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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 suggested that there is a close link between inositol phosphate (InsP) metabolism and cellular phosphate (Pi) homeostasis in eukaryotes; however, whether a common InsP species is deployed as an evolutionarily conserved metabolic messenger to mediate Pi signaling remains unknown. Here, using genetics and InsP profiling combined with Pi-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 hexakisphosphate; InsP6) synthesis, is indispensable for maintaining Pi homeostasis under Pi-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-InsP5; 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 from being 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 with 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 the InsP7 level. This study demonstrates a more nuanced picture of the intersection of InsP metabolism and Pi homeostasis and PSRs than has previously been elaborated, and additionally establishes intermediate steps to phytate biosynthesis in plant vegetative tissues.

Keywords: inositol phosphate, inositol pentakisphosphate 2-kinase (IPK1), inositol 1,3,4-trisphosphate 5/6-kinase 1 (ITPK1), phosphate homeostasis, D/L-inositol 3,4,5,6-tetrakisphosphate, phosphate starvation response, diphosphoinositol pentakisphosphate, phytate biosynthesis, Arabidopsis thaliana.

INTRODUCTION

Elemental phosphorus (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 from the rhizosphere in the form of Pi, where P 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; in seeds, Pi is reserved in phytate (inositol hexakisphosphate, InsP6) that accumulates to several percent of 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 the PSRs and store excess Pi in the vacuole to avoid cellular toxicity as a result of a surge of cytosolic Pi (Müller et al., 2004; Lin et al., 2013; Liu et al., 2015, 2016). 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; Chiu et al., 2006; Chiu 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,’ namely an agonist-responsive change in concentration that is recognized by a defined receptor (Shears et al., 2012), only a very few InsPs can be considered as 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 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., 2006; Bari 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 extensively assessed.

The first elaboration of the involvement of InsPs in Pi homeostasis in eukaryotes 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 (PUS) was identified to encode an InsP5 kinase (IP6K) that converts InsP5 to diphosphoinositol pentakisphosphates (PP-InsPs 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. Pli1p, Arg82p and Kcs1p) led to constitutive activation of a Pi-starvation-responsive phosphatase-coding gene, Pho5, under Pi-replete conditions (Auesukaree et al., 2005). Subsequent work showed that the synthesis of InsP5 by the other family of PP-InsP kinases (Vip1/PPIP5K), Vip1, is stimulated by Pi starvation (Lee et al., 2007) and InsP5 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 Pi-starvation-inducible genes (Lee et al., 2007, 2008). The synthesis of PP-InsPs is also metabolically linked to the synthesis of the main intracellular Pi 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 the availability of Pi and PP-InsP synthesis (Boer et al., 2010; Szigyarto et al., 2011; Choi et al., 2017), and it itself regulates the synthesis of PP-InsP (Voglimaier 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 Pi 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 Pi sensing and transport, with PP-InsPs showing the highest binding affinity (at submicromolar concentrations for yeast and animal protein) (Secco et al., 2012a,b; 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 Pi-related phenotypes of the Arabidopsis phosphate 1 (pho1) mutant (Wild et al., 2016). Despite the number of current investigations, the evidence for PP-InsPs as evolutionarily conserved messengers in eukaryotic Pi signaling is scattered, confounded by the absence of Pho80–Pho85–Pho81 homologs in other eukaryotic organisms and the contradictory responses of InsP levels to Pi 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, the current implication of InsP metabolism in regulation of Pi homeostasis comes from a study in which genetic disruption of the kinase responsible for InsP5 synthesis, inositol pentakisphosphate 2-kinase (IPK1), causes excessive accumulation of Pi (Stevenson-Paulik et al., 2005) as a result of elevated Pi uptake/allocation activities and activation of a subset of Pi-starvation-responsive genes (PSR genes) under Pi-replete conditions (Kuo et al., 2014). In addition to a decreased level of InsP5 ipk1 mutation causes a significant change in InsP composition, including accumulation of lower-phosphorylated InsP species (e.g. InsP3, InsP4 and InsP5) and decreased levels of PP-InsPs [InsP7 and InsP8 (bis-diphosphoinositol tetrakisphosphate)] (Stevenson-Paulik et al., 2005; Laha et al., 2015). The mechanism of modulation of Pi homeostasis by IPK1 and whether InsPs play a role in Pi-starvation signaling in plants is currently unknown.

Compared with the situation in other eukaryotic organisms, the investigation of biosynthesis of InsPs and their composition in the vegetative tissues of plants is more complicated than in other eukaryotes due to the presence of complex gene families of InsP biosynthetic enzymes. Mammalian InsP metabolism is dominated by receptor-coupled activation of phospholipase C (PLC) and subsequent metabolic conversion of Ins(1,4,5)P3 to multiple higher and lower InsPs (Irving and Schell, 2001), but few plant studies offer detailed identification of InsP species in vegetative tissues due to the limited levels of labeling achieved with myo-[3H]inositol. Nevertheless, specific
short-term non-equilibrium labeling with $[^{32}P]$Pi 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 when applied to vegetative tissues of plants has revealed a ‘lipid-independent’ pathway of InsP$_6$ synthesis (Brearley and Hanke, 1993; Brearley et al., 1997).

Here, using reverse genetics and InsP profiling by $[^{3}H]$inositol and $[^{32}P]$Ni labeling, 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 InsPs between ipk1-1, itpk1 and another mutant defective in InsP$_6$ synthesis, itpk4, reveals a correlation between elevated d/L-Ins(3,4,5,6)P$_4$ [Ins(1,4,5,6)P$_4$ and/or Ins (3,4,5,6)P$_4$] level and activation of P$_i$ uptake and PSR gene expression. However, the InsP profile in response to P$_i$ starvation is distinct from that of the ipk1-1 and ipk1 mutants and marks a shoot-specific increase in InsP$_7$ level accompanied by ATP increase. Our study reveals a complex relationship between InsP$_6$ metabolism and P$_i$ homeostasis in plants and identifies ITPK4 as a key enzyme in generating InsP$_4$ precursors for phytate biosynthesis.

