by itpk1 mutants (Figure 3c). In addition, several PSR genes were downregulated in ITPK1-overexpressing lines as compared with the WT (Figure 3a, e.g., PHT1;2, SPX1, AT4, IPS1 and PAP17). However, P\textsubscript{i}-uptake activity and PSR gene expression did not differ significantly between IPK1-overexpression lines and the WT (Figure 3a-d).

We drew additional support for the participation of ITPK1 and IPK1 in a common pathway regulating P\textsubscript{i} homeostasis in terms of their tissue-specific expression patterns and subcellular localization. Promoter-GUS activity assay and RT-PCR analysis demonstrated co-expression of ITPK1 and IPK1 throughout development and in specific tissues and cell types, such as vasculature, trichomes and guard cells (Figure 4a-k). In addition, neither gene was transcriptionally responsive to P\textsubscript{i} status (Figure 4l). The expression of ITPK1 native protein fused to yellow fluorescent protein (YFP), which restored P\textsubscript{i} content of the itpk1 mutant to the WT level (Figure S4b), demonstrated co-localization of ITPK1 and IPK1 in the nucleus and cytoplasm (Figures 4m and S1c) (Kuo et al., 2014).

We next examined the genetic interaction of ITPK1 and IPK1 with a genetic cross between ipk1-1 and itpk1 mutants. The ipk1-1 itpk1 double mutants exhibited more severe growth defects than single mutants (Figure 4n) and those that proceeded to the reproductive stage bore aborted seeds [Figure 4n(iv’), (v’)]. Tissue P\textsubscript{i} content was greater in ipk1-1 itpk1 double than single mutants, by 50% to 70%, which is likely attributed to the relative 50% to 80% reduction in fresh weight (Figure 4o). Notably, expression of PSR genes in ipk1-1 itpk1 double and single mutants was comparable (Figure 4p), which suggests IPK1 and ITPK1 function in a common regulatory pathway of P\textsubscript{i} homeostasis.
A common elevation of D/L-Ins(3,4,5,6)P_4 in itpk1 and ipk1-1 mutants

The observations that maintenance of Pi homoeostasis depends on (1) the kinase activity of IPK1, (2) an additional InsP kinase, ITPK1, and (3) the expression level of ITPK1 and IPK1 (i.e., contrary Pi-related phenotypes between mutants and overexpression lines), suggest the contribution of a stoichiometric alteration of InsP metabolites to Pi homeostasis regulation. To pinpoint the possible InsP molecules involved in such regulation, we compared InsP profiles of vegetative tissues of the relevant genotypes by in vivo labeling with [32P]Pi, and/or myo-[3H]inositol and HPLC analysis. As shown in Figure 5a, Figure 5b, and myo-[3H]inositol-labeled chromatogram in Supporting Figure S6a, the itpk1 mutant shared a significant reduction in InsP_6 (62 ± 2% WT) with the ipk1-1 mutant (17 ± 1% WT). To validate that reduced InsP_6 level is not a cause of misregulated Pi homeostasis, with the normal Pi-related phenotypes exhibited by another low-InsP_6 mutant mips1 (Murphy et al., 2008; Kuo et al., 2014), we analyzed the InsP profile of the mips1 mutant. Unexpectedly, mips1 mutants exhibited a WT level of InsP_6 (Figures 5a, b and S6a). For comparison, we also performed profile analysis of other itpk mutants and found that two itpk4 mutants (itpk4-1 and itpk4-2; Table S1) showed a strong reduction in InsP_6 level comparable to itpk1 and ipk1-1 mutants, by 50% and 80%, respectively (Figures 5a, b and S6a). Consistent with the previous report, itpk4 mutations also significantly reduced InsP_6 level in seeds, to a similar extent as ipk1-1 (Figure S7a) (Stevenson-Paulik et al., 2005; Kim and Tai, 2011). The itpk4 mutants did not show striking morphological phenotypes (Figures 2a and S3c) or Pi-related phenotypes, such as altered Pi content (Figures 2b and S3e, S7b), Pi uptake (Figure 2e), or altered PSR gene expression (Figure S7c). RT-PCR and promoter-GUS analysis indicated that ITPK4 was expressed in the same vegetative tissues as ITPK1 and IPK1 (Figure S7d-l), which suggests that ITPK4 is likely involved in the same tissue-specific pool of InsP_6 biosynthesis. In addition, YFP-tagged ITPK4, which complemented the seed-InsP_6 phenotype of the itpk4-1 mutant (Figure S7a), like ITPK1 and IPK1, was also localized to the nuclei and cytoplasm (Figure S7m). Hence,
reduced InsP₆ level alone is insufficient to alter Pᵢ homeostasis and ITPK4 is a key enzyme for InsP₆ biosynthesis in both vegetative tissues and seeds.

In accordance with decreased InsP₆ level, InsP₇ level was also decreased in the ipk1-1 mutant (Figure 5a) (Laha et al., 2015). Similarly, InsP₇ level was decreased in itpk1 and itpk4 mutants (Figure 5a), and therefore we could not draw a correlation between the reduced InsP₇ level and the Pᵢ-related phenotypes observed in ipk1-1 and itpk1. The ipk1-1 mutant shows significant accumulation of Ins(1,3,4,5,6)P₅ along with reduced InsP₆ level (InsP₅b in Figures 5a and S6a) (Stevenson-Paulik et al., 2005), but in contrast, there was no detectable accumulation in the corresponding InsP₅ in the itpk1 mutant. This finding suggests that the elevated Ins(1,3,4,5,6)P₅ level in the ipk1-1 mutant does not explain the misregulation of Pᵢ homeostasis.

