Biochemical Characterization of Arabidopsis Enzymes Involved in Inositol Pyrophosphate Biosynthesis

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ABSTRACT

To compensate for the sessile nature of plants, thousands of years of evolution have led to the development of many sophisticated signaling pathways that help plants sense and respond appropriately to different environmental cues. One such signaling pathway is called inositol phosphate signaling. This research dissertation focuses on the inositol phosphate signaling pathway in plants, with emphasis on elucidating how a new class of signaling molecules collectively referred to inositol pyrophosphates are synthesized. Inositol pyrophosphates are an emerging class of "high-energy" intracellular signaling molecules containing one or two diphosphate groups attached to an inositol ring, with suggested roles in bioenergetic homeostasis and inorganic phosphate sensing. Information regarding the biosynthesis of this unique class of signaling molecules in plants is scarce, however the enzymes responsible for their biosynthesis in other eukaryotes have been well described. This work aims to characterize the biochemical activity of the kinase domain (KD) of the Arabidopsis plant diphosphoinositol pentakisphosphate kinase enzymes (AtVIP1 and AtVIP2), and elucidate the biosynthesis pathway of inositol pyrophosphates in plants. Our data indicate that AtVIP1-KD and AtVIP2-KD function primarily as diphosphoinositol pentakisphosphate 5 kinases that phosphorylate this substrate at the 1-position. We also discovered a previously unreported inositol hexakisphosphate kinase activity for the Arabidopsis inositol(1,3,4) triphosphate 5/6kinase enzymes, that can convert $InsP_6$ to

$InsP_7$. Together, these enzymes can function in plants to produce inositol pyrophosphates, which have been implicated in signal transduction and phosphate sensing pathways. The significance and potential application of these findings in terms of reduced phytate content and phosphate pollution, improved plant fitness, and improved nutrient use efficiency are discussed. The future outlook of inositol phosphate signaling research is also discussed.

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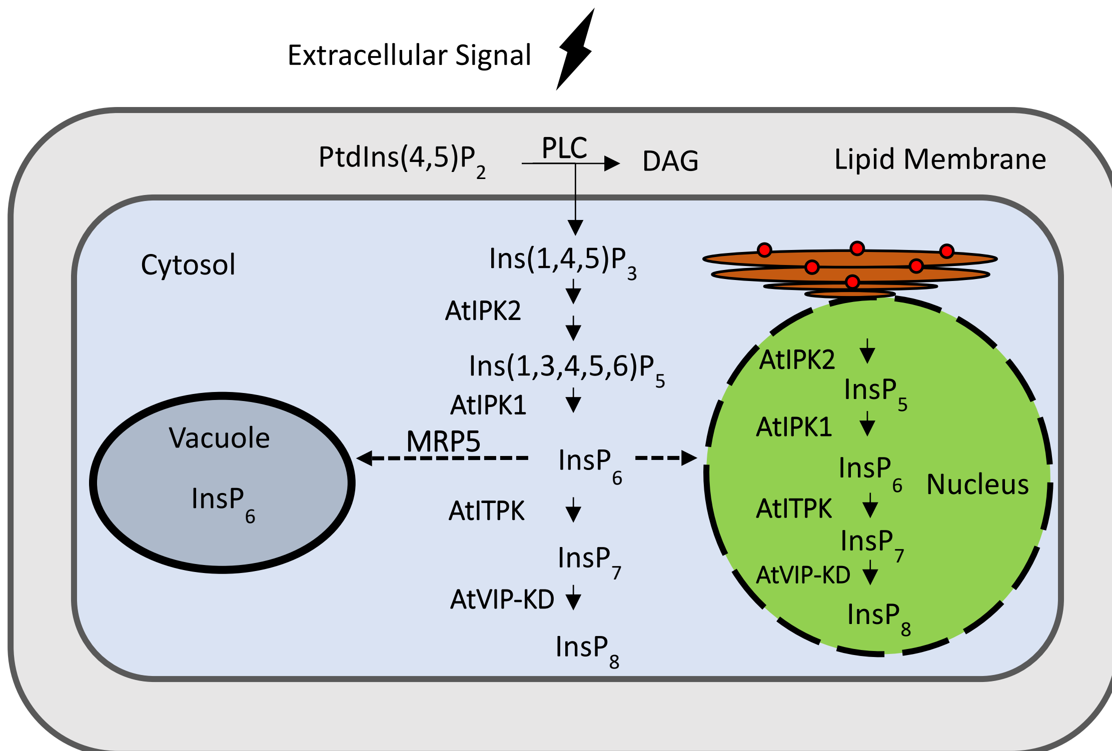
GRAPHICAL ABSTRACT



Inositol Pyrophosphate Biosynthesis and Subcellular Distribution of Enzymes. Notably, $InsP_6$ which represents the major precursor of PP-InsPs in plants is synthesized in the cytosol, however, it can also be transported into the vacuole by the ABC transporter MRP5. Subcellular localization of enzymes involved in PP-InsP biosynthesis including AtITPK and the kinase domains of AtVIP suggests that these molecules are present in the cytosol and nucleus, and to a smaller extent in the ER. Not shown are the full length and phosphatase domain of AtVIP, which are absent from the nucleus.

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TABLE OF ABBREVIATIONS

Ins	*myo*-inositol
InsP	Inositol phosphate
PPx-InsP	Inositol pyrophosphate
PP	Diphosphate
P*i*	Phosphate: as pertains to soil inorganic phosphate
PtdIns	Phosphatidylinositol
PtdInsP	Phosphatidylinositol phosphate
PLC	Phospholipase C
Ins(1,4,5)P_3	*myo*-inositol(1,4,5)-triphosphate
$InsP_6$	Inositol hexakisphosphate
$InsP_7$	Diphosphoinositol Pentakisphosphate
$InsP_8$	Bisdiphosphoinositol Tetrakisphosphate
HPLC	High-Performance Liquid Chromatography
WT	Wild Type
GFP	Green Fluorescent Protein
VIP	Very Important Protein
PPIP5K	Diphosphoinositol Pentakisphosphate 5-Kinase
KCS1	Protein Kinase C Suppressor 1, HsIP6K
At	*Arabidopsis thaliana*
KD	Kinase Domain
PD	Phosphatase Domain
OH	Hydroxyl
DAG	Diacyl Glycerol
PKC	Protein Kinase C
Ca^{2+}	Calcium II Ions
PI3K	Phosphatidylinositol 3-Kinase
PTEN	Phosphatase and Tensin Homolog
SAC	Suppressor of Actin

5PTase	Inositol Polyphosphate 5-Phosphatase
TGN	Trans Golgi Network
ER	Endoplasmic Reticulum
PdOH	Phosphatidic Acid
MIPS	*myo*-Inositol Phosphatase Synthase
G6P	Glucose-6-Phosphate
IMP	Inositol Monophosphatase
LPA	Low Phytic Acid
MIK	*myo*-Inositol Kinase
ITPK	Inositol Triphosphate-5/6 Kinase
IMPK	Inositol Multiphosphate Kinase
IPK	Inositol Polyphosphate Kinase

CHAPTER I

RATIONALE

The inositol phosphate (InsP) signaling pathway has been implicated in a diverse array of cellular processes in eukaryotic organisms. Inositol phosphates are intracellular signaling molecules built around a simple 6-carbon *myo*-inositol (inositol) scaffold used in signal transduction. The different patterns of phosphorylation of the inositol ring conveys specific cellular information, and several combinations of phosphorylation events are possible (York, 2006; for review, see Gillaspy, 2011; Williams et al., 2015;). In plants, this signaling pathway, and the molecules produced, has been linked with many cellular events critical for plant overall fitness and survival. Inositol pyrophosphates (PP-InsPs) are an emerging class of InsPs containing one or two diphosphate groups (PP) attached to the inositol ring. These PP-InsPs are gaining significant attention owing to their suggested roles in inorganic phosphate (P*i*) sensing and energetic metabolism. Elucidating the route for PP-InsP biosynthesis in plants is fundamentally critical to our understanding of their growing influence on plant fitness and survival and can help influence strategies aimed at improved crop productivity.

Given the importance and diversity of cellular processes linked with the different InsP molecules in plants, some of the genes that encode enzymes involved in their biosynthesis have been studied and characterized (Gillaspy, 2013). Inositol hexakisphosphate ($InsP_6$), which has all the hydroxyl (OH) groups on the inositol ring phosphorylated accumulates in plant tissues (Cosgrove & Irving, 1980; Raboy, 1997), and is a precursor for PP-InsP biosynthesis. The PP-InsPs are

regarded as “energetic signaling” molecules due to the presence of one or two pyrophosphate bonds in their chemical structure similar to ADP and ATP respectively (Stephens et al., 1993; York & Lew, 2008). The biosynthesis of PP-InsP have been well described in non-plant eukaryotes like yeast and mammals, and many physiological roles have been linked to these molecules (Shears, 2009; for review, see Williams et al., 2015). However, understanding of their biosynthesis in plants has lagged behind considerably, and their physiological relevance is only just emerging. This work attempts to fill the knowledge gap in plant PP-InsP biosynthesis through the biochemical characterization of the plant diphosphoinositol pentakisphosphate kinases (PPIP5Ks) enzymes as well as elucidation of the pathway for synthesizing inositol pyrophosphates in plants.

LITERATURE REVIEW

Overview of Phosphoinositide Signaling in Plants

Critical to the survival of an organism, is its capacity to respond appropriately to its immediate environment and adapt. A strategy that is well known is the use of cell surface receptors by organisms to bind extracellular signals, and their subsequent conversion into intracellular second messengers (Berridge, 2009). Second messengers control many cellular processes, and their cellular concentration is tightly regulated. This review focuses on a type of signal transduction system in plants which utilizes the simple cyclitol, *myo*-inositol (inositol) (Berridge, 2009), as the backbone for the biosynthesis of two classes of signaling molecules that convey specific information to the cell. This review gives some background information on the metabolism of the lipid-soluble phosphatidylinositol phosphates (PtdInsPs) and water-soluble inositol phosphates (InsPs), and highlights what is known about the biosynthesis and function of inositol pyrophosphates (PP-InsPs).

A complete understanding of PP-InsP metabolism requires a thorough knowledge of the early intermediates involved in its biosynthesis. PtdInsP and InsPs are intricately tied to PP-InsP biosynthesis, and as such an overview of these class of molecules is essential.

Phosphoinositide signaling encompasses two tightly interwoven and highly connected signaling pathways which despite their intricate connections, have clearly defined fates and influence different cellular processes (Gillaspy, 2013; for review, see Boss & Im, 2012; Mueller-Roeber & Pical, 2002). The two classes of signaling molecules used in phosphoinositide signaling are the

PtdInsPs, and the InsPs. Structurally, a common theme between these two classes of molecules is their use of a simple and stable naturally occurring cyclic carbohydrate; cis-1,2,3,5-trans-4,6-cyclohexanehexol (inositol), as well as a ubiquitous commonly used signaling device; phosphate (see **Figure 1.1**). The unique stereochemistry of inositol confers its unusual stability and capacity for many highly charged phosphate molecules within close proximity (Shears, 2015). An additional convenience for its use stems from the fact that inositol can be easily generated from intermediates within the glycolytic pathway (Sherman et al, 1977). Phosphate is a commonly used cellular signaling molecule, owing to its unique physicochemical properties (size and charge). These properties confer its ability in establishing specificity in protein-protein and protein-ligand interactions (Shears, 2015).

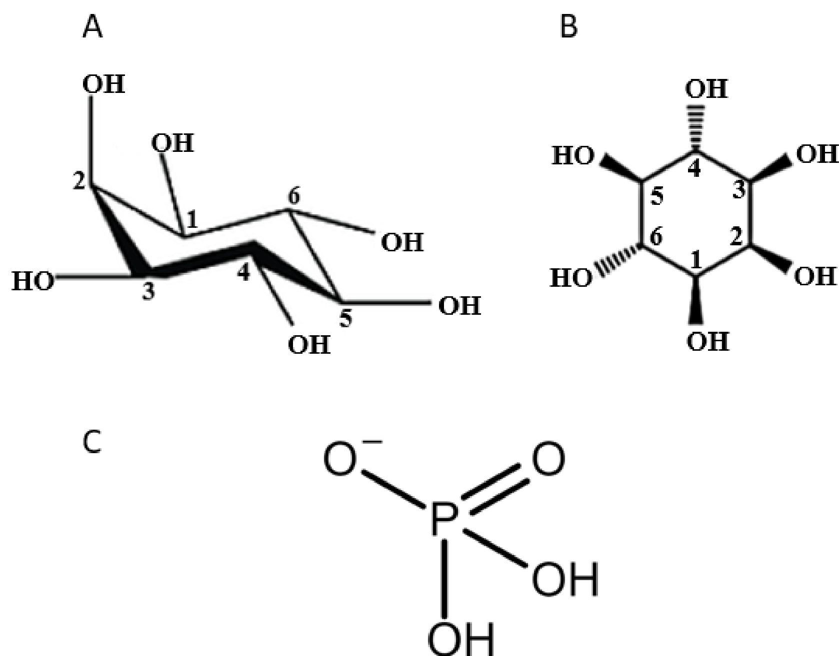

Figure 1. 1. Structure of D-*myo*-inositol and Phosphate.

A, Chair conformation of D-*myo*-inositol. B, Ring structure of D-*myo*-inositol showing replaceable hydroxyl groups. C, Phosphate group.

The hydrolysis of water soluble InsPs generate free inositol molecules, which serve as the headgroup of PtdInsPs. InsPs can be synthesized through the turnover of the lipid soluble PtdInsPs and serve as precursor for PP-InsP biosynthesis, highlighting their interdependence. Perturbation of the cellular levels of these molecules have been linked with different extracellular signals in plants and non-plant eukaryotes resulting in the transmission and amplification of intracellular signals that trigger a cellular response (Gillaspy, 2013). A classical example of phosphoinositide signaling in animal cells is the decrease in cellular concentration of the lipid-bound phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P_2] upon hydrolysis by activated phospholipase C (PLC) enzyme in response to an extracellular signal. This results in the generation of two second messengers; diacylglycerol (DAG) which remains anchored to the membrane, as well as inositol 1,4,5 triphosphate [Ins(1,4,5)P_3] (Berridge, 1993). The lipid-anchored DAG activates protein kinase C (PKC) in animal cells, which subsequently phosphorylates target proteins and results in a cellular response (Newton, 2010). The increase in cytosolic Ins(1,4,5)P_3 levels, on the other hand, results in the release of calcium II ions (Ca^{2+}) from intracellular stores which regulates many cellular processes (Berridge, 1993).