RESULTS

Kinase activity of IPK1 is required for maintenance of P$_i$ homeostasis

We previously demonstrated overaccumulation of P$_i$ in ipk1-1 mutants associated with activation of PSR genes involved in P$_i$ 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., 2013a,b), we examined whether regulation of P$_i$ 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 in the online Supporting Information) 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 InsP$_6$ content in ipk1-1 seeds, whereas InsP$_6$ 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.

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-

![Figure 1. Characterization of kinase-inactive inositol pentakisphosphate 2-kinase (IPK1) transgenic plants.](image)

(a) Relative inositol hexakisphosphate (InsP$_6$) content (% of wild type, 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 background. Error bars, SE of n = 3–12 independent experiments.

(b) Relative expression (to WT) of P$_i$-starvation response genes in roots.

(c) P$_i$ content in shoots of seedlings 14-days after germination. Asterisks indicate a significant differences from WT (Student’s $t$-test; **$P$ < 0.005).

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 Pi homeostasis in ipk1-1 is not caused by defective InsP₆-mediated mRNA export

In yeast, InsP₆ is required for export of mRNA 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 retention of mRNA 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 a reduced level of InsP₆ (Lee et al., 2015). To address whether defective mRNA export in the ipk1-1 mutant is a cause of the misregulation of Pi homeostasis, we examined Pᵢ-related phenotypes of the reported mRNA export mutants (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 Pᵢ content (Figure S2a, b) and PSR gene expression (Figure S2c). Furthermore, expression of variants of Gle1 (IS1 and IS2), which exhibit increased InsP₆ sensitivity to LOS4 stimulation and improved growth defects of ipk1-1 (Lee et al., 2015), did not reduce Pᵢ content or suppress PSR gene activation of the ipk1-1 mutant (Figure S2c, d). These results suggest that misregulation of Pᵢ homeostasis in ipk1-1 is not caused by defective mRNA export due to a reduced InsP₆ level.

Genetic dissection of the roles for InsP and PP-InsP biosynthesis enzymes in regulation of Pᵢ homeostasis

The dependence of Pᵢ 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 enzymes previously characterized in Arabidopsis, including myo-inositol-3-phosphate synthases (MIPS1-3) (Torabinejad and Gillaspy, 2006), Ins (1,4,5)P₃ 6/3-kinases (inositol phosphate multikinases; IPK2x and IPK2j) (Stevenson-Paulik et al., 2002), Ins(1,3,4)P₃ 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 InsP₆ 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, Figure S3a, b).

Morphologically, none of the mutants displayed growth defects as severe as ipk1-1 (stunted growth and leaf necrosis), although mips1, ipk1 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 ipk1 and mrp5-2 mutants (Figures 2a and S3c, d) (Kuo et al., 2014). Analysis of Pᵢ content in the shoot tissues revealed that only ipk1 accumulated excessive Pᵢ, comparable to ipk1-1 (Figure 2b), and this phenotype persisted to the mature stage (Figure S3e). Mildly (but significantly) elevated Pᵢ content was observed in mrp5-2 seedlings but was no longer seen at the mature stage (Figures 2b and S3e). Consistent with the elevated Pᵢ content, ipk1 exhibited elevated uptake of Pᵢ, activity comparable with that of ipk1-1, whereas all other mutants showed WT activities (Figure 2c–f). The excessive Pᵢ accumulation in ipk1 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 Pᵢ homeostasis.

In addition to the 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 PSRs in plants. Two families of kinases, IPK and Vip/PPIP5K, are involved in the synthesis of PP-InsP in eukaryotes (Wundenberg et al., 2014); however, only Vip1/PPIP5K homologs have been identified in plants and shown to be responsible for InsP₆ 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 a Pᵢ-content phenotype, but vip1-2 vip2-1 double mutants exhibited a lower Pᵢ content comparable with 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 the 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 indicate that the decreased level of InsP₆ in ipk1 mutants is not responsible for misregulation of Pᵢ homeostasis.
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 Pi content (Figure 3b). Correspondingly, ITPK1 overexpression significantly decreased Pi 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 compared with the WT (Figure 3a; e.g. PHT1;2, SPX1, AT4, IPS1 and PAP17). However, Pi 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 regulating Pi homeostasis in a common pathway from 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 Pi status (Figure 4l). The expression of ITPK1 native protein fused to yellow fluorescent protein (YFP), which restored the Pi 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 Pi content was greater in ipk1-1 itpk1 double than single mutants, by 50%–70%, which can probably be attributed to the relative 50%–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 Pi homeostasis.

Figure 2. Characterization of mutants defective in inositol phosphate (InsP) biosynthesis enzymes grown under phosphate (Pi)-replete conditions. (a) Morphology of plants 22 days after germination (DAG) plants grown in Pi-replete (1 mM) hydroponic medium. Scale bar = 1 cm. (b) Pi content in the shoots of 14-DAG seedlings grown on Pi-replete (1 mM) solid medium. Error bar: SE of n = 4–21 independent experiments. (c)–(f) Pi uptake activities of 14-DAG seedlings under Pi-replete (250 μM) growth conditions. Error bars: SE 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 wild type (WT; Student’s t-test; **P < 0.005).
A common elevation of d/L-Ins(3,4,5,6)P₄ in itpk1 and ipk1-1 mutants