Notably, the itpk1 mutant showed elevated level of an InsP₄ species with identical chromatographic mobility to that in the ipk1-1 mutant, which is predominantly Ins(3,4,5,6)P₄ (InsP₄* in Figures 5a and S6a) (Stevenson-Paulik et al., 2005). The InsP₄ species in the itpk1 mutant was further analysed by high-resolution HPLC separation and was co-eluted with D/L-Ins(3,4,5,6)P₄ standard (D/L enantiomers are not separable by existing chromatographic technologies) (Figure 5c). In addition to the increase in InsP₄ level, levels of earlier eluting InsP species were increased in both itpk1 and ipk1-1 mutants, which exhibited the chromatographic mobility of InsP₃ (Figures 5a and S6a). Because there are 20 possible InsP₃ isomers, being the most difficult InsP to resolve, isocratic HPLC analysis was performed under conditions designed for optimal resolution of these peaks (Wreggett and Irvine, 1989). As shown in Figure 5d, ipk1-1 and itpk1 mutations caused accumulation of distinct InsP₃ isomers that were not detectable in the WT. Inclusion of an internal standard of myo-[³H]Ins(1,4,5)P₃ revealed that these isomers are not Ins(1,4,5)P₃, which was shown to present only a trivial fraction of InsP₃ in plant tissues (Brearley and Hanke, 2000). We conclude that the only common change of InsP species associated with the Pᵢ-related phenotypes of ipk1-1 and itpk1 is the elevated D/L-Ins(3,4,5,6)P₄ level.

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**Pi starvation induced a shoot-specific increase of InsP7**

To address whether *itpk1* and *ipk1-1* mutants exhibit an InsP profile that shares a common feature with Pi-starvation responses, we investigated the change in InsP profiles in shoots and roots of WT plants in response to different Pi-starvation regimes. InsP profiles were analyzed in seedlings subjected to 1- and 3-day Pi starvation, when cellular Pi concentrations were significantly reduced and PSR genes induced (Figure S8a, b). To avoid unequal $[^{32}\text{P}]\text{Pi}$ labeling between Pi-replete (supplemented with 250 μM Pi) and Pi-deficient (supplemented with 10 μM Pi) conditions and subsequent biased-quantifications of InsPs, we performed a pulse-chase experiment with seedlings labeled with $[^{32}\text{P}]\text{Pi}$ before Pi starvation. Tissues were similarly radiolabeled in every pairwise '+P' vs. '-P' treatment, although more $[^{32}\text{P}]$ was allocated to shoots than roots (Figure 6a, b).

Overall, the chromatograms did not exhibit prominent profile changes in response to Pi starvation in either shoots or roots (Figures 6a and S9). Quantitative analysis indicated no significant change in InsP$_6$ level in response to Pi starvation in shoots or roots (Figure 6c). Despite no significant change in InsP$_7$ level in roots, shoots exhibited mild yet significant increase in InsP$_7$-to-InsP$_6$ ratio and InsP$_7$ level in response to 1- and 3-day Pi starvation (Figure 6d, e). We were unable to assess the InsP$_8$ level due to the detection limit in our analysis; however, depletion of InsP$_8$ caused by vih2 mutation not affecting the Pi-starvation response implied that this InsP species does not mediate Pi signaling (Figure S5c). Notably, the increase in D/L-Ins(3,4,5,6)P$_4$ level in *itpk1* and *ipk1-1* mutants was not observed in Pi-starved WT plants (Figures 6f, g and S9a-d), nor was the level of any InsP$_3$ isomer, including Ins(1,4,5)P$_3$, changed in response to Pi starvation (Figure S9e, f).

Because cellular adenylate energy is influenced by Pi availability (Boer et al., 2010; Alexova et al., 2017; Choi et al., 2017), and high energy phosphates delivered by ATP are required for pyrophosphorylation (Voglmaier et al., 1996), we examined whether phosphorylated adenine nucleotides are metabolically coordinated with the change in InsP$_7$ level in response to Pi starvation.
by LC/MS analysis. ATP increased along with InsP$_7$ level specifically in shoots during 1- and 3-day $P_i$ starvation, whereas AMP level remained steady (Figure 6h, I), which resulted in a significant increase of ATP/AMP ratio (0.68 ± 0.1 and 1.1 ± 0.1 for 3-day ‘$+$’ and ‘$-$’ treatment, respectively, $P=0.009$).

In conclusion, the changes in InsP profiles of WT seedlings in response to 1- and 3-day $P_i$ starvation distinctly differ from those in $ipk1$ and $ipk1$-1 mutants, which suggests that the mechanism of the ITPK1 and IPK1 contribution to $P_i$ homeostasis is distinct from the $P_i$-starvation response in WT plants.

**Discussion**

In this study, we demonstrated metabolism of distinct InsP species in correlation to $P_i$ homeostasis and $P_i$ limitation as summarized in Figure 7. Under $P_i$-replete conditions, the catalytic activity of IPK1 was required for maintenance of $P_i$ homeostasis, providing the first evidence of the involvement of InsP metabolism, as opposed to other possible aspects of IPK1 protein function (Figures 1 and S1). This notion is further supported by the identification of an additional InsP-synthesizing enzyme, ITPK1, with a comparable role to IPK1 (Figures 2, 3, S3 and S4). The epistatic relationship of IPK1 and ITPK1 in suppressing PSR genes under $P_i$-replete conditions, together with their co-expression pattern throughout development and their subcellular co-localization (Figure 4), indicate that ITPK1 and IPK1 constitute an InsP metabolic pathway maintaining $P_i$ homeostasis. InsP profiling revealed two distinct common features between $ipk1$-1 and $itpk1$ mutants: (1) decreases in InsP$_6$ and InsP$_7$ levels and (2) an increase in D/L-Ins(3,4,5,6)$P_4$ level (Figures 5 and S6). In contrast, $P_i$ starvation induced a distinct InsP profile from those with $ipk1$-1 and $itpk1$ mutations (Figure 6), which suggests that $ipk1$-1 and $itpk1$ mutations affect $P_i$ homeostasis by a mechanism other than $P_i$-starvation signaling.
Decrease in InsP₆, InsP₇ or InsP₈ level is not responsible for disturbed Pᵢ homeostasis in ipk1-1 and itpk1 mutants

The fact that itpk4 mutants did not exhibit Pᵢ-related phenotypes comparable to ipk1-1 and itpk1 mutants indicates that a decrease in InsP₆ or InsP₇ level did not cause the disturbed Pᵢ homeostasis under Pᵢ-replete conditions. The similar tissue/developmental expression pattern and subcellular localization of ITPK4 as ITPK1 and IPK1 suggest that these three enzymes control the same pool of vegetative InsP₆ and InsP₇ (Figure S7). While it is possible that radiolabeling does not entirely reflect metabolic (subcellular) pools of different InsP and PP-InsP metabolites, no other methods have been elaborated for measurement of these molecules in plants, never mind their subcellular fractionation. Although we were unable to determine the InsP₈ level, vih2 mutants mediating InsP₈ synthesis in planta (Laha et al., 2015) did not phenocopy ipk1-1 and itpk1 under Pᵢ-replete conditions and exhibited normal Pᵢ-starvation responses (Figures 2, S3 and S5), which suggests that InsP₈ is unlikely involved in the regulation of Pᵢ homeostasis.