Lipid soluble PtdInsPs and water soluble InsPs represent the two signaling molecules in phosphoinositide signaling, and more importantly are key players in the biosynthesis of PP-InsP. Regulatory mechanisms for tightly controlling the cellular concentrations of these signaling molecules is critical for efficient cellular communication. These three classes of signaling molecules (PtdInsPs, InsPs and PP-InsPs) are discussed in subsequent sections, with emphasis on the different molecular species found in plants, the enzymes responsible for their metabolism, its cellular distribution and regulation.

Phosphatidylinositol Phosphates

The lipid-soluble PtdInsPs are made via the phosphorylation of the inositol headgroup of the membrane-anchored lipid, phosphatidylinositol (PtdIns). The structural composition of PtdIns include an inositol headgroup that is linked to the C1-position of the lipid glycerol backbone via a phosphodiester bond, with the hydroxyl (OH) groups at positions 3, 4 and 5 of the headgroup available for phosphorylation (for review, see Heilmann, 2016) (see **Figure 1.2**). The unique architecture of PtdInsPs in which their glycerol backbone is embedded within the membrane and the phosphoinositol headgroup protrudes into the cytoplasm enables efficient information transfer from outside to inside of a cell (for review, see Heilmann, 2016).

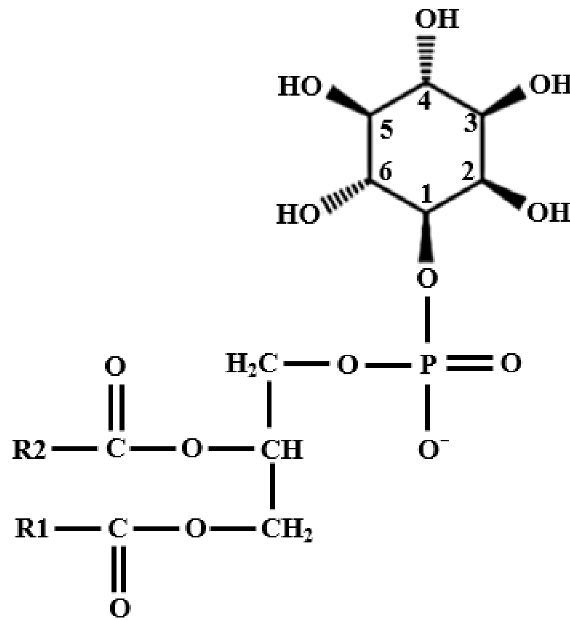

Figure 1. 2. Structure of Phosphatidylinositol.

The structure contains a glycerol backbone, linked to inositol headgroup via a phosphate group. R1 and R2 represents alky groups of long aliphatic chains.

PtdInsP Molecules and Enzymes Involved in their Biosynthesis

Different species of PtdInsPs that have been identified in plants include; phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4-phosphate (PtdIns4P), phosphatidylinositol 5-phosphate (PtdIns5P), phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (Munnik & Nielsen, 2011; Pical et al., 1999; Vanhaesebroeck & Waterfield, 1999; for review, see Mueller-Roeber & Pical, 2002). Some of the enzymes responsible for the biosynthesis of these molecules in plants have been described (for review, see Heilmann, 2016).

In plants, PtdIns3P is synthesized from PtdIns by the class III phosphatidylinositol 3 kinase (PI3K) (Foster, 2003; Lee et al., 2008), while the phosphatidylinositol 4-kinases (PI4K) phosphorylate PtdIns at the 4-OH position to generate PtdIns4P.

Enzymes involved in PtdInsP hydrolysis are less studied, however, a few families of PtdInsP phosphatases have been reported. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-related enzymes hydrolyze the 3-phosphate group of PtdIns monophosphates and bisphosphates (Pribat et al., 2012) which could explain how PtdIns5P is generated. Suppressor of actin (SAC) phosphatases hydrolyze PtdIns bisphosphates on the plasma membrane (for review, see Heilmann, 2016), the inositol polyphosphate 5-phosphatases (5PTases) which have a more flexible substrate preference, hydrolyze the 5-phosphate group of lipid-soluble PtdInsPs and the water-soluble InsPs (Gunesekera et al., 2007).

Another family of enzymes with PI-specificity are the phospholipase C (PLC) family of enzymes. The hydrolysis of PtdIns(4,5)P_2 by PLC represents a major step in the biosynthesis of the water-soluble InsPs (Abd-El-Haliem et al., 2016; Gillaspy, 2013). The rapid turnover of PtdInsPs by these complex network of kinases and phosphatases results in a dynamic cellular concentration of PtdInsPs that can be easily perturbed. This is one of the hallmarks of signaling molecules.

Intracellular Distribution of PtdInsPs

The distribution of these molecules within a cell have been studied by exploiting fluorescently tagged probes as biosensors, or fusion construct variant of proteins containing PtdInsPs-binding domains or enzymes involved in PtdInsP metabolism (Ischebeck et al., 2011; Ischebeck et al., 2013; Tejos et al., 2014). These studies have elucidated the intracellular distribution and dynamic nature of some of these molecules and enzymes involved in their regulation. PtdIns3P has been shown to concentrate in the endosomes, pre-vacuolar compartments and vacuoles (Lee et al., 2008; Vermeer et al., 2006); PtdIns4P is enriched in the Golgi apparatus, trans-Golgi network (TGN) and plasma membrane (Simon et al., 2014; Simon et al., 2016; Vermeer et al., 2009); PtdIns(4,5)P_2 is abundant in the plasma membrane (Simon et al., 2014; van Leeuwen et al., 2007), while the least abundant of the phosphoinositides PtdIns(3,5)P_2 is enriched in late endosomes and plasma membrane in root cells (Hirano et al., 2018; Hirano et al., 2017). It has been speculated that the least understood species of the phosphoinositides, PtdIns5P, could be enriched in the Golgi and plasma membrane (for review, see Heilmann, 2016).

Several studies have also reported on the subcellular distribution of enzymes involved in the metabolism of these highly dynamic molecules. A recent study reported the the localization of PI3K from tomato in the membrane and cytoplasm of guard cells in transgenic tobacco (Dek et al., 2017). Subcellular distribution of multiple isoforms of PI4K have been localized to different compartments highlighting the dynamic nature of these enzymes and molecules. The PI4Kβ1 isoform was localized to the TGN (Kang et al., 2011; Okazaki et al., 2015), in contrast, PI4Kα and PI4Kβ2 were localized to the cytosol (Okazaki et al., 2015). Subcellular localization experiments have reported a nuclear and plasma membrane presence for phosphatidylinositol 4-phosphate 5-kinase 2 (PI4P 5K 2) isomer (Gerth et al., 2017; Ischebeck et al., 2013; Tejos et al., 2014), however, there is no subcellular localization data reported for any plant PI3P 5K enzyme (for review, see Heilmann, 2016). The yeast PI5K is reportedly localized at the plasma membrane and involved in morphogenesis and vacuole formation (Cooke et al., 1998; Homma et al., 1998). Based on the localization pattern of enzymes involved in PtdInsP metabolism, it is reasonable to associate the different phosphorylation events with the dynamic delivery or retrieval of vesicles to and from the plasma membrane.

Other plant enzymes involved in PtdInsP metabolism for which subcellular localization data exists include: PI-PLC family of enzymes localized to the plasma membrane (Cao et al., 2007; Helling et al., 2006; Pokotylo et al., 2014), the SAC family of phosphatases that hydrolyze PtdIns bisphosphates at the 5-position which are localized to different compartments including the endoplasmic reticulum (ER), golgi and tonoplast (Despres et al., 2003; Novakova et al., 2014; Zhong, 2005). PTEN which hydrolyze both PtdIns monophosphate and bisphosphate at the 3-position, have been reported to localize to vesicles (Zhang et al., 2011), while the PtdInsPs and

InsPs hydrolyzing 5PTases have been reported to localize to the plasma membrane (Ercetin et al., 2008; Gunesekera et al., 2007). Overall, these subcellular localization data suggest that PtdInsPs synthesis and breakdown is intimately tied to membrane dynamics, and influence membrane trafficking and protein sorting.

Functions of PtdInsPs in Plants

Despite the relatively low abundance of phosphoinositides on biological membranes compared to other phospholipids, phosphoinositides influence a wide range of important cellular processes through their regulatory functions in eukaryotic cells. Several studies have highlighted some of these roles through experiments designed to perturb specific steps in the phosphoinositide biosynthesis pathway. For example; the Arabidopsis PI3Kinase homozygous loss-of-function mutant, in which a sequence of nucleotides from a transfer DNA (T-DNA) was inserted to disrupt the coding sequence of PI3K and PtdIns3P biosynthesis exhibited a lethal phenotype and the heterozygous mutant showed defects in pollen development, root hair development and vacuole morphology (Lee et al., 2008; Welters et al., 1994). It was shown that PI3K was vital for nuclear division and vacuole reorganization during pollen development as heterozygous mutants had a 2-fold higher proportion of dead pollen grains or reduced number of nuclei compared to wild-type plants. This study also reported abnormal vacuole size in the pollen of mutants compared to wild-type as a result of defective fission (Lee et al., 2008).

The rapid effect of PtdIns3P on root hair elongation was directly tested by growing Arabidopsis plants in media containing various concentrations of a PI3K inhibitor. The result showed a

concentration-dependent reduction in root hair length specific to tip elongation, and 80% shorter root hair length at 30 μM inhibitor concentration (Lee et al., 2008). Another study reported that upregulation of PI3K expression led to increased ethylene biosynthesis and flower senescence (Dek et al., 2017). In this study, PI3K transcript levels were significantly higher in detached flowers of PI3K overexpression lines than control plants, and there was an increase in PI3K protein level 48 hours post flower detachment relative to control. Overexpression of PI3K resulted in increased ethylene biosynthesis, accelerated senescence and reduced flower lifespan of Tobacco flower (Dek et al., 2017). These findings suggest a role for PI3K in plant senescence and a potential role in ethylene signal transduction, which could have potential agricultural applications.

PtdIns4P is the most abundant of the PtdInsPs in eukaryotes, serving both as a signaling molecule and the precursor for PtdIns(4,5)P_2 (Berridge & Irvine, 1989; Jung et al., 2002; Krinke et al., 2007). Genetic experiments using T-DNA insertion knockout or downregulation of different isoforms of Arabidopsis PI4Kinase (AtPI4KIIIα1, AtPI4Kβ2) with compromised PtdIns4P biosynthesis resulted in a homozygous lethal phenotype in the absence of AtPI4KIIIα1 protein which suggests a role in male or female sterility (Delage et al., 2012). In this study, the cold-triggered PLC activity was shown to be dependent on PI4K function as T-DNA double loss-of-function mutant *pi4kIIIβ*1β2 had a 40% decrease in phosphatidic acid (PdOH), and PtdIns(4,5)P_2 hydrolysis was impaired as a result of reduced PI-PLC activity (Delage et al., 2012). A recent study also reported the involvement of an isoform of PI4K from Arabidopsis in auxin biosynthesis and leaf margin development (Tang et al., 2016).

$PtdIns(3,5)P_2$ accounts for the least fraction of phosphoinositides in eukaryotic cells, accounting for only 0.05-0.1% of the total phospholipids (Hirano & Sato, 2019). Inhibition of $PtdIns(3,5)P_2$ biosynthesis resulted in defective morphology of late endosomes and vacuoles in plants (Hirano et al., 2017). A selective inhibitor of FYVE finger-containing phosphoinositide kinase (PIKfyve) involved in $PtdIns(3,5)P_2$ biosynthesis YM201636, was used to reduce $Ptds(3,5)P_2$ levels by as much as 70% in WT Arabidopsis. This reduction in $Ptdns(3,5)P_2$ level resulted in an abnormal membrane dynamics of plasma membrane proteins, Aux1 and Bor1, as well as pleiotropic defects in post-Golgi trafficking events and defects in late endosome morphology (Hirano et al., 2017). The connection between $Ptdns(3,5)P_2$ biosynthesis, auxin transporter (Aux1), boron transporter (Bor1), post-Golgi trafficking pathways including secretion, exocytosis, endocytosis, vacuole, recycling pathways, TGN and endosomes shows the influence of these class of molecules on a growing number of cellular processes (Hirano, et al., 2017).

In plants, $PtdIns(4,5)P_2$ levels is regulated by a family of PI4P 5-Kinases and phosphatases, including PtdIns-specific PLC and 5PTases that catalyze the biosynthesis and hydrolysis of $PtdIns(4,5)P_2$ (Gillaspy, 2013; for review, see Heilmann, 2016; Mueller-Roeber & Pical, 2002). Apart from its role as a precursor for water-soluble $Ins(1,4,5)P_3$ and diacylglycerol (DAG) biosynthesis (Hirayama et al., 1995; Shi et al., 1995; for review, see Mueller-Roeber & Pical, 2002), perturbation of levels of $PtdIns(4,5)P_2$ has been observed in plants under various stress conditions, suggesting a role for the molecule in stress response (DeWald, 2001; Mishkind et al., 2009; Mosblech et al., 2011; Williams et al., 2005). Other roles reported for $PtdIns(4,5)P_2$ in plants include stomata opening, root hair development, pollen tube development, membrane

destabilization through its interaction with protein targets (Ischebeck et al., 2011; Ischebeck et al., 2008; Lee et al., 2007; Payne et al., 2016).

Some of the roles described for PtdInsPs are exerted by modulating the properties of membranes in which they are embedded, thereby altering their structural properties (Lundbæk et al., 2010). They can also interact with target proteins containing PtdInsPs-binding domain, thereby regulating their function through sequestration or localization, or their role as precursors for the biosynthesis of a different class of water-soluble signaling molecules referred to InsPs (Eyster, 2007; Lemmon, 2003; Takenawa, 2010; for review, see Heilmann, 2016). From these different reports, it is evident that perturbation of cellular levels of different PtdInsPs species result in pleiotropic phenotypes, owing in part to their interaction with different protein targets which regulate a broad range of cellular processes, as well as their overarching influence of membrane dynamics and intracellular transport.