The observations that maintenance of Pᵢ homoeostasis depends on (i) the kinase activity of IPK1, (ii) an additional InsP kinase, ITPK1, and (iii) the expression level of ITPK1 and IPK1 (i.e. contrary Pᵢ-related phenotypes between mutants and overexpression lines), suggest that a stoichiometric alteration of InsP metabolites contributes to the regulation of Pᵢ homeostasis. To pinpoint the possible InsP molecules involved in such regulation, we compared the

Figure 3. Phenotypic similarities between itpk1, ipk1-1 mutants and overexpression lines.
(a) Relative expression (to the wild type, WT) of phosphate (Pᵢ)-starvation response (PSR) genes in roots of itpk1, ipk1-1; IPK1-overexpression (OxIPK1) and ITPK1-overexpression (OxITPK1) lines at 14 days after germination (DAG) grown under Pᵢ-replete (1 mM) conditions (see Table S3 for quantitative (q)PCR raw data and the SE of three independent experiments). Note that qPCR primers for ITPK1 are located 5' to the T-DNA insertion site.
(b) Pᵢ content in shoots of 14-DAG T₂ transgenic lines overexpressing ITPK1 or IPK1 compared with WT, itpk1 and ipk1-1 mutants grown under Pᵢ-replete (250 μM) conditions. Error bars, SE of n = 6–12 independent experiments.
(c), (d) Pᵢ uptake activities of 14-DAG seedlings grown under Pᵢ-replete (250 μM Pᵢ) conditions. Error bars, SE of n = 6–12 independent experiments. Asterisks denote significant differences from the WT (Student's t-test; **P < 0.005).

A common elevation of d/L-Ins(3,4,5,6)P₄ in itpk1 and ipk1-1 mutants

The observations that maintenance of Pᵢ homoeostasis depends on (i) the kinase activity of IPK1, (ii) an additional InsP kinase, ITPK1, and (iii) the expression level of ITPK1 and IPK1 (i.e. contrary Pᵢ-related phenotypes between mutants and overexpression lines), suggest that a stoichiometric alteration of InsP metabolites contributes to the regulation of Pᵢ homeostasis. To pinpoint the possible InsP molecules involved in such regulation, we compared the

Figure 4. Tissue-specific expression and protein subcellular localization of inositol 1,3,4-trisphosphate 5/6-kinase 1 (ITPK1) and inositol pentakisphosphate 2-kinase (IPK1), and phenotypes of itpk1, ipk1-1 and itpk1-1 double mutants.
(a)-(j) Promoter activities of IPK1 and ITPK1 at different developmental stages. (a) 3 days after germination (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) Silicules; scale bar = 0.5 mm. (k), (l) RT-PCR analysis of tissue-specific expression of ITPK1 and IPK1 at different developmental stages (k) and in response to phosphate (Pᵢ) status (l). S, shoot; R, root; LF, rosette leaves; FS, florescence stem; FL, flower; SL, silique; +P, 250 μM Pᵢ; −P, 10 μM Pᵢ; PCR amplification cycles for ITPK1, 32; IPK1, 32; ACTIN2, 22. (m) Subcellular localization of C-terminus YFP-tagged IPK1 and ITPK1 protein in roots of 10-DAG itpk1-1 and itpk1-1 double mutants, respectively; scale bar = 10 μm. Arrows, cytoplasm, arrowheads, nucleus. (n) Morphology of 25-DAG itpk1-1 mutants grown under Pᵢ-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 = 1 cm, 1 mm and 100 μm for the whole plant, insets (a–d) and inset (e), respectively. (o) Tissue-specific Pᵢ content and (p) relative expression of Pᵢ-starvation response genes of 16-DAG seedlings grown on Pᵢ-replete (250 μM) solid medium. Error bar, SE of n = 3–6 independent experiments. Asterisks denote significant differences from the wild type (WT; Student’s t-test; *P < 0.05; **P < 0.005).
InsP profiles of vegetative tissues of the relevant genotypes by in vivo labeling with $[^{32}\text{P}]\text{Pi}$ and/or myo-$[^{3}\text{H}]$inositol and HPLC analysis. As shown in Figure 5(a) and (b), and the myo-$[^{3}\text{H}]$inositol-labeled chromatogram in Figure S6(a), the itpk1 mutant shared a significant reduction in InsP$_6$ (62 ± 2% of the WT level) with the ipk1-1 mutant (17 ± 1% of the WT level). To validate that the reduced InsP$_6$ level is not a cause of misregulated P$_i$ homeostasis, with normal P$_i$-related phenotypes being 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

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that two *ippk4* mutants (*ipkk4-1 and ipkk4-2; Table S1) showed a strong reduction in InsP$_6$ level comparable to *ipkk1* and *ipkk1-1* mutants of 50% and 80%, respectively (Figures 5a, b and S6a). Consistent with the previous report, *ipkk4* mutations also significantly reduced the InsP$_6$ level in seeds, to a similar extent as *ipkk1-1* (Figure S7a) (Stevenson-Paulik *et al.*, 2005; Kim and Tai, 2011). The *ipkk4* mutants did not show striking morphological phenotypes (Figures 2a and S3c) or P$_i$-related phenotypes, such as altered P$_i$ content (Figures 2b, S3e and S7b), P$_i$ uptake (Figure 2e) or altered PSR gene expression (Figure S7c). Promoter–GUS analysis and RT-PCR indicated that *ITPK4* was expressed in the same vegetative tissues as *ITPK1* and *IPK1* (Figure S7d–f), which suggests that *ITPK4* is probably 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 *ipkk4-1* mutant (Figure S7a), like ITPK1 and IPK1, was also localized to the nucleus and cytoplasm (Figure S7m). Hence, a reduced InsP$_6$ level alone is insufficient to alter P$_i$ homeostasis and ITPK4 is a key enzyme for InsP$_6$ biosynthesis in both vegetative tissues and seeds.