We have also ruled out that misregulated Pᵢ homeostasis is a secondary consequence of mitigated InsP₆-mediated mRNA export by demonstrating that mutations compromising or enhancing InsP₆-Gle1-Los4 mRNA machinery neither caused comparable Pᵢ-related phenotypes of ipk1-1 nor complemented ipk1-1 (Figure S2). The identification of two itpk4 alleles with similar reduction in InsP₆ (and InsP₇) level in ipk1-1 and itpk1, respectively, without showing Pᵢ-related phenotypes, also argues against a role for InsP₆-mediated mRNA export in regulating Pᵢ homeostasis (Figures 2, 5 and S7). Of note, although growth retardation of ipk1-1 is attributed to defective InsP₆-mediated mRNA export (Lee et al., 2015), itpk4 mutants did not exhibit growth defects comparable to ipk1-1 or itpk1 (Figure 2a). Thus, InsP₆ reduction may not be the sole cause for the growth defect observed in the ipk1-1 and itpk1 mutants.
Correlation between the increased level of D/L-Ins(3,4,5,6)P_4 and misregulation of P_i homeostasis in ipk1-1 and itpk1 mutants

Aside from the reduced levels of InsP_6 and InsP_7, the most significant common InsP profile change between itpk1 and ipk1-1 is the increased accumulation of the InsP_4 species, shown to predominantly consist of Ins(3,4,5,6)P_4 in the ipk1-1 mutant (Stevenson-Paulik et al., 2005). The isomeric identity of the InsP_4 species in the itpk1 mutant remains to be determined, but human ITPK1 was found a reversible InsP 1-kinase/phosphatase that regulates the level of Ins(3,4,5,6)P_4, an inhibitor of Ca^{2+}-activated chloride channels in the plasma membrane (Vajanaphanich et al., 1994; Yang et al., 1999; Ho et al., 2002; Saiardi and Cockcroft, 2008). In tobacco, Ins(3,4,5,6)P_4 is also linked to chloride transport, regulating growth and cell volume in pollen tubes (Zonia et al., 2002). We attempted to test the effect of Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4 on P_i homeostasis of Arabidopsis seedlings by using membrane-permeant bioactivatable analogues of these two InsP isomers [Bt2-Ins(1,4,5,6)P_4/PM and Bt2-Ins(3,4,5,6)P_4/PM] (Vajanaphanich et al., 1994) but did not observe significant effects on tissue P_i accumulation or PSR gene expression. However, the effectiveness of intracellular delivery and metabolism of these InsP analogs on plant tissues remains to be assessed.

In addition to InsP_4, InsP_3 showed changes in ipk1-1 and itpk1 mutants (Figure 5d). In plants, Ins(1,4,5)P_3 (assayed by a competitive InsP_3-receptor binding assay) has been linked to several physiological responses, such as gravitropism, salt and drought stresses (Perera et al., 2001; Xiong et al., 2001; Perera et al., 2006; Perera et al., 2008). We demonstrated that neither ipk1-1 nor itpk1 mutation affected the levels of Ins(1,4,5)P_3, as measured by radiolabelling approaches. Species that co-elute with this isomer are barely detectable in WT plants (Figure 5d) (Brearley and Hanke, 2000). Because the two mutants showed distinctive InsP_3 profiles, and neither accumulated Ins(1,4,5)P_3, we did not find any association between changes in specific InsP_3 and P_i homeostasis.
Because InsP lipids, called polyphosphoinositides (PPIs), also play important roles in cellular signaling and InsP metabolism (Munnik and Vermeer, 2010; Munnik and Nielsen, 2011), we examined whether PPI levels were altered in ipk1-1 and itpk1 mutants and found elevated levels of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] in both ipk1-1 and itpk1 (Figure S10a, b). We further examined P1-related phenotypes in mutants or transgenic lines with elevated levels of PtdInsP2, i.e., phosphatidylinositol-phospholipase C2 (plc2), suppressor of actin 9 (sac9), and a PHOSPHATIDYLINOSITOL PHOSPHATE 5-KINASE 3 (PPIP3)-overexpression line (Williams et al., 2005; Kusano et al., 2008; Stenzel et al., 2008; Kanehara et al., 2015). None of these lines were comparable to the ipk1-1 mutant (Figure S10c, d), which suggests that the increased PtdIns(4,5)P2 levels in ipk1-1 and itpk1 mutants are not likely attributable to the misregulated P1 homeostasis.

P1 starvation induced a change in InsP profile distinct from those caused by itpk1 and ipk1-1 mutations

Although ipk1-1 and itpk1 mutants exhibited characteristic phenotypes of P1-starvation responses under P1-replete conditions, their InsP profiles were distinct from those under P1 starvation, notably the contrasting levels of D/L-Ins(3,4,5,6)P4, InsP6, and InsP7 (Figures 5a, 6f, 6g and S9a-d). The level of D/L-Ins(3,4,5,6)P4 not being altered by P1 starvation suggests these InsP species are not involved in P1-starvation signaling in WT plants. The disparate InsP profiles in response to P1 starvation versus that caused by ipk1-1 and itpk1 mutations imply two distinct P1 signaling pathways. In support of this notion, the P1-starvation responses persisted in the ipk1-1 and itpk1 mutants, in which PSR genes remained inducible under P1 starvation (Figure S8c). We observed no distinct alteration of InsP profile in response to P1 starvation except for a significant increase in InsP7 level of unknown isomeric identity in the shoot of P1-starved plants but not in the root (Figure 6d, e), where P1-starvation responses also take place. Shoot tissues are more responsive to P1 starvation than are roots (Huang et al., 2008; Lin et al., 2008), which has led to a hypothesis that the shoot is the tissue where P1 starvation is sensed and the signal initiated (Hammond and White, 2008; Lin et al., 2008).
Alternatively, because P_i starvation triggers differential transcriptional and metabolic responses between shoots and roots (Wu et al., 2003; Pant et al., 2015), the shoot-specific increase in InsP_7 level may have tissue-specific physiological significance under P_i starvation conditions. It will be important to identify the kinase responsible for InsP_7 synthesis in plants to address these speculations.