Regulation of PtdInsPs

Given the diverse cellular processes influenced by this class of molecules, cytoskeletal dynamics, guard cell physiology, regulation of intracellular Ca^{2+} levels, pollen tube growth, hormone signaling, stress response, vesicle trafficking, transcription regulation (Alexandra et al., 1990; Blatt et al., 1990; Bunney et al., 2000; Drobak et al., 1994; Gilroy et al., 1990; Kim et al., 2001; Kost et al., 1999; Munnik et al., 1998; Pical et al., 1999), it is critical their cellular concentration is tightly regulated.

One mechanism suggested for the regulation of cellular levels of PtdInsPs is the post-translational regulation of PI4K activity through its phosphorylation by protein interactors. PI4K is a key enzyme involved in the biosynthesis of PtdIns4P which itself is a precursor for PtdIns(4,5)P_2 and therefore represents a key step in PtdInsPs metabolism (Boss et al., 1995; for review, see Mueller-Roeber & Pical, 2002). This phosphorylation event has also been reported to lead to trafficking of PI4K, therefore affecting the localization of PI4K (for review, see Mueller-Roeber & Pical, 2002). PtdIns3P was reported to traffick proteins (Kim et al., 2001), such trafficking events can help sequester enzymes into specific compartments within the cell where they are inaccessible to their target substrates. Specificity of enzymes involved in PtdInsPs biosynthesis also play a role in the cellular abundance of these molecules and could be a mechanism for their regulation.

One major difference between Arabidopsis PLC and the animal PLC genes is the lack of specificity for PtdIns(4,5)P_2 (Gillaspy, 2013), and this lack of specificity could result in the hydrolysis of other PtdInsPs species thereby regulating the cellular concentration of different PtdInsPs species and their downstream effect.

Inositol Phosphates

Inositol phosphates (InsPs) represent the water-soluble class of signaling molecules used in phosphoinositide signaling. InsPs are similar to the PtdInsPs in that inositol is the structural scaffold on which several different species of InsPs are constructed (Michell, 2007). Based on the number of phosphates attached to the inositol ring and the different combinatorial patterns, a

large number of InsP species is possible, with over 37 different species having been identified in biological systems (Alcázar-Román & Wente, 2008; for review, see Williams et al., 2015). This richness in diversity enables the use of InsPs in signal transduction and intracellular communication, as they can influence a diverse array of cellular processes. Unlike the PtdInsPs that are tethered to the membrane via fatty-acyl chains, InsPs are water soluble and are present in the cytosol (see **Figure 1.3**).

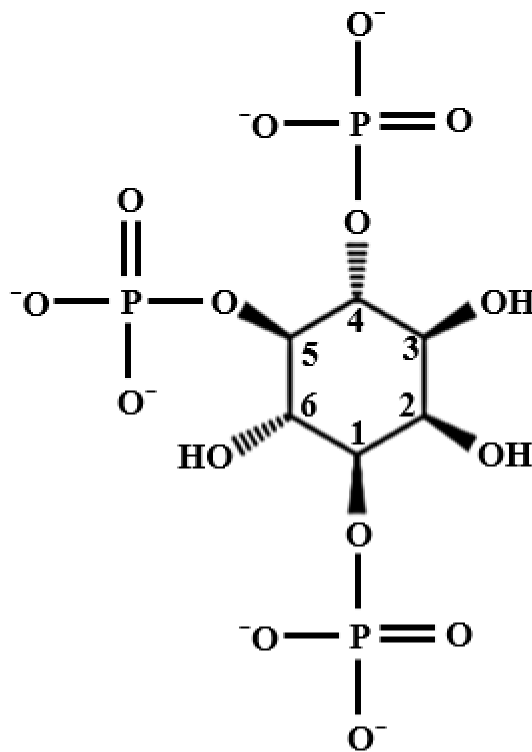

Figure 1. 3. Structure of Inositol Phosphates.

Inositol(1,4,5)P_3, an example of inositol phosphates with phosphate groups attached to the inositol ring at positions 1, 4, and 5.

InsP Molecules and Enzymes Involved in their Biosynthesis

Given the technical challenges in plant InsPs research, the exact number of molecular species of InsPs present in plants, or that is realistically possible, is unknown. However, with growing information on characterized genes and enzymes involved in InsP metabolism, as well as discovery of new players involved in InsP metabolism, it is safe to assume that the number of InsPs species will continue to rise.

Of the different InsP molecules described in plant tissues, the fully phosphorylated $InsP_6$ is the most abundant where it accumulates in plant seeds complexed with metal ions (Gillaspy, 2013; Victor Raboy & Bowen, 2006). In this form, $InsP_6$ also referred to as phytate or phytic acid, which accounts for 65-95% of total P*i* in mature seeds, where it serves as a store of phosphorus, minerals and inositol (Cosgrove & Irving, 1980; Raboy, 1997; Raboy et al., 2001). In yeast, it is suggested that $Ins(1,4,5)P_3$ does not act as a second messenger involved in Ca^{2+} release from intracellular stores, and is instead used specifically for $InsP_6$ biosynthesis (York, 1999). Nuclear roles that have been attributed to $InsP_6$ in yeast include mRNA export, chromatin remodeling and telomere length (Monserrate & York, 2010). Some studies in plants have also suggested that $InsP_6$ generated from $Ins(1,4,5)P_3$ serves as a second messenger and is more effective in stimulating Ca^{2+} from intracellular stores (Lemtiri-Chlieh et al., 2000; Lemtiri-Chlieh et al., 2003).

The accumulation of $InsP_6$ in plant seeds also has some detrimental effects. $InsP_6$ has anti-nutrient properties in humans and monogastric animals are incapable of digesting the molecule.

The highly electronegative $InsP_6$ chelates positively charged metal ions such as Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, and makes these minerals inaccessible (Cowieson et al., 2006; Raboy, 2001).

Undigested $InsP_6$ that is excreted is also a major environmental concern as this leads to phosphorus pollution, eutrophication and poor water quality (Abelson, 1999; Sharpley et al., 1994). These existing issues have led to several research efforts at generating crop plants with reduced phytic acid content (Abelson, 1999; Raboy, 2006; Raboy, 2007). $InsP_6$ has also been reported to have some anticancer properties (Kapral et al., 2017; Shamsuddin et al., 1997; Shamsuddin, 1995). Given the presence of $InsP_6$ in several organisms and its abundance in plant seeds, as well as the interesting cellular roles $InsP_6$ has been linked to, it is not surprising that a great deal of research has focused on understanding its metabolism and regulation.

The biosynthesis of $InsP_6$ in plants is suggested to proceed via two routes; the lipid-independent and the lipid-dependent pathway (see **Figure 1.4**). Unlike the lipid-dependent pathway that is initiated by PLC hydrolysis of PtdIns(4,5)P_2, the lipid-independent pathway involves *de novo* $InsP_6$ biosynthesis via sequential phosphorylation of the inositol ring by kinases, some of which are unique to plants (Gillaspy, 2013).

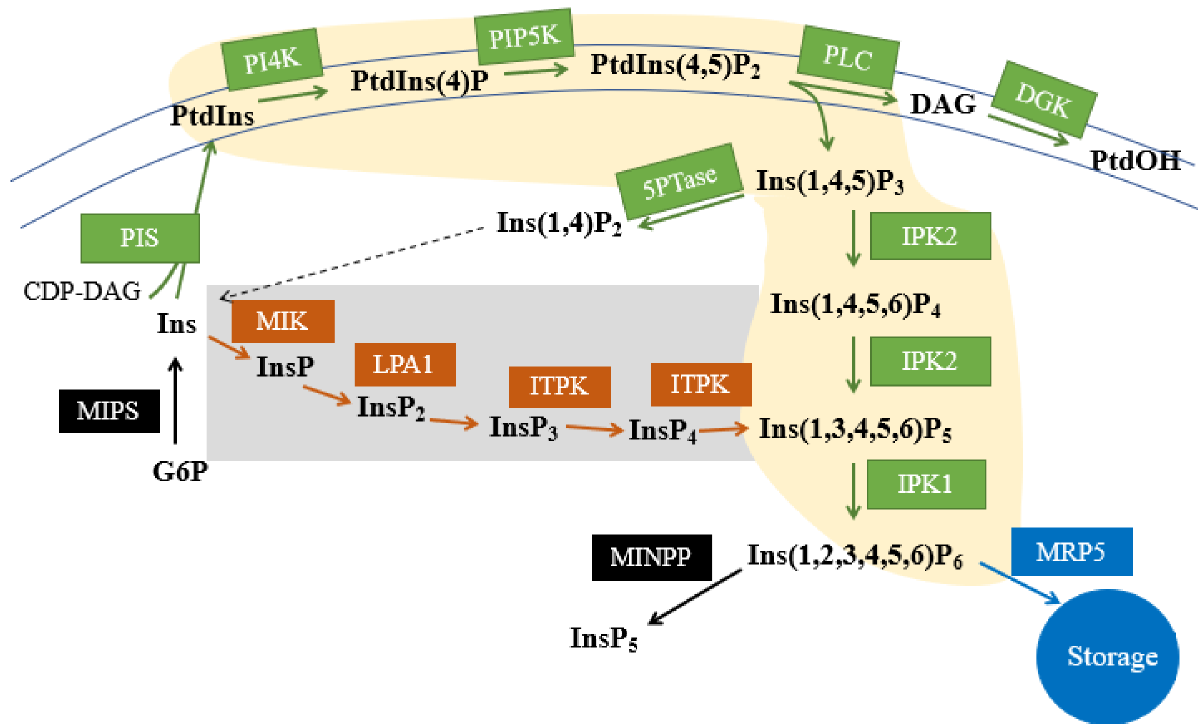

Figure 1. 4. Lipid-dependent and Lipid-Independent Pathway for $InsP_6$ Biosynthesis.

The route for the lipid-dependent pathway of $InsP_6$ biosynthesis is shown in cream, and the lipid-independent pathway is depicted in grey. Enzymes involved in the lipid-independent pathway are shown in brown boxes, and those involved in the lipid-dependent pathway are shown in green boxes. MIPS, *myo*-inositol phosphate synthase; CDP-DAG, cytidine diphosphate diacylglycerol; PIS, phosphatidylinositol synthase; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol phosphate 5-kinase; PLC, phospholipase C; DAG, diacylglycerol; DGC, diacylglycerol kinase; 5PTase, inositol polyphosphate 5-phosphatase; IPK2, inositol polyphosphate kinase 2, IPK1, inositol polyphosphate kinase 1; MRP5, multidrug resistant protein 5; MINPP, multiple inositol polyphosphate phosphatase; MIK, *myo*-inositol kinase; LPA1, low phytic acid 1; ITPK, inositol triphosphate 5/6 kinase.

Lipid-Independent Pathway of $InsP_6$ Biosynthesis

The Lipid-Independent pathway of $InsP_6$ biosynthesis begins with free inositol generated from glucose-6-phosphate (G6P). The inositol phosphate synthase (MIPS) converts G6P to inositol-3-phosphate (Ins3P), and then inositol monophosphatase (IMP) converts Ins3P to free inositol (Loewus & Murthy, 2000; Loewus & Loewus, 1982; Torabinejad & Gillaspy, 2006; Yoshida et

al., 1999). MIPS genes in crop plants were identified in efforts to generate and characterize so-called low phytic acid (LPA) mutants (Raboy, 2007). The *lpa1* mutant from maize has a disrupted MIPS gene that causes a significant reduction in $InsP_6$ levels (Dorsch et al., 2003; Raboy et al., 2000). In the second step of the lipid-independent pathway of $InsP_6$ biosynthesis, free inositol in the cell is phosphorylated by an inositol kinase (MIK), first identified in maize as encoded by the *LPA3* gene (Shi et al., 2005; Raboy, 2007). Loss-of-function maize *lpa3* mutants have reduced $InsP_6$ and elevated inositol levels in the seeds (Shi et al., 2005).

The product of MIK action on inositol generates Ins3P (English et al. 1966; Loewus et al., 1982; Shi et al., 2005), which undergoes two further phosphorylation steps by yet to be determined plant kinases to produce inositol trisphosphate ($InsP_3$) (Gillaspy, 2013). Studies in the slime mold *Dictyostelium discoideum* and duckweed *Spirodela polyrhiza* have suggested similar pathways of $InsP_6$ biosynthesis via sequential phosphorylation of inositol and defined InsP intermediates (Brearley & Hanke, 1996; Stephens & Irvine, 1990). The sequence of phosphorylation events in duckweed from inositol to $InsP_6$ was described to proceed via Ins3P - $Ins(3,4)P_2$ - $Ins(3,4,6)P_3$ - $Ins(3,4,5,6)P_4$ - $Ins(1,3,4,5,6)P_5$ - $InsP_6$ (Brearley & Hanke, 1996). These findings demonstrate that the enzyme action of MIK is vital for the lipid-independent synthesis of $InsP_6$ from free inositol in plants, and disruption of the gene alters $InsP_6$ levels.

The third gene that was identified in LPA screens of mutant maize plants is the *LPA2* gene, which encodes an inositol trisphosphate -5/6Kinase (ITPK). The recombinant purified ITPK enzyme showed broad specificity and catalyzed the phosphorylation of $Ins(1,3,4)P_3$, $Ins(3,5,6)P_3$,

$Ins(3,4,5,6)P_4$ and $Ins(1,2,5,6)P_4$ *in vitro*. The *lpa2* mutant had a 30% reduction in seed $InsP_6$ level and a 3-fold increase in P*i* (Shi, 2003). The Arabidopsis homologue of *ZmLPA2* is referred to as inositol(1,3,4) triphosphate 5/6-kinase (ITPK) and is encoded by four genes (AtITPK1-4) (Sweetman et al., 2007; Wilson & Majerus, 1997). These multifunctional enzymes can catalyze the conversion of $Ins(1,3,4)P_3$ to $Ins(1,3,4,5)P_4$ and $Ins(1,3,4,6)P_4$, and subsequently to $Ins(1,3,4,5,6)P_5$. AtITPK2 and AtITPK3 also showed activity with other isomers of $InsP_3$ including $Ins(1,4,6)P_3$ and $Ins(3,4,6)P_3$ producing $InsP_4$. The enzyme also possesses a 1-kinase activity and will catalyze the conversion of $Ins(3,4,5,6)P_4$ to $Ins(1,3,4,5,6)P_5$. ITPK4 was considered an outlier within the family as it showed some unusual isomerase activity with $InsP_4$ interconverting different $InsP_4$ species without detectable $InsP_5$ species due to its lack of a 1-kinase activity (Sweetman et al., 2007).