In accordance with the decreased level of InsP$_6$, the level of InsP$_5$ was also decreased in the *ipkk1-1* mutant (Figure 5a) (Laha *et al.*, 2015). Similarly, the InsP$_5$ level was decreased in *ipkk1* and *ipkk4* mutants (Figure 5a), and therefore we could not draw a correlation between the reduced InsP$_6$ level and the P$_i$-related phenotypes observed in *ipkk1-1* and *ipkk1*. The *ipkk1-1* mutant shows significant accumulation of Ins(1,3,4,5,6)P$_5$ along with a reduced InsP$_6$ level (InsP$_{50}$ in Figures 5a and S6a) (Stevenson-Paulik *et al.*, 2005); in contrast, there was no detectable accumulation in the corresponding InsP$_5$ in the *ipkk1* mutant. This finding suggests that the elevated Ins(1,3,4,5,6)P$_5$ level in the *ipkk1-1* mutant does not explain the misregulation of P$_i$ homeostasis.

Notably, the *ipkk1* mutant showed an elevated level of an InsP$_5$ species with identical chromatographic mobility to that in the *ipkk1-1* mutant, which is predominantly Ins(3,4,5,6)P$_4$ (InsP$_{5*}$ in Figures 5a and S6a) (Stevenson-Paulik *et al.*, 2005). The InsP$_5$ species in the *ipkk1* mutant was further analysed by high-resolution HPLC separation and was co-eluted with a d/L-Ins(3,4,5,6)P$_4$ standard (d/L-enantiomers are not separable by existing chromatographic technologies) (Figure 5c). In addition to the increase in level of InsP$_6$, levels of earlier eluting InsP species were increased in both *ipkk1* and *ipkk1-1* mutants, which exhibited the chromatographic mobility of InsP$_3$ (Figures 5a and S6a). Because there are 20 possible InsP$_5$ isomers, InsP$_5$ 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 5(d), *ipkk1-1* and *ipkk1* mutations caused accumulation of distinct InsP$_3$ isomers that were not detectable in the WT. Inclusion of an internal standard of myo-[H]$^{3}$H]Ins(1,4,5)P$_3$ revealed that these isomers are not Ins(1,4,5)P$_3$, which was shown to represent only a trivial fraction of InsP$_3$ in plant tissues (Brearley and Hanke, 2000). We conclude that the only common change of InsP species associated with the P$_i$-related phenotypes of *ipkk1-1* and *ipkk1* is the elevated D/L-Ins(3,4,5,6)P$_4$ level.

**P$_i$ starvation induced a shoot-specific increase of InsP$_5$**

To address whether *ipkk1* and *ipkk1-1* mutants exhibit an InsP profile that shares a common feature with PSR mutants, we investigated the change in InsP profiles in shoots and roots of WT plants in response to different P$_i$-starvation regimes. InsP profiles were analyzed in seedlings subjected to 1- and 3-day P$_i$ starvation, when cellular P$_i$ concentrations were significantly reduced and PSR genes induced (Figure S8a, b). To avoid unequal [$^{32}$P] labeling between Pi-replete (supplemented with 250 μM P$_i$) and P$_i$-deficient seedlings (11 DAG) was spiked with a hydrolysate of InsP$_6$ and separated on a CarboPac PA200 column with post-column colorimetric detection of InsP peaks as described (Phillipps and Bland, 1988). Upper panel, [$^{32}$P]-radioactivity obtained from this extract. Ins(1,4,5,6)P$_4$/Ins(3,4,5,6)P$_4$ is the latest eluting InsP$_4$ species associated with the Pi-related phenotypes of *ipkk1* and *ipkk1* is the elevated D/L-Ins(3,4,5,6)P$_4$ level.

Figure 5. Inositol phosphate (InsP) profiles of various genotypes.

(a) HPLC analysis of root extracts from seedlings of various genotypes at 11 days after germination (DAG) labeled with [$^{32}$P]P$_i$. InsP$_{50}$, Ins(1,2,4,5,6)P$_5$ and/or Ins(2,3,4,5,6)P$_5$ and/or Ins(1,2,3,4,6)P$_5$ (these three isomers are not resolved on the Partispher SAX HPLC (Brearley and Hanke, 1996); InsP$_{50}$, InsP$_{5*}$, InsP$_{1*}$, Ins(1,4,5,6)P$_5$ and/or Ins(3,4,5,6)P$_5$; InsP$_{40}$, peaks with the chromatographic mobility of InsP$_{40}$. Insets show expanded chromatograms of more polar InsPs, obtained by counting 1-min fractions collected from the Flo-Detector eluted from a retention time of 50 min onwards. The ordinate is scaled by the same factor in (a).

(b) Quantification of relative InsP$_5$ content (% of total radioactivity per HPLC run recovered in the integrated InsP$_5$ peak) in 11-DAG [$^{32}$P]P$_i$-labeled seedlings. Error bar, SE 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$_{5*}$ in the *ipkk1* mutant. An aliquot of an extract of [$^{32}$P]P$_i$-labeled ipkk1 seedlings (11 DAG) was spiked with a hydrolysate of InsP$_5$ and separated on a CarboPac PA200 column with post-column colorimetric detection of InsP peaks as described (Phillipps and Bland, 1988). Upper panel, [$^{32}$P]-radioactivity counted inline on the Flo Detector. Note that InsP$_{50}$ and InsP$_{5*}$ 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$_5$/Ins(3,4,5,6)P$_5$ is the latest eluting InsP$_5$ on this column and elutes before Ins(1,2,3,4,6)P$_5$. Lower panel, UV trace overlaid with [P] 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 [P] peaks (compared with 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 InsP$_5$ isomers elute before 20 min: InsP$_5$(5-OH), Ins(1,2,3,4,6)P$_5$; InsP$_5$(4/6-OH), Ins(1,2,3,4,5)P$_5$ and/or Ins(1,2,3,4,5)P$_5$; InsP$_5$(1,3-OH), Ins(1,2,4,5,6)P$_5$ and/or Ins(2,3,4,5,6)P$_5$; InsP$_5$(2-OH), Ins(1,3,4,5,6)P$_5$. Note that a single peak of InsP$_{5*}$ was detected in *ipkk1* in (a).