Adenylate energy has been shown to regulate PP-InsPs synthesis, with increased ATP/ADP ratio promoting mammalian IP6K kinase activity (Wundenberg et al., 2014). We observed that the shoot-specific increase in InsP_7 level was associated with a shoot-specific increase in ATP and ATP/AMP ratio during 1- and 3-day P_i starvation (Figure 6h, i). Increases in ATP level in response to P_i starvation has been noted in barley leaves (Alexova et al., 2017), which contrasts with the decrease in ATP level during P_i starvation reported in yeast (Boer et al., 2010; Choi et al., 2017). P_i starvation-induced ATP decreases have been shown in other plant species (Duff et al., 1989; Rao et al., 1989), but concentration ratios of ATP to ADP (or AMP), which control kinetics of cellular metabolism (Pradet and Raymond, 1983), remained unchanged or was increased in those studies. Whether the elevated ATP/AMP ratio drives InsP_7 accumulation in P_i-starved shoots awaits further characterization of the InsP_7 synthesis enzyme. Of note, multiple enzymes involved in adenine nucleotide metabolism have been genetically identified to act upstream of the Pho80/Pho85/Pho81 complex as negative regulators of PHO signaling (Huang and Shea, 2005; Choi et al., 2017). Despite the inter-species difference in strategies for the P_i-starvation response, accumulating evidence has pointed to a close relationship between adenylate energy status and P_i signaling. PP-InsPs are proposed to be ‘metabolic messengers’ that mediate pyrophosphorylation of proteins involved in multiple cellular metabolism, including phosphorylation-based signal transduction pathways in yeast (Saiardi, 2012; Wu et al., 2016). Whether the shoot-specific P_i starvation-stimulated InsP_7 observed in this study has a role in P_i signaling by such protein pyrophosphorylation remains speculative.
Significant roles of ITPK family of enzymes in phytate biosynthesis in plant vegetative tissues

Mutation of IPK1 leads to substantively reduced InsP₆ level in seeds (Stevenson-Paulik et al., 2005) and vegetative tissues (Stevenson-Paulik et al., 2005; Nagy et al., 2009). The concomitant accumulation of Ins(1,3,4,5,6)P₅ in these tissues/organs (Stevenson-Paulik et al., 2005; Nagy et al., 2009) strongly indicates the dominant contribution of the Ins(1,3,4,5,6)P₅ 2-kinase activity of IPK1 to InsP₆ synthesis. The coincident accumulation of Ins(3,4,5,6)P₄ in vegetative tissues and seeds (Stevenson-Paulik et al., 2005) may be explained by mass action effects (Hanke et al., 2012), possibly indicating reversibility of the detected Ins(3,4,5,6)P₄ 1-kinase activity (Brearley and Hanke, 2000). The enzyme(s) responsible for producing Ins(3,4,5,6)P₄ in plants are not well defined. In avian erythrocytes, Ins(3,4,5,6)P₄ is the product of 5-phosphorylation of Ins(3,4,6)P₃ and is itself the precursor of Ins(1,3,4,5,6)P₅ (Stephens and Downes, 1990).

In nucleated mammalian cells, the origins of Ins(3,4,5,6)P₄ have not been tested by the methods of Stephens and Downes (Stephens and Downes, 1990), but the single mammalian ITPK1 is a multifunctional kinase and phosphotransferase that interconverts Ins(3,4,5,6)P₄ and Ins(1,3,4,5,6)P₅ (Chamberlain et al., 2007). The existence in Arabidopsis of a gene family of four inositol tris/tetrakisphosphate kinases (ITPK1-4) complicates study of InsP metabolism. Our identification of significant contributions of ITPK1 and ITPK4 to InsP₆ synthesis in vegetative tissues focuses attention on the contribution of these enzymes to not just InsP₆ synthesis but also physiological processes regulated by the intermediate InsPs. ITPK1 mutation reduces labeling of InsP₆ by 50%, with concomitant accumulation of D/L-Ins(3,4,5,6)P₄, but because it does so without affecting Ins(1,3,4,5,6)P₅ level (Figures 5a and S6a) suggests that ITPK1 does not likely act as an Ins(1,3,4,5,6)P₅ 1-phosphatase. ITPK1 may be acting at the level of InsP₄-InsP₅ interconversion. Remarkably, our studies show that ITPK4, which contributes to nearly 90% of vegetative InsP₆, and more in seeds, has no effect on the P₇-starvation response. Our labeling studies showed no increased InsP₄ accumulation in vegetative tissues (Figures 5a and S6a). This implies that most of the InsP₄...
precursors for InsP₆ synthesis are generated by this enzyme and the contribution of ITPK4 may lie in its InsP₃ kinase activity rather than its InsP₄ isomerase/mutase activity (Sweetman et al., 2007).