These broad substrate selectivity has also been demonstrated in soybean ITPKs (GmITPK1-4) (Stiles et al., 2008). The Arabidopsis ITPK1 and ITPK4 were also found to impact seed phytic acid content. In this study, *Atitpk1* and *Atitpk4* loss of function T-DNA insertion mutants had reduced $InsP_6$ levels (Kim & Tai, 2011), and a separate study reported an accumulation of $Ins(3,4,5,6)P_4$ in the *Atitpk1* mutant accompanied by reduced $InsP_6$ levels (Kuo et al., 2018). An insertion mutation of *Atitpk2* resulted in a slight decrease in seed $InsP_6$ levels, accompanied by defects in seed coat formation (Tang et al., 2013). Mutation of rice ITPK6 (*ositpk6*) had between 10% - 32% reduction in seed $InsP_6$ levels as well as defects in growth and reproduction (Jiang et al., 2019). There is no reported ITPK activity in yeast, and the human enzyme (HsITPK) has been reclassified as inositol(3,4,5,6) tetrakisphosphate 1-kinase/inositol(1,3,4,5,6) pentakisphosphate 1-phosphatase (Ho et al., 2002; Wilson & Majerus, 1996). The presence of

multiple ITPK genes and functional redundancy in plants compared to their human counterparts suggests that the plant genetic product may be more important for overall plant fitness, and the lipid-independent pathway is a more favored route for $InsP_6$ biosynthesis in plants.

The ITPKs are structurally classified as a family of ATP-grasp fold proteins containing a globular fold with three distinct domains surrounding the ATP binding cleft: N-terminal domain, the central and C-terminal domain which are all primarily made up of β sheets (Cheek et al., 2002; Miller et al., 2005). The crystal structure of the *Entamoeba histolytica* ITPK shows that the inositol ring faces ATP edge-on and the 5- and 6-hydroxyl groups are nearly equidistant from the γ-phosphate of ATP such that the phosphorylation of either hydroxyl group is catalytically plausible. This was suggested as the reason for the dual-site specificity of the enzyme (Miller et al., 2005). The biosynthesis of Ins(1,3,4,5,6)P_5 signifies the last unique step of the lipid-independent pathway of $InsP_6$ biosynthesis, as it is generally accepted that the same $InsP_5$ isomer is generated from both lipid-independent and lipid-dependent pathway, and a common enzyme catalyzes its conversion to $InsP_6$ in plants and non-plant eukaryotes (Raboy & Bowen, 2006; Stevenson-Paulik et al., 2005).

Lipid-Dependent Pathway of $InsP_6$ Biosynthesis

The lipid-dependent pathway of $InsP_6$ biosynthesis has been described in many organisms, including yeast (Odom et al., 2000; Perera et al., 2004; Saiardi et al., 2000a; York, 1999) and animals (Fujii & York, 2005; Seeds et al., 2004). Homologues of the enzymes involved in $InsP_6$ biosynthesis through this pathway exist in plants and have been well characterized (Stevenson-

Paulik et al., 2005; Stevenson-Paulik et al., 2002; Xia et al., 2003). A list of key enzymes in the lipid-dependent and lipid-independent pathway of InsP6 biosynthesis is shown in the table below.

Table 1. 1. Enzymes of the Lipid-Dependent and Lipid-Independent Pathway.

Pathway	Enzyme	Substrate(s)	Product(s)
Lipid-Dependent Pathway	PLC	PtdIns(4,5)P_2	Ins(1,4,5)P_3 + DAG
	IPK2	Ins(1,4,5)P_3	Ins(1,4,5,6)P_4
		Ins(1,4,5,6)P_4	Ins(1,3,4,5,6)P_5
	IPK1	Ins(1,3,4,5,6)P_5	Ins(1,2,3,4,5,6)P_6
Lipid-Independent Pathway	MIK	Inositol	Ins3P
	ITPK	Ins(1,3,4)P_3	Ins(1,3,4,5)P_4 ; Ins(1,3,4,6)P_4
		Ins(1,3,4,5)P_4	Ins(1,3,4,5,6)P_5
		Ins(1,3,4,6)P_4	Ins(1,3,4,5,6)P_5

This pathway is initiated by the hydrolysis of PtdIns(4,5)P_2 by PLC to produce Ins(1,4,5)P_3, which is sequentially phosphorylated by the dual specific inositol polyphosphate multikinase (IPMK) to generate $InsP_5$. These enzymes have a reported 6/3-kinase activity and catalyze the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5,6)P_5 via Ins(1,4,5,6)P_4. In *Saccharomyces cerevisiae*, this enzyme referred to as Arg82 or IPK2 or IPMK is encoded by *ARGRIII* gene, and the loss of function mutant *argrIII* has a 100-fold decrease in $InsP_6$, and a 170-fold increase in cellular $InsP_3$ concentration, indicating reduced phosphorylation of $InsP_3$ to $InsP_6$. It was also reported that the mutant exhibited defects in nuclear mRNA export suggesting possible nuclear roles for $InsP_6$ in yeast (Odom et al., 2000; Saiardi et al., 2000a; York et al., 1999).

Studies in *Drosophilia melanogaster* showed that the DsIPK2 enzyme was also multifunctional having in addition to its 6/3-kinase activity, a 5-kinase activity towards Ins(1,3,4,6)P_4. DsIPK2

also complemented an *ipk2* mutant yeast restoring $InsP_6$ biosynthesis and *dmipk2* knockdowns had reduced $InsP_6$ levels (Seeds et al., 2004). Similar studies in rat cells reveal the presence of a dual-specific inositol polyphosphate kinase, and established the importance of rIPK2 in maintaining normal $InsP_6$ levels (Fujii & York, 2005).

In Arabidopsis, two IPK2 enzymes also regarded as inositol multiphosphate kinase (IMPK) are present (AtIPK2α and AtIPK2β). These enzymes are encoded by two distinct genes with the proteins sharing a 70% primary sequence identity between themselves and a 12% - 18% identity with the yeast and mammalian IPK2 respectively. The recombinant AtIPK2α and AtIPK2β enzymes like IPK2s from other species are multifunctional having a 6/3-kinase activity that can catalyze the conversion of $Ins(1,4,5)P_3$ to $Ins(1,4,5,6)P_4$ and $Ins(1,3,4,5,6)P_5$. An additional 5-kinase activity towards $Ins(1,3,4,6)P_4$ and $Ins(1,2,3,4,6)P_5$ was also reported for the plant enzymes (Stevenson-Paulik et al., 2002). Later studies also reported that *AtIPK2β* gene complemented a yeast *ipk2* mutant (Xia et al., 2003), and mutant *atipk2β* had a 35% reduction in $InsP_6$ in seed extracts (Stevenson-Paulik et al., 2005).

IPMKs belong to the inositol phosphate kinase superfamily which also consists of inositol triphosphate 3-kinases ($InsP_3$ 3-kinases) and inositol hexakisphosphate kinases (IP6Ks) (Holmes & Jogl, 2006). The crystal structure of Arabidopsis IPMK (IPK2α) is similar to the yeast IPK2 homologue as well as the binding pocket architecture of human $InsP_3$ 3-kinase despite the low sequence identity . The secondary and tertiary structure of the enzyme is reported to be similar to members of the ATP-grasp fold family (Endo-Streeter et al., 2012; Murzin, 1996), and the basis

for its substrate specificity was established. The enzyme was reported to discriminate between InsP species based on a combination of steric effects and the filling of specific phosphate binding pockets. Mutations of specific residues led to specificity restrictions in the Arabidopsis IPMK where its Ins(1,4,5)P_3 6-kinase activity remained intact but its Ins(1,4,5,6)P_4 3-kinase activity was reduced (Endo-Streeter et al., 2012). The role of multifunctional IPK2 in the lipid-dependent biosynthesis of $InsP_6$ is well established across multiple species, and its production of Ins(1,3,4,5,6)P_5 signifies the last divergent step in the lipid-independent and lipid-dependent pathway of $InsP_6$ biosynthesis.

With the biosynthesis of a single Ins(1,3,4,5,6)P_5 isomer from both the lipid-independent and lipid-dependent pathway, it is the general consensus that the conversion of Ins(1,3,4,5,6)P_5 to the fully phosphorylated $InsP_6$ is catalyzed by the inositol pentakisphosphate 2-kinase enzyme ($InsP_5$ 2-kinase or IPK1) (Shears, 2001; Sweetman et al., 2006). A single Arabidopsis gene, *AtIPK1*, encodes this enzyme and the biochemical characterization of the recombinant protein shows that the enzyme phosphorylates the 2-OH position of Ins(1,3,4,5,6)P_5 (Stevenson-Paulik et al., 2005). Loss of IPK1 function results in 83% reduction in seed $InsP_6$ levels and the combined loss of IPK1 and IPK2β resulted in ablation of seed $InsP_6$ emphasizing the importance of the gene in $InsP_6$ biosynthesis (Stevenson-Paulik et al., 2005). The Arabidopsis IPK1 enzyme was only 27% identical to the human IPK1 and 18% identical to the yeast ScIPK1, however, several residues were conserved among the proteins (Sweetman et al., 2006).

Apart from its physiological $InsP_5$ 2-kinase activity, minor catalysis was observed when IPK1 was incubated with Ins(1,3,4,6)P_4 generating an Ins(1,2,3,4,6)P_5 product (Stevenson-Paulik et al., 2005), and this activity together with the conversion of Ins(1,4,5,6)P_4 to Ins(1,2,4,5,6)P_5 was also reported for maize IPK1 in a different study (Stevenson-Paulik et al., 2005; Sun et al., 2007). Complementation assays also revealed that the *AtIPK1* gene was able to complement a yeast *Scipk1* mutant, restore $InsP_6$ levels, and rescue the mutant's temperature-sensitive growth phenotype (Sweetman et al., 2006). A common theme in the role of IPK1s from different species is their ability to complement and restore $InsP_6$ biosynthesis regardless of their low overall conservation (Shi, 2003; Verbsky et al., 2002).

The solved crystal structure of AtIPK1 has established the uniqueness of this inositol phosphate kinase enzyme in its capacity to phosphorylate the axial 2-OH position on the inositol ring, while other enzymes phosphorylate equatorial OH groups (González et al., 2010). It was reported that the AtIPK protein showed some remote similarities with the inositol polyphosphate kinase (IPK) family to which IPK2s belong, however, AtIPK1 houses a uniquely large InsP binding pocket containing an Asp368 residue that impacts recognition of the axial 2-OH position. Mutation of Asp368 results in an inactive enzyme (González et al., 2010).

To summarize, both pathways for $InsP_6$ biosynthesis involve several specialized kinases catalyzing specific steps of the pathway. The lipid-independent pathway, which is not completely characterized as yet, makes use of MIK, the ITPKs and yet to be determined inositol phosphate kinases that catalyze two missing steps in $InsP_6$ biosynthesis. The lipid-dependent pathway on

the other hand uses PLC, IPMK (IPK2) and IPK1 for $InsP_6$ biosynthesis. A list of the enzymes involved in each pathway, and their specificities are summarized in the table below.

Apart from the technical challenges involved in plant InsP research, a further complication in cataloguing the identity of all InsPs species present in plant cells arise due to lack of sufficient biochemical data on InsPs phosphatases capable of InsPs hydrolysis. It is therefore reasonable to assume that many more molecular players and InsP species will be identified in due course.

Intracellular Distribution of InsPs

Given the polar nature of these molecules, one would expect InsPs to be enriched primarily in the cytosol. There is limited information on the subcellular localization of InsP species present in eukaryotes. Recent work on the subcellular localization of GFP-fusion constructs of genes encoding enzymes (kinases) involved in InsP biosynthesis reveal a significant nuclear and cytoplasmic presence (Kuo et al., 2018; Xia et al., 2003; Adepoju et al., Submitted) . Localization of Arabidopsis AtIPK2α and AtIPK2β was reported in the cytoplasm and nucleus of mature pollen tube (Xia et al., 2003; Zhan et al., 2015). AtIPK1 has been reported to localize to the cytoplasm and nuclear envelope (Kuo et al., 2014; Lee et al., 2015) Our data also suggests that GFP fusion constructs of AtIPK2α, AtIPK2β, AtIPK1, AtITPK1 proteins and perhaps the InsP substrates ($InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$) they produce, are enriched primarily in the cytosol and nucleus of plant cells where they mediate various cellular processes (Adepoju et al. Submitted).

Phytases are phosphatases that hydrolyze $InsP_6$, and an earlier study of the localization of three phytase isoforms from maize root reported an enrichment in the endodermis (Hubel & Beck, 1996). MRP5 is an ABC-transporter involved in the transport of $InsP_6$ from the cytosol to the vacuoles where it is sequestered. MRP5 has been reported to be localized in the vacuolar membrane (Nagy et al., 2009).

Other studies have reported similar results of a nuclear and cytosolic localization for enzymes involved in InsP biosynthesis in non-plant organisms including PLC, IPK2 and IPK1 (Brehm et al., 2007; Odom, 2000; York et al., 1999). For example, FLAG-tagged Drosophila IPK2 (DmIPK2) localized to the nucleus suggesting DmIPK2-dependent $InsP_6$ biosynthesis is nuclear (Seeds et al., 2004), yeast IPK2 and IPK1 (ScIPK2, ScIPK1) were also reported to be predominantly nuclear (Odom et al., 2000).