(d) Isocratic separation and counting of collected fractions for analysis of InsP$_5$ isomers. For better presentation, the chromatogram of each genotype was shifted by 200 c.p.m.
(supplemented with 10 μM P₃) conditions and subsequent biased-quantifications of InsPs, we performed a pulse-chase experiment with seedlings labeled with [³²P]Pi before P, starvation. Tissues were similarly radiolabeled in every pairwise ‘+P’ vs. ‘−P’ treatment, although more [³²P]Pi was allocated to shoots than roots (Figure 6a, b).
Figure 6. Tissue-specific inositol phosphate (InsP) profiles in response to 1- and 3-day phosphate (P) starvation.

(a) Chromatograms of HPLC analysis of [32P]Pi-labeled wild-type (WT) seedlings after 1- and 3-day P-replete (+P, 250 μM) or P-deficient (−P, 10 μM) treatments. At 8 days after germination (DAG) seedlings were labeled with [32P]Pi under P-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 a retention time of 50 min upwards. For clearer presentation, [32P] radioactivity signals of Pi-starvation treatment (S-P and R-P) were shifted by 100 c.p.m. InsP5a, Ins(1,2,4,5,6)P5 and/or Ins(2,3,4,5,6)P5. (b) Total [32P], 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 a percentage of total radioactivity across the gradient recovered in the integrated peak. (d) InsP7:InsP6 ratio determined from counting of fractions in the inset (a). (e) InsP7 level as per mille total radioactivity derived from the InsP7:InsP6 ratio (derived in d) multiplied by the InsP6 level in (c). (f) InsP4*:InsP6 ratio determined by scintillation counting of fractions as shown in Figure S9A. InsP4* shares a common retention time with the elevated peak detected in the ipk1-1 and ipk1 mutants (Figure 5). (g) InsP4* level as per mille total radioactivity derived from the InsP4*:InsP6 ratio (Figure S9A–D) multiplied by the InsP6 level in (c). Error bars in (b–g), SE of three independent labeling experiments. (h), (i) Relative ATP and AMP level per mg fresh weight (FW) derived from normalization to the internal standard ribitol. Error bars, SE of n = 9 and n = 7 independent experiments for the shoots and roots, respectively. Asterisks indicate significant differences from the WT (Student’s t-test; *P < 0.05).
Overall, the chromatograms did not exhibit prominent changes in profile in response to P_i starvation in either shoots or roots (Figures 6a and S9). Quantitative analysis indicated no significant change in InsP_8 level in response to P_i starvation in shoots or roots (Figure 6c). Despite there being no significant change in the InsP_7 level in roots, shoots exhibited mild yet significant increase in the InsP_7: InsP_6 ratio and InsP_7 level in response to 1- and 3-day P_i starvation (Figure 6d, e). We were unable to assess the InsP_8 level due to the detection limit in our analysis; however, the fact that depletion of InsP_8 caused by vihu2 mutation did not affect the PSRs implied that this InsP species does not mediate P_i signaling (Figure S5c). Notably, the increase in the ω/L-Ins(3,4,5,6)P_4 level in ipk1 and ipk1-1 mutants was not observed in P_i-starved WT plants (Figures 6f, g and S9a-d), nor was the level of any InsP_2 isomer, including Ins(1,4,5)P_3, changed in response to P_i starvation (Figure S9e, f).

Because cellular adenylate energy is influenced by the availability of P_i (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 used LC/MS analysis to examine whether phosphorylated adenine nucleotides are metabolically coordinated with the change in InsP_7 level in response to P_i starvation. ATP increased along with the InsP_7 level specifically in shoots during 1- and 3-day P_i starvation, whereas the AMP level remained steady (Figure 6h, i), which resulted in a significant increase in the ATP/AMP ratio (0.68 ± 0.1 and 1.1 ± 0.1 for 3-day ‘+P’ and ‘−P’ 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 ipk1-1 and ipk1-1 mutants, which suggests that the mechanism of the contribution of ITPK1 and IPK1 to P_i homeostasis is distinct from the PSRs in WT plants.

**DISCUSSION**

In this study we have demonstrated metabolism of distinct InsP species in correlation with 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: (i) decreases in InsP_6 and InsP_7 levels and (ii) an increase in the ω/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.

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**Figure 7.** Intersections between inositol phosphate (InsP) metabolism and phosphate (P_i) homeostasis.

Biochemical pathways for the synthesis of InsP_6 and diphosphoinositols (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., 2002; Stevenson-Paulik et al., 2005; Kim and Tai, 2011; Munnik and Nielsen, 2011; Desai et al., 2014; Laha et al., 2015) and a ‘lipid-independent pathway’ identified in the duckweed Spirodea polyrhiza (Brearley and Hanke, 1993; Brearley et al., 1997). For the ‘alternative lipid-dependent pathway’, an alternative substrate, PtdIns4P, is proposed for phospholipase C (PLC) based on the enzyme’s capacity to hydrolyse this lipid, the physiological levels of PtdIns4P, while PtdIns(4,5)P_2 is hardly detectable, and the fact that plant PLCs lack a PH domain to bind PtdIns(4,5)P_2 (Munnik, 2014). The identity and origin of the InsP_2 substrate for ITPK4 is unknown. IMP; inositol monophosphatase; MIK, myo-inositol kinase; INT2 and 4, INOSITOL TRANSPORTER 2 and 4 (Schneider, 2015); PIS, phosphatidylinositol synthase; IPK4, phosphatidylinositol 4-kinase.
A decrease in the InsP$_6$, InsP$_7$, or InsP$_8$ level is not responsible for disturbed P$_i$ homeostasis in ipk1-1 and ipk1 mutants