**Implication of InsP metabolism in regulating Pᵢ homeostasis**

Across eukaryotic kingdoms, the SPX domains of a large family of proteins involved in Pᵢ sensing and transport have been shown to bind InsPs, thereby regulating SPX-protein activities and their interaction with other proteins (Wild et al., 2016). Although InsP₆ and PP-InsPs at sub-micromolar concentration exhibited the highest binding affinity to the SPX domains, the lower InsP levels also exhibited physiologically relevant binding affinity at a micromolar range (Wild et al., 2016). Our study has pointed to a significant association between the level of D/L-Ins(3,4,5,6)P₄ and maintenance of Pᵢ homeostasis under Pᵢ-replete conditions but not the Pᵢ-starvation response. It remains speculative how increases in InsP₄ level is associated with elevated Pᵢ uptake and PSR-gene expression and the future identification of the enantiomerism of D/L-Ins(3,4,5,6)P₄ in the itpk1 mutant and its interacting protein targets, such as by using InsP affinity screens (Wu et al., 2016), should provide further mechanistic insights. The confounding effects on PHO signaling of Kcs1p (negative) and Vip1p (positive) (Auesukaree et al., 2005; Lee et al., 2007), together with a Vip1-independent Pᵢ-starvation signaling pathway (Choi et al., 2017), suggest the regulatory mechanisms that control Pᵢ homeostasis likely involve multiple InsP and PP-InsP species. Different InsP and PP-InsP species may regulate Pᵢ homeostasis via their competitive interaction with a spectrum of SPX-domain protein(s). For example, the binding of InsP₆ and 5-InsP₇ to OsSPX4/OsPHR2 yielded $K_d$ of ~50 µM and 7 µM respectively (Wild et al., 2016), suggesting that competition between the more abundant InsP₆ and less abundant PP-InsPs are relevant considerations in SPX function (Wild et al., 2016). Consequently, it will be important to consider the prevailing physiological concentration of potential InsP and PP-InsP competitors. Together with the diverse functions of SPX proteins at different levels of Pᵢ homeostasis regulation (Secco et al., 2012; Azevedo and Saiardi,
2017) and our findings presented here, InsP₇ may not be a general (or conserved) signal, and the role of other InsP intermediates in regulating Pᵢ homeostasis need to be considered.

**Experimental procedures**

**Plant materials and growth conditions**

*Arabidopsis thaliana* mutant lines and their sources are listed in S1 Table; the wild-type line (WT) indicates Col-0 unless specified otherwise. Seeds were surface-sterilized, stratified at 4°C for 1-3 days, and germinated on agar medium of half-strength modified Hoagland nutrient solution containing 250 μM KH₂PO₄, 1% sucrose, and 0.8% Bacto agar (Aung et al., 2006). The Pᵢ-replete ('+P') and Pᵢ-deficient ('-P') media were supplemented with 250 μM (or 1 mM as specified) and 10 μM KH₂PO₄, respectively. For hydroponic growth, seedlings were germinated and grown on solid media for 10 days before being transferred to half-strength modified Hoagland nutrient solution with sucrose omitted. Plants were grown at 22°C under a 16-h photoperiod with cool fluorescent white light at 100 to 150 μE m⁻² s⁻¹. For generating *ipk1-1 itpk1* double mutants, both double mutants and isogenic WT progenies were recovered from the F₂ population at an equivalent yet lower segregation rate (1%) than expected (6%). Because these two loci are located on different arms of chromosome 5, the reason for this segregation distortion is unknown.

**Measurement of Pᵢ content and Pᵢ uptake activity**

Total Pᵢ content and Pᵢ uptake activity were measured as described previously (Chiou et al., 2006). To measure the root-to-shoot Pᵢ translocation activity, pulse-chase labeling was performed. 14-day after germination seedlings were first incubated in Pᵢ-replete nutrient solution (half-strength modified Hoagland solution supplemented with 250 μM KH₂PO₄) containing ³²P]orthophosphate (Pᵢ) for 3 h (‘pulse’ treatment), then transferred to Pᵢ-replete nutrient solution without ³²P]Pᵢ for
indicated times ('chase' treatment). $^{33}\text{P}$ radioactivity in the plants tissues was measured as the $\text{P}_i$ uptake assay and the root-to-shoot $\text{P}_i$ translocation activity was measured by shoot-to-root ratio of $^{33}\text{P}$ count.

**Genotype analysis, transgene construction and plant transformation**

Primers used for genotyping of T-DNA insertional lines were designed according to SIGnAL (http://signal.salk.edu/tdnaprimers.2.html) and are listed in Supporting Table S2. For constructing kinase-inactive IPK1, nucleotide substitutions were introduced in the primers (5’ phosphorylated; Supporting Table S2) used for PCR amplification by using a vector (pMDC32) containing the IPK1 CDS sequence driven by the 35S promoter as template. PCR product was ligated before transformation and sequences were confirmed before recombination into the Gateway destination vector pK7YWG2.0 (C’-YFP) (Karimi et al., 2007) via LR Clonase enzyme mix (Invitrogen). For complementation analysis, the genomic sequence of ITPK1, including 1 kb upstream of ATG start codon, was amplified by PCR (primers listed in Supporting Table S2) and cloned into pCR8/GW/TOPO (Invitrogen) followed by recombination into the Gateway destination vectors. pMDC99, pMDC32 (Curtis and Grossniklaus, 2003), and pK7YWG2.0 were chosen as destination vectors for complementation, promoter::GUS activity and YFP fluorescence analysis, respectively. All cloned constructs were validated by sequencing analysis before being introduced into Arabidopsis by the floral-dip transformation method (Clough and Bent, 1998).

**RNA isolation, RT-PCR, and qRT-PCR**

Total RNA was isolated by using RNAzol reagent (Molecular Research Center) and cDNA was synthesized from 0.5 to 1 $\mu$g total RNA by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen) and oligo(dT) primers. Sequences of primers used for RT-PCR and qRT-PCR are in Supporting Table S2. qRT-PCR involved use of the Power SYBR Green PCR Master Mix kit (Applied Biosystems) on a 7500 Real-Time PCR system as instructed. Gene expression was

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normalized by subtracting the Ct value of UBQ10 (∆Ct) from that of the gene studied and presented as $2^{\Delta\Delta Ct}$. The expression relative to the WT (i.e., fold change relative to the WT) is presented as $2^{\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct - \Delta Ct^{WT}$). qPCR raw data is provided in Supporting Table S3.

**GUS staining and fluorescence microscopy**

GUS activity of transgenic T2 plants was detected as described (Lin et al., 2005), and the signal was observed under an Olympus SZX12 or a Zeiss AxioSkop microscope. Confocal microscopy images of the YFP signal were obtained by using a Zeiss LSM 510 META NLO DuoScan with LCI Plan-Neofluar x63/1.3 Immersion and Plan-Apochromat x100/1.4 oil objectives. Excitation/emission wavelengths were 514 nm/520 to 550 nm for YFP.