Functions of InsPs in Plants

The signaling roles played by InsPs impacts several cellular processes intricately tied to plant growth and development. In animals, $Ins(1,4,5)P_3$ along with DAG are second messengers released upon PLC hydrolysis of $PtdIns(4,5)P_2$ in response to stimuli such as neurotransmitters, hormones and growth factors (Berridge, 1993). The cytosolic $Ins(1,4,5)P_3$ binds $InsP_3$ receptor causing a conformational change in the receptor, opening of $Ins(1,4,5)P_3$-sensitive calcium channels and release of Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER) into the cytosol (Berridge, 1993). Despite the debate surrounding the existence of a plant $Ins(1,4,5)P_3$

receptor, there have been reports of Ins(1,4,5)P_3 regulation of Ca^{2+} affecting downstream cellular processes (Gillaspy, 2013). An example is how transient increases in Ins(1,4,5)P_3 concentration upon abscisic acid (ABA) exposure causes a rise in intracellular levels of Ca^{2+} and a physiological change in guard cells (Gilroy et al., 1990; Lee et al., 1996). Other roles linked to Ins(1,4,5)P_3 in plants include drought tolerance, root hair development, gravitropism, oxidative stress signaling (Jones et al., 2006; Kaye et al., 2011; Khodakovskaya et al., 2010; Wang et al., 2009).

Genetic studies investigating the roles of genes involved in InsP biosynthesis and metabolism have highlighted the importance of this class of molecules on plant growth, development and physiology. Using loss of function mutant lines of Arabidopsis *IPK2α* and *IPK2β*, the roles of these genes in pollen development, pollen tube guidance and embryogenesis was established, suggesting a role for the enzyme products Ins(1,4,5,6)P_4 and or Ins(1,3,4,5,6)P_5 in these physiological processes (Zhan et al., 2015). The expression of *AtIPK2β* was detected in leaves, flowers, root and stem with no huge variation in expression level across tissues. β-glucuronidase (GUS) reporter experiment showed the expression of *AtIPK2β-GUS* in mature pollen but not in immature pollen grains and *AtIPK2β* also complemented a yeast loss-of-function *ipk2* (*arg82*) mutant (Xia et al., 2003). The expression of *IPK2α* was similarly reported across different tissues including roots, root hair, stem, leaf, pollen tubes, flowers and siliques (Xu, 2005). The expression of *GUS-IPK2α* was shown across different developmental stages in seedlings, siliques, flowers and persisted in pistil post fertilization (Xu, 2005). High *IPK2α-GUS* expression was seen in roots and pollen grains.

The use of *Atipk2α* and *Atitpk2β* single and double loss-of-function mutants demonstrated the functional redundancy of these genes in regulating male gametophyte and embryogenesis (Zhan et al., 2015). The single mutants showed no phenotype, however the *Atipk2α Atitpk2β* double mutant was defective in genetic transmission of male gametophyte, pollen tube development and embryogenesis (Zhan et al., 2015). It was also demonstrated that the phenotypic consequence of disrupting *AtIPK2α* and *AtIPK2β* is as a result of their kinase activity (Zhan et al., 2015). These findings suggest that Arabidopsis IPK2α and AtIPK2β and their InsPs catalytic products are important in normal plant growth and the different developmental transitions they undergo.

One interesting development in InsP signaling is their binding of plant hormone receptors and potential functional roles as cofactors regulating plant hormone signaling (Laha et al., 2015; Sheard et al., 2010; Tan et al., 2007). Auxin is an important plant hormone controlling various aspects of plant growth in response to developmental and environmental cues (Teale et al., 2006; Woodward, 2005). Auxin modulates gene expression by binding to the auxin receptor, transport inhibitor response 1 (TIR1) an F-box protein, and mediates the SCF ubiquitin-ligase-catalyzed proteolysis of AUX/IAA transcriptional repressors (Gray et al., 2001; Tan & Zheng, 2009).

The crystallization of Arabidopsis TIR1 protein complex identified a tightly bound $InsP_6$ in the leucine-rich repeat (LRR) domain of TIR1 and suggested $InsP_6$ as a specific structural and functional cofactor of the auxin receptor (Tan et al., 2007). Some of the observations reported in this study included the robust co-purification of $InsP_6$ with TIR1 complex suggesting a tight

binding interaction. Out of the ten positively charged TIR1 residues interacting with $InsP_6$, eight are highly conserved among Arabidopsis C3 subfamily of LRR-containing F-box proteins suggesting that $InsP_6$ binding is important for this subfamily of proteins. $InsP_6$ also interacts with several structural elements of TIR1 important for its function (Tan et al., 2007). Clearly, $InsP_6$ plays some role in auxin perception and signaling in plants and based on the important role auxin plays in plant growth and development, it is reasonable to imagine the pleiotropic effects that perturbation of cellular levels of $InsP_6$ will have on a plant.

A later study also identified another InsP molecule Ins(1,2,4,5,6)P_5 in the crystal structure of a homologous plant hormone- jasmonic acid (JA) co-receptor (Sheard et al., 2010). JA is a plant hormone that regulates different aspects of plant physiology including growth processes, normal developmental as well as defense responses to environmental and pathogenic stressors (Browse, 2009). The bioactive form of JA (JA-Ile) is generated upon specific conjugation to the amino acid isoleucine (Fonseca et al., 2009). Similar to the auxin receptor TIR1, the JA co-receptor complex houses an F-box protein -coronatine-insensitive 1 (COI1), and jasmonate zim domain (JAZ) which is a family of transcriptional repressors (Chini et al., 2007). Analogous to auxin signaling regulation of gene expression, JA regulation of gene expression involves the hormone-mediated binding and degradation of JAZ proteins, relieving JAZ repression of MYC2 and other transcription factors (Sheard et al., 2010).

The binding of Ins(1,2,4,5,6)P_5 to COI1-JAZ co-receptor is similar to $InsP_6$ binding to TIR1 in terms of the location of electron densities adjacent of the ligand-binding pocket, and the interaction with multiple positively charged residues in COI-JAZ (Sheard et al., 2010). The specificity and functional role of Ins(1,2,3,4,5,6)P_5 for COI1-JAZ binding was tested by stripping the co-purified $InsP_5$ from the COI1-JAZ protein complex, and testing the complex formation in a ligand-binding reconstitution assays. Formation of COI1-JAZ1 complex from the individual proteins was restored with Ins(1,2,4,5,6)P_5 in a dose-dependent manner suggesting that $InsP_5$ binding is crucial for the JA co-receptor formation (Sheard et al., 2010). Binding assays showed that Ins(1,2,4,5,6)P_5 has the highest affinity, and while Ins(1,4,5,6)P_4 will also support COI1-JAZ1 activity, Ins(1,4,5)P_3 and $InsP_6$ will not (Sheard et al., 2010). A similar binding study reported that PP-InsPs have a stronger binding affinity than InsP (Laha et al., 2015). The independent co-purification of two different InsPs [InsP(1,2,4,5,6)P_5 and $InsP_6$] which specifically bind to similar plant hormone receptors- COI1-JAZ1, TIR1 respectively suggests that InsPs might be intricately tied to hormone signaling in plants.

$InsP_6$ biosynthesis represents a unique point in the InsP biosynthesis pathway in plants. It is the most abundant InsP molecule in plants and several functions have been associated with genes involved in $InsP_6$ synthesis in plants. For example, Arabidopsis *IPK1* and *ITPK1* are InsP kinases involved in $InsP_6$ biosynthesis, both these genes have been implicated in normal leaf development and inorganic phosphate homeostasis (Kuo et al., 2014, 2018; Stevenson-Paulik et al., 2005). Whether or not the different phenotypes reported in studies involving disruption of genes in InsP metabolism is a direct result of perturbations in cellular levels of $InsP_6$, it is clearly evident that $InsP_6$ plays a significant role in normal plant growth and development. One

interesting role of $InsP_6$ that is gaining considerable attention is their use as precursors for the biosynthesis of PP-InsP, an emerging class of signaling molecules in eukaryotes.

Regulation of InsP

Regulation of the cellular concentrations of InsPs in plants is not well reported, however, it is reasonable to expect a tight control of the levels of these molecules given their diverse roles in signal transduction in plants. One plausible mechanism of controlling InsPs levels is the regulation of activity of enzymes involved in InsP metabolism. The plant membrane associated PI-PLCs are involved in the lipid-dependent $InsP_6$ biosynthesis. The activity and specificity of this class of enzymes can be regulated by cellular levels of Ca^{2+}, requiring micromolar amounts of Ca^{2+} for a preference for PtdIns(4,5)P_2 hydrolysis to generate $InsP_3$, and millimolar concentrations for a preference for PtdIns and PtdInsP hydrolysis (Drøbak, 1992; Helsper et al., 1987; Munnik et al., 1998). In non-plant eukaryotes, PI-PLC activation by heterotrimeric G-protein-coupled receptor (GPCR) has been reported (Rupwate & Rajasekharan, 2012), and a similar mechanism may exist in plants, based on the finding that recombinant Tobacco PI-PLC (NtPLC3) interact *in vitro* with a G-protein, NtRac5 (Helling et al., 2006).

Another connection between PI-PLC and GPCR was reported in a study showing that a putative GPCR1 enhanced DNA synthesis through activation of PI-PLC and synthesis of Ins(1,4,5)P_3 in Tobacco (Apone et al., 2003). In the lipid-independent pathway of $InsP_6$ biosynthesis in plants, Ins is a precursor for the biosynthesis of higher phosphorylated InsPs. The rate-limiting step of inositol biosynthesis is catalyzed by MIPS (Loewus & Loewus, 1980; Loewus et al., 1980). This

gene is an ideal target for regulating InsP levels and it was reported that the different MIPs genes in Arabidopsis is spatially and temporally regulated (Donahue et al., 2010) suggesting that the level of inositol, and invariably InsPs may vary depending on tissue type and developmental stage of a plant.

The presence of InsP-specific phosphatases capable of InsP hydrolysis ensures that the cellular concentration of these molecules is tightly controlled, and the cellular response they initiate is effectively turned off, however, there is limited information available on the plant phosphatases involved in InsP metabolism. It has been suggested in non-plant eukaryotes that Ins(1,3,4,5)P_4 can bind to, and modify the structure of the endoplasmic reticulum (E.R), regulating Ins(1,4,5)P_3 mediated Ca^{2+} release (Loomis-Husselbee et al., 1996). It was also reported that mammalian ITPKs are regulated by both phosphorylation and acetylation which altered the activity and stability of the protein (Zhang et al., 2012).

There have been reports of a strong connection between the P*i* metabolic status of a cell and regulation of genes involved in InsPs metabolism (Kuo et al., 2018; McKnight, 2003). The presence of an ABC transporter, MRP5 in plants that is able to transport $InsP_6$ across membranes (Nagy et al., 2009; Shi et al., 2007) suggests that this transporter is key to InsPs regulation, sequestering $InsP_6$ away from phytases that can hydrolyze the molecule under specific conditions. Taken together, it is evident that the regulation of steady-state cellular levels of InsPs depend on the transcriptional control of genes involved in InsPs metabolism, the localization and compartmentation of kinases and phosphatases that hydrolyze InsPs, as well as the activity and

stability of these enzymes. The maintenance of steady-state levels of these intracellular molecules is vital for their signaling roles which is triggered by a rapid and transient increase in their cellular concentration.

Inositol Pyrophosphates

The PP-InsPs are a unique class of molecules with emerging roles in signal transduction and cellular metabolism. These molecules are present in most eukaryotes including plants, and are considered “highly energetic” because of the presence of one or two diphosphate groups in addition to the multiple monophosphate groups around its inositol headgroup (Shears, 2015; for review, see Williams et al., 2015). The standard free energy of hydrolysis released upon cleavage of their diphosphate group is relatively high and comparable to that of ADP and ATP, however, a significant amount of cellular energy is also required for their biosynthesis (Stephens et al., 1993). These molecules have a low cellular abundance and high turnover rate as a consequence of coupled kinase/phosphatase cycles which is a common feature of signaling molecules (Draškovič et al., 2008). The cellular concentrations of PP-InsPs in yeast and mammals is in the 1-2 μM range (Ingram et al., 2003), however, it can be as high as 100-200 μM in slime molds during specific environmental challenges (Laussmann et al., 2000).

Given the highly dynamic nature of these molecules, a significant metabolic investment is necessary for maintaining their steady-state levels within the cell (Shears, 2015). Despite the accumulation of the PP-InsP precursor, $InsP_6$ in plant seeds, the identification of these class of molecules in plants is quite recent (Desai et al., 2014; for review, see Williams et al., 2015), and

the functional roles are just beginning to be determined. Most of the information available on PP-InsP metabolism, distribution, cellular function and mechanism of action come from extensive scientific work in non-plant eukaryotes (for review, see Shears, 2018), and plant scientists are trying to fill the knowledge gap that exists in PP-InsP metabolism.

PP-InsP Molecules and Enzymes Involved in their Biosynthesis

Since the discovery of the PP-InsPs over two decades ago in non-plant eukaryotes (Menniti et al., 1993; Stephens et al., 1993), a great deal of interest by the scientific community has culminated in the generation of a wealth of information about their biosynthesis and identity. PP-InsPs containing a total of 6 phosphates, 7 phosphates and 8 phosphates have been described (see **Figure 1.5**), which has led to their description as the most concentrated three-dimensional array of phosphate groups in nature (for review, see Shears, 2018). Only a few molecular species of PP-InsPs have been identified across eukaryotes, most originating from the phosphorylation of $InsP_6$ which represent an abundant pool of precursor for PP-InsPs. However, there have also been reports of PP-InsPs whose precursor is $InsP_5$ (for review, see Shears, 2018).