The fact that itpk4 mutants did not exhibit P$_r$-related phenotypes comparable to ipk1-1 and itpk1 mutants indicates that a decrease in the InsP$_6$ or InsP$_7$ level did not cause the disturbed P$_i$ homeostasis under P$_r$-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 InsP$_6$ and InsP$_7$ (Figure S7). While it is possible that radiolabeling does not entirely reflect the 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 InsP$_6$ level, vih2 mutants mediating InsP$_6$ synthesis in planta (Laha et al., 2015) do not phenocopy ipk1-1 and itpk1-1 under P$_r$-replete conditions and exhibit normal PSRs (Figures 2, S3 and S5), which suggests that InsP$_6$ is unlikely to be involved in the regulation of P$_i$ homeostasis.

We have also ruled out that misregulated P$_i$ homeostasis is a secondary consequence of mitigated InsP$_6$-mediated mRNA export by demonstrating that mutations compromising or enhancing InsP$_6$-Gle1-Los4 mRNA machinery neither caused comparable P$_r$-related phenotypes of ipk1-1 nor complemented ipk1-1 (Figure S2). The identification of two itpk4 alleles with a similar reduction in InsP$_6$ (and InsP$_7$) level in ipk1-1 and itpk1, respectively, and not showing P$_r$-related phenotypes, also argues against a role for InsP$_6$-mediated mRNA export in regulating P$_i$ homeostasis (Figures 2, 5 and S7). It is notable that although growth retardation of ipk1-1 is not due to defective InsP$_6$-mediated RNA export (Lee et al., 2015), itpk4 mutants did not exhibit growth defects comparable to ipk1-1 or itpk1 (Figure 2a). Thus, reduction of InsP$_6$ may not be the sole cause of 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

Apart from the reduced levels of InsP$_6$ and InsP$_7$, the most significant common change in InsP profile between itpk1 and ipk1-1 is the increased accumulation of the InsP$_6$ 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$_6$ species in the ipk1-1 mutant remains to be determined, but human ITPK1 was found to be 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 and the regulation of 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-permeable 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$_6$, InsP$_7$ showed changes in the 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 and salt and drought stresses (Perera et al., 2001; Xiong et al., 2001; Perera et al., 2006, 2008). We demonstrated that neither the ipk1-1 nor the itpk1 mutation affected the levels of Ins(1,4,5)P$_3$, as measured by radiolabeling 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 phosphatidylinositols 4,5-bisphosphate [PtdIns(4,5)P$_2$] in both ipk1-1 and itpk1 (Figure S10a, b). We further examined P$_r$-related phenotypes in mutants or transgenic lines with elevated levels of PtdInsP$_2$, i.e. phosphatidylinositol-phospholipase C2 (plc2), suppressor of actin 9 (sac9) and a PHOSPHATIDYLINOSITOL PHOSPHATE 5-KINASE 3 (PIP5K3)-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)P$_2$ levels in ipk1-1 and itpk1 mutants are probably not attributable to the misregulated P$_i$ homeostasis.

P$_i$ 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 PSRs under P$_r$-replete conditions, their InsP profiles were distinct from those under P$_i$ starvation, notably the contrasting levels of D/L-Ins(3,4,5,6)P$_4$, InsP$_6$ and InsP$_7$ (Figures 5a, 6f and S9a–d). The level of D/L-Ins(3,4,5,6)P$_4$ not being altered by P$_i$ starvation suggests that these InsP species are not involved in P$_i$ starvation signaling in WT plants. The disparate InsP profiles in response to...
P$_i$ starvation versus those caused by *ipk1-1* and *itpk1* mutations imply two distinct P$_i$ signaling pathways. In support of this notion, the PSRs persisted in the *ipk1-1* and *itpk1* mutants, in which PSR genes remained inducible under P$_i$ starvation (Figure S8c). We observed no distinct alteration of the InsP profile in response to P$_i$ starvation except for a significant increase in InsP$_7$ level of unknown isomeric identity in the shoot of P$_i$-starved plants but not in the root (Figure 6d, e), where PSRs also take place. Shoot tissues are more responsive to P$_i$ 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 P$_i$ 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 synthesis of InsP$_7$ in plants to address these speculations.

Adenylate energy has been shown to regulate synthesis of PP-InsPs, with an 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 the ATP/AMP ratio during 1- and 3-day P$_i$ starvation (Figure 6h, i). Increases in ATP level in response to P$_i$ starvation have 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 decreases in ATP 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 the kinetics of cellular metabolism (Pradet and Raymond, 1993), remained unchanged or was increased in those studies. Whether the elevated ATP/AMP ratio drives the accumulation of InsP$_7$ 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 PSRs, accumulating evidence has pointed to a close relationship between adenylate energy status and P$_i$ signaling. The PP-InsPs are proposed to be ‘metabolic messengers’ that mediate pyrophosphorylation of proteins involved in multiple cellular metabolic pathways, 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 the ITPK family of enzymes in phytate biosynthesis in plant vegetative tissues**

Mutation of *IPK1* leads to a substantially reduced InsP$_6$ 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)P$_5$ 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)P$_5$ 2-kinase activity of IPK1 to InsP$_6$ synthesis. The coincident accumulation of Ins(3,4,5,6)P$_4$ 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)P$_4$ 1-kinase activity (Brearley and Hanke, 2000). The enzyme(s) responsible for producing Ins(3,4,5,6)P$_4$ in plants are not well defined. In avian erythrocytes, Ins(3,4,5,6)P$_4$ is the product of 5-phosphorylation of Ins(3,4,6)P$_3$ and is itself the precursor of Ins(1,3,4,5,6)P$_5$ (Stephens and Downes, 1990).