**InsP profiling of Arabidopsis seedlings and seeds**

For InsP profile analysis of Arabidopsis vegetative tissue, seedlings (8-11 DAG) were labelled with myo-[2-$^3$H]inositol (19.6 Ci mmol$^{-1}$, Perkin Elmer NET114A00; 0.4 mCi mL$^{-1}$ for 5 days) or [$^{32}$P]P$_i$ (8500-9120 Ci mmol$^{-1}$, Perkin Elmer NEX05300; 0.02 mCi mL$^{-1}$ for 1-3 days accordingly) in half-strength Hoagland’s medium supplemented with P$_i$ at levels specified in the text. InsP was extracted from the radiolabeled tissues, roots, shoots or whole seedlings as described (Azevedo and Saiardi, 2006). Extracts were resolved on a 250 x 4.6 mm Whatman Partisphere SAX WVS column fitted with guard cartridge of the same material at a flow rate of 1 mL min$^{-1}$ with a gradient derived from buffer reservoirs containing A, water; B, 1.25M (NH$_4$)$_2$HPO$_4$, adjusted to pH 3.8 with H$_3$PO$_4$, mixed according to the following gradient: time (min), %B; 0, 0; 5, 0; 65, 100; 75, 100. Isocratic separations of InsP$_3$ species were performed at the same flow rate on the same column eluted with 20% buffer B. For myo-[$^3$H]inositol labeling, fractions were collected every minute from retention time 0 to 30 min and every 0.5 min from 30 min onward, followed by scintillation counting (1:4 ratio column eluent to scintillation cocktail; Perkin-Elmer; ULTIMA-FLO AP). For [$^{32}$P]P$_i$ labeling,
radioactivity was measured by Cherenkov counting on a Canberra Packard Radiomatic A515 Flow Detector fitted with a 0.5-mL flow cell with an integration interval of 0.1 min (Brearley et al., 1997).

myo-[3H]inositol and [32P]Pi exhibited different allocation between tissues in planta, with greater [3H] labeling of roots (Figure S6b), whereas [32P]Pi labeled shoots more strongly (Figure 6b). With the exception of experiments to compare the extent of labeling of InsP6 between a wide range of genotypes (Figure 5b), performed with whole seedlings, the shoot and root tissues were analyzed independently. Aside from stoichiometric differences of specific InsPs, the InsP profile was in general similar between these two tissues (Figures 6a and S6b).

For analysis of InsPs in seeds, 2 mg seed was homogenized in 500 μl of ice-cold 0.6 N HCl before centrifugation for 15 min to remove cell debris. Aliquots (20 μL) were injected onto a 3 mm i.d. x 200 mm Carbo Pac PA200 HPLC column (Dionex) fitted with a 3 mm x 50 mm guard column of the same material. The column was eluted at a flow rate of 0.4 mL/min with a gradient of methane sulfonic acid (Acros Organics) delivered from buffer reservoirs containing: A, water; B, 600 mM methane sulfonic acid according to the following schedule: time (minutes), % B; 0, 0; 25, 100; 38, 100; 39, 0; 49, 0. The column eluate was mixed by using a mixing tee with a solution of 0.1% w/v ferric nitrate in 2% w/v perchloric acid (Phillippy and Bland, 1988) delivered at a flow rate of 0.2 mL/min, before passage through a 194-μL volume knitted reaction coil (4 m x 0.25 mm i.d.) obtained from Biotech AB, Sweden. The column, mixing tee and reaction coil were held at 35°C. Peaks of InsP were detected at 290 nm with a Jasco UV-2077 Plus UV detector. Chromatographic data were integrated in ChromNav (Jasco) software. The position of elution of different stereoisomers of the different classes of InsPs was determined by the inclusion at regular intervals of a set of standards obtained by extended acid treatment of phytic acid (middle panel in Figure 5c).
ATP and AMP analysis

Adenylates from plant tissues were extracted as described (Cho et al., 2016). Tissues were homogenized in liquid nitrogen and re-suspended in 2.3% (v/v) TCA containing 200 μg/ml ribitol (250 μl per 100 mg tissue). Homogenates were centrifuged at 13,000 rpm at 4°C for 15 min, and supernatants were recovered and neutralized to pH 6.5-7 by KOH, followed by 30-min incubation on ice. Extracts were centrifuged at 13,000 rpm at 4°C for 15 min and the supernatants were collected for LC/MS quantification with an ultra-performance liquid chromatography (UPLC) system (ACQUITY UPLC, Waters, Millford, MA). The sample was separated with a ZIC-chILIC column (3-μm particle size, 2.1 × 100 mm, Merck-Millipore). The UPLC system was coupled online to the Waters Xevo TQ-S triple quadruple mass spectrometer. Ribitol was used as internal standard. Characteristic MS transitions were monitored by the negative multiple reaction monitoring (MRM) mode for ATP (m/z, 506→159), AMP (m/z, 346→79), and ribitol (m/z, 151→71). Data acquisition and processing involved use of MassLynx v4.1 and TargetLynx software (Waters Corp.), with intensities of ATP and AMP normalized to ribitol.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
H.-F.K. and T.-J.C. conceived the project; H.-F.K., C.B. and T.-J.C. designed the experiments; H.-F.K., Y.-Y.H., W.-C.L., K.-Y.C., T.M. and C.B. performed the research; H.-F.K., C.B., T.M. and T.-J.C. interpreted the results; H.-F.K. and C.B. wrote the manuscript; H.-F.K., T.M., C.B. and T.-J.C. contributed to the final version of this article.
Supporting Information

Supporting Table S1. Mutant lines used in this study

Supporting Table S2. Primers used in this study

Supporting Table S3. RT-qPCR data for gene expression

Supporting Figure S1. Characterization of kinase-inactive IPK1 transgenic plants

Supporting Figure S2. The role of Gle1-InsP₆-Los4 mRNA export machinery in ipk1-mediated Pᵦ-related phenotypes

Supporting Figure S3. Genetic and phenotypic characterization of mutants of InsP biosynthesis enzymes

Supporting Figure S4. Complementation of itpk1 phenotypes

Supporting Figure S5. Pᵦ allocation activities and PSR gene expression in the vip/vih mutants.

Supporting Figure S6. InsP profile of genotypes labeled with myo-[^3]H]inositol

Supporting Figure S7. Characterization of itpk4 mutants and expression pattern of ITPK4.