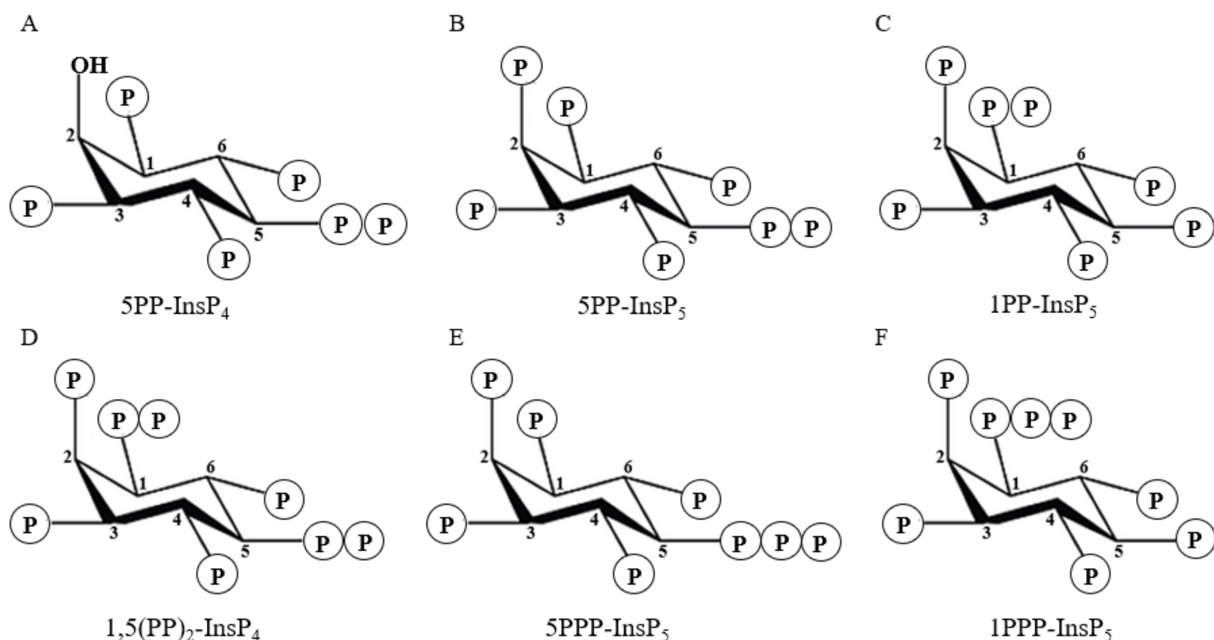

Figure 1. 5. A Few Naturally Occurring Inositol Pyrophosphate Molecules.

A, 5PP-InsP_4. B, 5PP-InsP_5. C, 1PP-InsP_5. D, 1,5(PP)$_2$-InsP_4. E, 5PPP-InsP_5. F, 1PPP-InsP_5**.**

The biosynthesis of PP-InsP from InsP_6 involves two classes of specialized and evolutionarily conserved kinases that catalyze the pyrophosphorylation of different positions on the inositol ring. The InsP_6 kinases (IP6Ks), also referred to as KCS1 in yeast, are encoded by three genes in the human genome (*HsIP6K1*, *HsIP6K2*, *HsIP6K3*) (Saiardi et al., 1999; Saiardi et al., 2001), a single gene in yeast (*KCS1*) and Dictyostelium. However, no plant IP6K homologue was identified using sequence homology searches (Bennett et al., 2006; Desai et al., 2014; Luo et al., 2003). The predominant enzymatic role associated with IP6Ks is the ATP-dependent phosphorylation of InsP_6 at the 5 position using their 5-kinase activity, to generate a 5-diphosphoinositol 1,2,3,4,6- pentakisphosphate (5PP- InsP_5) containing seven total phosphates (5 monophosphates and 1 diphosphate at C5), as well as the phosphorylation of 1-diphosphoinositol 2,3,4,5,6- pentakisphosphate (1PP- InsP_5) at the 5 position to generate 1,5-bis-diphosphoinositol

2,3,4,6-tetrakisphosphate [1,5-$(PP)_2$-$InsP_4$], containing eight total phosphates (4 monophosphates and 2 diphosphates) (Saiardi et al., 2000b; Saiardi et al., 1999; Saiardi et al., 2001; Schell et al., 1999; Voglmaier et al., 1996; for review, see Shears, 2018).

Other *in vitro* enzymatic activities have been reported for mammalian and Dictyostelium IP6Kinases, including the biosynthesis of two other isomers of PP-InsPs containing eight total phosphates; 5-triphosphoinositol 1,2,3,4,6-pentakisphosphate (5PPP- $InsP_5$) containing 5 monophosphates and one triphosphate, as well as the biosynthesis of 5,6-diphosphoinositol 1,2,3,4-tetrakisphosphate (5,6$[PP]_2$-$InsP_4$) (Draškovič et al., 2008; Laussmann et al., 1998). It has been suggested that there is little physiological relevance to these other enzymatic activities in most eukaryotes excluding Dictyostelium (Shears, 2009; for review, see Shears, 2018). The biosynthesis of 5-diphosphoinositol 1,3,4,6- tetrakisphosphate (5PP-$InsP_4$) from a minor IP6K substrate, $InsP_5$, has been described in yeast and mammals (Draškovič et al., 2008; Lin et al., 2009; Saiardi et al., 2000b), the product of the reaction was reported to undergo an additional phosphorylation step catalyzed *in vitro* by IP6K to generate 5PPP-$InsP_4$ (Draškovič et al., 2008). Interestingly, it has been reported that in addition to the phosphorylation of $InsP_6$, the *Entamoeba histolytica* IP6K (EhIP6KA) can also catalyze the phosphorylation of Ins(1,4,5)P_3 at the 6-OH position of the inositol ring (Löser et al., 2012). This plasticity in substrate use is similar to what holds in the IPMKs, which is not surprising given that the IP6Ks and InsP3Ks belong to the same enzyme superfamily that share a PxxxDxKxG ("PDKG") catalytic motif (Odom, 2000; Saiardi et al., 1999).

The crystal structure of EhIP6KA reveals that the use of such structurally different substrates in terms of size and charge is due largely to two structural elements in the binding pocket of the enzyme which enable a 55 degree rotation of bound Ins(1,4,5)P_3 relative to $InsP_6$ for establishing the necessary contacts with the protein (Wang et al., 2014). Clearly, the IP6Ks have a broad range of secondary substrates outside $InsP_6$ that could be phosphorylated *in vitro*, however the physiological significance of some of these activities remain to be established, except for a few reports which have linked the PP-InsPs generated from $InsP_5$ to maintenance of telomere length (Saiardi et al., 2005; York et al., 2005).

The second class of enzymes involved in the biosynthesis of PP-InsPs are referred to as the diphosphoinositol-pentakisphosphate kinase (PPIP5Ks), also known as the VIPs). These enzymes have a 1-kinase activity and their primary enzymatic activity is the ATP-dependent phosphorylation of 5PP- $InsP_5$ at the 1-position to generate 1,5$(PP)_2$-$InsP_4$, and a secondary substrate is $InsP_6$ which they are capable of phosphorylating to a lower extent to generate 1PP-$InsP_5$ containing a diphosphate group at the 1-position (Choi et al., 2007; Fridy et al., 2007; Lin et al., 2009; Mulugu et al., 2007; Weaver et al., 2013). 1PP- $InsP_5$ is considered to be the least abundant of any InsPs species in yeast and mammalian cells, with their steady state levels around 0.05 μM (for review, see Shears, 2018). This also supports the fact that $InsP_6$ phosphorylation by PPIP5Ks is a minor reaction in the PP-InsP biosynthesis pathway (Gu et al., 2016; Lin et al., 2009), and the 1PP-$InsP_5$ product is a minor route for the biosynthesis of 1,5$(PP)_2$-$InsP_4$ (for review, see Shears, 2018). There are two mammalian *PPIP5K* genes encoding large HsPPIP5K1 and HsPPIP5K2 enzymes of 160 kDa and 140 kDa molecular weight respectively (Choi et al., 2007; Fridy et al., 2007). *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*) have one orthologue, and two Arabidopsis genes (*AtVIP1 and AtVIP2*) encode

similar proteins (Desai et al., 2014; Fridy et al., 2007; Laha et al., 2015; Mulugu et al., 2007). Interestingly, each class of PP-InsP biosynthesis enzyme (IP6Ks and VIPs/PPIP5Ks) can phosphorylate the $InsP_7$ product of each other, to generate a $1,5(PP)_2$-$InsP_4$ product in mammals and yeast (Lin et al., 2009) (see **Figure 1.6**). The lack of an identifiable IP6K gene in plants suggests that either the $InsP_8$ in plants is a different isomer from what exists in yeast and mammals, or existing enzymes involved in InsP biosynthesis possess a yet to be identified enzymatic activity.

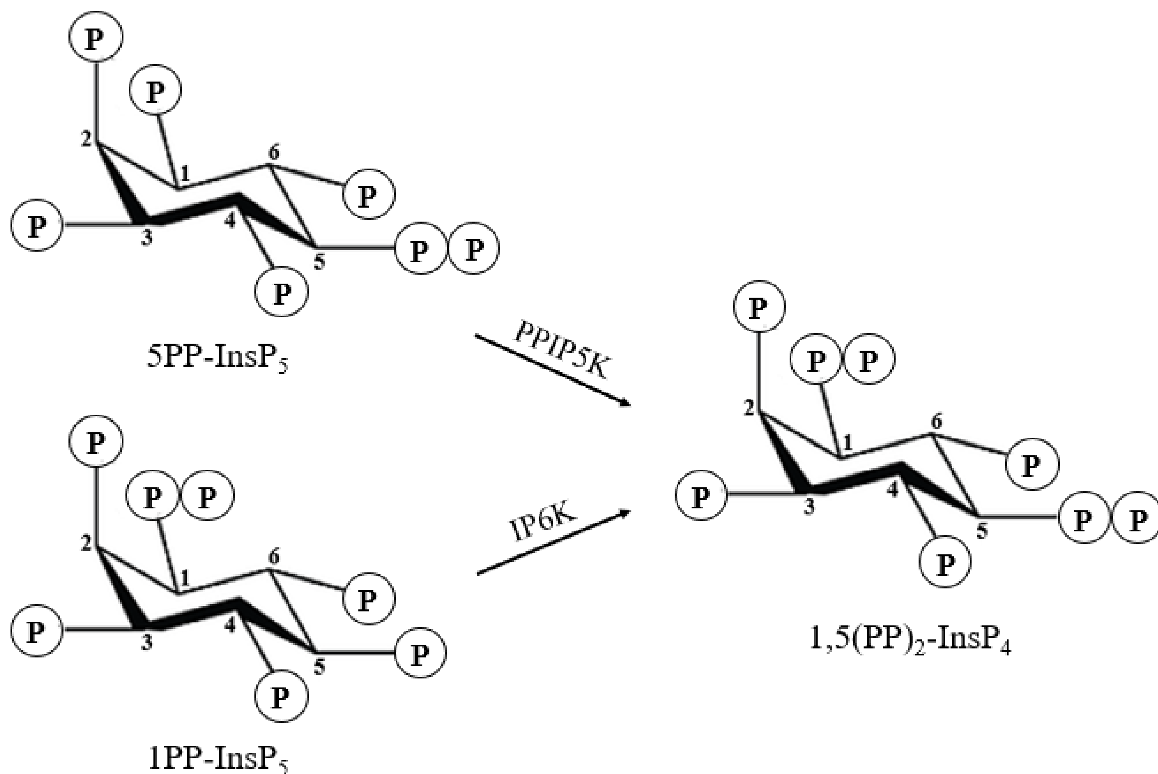

Figure 1. 6. Phosphorylation of Different Isomers of $InsP_7$ by IP6Ks and PPIP5Ks.

5PP-$InsP_5$ product of $InsP_6$ and HsIP6K can be phosphorylated by PPIP5Ks to generate $InsP_8$. Likewise, 1PP-$InsP_5$ product of $InsP_6$ and PPIP5K can be phosphorylated by HsIP6K to generate $InsP_8$.

Structurally, the IP6Ks belong to the inositol phosphate kinase family including IPMKs and IP3Ks with conserved N and C domains described as the ATP-binding domain of this family of proteins (Endo-Streeter et al., 2012; González et al., 2004; Holmes & Jogl, 2006; Miller & Hurley, 2004; Wang et al., 2014). The InsP binding site of the EhIP6K crystal structure is described as a shallow depression in the surface of the enzyme that is similar to an "open-clamshell" that is absent in other members of the family, and which allows its unique substrate binding (Wang et al., 2014).

In contrast to the IP6Ks, the overall structural fold of the PPIP5Ks was described as containing an N-terminal domain that binds ATP between two sets of anti-parallel beta-sheets referred to as the "ATP-grasp" domain (Fridy et al., 2007; Mulugu et al., 2007; Wang et al., 2011). Additional structural elements in the kinase domain include a sterically constraining pocket that restricts the entry of substrates other than 5PP-$InsP_5$ and $InsP_6$ into the active site of the enzyme, as well as a ligand binding site on the protein surface that is capable of catching and flipping a substrate into the active site of the enzyme (Wang et al., 2011; Wang et al., 2014). This unusual mechanism of reaction termed catch-and-pass is suggested to enhance the specificity of the enzyme.

Also, the presence of a phosphatase domain in this family of enzymes is a structural feature that has been reported (Desai et al., 2014; Fridy et al., 2007; Mulugu et al., 2007) (see **Figure 1.7**). The phosphatase domain is reported to catalyze the specific hydrolysis of the β-phosphate of 1PP- $InsP_5$ and 1,5$(PP)_2$-$InsP_4$ to generate $InsP_6$ and 5PP- $InsP_5$ respectively (Gu et al. 2017).

Given the uncommon dual domain architecture of this family of enzymes which has been reported in yeast, plants and mammals (Desai et al., 2014; Mulugu et al., 2007), as well as the known or predicted specificity of their kinase and phosphatase domains (1-kinase /1-phosphatase), there is interest in the interconversion of 5PP-InsP$_5$ and 1,5(PP)$_2$-InsP$_4$ as a type of substrate cycling. Investigation of the kinetic parameters of the kinase and phosphatase activity of mamm[illegible]ed tha[illegible]inase activity towards 5PP-InsP$_5$ than InsP$_6$, as well as a 4-fold higher maximum rate (Vmax) of phosphatase activity towards 1,5(PP)$_2$-InsP$_4$ than 1PP-InsP$_5$ (Gu et al., 2017). These observations suggest that substrate cycling through 5PP- InsP$_5$ and 1,5(PP)$_2$-InsP$_4$ is quantitatively more significant than cycling through InsP$_6$ and 1PP-InsP$_5$, and supports the idea that the major route of 1,5(PP)$_2$-InsP$_4$ biosy[illegible] and [illegible]abhan et al., 2009; Saiardi, 2012).