In nucleated mammalian cells, the origins of Ins(3,4,5,6)P$_4$ have not been tested by the methods of Stephens and Downes (1990), but the single mammalian ITPK1 is a multifunctional kinase and phosphotransferase that interconverts Ins(3,4,5,6)P$_4$ and Ins(1,3,4,5,6)P$_5$ (Chamberlain et al., 2007). The existence in Arabidopsis of a gene family of four inositol tris/tetrakisphosphate kinases (ITPK1–4) complicates the study of InsP metabolism. Our identification of significant contributions of ITPK1 and ITPK4 to InsP$_6$ synthesis in vegetative tissues focuses attention on the contribution of these enzymes not just to InsP$_6$ synthesis but also to physiological processes regulated by the intermediate InsPs. *ITPK1* mutation reduces labeling of InsP$_6$ by 50%, with concomitant accumulation of α/β-Ins(3,4,5,6)P$_4$, but because it does so without affecting the level of Ins(1,3,4,5,6)P$_5$ (Figures 5a and S6a) suggests that *ITPK1* probably does not act as an Ins(1,3,4,5,6)P$_5$ 1-phosphatase. ITPK1 may be acting at the level of InsP$_6$–InsP$_4$ interconversion. Remarkably, our studies show that ITPK4, which contributes to nearly 90% of vegetative InsP$_6$ more in seeds, has no effect on the PSRs. Our labeling studies showed no increase in InsP$_4$ accumulation in vegetative tissues (Figures 5a and S6a). This implies that most of the InsP$_4$ precursors for InsP$_6$ synthesis are generated by this enzyme and the contribution of ITPK4 may lie in its InsP$_3$ kinase activity rather than its InsP$_4$ isomerase/mutase activity (Sweetman et al., 2007).

**Implications of the role of InsP metabolism in regulating P$_i$ homeostasis**

Across eukaryotic kingdoms, the SPX domains of a large family of proteins involved in P$_i$ sensing and transport have been shown to bind InsPs, thereby regulating the activities of SPX proteins and their interaction with other
proteins (Wild et al., 2016). Although InsP₆ and PP-InsPs at submicromolar concentrations exhibited the highest binding affinity to SPX domains, the lower InsP levels also exhibited physiologically relevant binding affinity in the micromolar range (Wild et al., 2016). Our study has pointed to a significant association between the level of D/L-InsP₄ and maintenance of Pi homeostasis under Pi-replete conditions but not the PSRs. It remains speculative how increases in InsP₆ level are associated with elevated Pi uptake and PSR gene expression, and the future identification of the enantiomerism of D/L-InsP₄ in the itpk1 mutant and its interacting protein targets, such as by the use of 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 that the regulatory mechanisms that control Pi homeostasis probably 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-InsP₃ to OsSPX4/ OsPHR2 yielded Kₘs of about 50 μM and 7 μM, respectively (Wild et al., 2016), suggesting that competition between the more abundant InsP₆ and less abundant PP-InsPs is a relevant consideration 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. Taking into account the diverse functions of SPX proteins at different levels of regulation of Pi homeostasis (Secco et al., 2012a, Azevedo and Saiardi, 2017) and our findings presented here, InsP₆ may not be a general (or conserved) signal, and the role of other InsP intermediates in regulating Pi homeostasis needs to be considered.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

*Arabidopsis thaliana* mutant lines and their sources are listed in Table S1; the WT line is 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 (half-strength modified Hoagland solution supplemented with 250 μM KH₂PO₄ containing ³²P-orthophosphate (P₀) for 3 h (‘pulse’ treatment), then transferred to Pi-replete nutrient solution without ³²P for the indicated times (‘chase’ treatment). The [³²P] radioactivity in the plants tissues was measured as the Pi uptake assay and the root-to-shoot Pi translocation activity was measured by the shoot-to-root ratio of ³²P count.

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

Total Pi content and Pi uptake activity were measured as described previously (Chiu et al., 2006). To measure the root-to-shoot Pi translocation activity, pulse-chase labeling was performed. Seedlings at 14 days after germination were first incubated in Pi-replete nutrient solution (half-strength modified Hoagland solution supplemented with 250 μM KH₂PO₄ containing ³²P-orthophosphate (P₀) for 3 h (‘pulse’ treatment), then transferred to Pi-replete nutrient solution without ³²P for the indicated times (‘chase’ treatment). The [³²P] radioactivity in the plants tissues was measured as the Pi uptake assay and the root-to-shoot Pi translocation activity was measured by the shoot-to-root ratio of ³²P 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 Table S2. For constructing kinase-inactive IPK1, nucleotide substitutions were introduced in the primers (5’ phosphorylated; Table S2) used for PCR amplification by using a vector (pMDC32) containing the IPK1 coding sequence driven by the 3SS promoter as a template. The PCR product was ligated before transformation and sequences were confirmed before recombination into the Gateway destination vector pK7WG2.0 (C-YFP) (Karimi et al., 2007) via LR Clonase enzyme mix (Invitrogen, http://www.invitrogen.com/). For complementation analysis, the genomic sequence of ITPK1, including 1 kb upstream of the ATG start codon, was amplified by PCR (primers listed in Table S2) and cloned into pcr8/GW/TOPO (Invitrogen) followed by recombination into the Gateway destination vectors. pMDC99, pMDC32 (Curtis and Grossniklaus, 2003) and pK7WG2.0 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, https://www.mrcgene.com/) and cDNA was synthesized from 0.5 to 1 μg 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 given in Table S2. Quantitative qRT-PCR involved use of the Power SYBR Green PCR Master Mix kit (Applied Biosystems, https://www.thermofisher.com/twzt/home/brands/applied-biosystems.html) on a 7500 Real-Time PCR system as instructed. Gene expression was normalized by subtracting the Ct value of *UBQ10* (ΔCt) from that of the gene studied and presented as 2⁻ΔΔCt. The expression relative to the WT (i.e. fold change relative to the WT) is presented as 2⁻ΔΔΔCt (where ΔΔΔCt = ΔCt – ΔCtWT). The raw qPCR data are provided in Table S3.