Supporting Figure S8. Pᵦ starvation responses of WT and various genotypes under different regimes of Pᵦ starvation

Supporting Figure S9. Tissue-specific InsP profiles in response to 1- and 3-day Pᵦ starvation.

Supporting Figure S10. PPI composition in itpk1, ipk1-1 and OxITPK1 lines, and Pᵦ content in mutants exhibiting elevated PtdIns(4,5)P₂ levels
References


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### Figure legends

**Figure 1. Characterization of kinase-inactive IPK1 transgenic plants.**

(a) Relative InsP$_6$ content (% of WT) in seeds of *ipk1*-1 mutants and homozygous transgenic lines expressing C-terminus YFP-tagged wild-type *IPK1* (*IPK1*-YFP), *IPK1$^{K168A}$* (*IPK1$^{K168A}$-YFP), or *IPK1$^{D368A}$* (*IPK1$^{D368A}$-YFP) coding sequences in the *ipk1*-1 mutant background. Error bars, S.E. of n=3-12 independent experiments. (b) Relative expression (to WT) of PSR genes in roots, and (c) P$_i$ content in shoots of 14-days after germination (DAG) seedlings. Asterisks indicate significant differences from WT (Student’s t-test; **, *P* < 0.005).

**Figure 2. Characterization of mutants defective in InsP biosynthesis enzymes grown under P$_i$-replete condition.**

(a) Morphology of 22-DAG plants grown in P$_i$-replete (1 mM) hydroponic medium. Scale bar, 1 cm. (b) P$_i$ content in the shoots of 14-DAG seedlings grown on P$_i$-replete (1 mM) solid medium. Error bar, S.E. of n=4-21 independent experiments. (c-f) P$_i$ uptake activities of 14-DAG seedlings under P$_i$-replete (250 μM) growth conditions. Error bars, S.E. of n=3-24 independent experiments. Uptake
activities of genotypes in (a-c) were measured in overlapping sets of experiments and plotted separately for clear presentation. Asterisks denote significant differences from the WT (Student’s t-test; **, P < 0.005).

Figure 3. Phenotype similarities between itpk1, ipk1-1 mutants, and overexpression lines.

(a) Relative expression (to WT) of PSR genes in roots of 14-DAG itpk1, ipk1-1, IPK1-overexpression (OxIPK1) and ITPK1-overexpression (OxITPK1) lines grown under P<sub>r</sub>-replete (1 mM) conditions (see Supporting Table S3 for qPCR raw data and S.E. of 3 independent experiments). Note that qPCR primers for ITPK1 are located 5’ to the T-DNA insertion site. (b) P<sub>r</sub> content in shoots of 14-DAG T2 transgenic lines overexpressing ITPK1 or IPK1 compared to WT, itpk1 and ipk1-1 mutants grown under P<sub>r</sub>-replete (250 μM) condition. Error bars, S.E. of n=6-12 independent experiments. (c and d) P<sub>r</sub> uptake activities of 14-DAG seedlings grown under P<sub>r</sub>-replete (250 μM P<sub>r</sub>) condition. Error bars, S.E. of n=6-12 independent experiments. Asterisks denote significant differences from the WT (Student’s t-test; **, P < 0.005).

Figure 4. Tissue-specific expression and protein subcellular localization of ITPK1 and IPK1, and phenotypes of itpk1, ipk1-1 and itpk1 ipk1-1 double mutants.

(a-j) Promoter activities of IPK1 and ITPK1 at different developmental stages. (a) 3-DAG; scale bar, 10 μm. (b) 5-DAG; scale bar, 1 mm. (c) 7-DAG; scale bar, 1 mm. (d) 14-DAG; scale bar, 1 cm. (e) Cross section of 14-DAG root; scale bar, 10 μm. (f) Guard cells of 14-DAG leaves; scale bar, 10 μm. (g) Trichome of 14-DAG leaves; scale bar, 0.1 mm. (h) 22-DAG floral tissues; scale bar, 0.5 cm. (i) 22-DAG flowers; scale bar, 0.5 mm. (j) Siliques; scale bar, 0.5 mm. (K and L) RT-PCR analysis of tissue-specific expression of ITPK1 and ITPK4 at different developmental stages (k) and in response to P<sub>r</sub> status (l). S, shoot; R, root; LF, rosette leaves; FS, florescence stem; FL, flower; SL, silique; +P, 250 μM P<sub>r</sub>; -P, 10 μM P<sub>r</sub>. PCR amplification cycles for ITPK1, 32; ITPK4, 32; ACTIN2, 22. (m) Subcellular localization of C-terminus YFP-tagged IPK1 and ITPK1 protein in roots of 10-DAG ipk1-1 and itpk1 mutants.
respectively; scale bar, 10 μm. Arrows, cytoplasm, arrowheads, nucleus. (n) Morphology of 25-DAG itpk1 ipk1-1 mutants grown under P\textsubscript{i}-replete (250 μM) conditions. Insets show enlarged images of floral tissues (i'), rosette leaves (ii'), roots (iii'), mature siliques (iv') and aborted seeds (v'). Scale bars are 1 cm, 1 mm and 100 μm for the whole plant, insets (a-d) and inset (e), respectively. (o) Tissue-specific P\textsubscript{i} content and (p) relative expression of PSR genes of 16-DAG seedlings grown on P\textsubscript{i}-replete (250 μM) solid medium. Error bar, S.E. of n=3-6 independent experiments. Asterisks denote significant differences from the WT (Student's t-test; *, P < 0.05; **, P < 0.005).

Figure 5. InsP profiles of various genotypes.