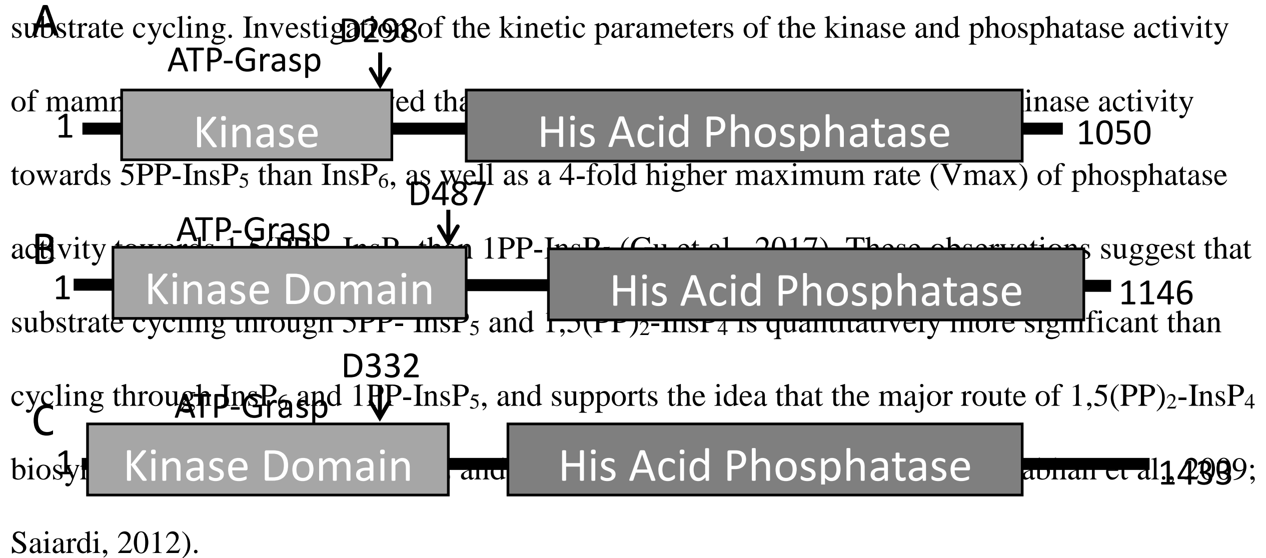

Figure 1. 7. Dual Domain Organization of PPIP5Kinases.

A, PPIP5K1 from *Arabidopsis thaliana* showing the kinase and phosphatase domain, with the catalytic Aspartate 298 residue depicted with an arrow. B, PPIP5K1 from *Saccharomyces cerevisiae*. C, PPIP5K1 from *Homo sapiens*.

Intracellular Distribution of PP-InsPs

In a study investigating the regulation of inositol biosynthesis in mammalian embryonic fibroblast cells, it was reported that the mammalian IP6K1 has a dual localization, cytoplasmic and nuclear, and that phosphatidic acid (PA) binds with high affinity to the enzyme, promoting its nuclear localization (Yu et al., 2016). It was also suggested that nuclear localization of IP6K1 and its kinase activity was important for regulation of gene expression (Yu et al., 2016).

A different study investigating the intracellular distribution of the mammalian IP6K isoforms reported a nuclear and cytoplasmic distribution for IP6K1, an exclusively nuclear localization for IP6K2 and an exclusively cytoplasmic localization for IP6K3, supporting nuclear and cytoplasmic roles for these enzymes and their PP-InsP product (Choi et al., 2007). In support of these observations, several studies have reported nuclear roles for PP-InsPs including regulation of non-homologous end joining repair of double strand DNA breaks (Hanakahi et al., 2000; Jadav et al., 2013; Luo et al., 2002), interferon-β-induced apoptosis is a nuclear process in which IP6K2 has been suggested to play a role (Morrison et al., 2001). Also, cytoplasmic roles have been reported for PP-InsPs including roles in vesicle trafficking (Saiardi et al., 2000b; Saiardi et al., 2002; Ye et al., 1995), supporting the cytoplasmic distribution of these molecules.

Subcellular localization of mammalian PPIP5K1 and PPIP5K2 fused with an N-terminal CFP and FLAG tag expressed in human embryonic kidney (HEK) cells showed that both enzymes

were restricted to the cytosol and excluded from the nucleus (Choi et al., 2007). It was noted in the study that the enzymes were expressed at a low level due to toxicity issues, this may have an influence on the limited distribution of the protein, however, they also reported a 30-fold and 8-fold increase in 1,5$(PP)_2$-$InsP_4$ concentration in HEK cells expressing PPIP5K1 and PPIP5K2 respectively (Choi et al., 2007).

In plants the expression pattern of Arabidopsis PPIP5Ks show that AtVIP1 was highly expressed in vegetative tissues including shoots of seedlings, mature leaves and stem. AtVIP2 on the other hand was mostly expressed in roots and reproductive tissues (Desai et al., 2014; Laha et al., 2015).

PP-InsP Function in Plants

Diverse cellular roles have been linked to PP-InsPs in non-plant eukaryotes, and the functional roles of this class of molecules is just emerging in plants. It has been suggested that the capacity of PP-InsPs to influence a diverse array of cellular processes stem from their actions, which is intricately tied to cell signaling and metabolism (for review, see Shears, 2018). In keeping with this idea, several studies have suggested that the metabolic status of a cell can influence the signaling activities of PP-InsPs, likewise, PP-InsPs can regulate cellular metabolism (Bhandari et al., 2008; Chakraborty et al., 2010; Saiardi et al., 1999; Szijgyarto et al., 2011; Voglmaier et al., 1996).

Genetic studies involving knockout or overexpression of genes involved in PP-InsP metabolism have helped in identifying the cellular roles played by these molecules. The targeted deletion of mice IP6K resulted in a loss of its kinase activity, ablation of 5PP-InsP$_5$ and 1,5(PP)$_2$-InsP$_4$ from cytosolic extracts and significantly reduced PP-InsPs in nuclear extracts from mouse embryonic fibroblasts (MEF) (Bhandari et al., 2008). This perturbation in cellular PP-InsP levels resulted in defects in spermatogenesis and male fertility, as well as a decrease in body weight as a result of 70% reduction in plasma insulin. The decrease in circulating insulin levels in *ip6k1* knockout is consistent with a role for PP-InsPs in vesicular insulin secretion. It is interesting to note that the study also reported a normal blood glucose level in *ip6k1* mutant mice regardless of being fed or fasted as a result of insulin hypersensitivity. This finding also supports a role for PP-InsPs in regulating metabolic homeostasis.

Similar studies of genetic knockout of *IP6K2* and *IP6K3* did not show overlapping phenotypes, with *ip6k2* mutant mice having normal insulin levels and were fertile (Morrison et al., 2009), while *ip6k3* mutant mice were also fertile and lower blood glucose (Moritoh et al., 2016). These findings suggest that these genes may be differentially or temporally regulated. The connection between PP-InsPs and cellular metabolism was also demonstrated in genetic study of human and mouse *IP6K3* which showed that both genes are highly expressed in muscle cells, and mouse *IP6K3* transcription was upregulated during fasting and diabetic conditions (Moritoh et al., 2016). It was also shown that a synthetic glucocorticoid, dexamethasone, which inhibits glucose metabolism in skeletal muscle cells upregulated human *IP6K3* transcription by 3.5-fold. In support of a cellular signaling role for mouse *IP6K3* and PP-InsPs, mouse *ip6k3* mutant

displayed reduced phosphorylation of a ribosomal protein in heart muscle relative to wild-type. This suggests a signaling effect as mouse *IP6K3* expression was restricted to skeletal muscle, and this event was suggested to contribute the extended lifespan of mouse *ip6k3* (Moritoh et al., 2016). These studies clearly demonstrate the influence of PP-InsPs on cellular processes, however, due to the use of multiple substrates by IP6Ks, the identity of PP-InsP species responsible for the observed phenotypes remain to be determined.

Regulation of PP-InsP

One mechanism that has been suggested for the regulation of cellular levels of PP-InsPs involves the inhibition of the kinase activity of human IP6K2 through its binding interaction with heat-shock protein (HSP90) (Chakraborty et al., 2008). It was reported in the study, that HSP90 specifically binds a 12-amino acid motif present in human IP6K2 but not IP6K1, and mutations of specific residues within the binding motif diminished or abolished the binding interaction. Quantitative analysis estimated a 4-fold increase in IP6K2 activity in HSP90 RNAi knockdown HEK cells, and overexpression of HSP90 significantly reduced $InsP_7$ levels, supporting a role for HPS90 in regulating the cellular level of PP-InsPs (Chakraborty et al., 2008).

In separate studies involving yeast *S. pombe* recombinant Asp1 protein, a functional PPIP5K enzyme, it was reported that the kinase domain of the enzyme was negatively regulated by its C-terminal phosphatase domain (Pöhlmann & Fleig, 2010; Wang et al., 2015). One of the studies used different variants of Asp1 protein expressed in different strains of *S. pombe*. A wild-type *S. pombe* strain housing both kinase and phosphatase domain (*asp1*$^{+}$), and mutant *S. pombe* strains:

asp1^{D33A} a kinase-dead mutant and *asp1*^{H397A} a kinase-only mutant (Pöhlmann & Fleig, 2010). It was reported that expression of these different mutants affected flocculation and cell-cell adhesion, with *asp1*^{H397A} showing constant flocculation, *asp1*$^{+}$ showing rare flocculation and *asp1*^{D33A} showing no flocculation. It was also shown that overexpression of Asp1 kinase domain (Asp1$^{1\text{-}364}$) or expression of Asp1^{H397A} resulted in increased flocculation, suggesting that the phosphatase domain plays a biological role and negatively regulates the kinase domain whose activity is required for cell-cell adhesion in *S. pombe*. It has been reported that, *in vitro*, the phosphatase domain in recombinant, full-length Asp1 is sufficient to reduce the amount of $InsP_7$ synthesized by the kinase domain (Pöhlmann et al., 2014), and a regulatory role was also proposed earlier for the C-terminal phosphatase domain of PPIP5Ks (Shears, 2009).

With the subsequent characterization of *S. pombe* Asp1 phosphatase domain (Asp1$^{371\text{-}920}$) as a phosphohydrolase that specifically hydrolyzes the 1-diphosphate from 1PP-$InsP_5$ and 1,5(PP_2)-$InsP_4$ (Wang et al., 2015), it was shown that the recombinantly purified phosphatase domain contained an incomplete iron-sulphur cluster (Fe-S) that inhibited its phosphatase activity and increased its kinase activity. Fe-S clusters are found in diverse proteins for structural stability, and they also play key roles in electron transport, enzyme catalysis, iron homeostasis and cellular regulation (Beinert, 1997; Johnson & Smith, 2006; Rouault, 2015). *In vitro* anaerobic reconstitution of a complete Fe-S cluster ($[2Fe\text{-}2S]^{2+}$) in the purified recombinant Asp1$^{371\text{-}920}$ resulted in a 95% lower phosphatase activity relative to the non-reconstituted purified enzyme, and the incubation of 5PP- $InsP_5$ with non-reconstituted Asp1 full-length enzyme in the presence of ATP, produced very little amount of 1,5$(PP)_2$-$InsP_4$ product relative to the reconstituted Asp1

full-length enzyme, which generated a considerable amount of 1,5$(PP)_2$-$InsP_4$ product (Wang et al., 2015). This data supports a role for Fe-S cluster in the negative regulation of the phosphatase domain of Asp1, which results in an unmasking of its kinase activity. This intricately tied connection between the activity of N-terminal kinase domain and C-terminal phosphatase domain of *S. pombe* points to a sophisticated mode of regulating the PP-InsPs levels in this organism.

The regulation of inositol biosynthesis in mouse embryonic fibroblasts by PP-InsP product of mammalian *IP6K1* has been reported (Yu et al., 2016). In this study, disruption of *IP6K1* led to increased transcription and altered DNA methylation of mammalian homologue of yeast *INO1* (*mIno1*) which catalyzes the rate-limiting synthesis of inositol. A similar study in yeast was reported, however PP-$InsP_4$ synthesized by Kcs1 positively regulated *INO1* transcription (Ye et al., 2013). It is plausible that the change in inositol levels reported in these studies directly or indirectly alters the cellular levels of PP-InsPs.

In plants, regulation of PP-InsPs levels is not yet well understood, and it remains to be determined if such a sophisticated regulatory mechanism exists. However, the presence of a putative phosphorylated serine residue adjacent to the phosphatase domain of Arabidopsis PPIP5Kinases has been predicted, and suggested as a possible regulatory mechanism for the plant enzymes (Desai et al., 2014). Also, it is speculated that the kinase and phosphatase domain of Arabidopsis PPIP5Kinases work in concert to regulate cellular PP-InsP levels, and control P*i* homeostasis (Zhu et al., 2018). Using different genetic constructs, it was shown that plants

constitutively overexpressing full-length AtVIP1 in wild-type background looked exactly like wild-type plants, however, overexpression of either a kinase-dead mutant ($AtVIP1^{KD/AA}$) or a phosphatase-dead mutant ($AtVIP1^{RH/AA}$) results in severe growth defects. This suggests that both domains are necessary for the proper plant growth. It was also reported that the shoot Pi levels in $AtVIP1^{KD/AA}$ is significantly higher than wild-type levels, while $AtVIP1^{RH/AA}$ has lower Pi levels relative to wild-type plants. This observation suggests that the kinase and phosphatase activity of AtVIP1 work antagonistically to maintain steady state P*i* levels in the plant (Zhu et al., 2018).

Incubation of purified recombinant full-length *Saccharomyces cerevisiae* PPIP5Kinase1 (ScVIP1) containing both kinase and phosphatase domains with 5PP-$InsP_5$ and varying concentrations of ATP, showed differential stimulation of each domain. At low ATP concentrations, the phosphatase activity predominates, generating $InsP_6$. At high ATP concentrations, the kinase activity of the enzyme predominates, producing 1,5$(PP)_2$-$InsP_4$. At intermediate ATP concentrations, neither the kinase nor phosphatase activity predominates, and there was no net production of $InsP_6$ or 1,5$(PP)_2$-$InsP_4$. The data suggests that cellular levels of ATP may influence the kinase and phosphatase activity of ScVIP1 in regulation of cellular levels of PP-InsPs.

From these studies, it is evident that cellular levels of PP-InsPs is tightly controlled by sophisticated mechanisms encompassing both the kinase and phosphatase domain of eukaryotic

PPIP5Kinases. However, more detailed mechanistic studies are required to fully understand how these signaling molecules are regulated and their cellular levels maintained.