**GUS staining and fluorescence microscopy**

The GUS activity of transgenic T₂ plants was detected as described (Lin et al., 2005), and the signal was observed under an Olympus SZX12 (https://www.olympus-lifescience.com/) or a Zeiss AxioSkop (https://www.zeiss.com/) microscope. Confocal
microscopy images of the YFP signal were obtained using a Zeiss LSM 510 META NLO DuoScan with LCI Plan-Neofluar ×63/1.3 Immersion and Plan-Apochromat ×100/1.4 oil objectives. Excitation/emission wavelengths were 514 nm/520-550 nm for YFP.

**InsP profiling of Arabidopsis seedlings and seeds**

For InsP profile analysis of Arabidopsis vegetative tissue, seedlings (8-11 days after germination) were labeled with myo-[2-3H]inositol (19.6 Ci mmol⁻¹, Perkin Elmer NET114A00, http://www.perkinelmer.com/; 0.4 mCi ml⁻¹ for 5 days) or [32P]Pi (8500-9120 Ci mmol⁻¹, Perkin Elmer NEX05300; 0.02 mCi ml⁻¹ for 1-3 days) in half-strength Hoagland’s medium supplemented with P, 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 mm × 4.6 mm Whatman Partisax SAX WXS column (https://www.sigmaaldrich.com/analytical-chromatography/hplc.html) 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 and (B) 1.25 mM (NH₄)₂HPO₄ adjusted to pH 3.8 with H₃PO₄, mixed according to the following gradient: time (min), %B; 0; 0; 5; 0; 65; 100; 75, 100. Isotopic separations of InsP species were performed at the same flow rate on the same column eluted with 20% buffer B. For myo-[3H]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 [32P]Pi labeling, radioactivity was measured by Cherenkov counting on a Canberra Packard Radiomatic A515 Flow Detector (http://www.cpcnet.net/) 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 allocations between tissues in planta, with greater [3H] labeling of roots (Figure S6b), whereas [32P]Pi labeled shoots more strongly (Figure S6b). With the exception of experiments to compare the extent of labeling of InsPs between a wide range of genotypes (Figure 5b), performed with whole seedlings, the shoot and root tissues were analyzed independently. Apart 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 of seeds was homogenized in 500 μl of ice-cold 0.6 N HCl before centrifugation at 16 000 g for 15 min to remove cell debris. Aliquots (20 μl) were injected onto a 3 mm i.d. × 200 mm Carbo Pac PA200 HPLC column ( Dionex, https://www.thorofisher.com.tw/z/home/industrial/ chromatography/dionex.html) fitted with a 3 mm × 50 mm guard column of the same material. The column was eluted at a flow rate of 0.4 ml min⁻¹ with a gradient of methane sulfonic acid (Acros Organics, https://www.acros.com/) delivered from buffer reservoirs containing: (A) water and (B) 600 mM methane sulfonic acid according to the following schedule: time (min), %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⁻¹, before passage through a knitted reaction coil (194-μl volume, 4 mm × 0.25 mm i.d.) obtained from Bio-tech AB (https://www.biotech.se/). The column, mixing tee and reaction coil were held at 35°C. Peaks of InsPs were detected at 290 nm with a Jasco UV-2077 Plus UV detector (https://jasoinc.co.jp/). 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) trichloroacetic acid containing 200 μg ml⁻¹ ribitol (250 μl per 100 mg tissue). Homogenates were centrifuged at 16 000 g at 4°C for 15 min, and supernatants were recovered and neutralized to pH 6.5-7 by KOH, followed by a 30-min incubation on ice. Extracts were centrifuged at 16 000 g 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, http://www.waters.com/). The sample was separated with a ZIC-HILIC column (3-μm particle size, 2.1 × 100 mm, Merck-Millipore, http://www.merckmillipore.com/). The UPLC system was coupled online to the Waters Xevo TQ-S triple quadrupole mass spectrometer. Ribitol was used as the 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 v.4.1 and TargetLynx software (Waters Corp.), with intensities of ATP and AMP normalized to ribitol.

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Additional Supporting Information may be found in the online version of this article.

Table S1. Mutant lines used in this study

Table S2. Primers used in this study

Table S3. RT-qPCR data for gene expression

Figure S1. Characterization of kinase-inactive IPK1 transgenic plants

Figure S2. The role of Gle1-InsP6-Los4 mRNA export machinery in ipk1-mediated P1-related phenotypes

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

Figure S4. Complementation of ipk1 phenotypes

Figure S5. P1 allocation activities and PSR gene expression in the vip/vih mutants.

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

Figure S7. Characterization of ipk4 mutants and expression pattern of ITPK4.

Figure S8. P-starvation responses of the wild type and various genotypes under different regimes of P1 starvation

Figure S9. Tissue-specific InsP profiles in response to 1- and 3-day P1 starvation.

Figure S10. Polyphosphoinositide composition in itpk1, ipk1-1 and OxtITPK1 lines, and P1 content in mutants exhibiting elevated PtdIns(4,5)P2 levels

REFERENCES