(a) HPLC analysis of roots extracts from 11-DAG seedlings of various genotypes labeled with [\textsuperscript{32}P]P\textsubscript{i}. InsP\textsubscript{sa}, Ins(1,2,4,5,6)P\textsubscript{3} and/or Ins(2,3,4,5,6)P\textsubscript{3} and/or Ins(1,2,3,4,6)P\textsubscript{3} (these three isomers are not resolved on Partisphere SAX HPLC (Brearley and Hanke, 1996); InsP\textsubscript{sb}, Ins(1,3,4,5,6)P\textsubscript{5}; InsP\textsubscript{a}*, Ins(1,4,5,6)P\textsubscript{4} and/or Ins(3,4,5,6)P\textsubscript{4}; InsP\textsubscript{b}, peaks with the chromatographic mobility of InsP\textsubscript{sa}. Insets show expanded chromatograms of more polar InsPs, obtained by counting 1-min fractions collected from the Flo-Detector eluted from retention time of 50 min onwards. The ordinate is scaled by the same factor for the different genotypes, representing a constant fraction of the largest (P\textsubscript{i}) peak in each chromatogram. (b) Quantification of relative InsP\textsubscript{6} content (% of total radioactivity per HPLC run recovered in the integrated InsP\textsubscript{6} peak) in 11-DAG [\textsuperscript{32}P]-labeled seedlings. Error bar, S.E. of n=3-5 independent experiments. Double asterisks denote a significant difference from the WT (Student's t-test, P < 0.005). (c) Identity of InsP\textsubscript{a}* in itpk1 mutant. An aliquot of extract of [\textsuperscript{32}P]-labeled itpk1 seedlings (11-DAG) was spiked with a hydrolysate of InsP\textsubscript{6} and separated on a CarboPac PA200 column with post-column colourimetric detection of InsP peaks as described (Phillippy and Bland, 1988). Upper panel, [\textsuperscript{32}P]-radioactivity counted inline on the Flo Detector. Note that InsP\textsubscript{4} and InsP\textsubscript{6} are below the level of detection on the Flo Detector, and therefore corresponding fractions (0.5 min) were collected for static counting (lower panel). Middle panel, UV trace obtained from this extract. Ins(1,4,5,6)P\textsubscript{4}/Ins(3,4,5,6)P\textsubscript{4} is the latest eluting InsP\textsubscript{4} on this column.
and elutes before Ins(1,2,3,4,6)P₅. Lower panel, UV trace overlaid with [³²P] counts of collected fractions. The retention time of the fractions in the upper and lower panel is corrected for the plumbing delay between the UV detector and the Flo Detector or the fraction collector. The broadness of the [³²P] peaks (compared to the sharp UV peaks) is a consequence of band broadening after the UV detector (in the Flo Detector and the collected fractions). All the other Ins₄ isomers elute before 20 min. InsP₅[5-OH], Ins(1,2,3,4,6)P₅; InsP₅[4/6-OH], Ins(1,2,3,5,6)P₅ and/or Ins(1,2,3,4,5)P₅; InsP₅[1/3-OH], Ins(1,2,4,5,6)P₅ and/or Ins(2,3,4,5,6)P₅; InsP₅[2-OH], Ins(1,3,4,5,6)P₅.

Note that a single peak of InsP₄ was detected in itpk1 in (a). (d) Isocratic separation and counting of collected fractions for analysis of InsP₃ isomers. For better presentation, the chromatogram of each genotype was shifted by 200 CPM.

**Figure 6. Tissue-specific InsP profiles in response to 1- and 3-day Pi starvation.**

(a) Chromatograms of HPLC analysis of [³²P]Pi-labeled WT seedlings after 1- and 3-day Pi-replete (+Pi, 250 μM) or Pi-deficient (-Pi, 10 μM) treatments. 8-DAG seedlings were labeled with [³²P]Pi, under Pi-replete conditions for 3 days (“pulse”) before transfer to unlabeled media (“chase”). Insets show enlarged chromatograms of more polar InsPs, plotted from scintillation counts of 1-min fractions collected from retention time 50 min onwards. For clearer presentation, [³²P] radioactivity signals of Pi starvation treatment (S-P and R-P) were shifted by 100 CPM. InsP₃a, Ins(1,2,4,5,6)P₅ and/or Ins(2,3,4,5,6)P₅. (b) Total [³²P]Pi recovered in metabolites of tissues after pulse-chase labeling determined by integration of peaks from in-line flow detection. Error bars, SE of three independently labeled populations of seedlings. (c) InsP₆ level as percentage of total radioactivity across the gradient recovered in the integrated peak. (d) InsP₅-to-InsP₆ ratio determined from counting of fractions in inset (a). (e) InsP₇ level as per mille total radioactivity derived from InsP₅-to-InsP₆ ratio [derived in (d)] multiplied by the InsP₆ level in (c). (f) InsP₄*-to-InsP₆ ratio determined by scintillation counting of fractions as shown in Supporting Figure S9A. InsP₄* shares common retention time with the elevated peak detected in the ipk1-1 and itpk1 mutants (Figure 5). (g) InsP₄* level as per mille
total radioactivity derived from InsP₄*-to-InsP₆ ratio (Figure S9A-D) multiplied by the InsP₆ level in (c). Error bars in (b-g), S.E. of three independent labeling experiments. (h and i) Relative ATP and AMP level per mg fresh weight (FW) derived from normalization to the internal standard ribitol. Error bars, S.E. of n=9 and 7 independent experiments for the shoots and roots, respectively. Asterisks indicate significant differences from the WT (Student’s t-test; *, P < 0.05).

**Figure 7. Intersections between InsP metabolism and P, homeostasis.** Biochemical pathways for the synthesis of InsP₆ and PP-InsPs in vegetative tissues of Arabidopsis, consisting of a ‘lipid-dependent pathway’ based on biochemical activities of homologous enzymes (Raboy, 2003; Stevenson-Paulik et al., 2003; Stevenson-Paulik et al., 2005; Kim and Tai, 2011; Munnik and Nielsen, 2011; Desai et al., 2014; Laha et al., 2015) and ‘lipid-independent pathway’ identified in the duckweed *Spirodela polyrhiza* (Brearley and Hanke, 1993; Brearley et al., 1997). For the ‘alternative lipid-dependent pathway’, an alternative substrate, PtdIns4P, for PLC is proposed based on enzyme’s capacity to hydrolyse this lipid, the physiological levels of PtdIns4P while PtdIns(4,5)P₂ is hardly detectable, and the fact that plant PLCs lack a PH domain to bind PtdIns(4,5)P₂ (Munnik, 2014). The identity and origin of InsP₃ substrate for ITPK4 is unknown. IMP, inositol monophosphatase; MIK, myo-inositol kinase; INT2 & 4, INOSITOL TRANSPORTER 2 & 4 (Schneider, 2015); PIS, phosphatidylinositol synthase; PI4K, phosphatidylinositol 4-kinase.
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