Several years of research has expanded our knowledge and understanding of phosphoinositide signaling. Many signaling molecules have been identified, and the specific signaling event they modulate have been unraveled. A combination of genetic and biochemical approaches have been utilized in determining the roles of specific genes and gene products in the metabolism of phosphoinositide signaling molecules. Also, physiological approaches and analytical techniques have expanded our understanding of the signals that perturb cellular levels of these signaling molecules. Questions remain in our understanding of how phosphoinositide signaling is regulated; how the biosynthesis and turnover of these signaling molecules is controlled? how the different isomers of a molecule specifically perturb different cellular events? the role of PP-InsPs in plant cells? and more.

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CHAPTER II

"Inositol Trisphosphate Kinase and Diphosphoinositol Pentakisphosphate Kinase Enzymes Constitute the Inositol Pyrophosphate Synthesis Pathway in Plants"

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SUMMARY

Inositol pyrophosphates (PP-InsPs) are an emerging class of "high-energy" intracellular signaling molecules containing one or two diphosphate groups attached to an inositol ring, with suggested roles in bioenergetic homeostasis and inorganic phosphate (P*i*) sensing. Information regarding the biosynthesis of these unique class of signaling molecules in plants is scarce, however the enzymes responsible for their biosynthesis in other eukaryotes have been well described. Here we report the characterization of the two Arabidopsis VIP kinase domains, a newly discovered activity of the Arabidopsis ITPK1 and ITPK2 enzymes, and the subcellular localization of the enzymes involved in the synthesis of $InsP_6$ and PP-InsPs. Our data indicate that AtVIP1-KD and AtVIP2-KD act primarily as 1PP-specific Diphosphoinositol Pentakisphosphate Kinases (PPIP5) Kinases. The AtITPK enzymes, in contrast, can function as $InsP_6$ kinases, and thus are the missing enzyme in the plant PP-InsP synthesis pathway. Together, these enzyme classes can function in plants to produce PP-InsPs, which have been implicated in signal transduction and P*i* sensing pathways. We measured a higher

$InsP_7$ level (increased $InsP_7$/$InsP_8$ ratio) in *vip1/vip2* double loss-of-function mutants, and an accumulation of $InsP_8$ (decreased $InsP_7$/$InsP_8$ ratio) in the 35S:*VIP2* overexpression line relative to wild-type plants. We also report that enzymes involved in the synthesis of InsPs and PP-InsPs accumulate within the nucleus and cytoplasm of plant cells. Our work defines a molecular basis for understanding how plants synthesize PP-InsPs which is crucial for determining the roles of these signaling molecules in processes such as P*i* sensing.

SIGNIFICANCE STATEMENT

Inositol pyrophosphate signaling molecules are of agronomic importance as they can control complex responses to the limited nutrient, phosphate. This work fills in the missing steps in the inositol pyrophosphate synthesis pathway and points to a role for these molecules in the plant cell nucleus. This is an important advance that can help us design future strategies to increase phosphate efficiency in plants.

INTRODUCTION

Inositol phosphates (InsPs) are part of a chemical signaling language used by most eukaryotic organisms, including plants (for review, see Gillaspy, 2011). The InsP synthesis pathway makes use of a collection of inositol phosphate kinases that function to phosphorylate specific substrates (Shears and Wang, 2019), resulting in the production of InsP signaling molecules that convey signaling information within the cell (Irvine and Schell, 2001, Hatch and York, 2010, Shears et al., 2012). In plants, InsPs have been studied in connection with several different developmental processes (for review, see Gillaspy, 2011), auxin and jasmonic acid pathways via binding to receptors (Tan et al., 2007, Sheard et al., 2010, Laha et al., 2015), and more recently in inorganic phosphate (P*i*) sensing (Kuo et al., 2014, Wild et al., 2016, Kuo et al., 2018).

A unique subclass of InsP signaling molecules containing one or two diphosphate groups are gaining significant attention as important players in signal transduction and regulation of cellular metabolism (Shears, 2007, Shears, 2015, Williams et al., 2015). These molecules are collectively named Inositol Pyrophosphates (PP-InsPs), and are considered "high energy" signaling molecules due to the considerable amount of free energy released upon hydrolysis of their pyrophosphate groups (Stephens et al., 1993). PP-InsPs are derived from phosphorylation of inositol hexakisphosphate ($InsP_6$), one of the most abundant InsPs in the plant cell **(see Figure 2.1)** (Raboy, 2003), and a major phosphate sink in plant seeds (i.e. as phytate) (Raboy, 2007). The most common PP-InsPs contain 7 or 8 phosphates and hence are referred to as $InsP_7$ and $InsP_8$ (Shears, 2015).

We previously showed that plants synthesize and accumulate both $InsP_7$ and $InsP_8$, and along with others identified the Arabidopsis *Vip/Vih* genes as capable of complementing yeast mutants defective in PP-InsP synthesis (Desai et al., 2014, Laha et al., 2015). The Arabidopsis genome contains two *Vip/Vih* genes (Desai et al., 2014, Laha et al., 2015) that encode enzymes predicted to function as Diphosphoinositol Pentakisphosphate Kinases (PPIP5Ks) (Choi et al., 2007). The Arabidopsis VIP1 and VIP2, share 94% similarity at the amino acid level (Desai et al., 2014). AtVIP1 share a 59% similarity with the human VIP1 (Fridy et al., 2007), and 50% similarity with the yeast VIP1 (Mulugu et al., 2007) respectively. Additionally, they share a conserved dual domain architecture housing an N-terminal ATP-grasp kinase domain and a C-terminal histidine phosphatase domain (Mulugu et al., 2007). VIPs phosphorylate 5PP-Ins(1,2,3,4,6)P_5 at the 1-position, resulting in a 1-pyrophosphate group (1PP) (Lin et al., 2009, Wang et al., 2011). In other organisms, the conversion of $InsP_6$ to $InsP_7$ is catalyzed by $InsP_6$ Kinases that form a pyrophosphate at the 5-position (5PP) (Saiardi et al., 1999, Saiardi et al., 2001, Draskovic et al.,

2008). Since all plant genomes studied to date do not contain genes predicted to encode $InsP_6$ Kinases, and the *AtVip* genes can restore mutant yeast synthesis of $InsP_7$ (Desai et al., 2014, Laha et al., 2015), we previously hypothesized that the AtVIPs catalyze the two penultimate synthesis steps in the pathway, converting $InsP_6$ to $InsP_7$, and $InsP_7$ to $InsP_8$ (Desai et al., 2014).

Given the importance of the PP-InsPs in pathways such as P*i* sensing (Azevedo and Saiardi, 2017, Jung et al., 2018), we sought to characterize the biochemistry and subcellular locations of AtVIP1 and AtVIP2, and other enzymes acting in this pathway. To do this, we established a system to enzymatically produce substrates in the pathway, and used recombinant enzymes *in vitro* to show that the VIPs and another class of enzymes, the Inositol Trisphosphate Kinases (ITPKs) (Abdullah et al., 1992, Shears, 2009), most likely act in concert to catalyze the last two reactions in the PP-InsP synthesis pathway. InsP profiling experiments of a *vip1/vip2* double mutant and a VIP2 overexpressor (OX) line support these roles. We also investigated the subcellular distribution of many enzymes involved in the PP-InsP synthesis pathway, by using a transient expression system and confocal imaging. It has been proposed that regulation of PP-InsP synthesis may be specific for different developmental stages and/or takes place in different subcellular compartments within the cell. Our results suggest that the entire PP-InsP synthesis pathway may function in the nucleus and cytoplasm, and provides a new paradigm for understanding this critical signaling pathway.

RESULTS

Purification of AtVIP1-KD and AtVIP1-KD Recombinant Proteins

To determine the substrate preference of the AtVIP enzymes, we sought to express AtVIP full-length recombinant proteins in *E. coli.* As has been the case with other vertebrate VIPs, this approach was not successful in producing intact, soluble protein. We then opted to express and purify recombinant proteins corresponding to residues 1-361 for AtVIP1, and residues 1-362 for AtVIP2 as Glutathione S-Transferase (GST) fusions. These regions each contain a RimK/ATP-grasp domain, which is predicted to encode the VIP kinase domain (KD) (Fridy et al., 2007, Mulugu et al., 2007). Recombinant proteins were expressed and purified by affinity chromatography. SDS-PAGE analysis of these purified fusion proteins reveals the expected sizes of 68.6 kDa and 68.7 kDa for the AtVIP1-KD and AtVIP2-KD, respectively **(Figure 2.12A)**. We also identified lower molecular weight proteins that immuno-reacted with the anti-glutathione (α-GST) antibody that we predict to be free GST or breakdown products **(Figure 2.12B)**.

Substrate Synthesis for VIP Activity Assays

To test the inositol phosphate kinase activity of recombinant AtVIP1-KD and AtVIP2-KD fusion constructs, we utilized an *in vitro* substrate synthesis system with purified enzymes and commercially available [^{3}H]Ins(1,4,5)P_3, which has been described and used by others (Fridy et al., 2007, Mulugu *et al.*, 2007, Weaver *et al.*, 2013). We expressed and purified recombinant fusion proteins with inositol phosphate kinase activities including Inositol Polyphosphate Multikinase 2 alpha (AtIPK2α) (Stevenson-Paulik et al., 2002), Inositol Pentakisphosphate 2-

kinase (AtIPK1) (Stevenson-Paulik et al., 2005), the human Inositol Hexakisphosphate Kinase 1 (HsIP6K) (Saiardi et al., 1999), and the kinase domain of the human PPIP5K (HsVIP1-KD) (Fridy et al., 2007) **(Figure 2.1)**. Specifically, we incubated commercial [^{3}H]Ins(1,4,5)P_3 with purified AtIPK2α, resulting in the production of [^{3}H]Ins(1,3,4,5,6)P_5 **(Figure 2.2A)**. This product was incubated with AtIPK1, resulting in the formation of [^{3}H]InsP_6, **(Figure 2.2B)**. For synthesis of PP-InsPs, we incubated [^{3}H]InsP_6 with HsIP6K1, to generate [^{3}H]5PP-InsP_5 (herein referred to as 5-InsP_7) **(Figure 2.2C)**. To generate [^{3}H]1PP-InsP_5 (herein referred to as 1-InsP_7), we incubated the HsVIP1KD with [^{3}H]InsP_6 . To generate [^{3}H]1,5-PP_2-InsP_4 (InsP_8), we used reactions containing 5-InsP_7 with HsVIP1-KD **(Figure 2.2D)**.

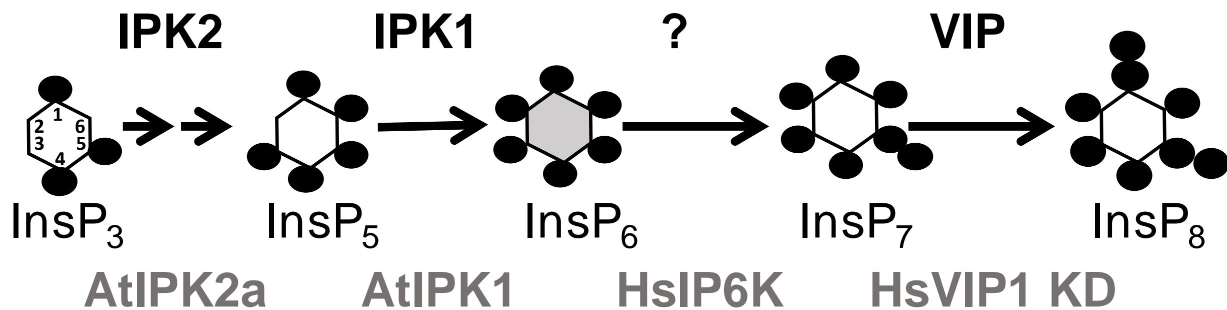
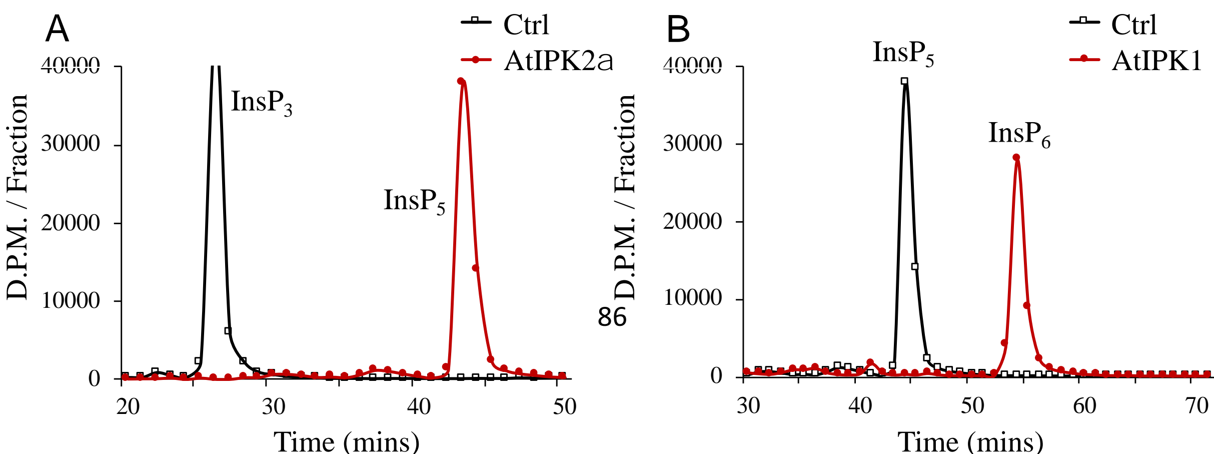


Figure 2. 1. PPx-InsP Synthesis Pathway.

Schematic representation of PPx-InsP synthesis pathway starting from Ins(1,4,5)P_3. Locants are given for Ins(1,4,5)P_3 and other molecules are similarly oriented. Enzymes (in black) on top function in the plant synthesis pathway: IPK2 = Inositol Polyphosphate Multikinase, IPK1 = Inositol Pentakisphosphate 2-Kinase, VIP or PPIP5K = Diphosphoinositol Pentakisphosphate Kinase. The question marks denotes the lack of an identified Inositol Hexakisphosphate Kinase (IP6K) enzyme in plants. Note that the structure of InsP_7 and InsP_8 from plants has not yet been determined. Synthesis of substrates for this study are on the bottom in gray.



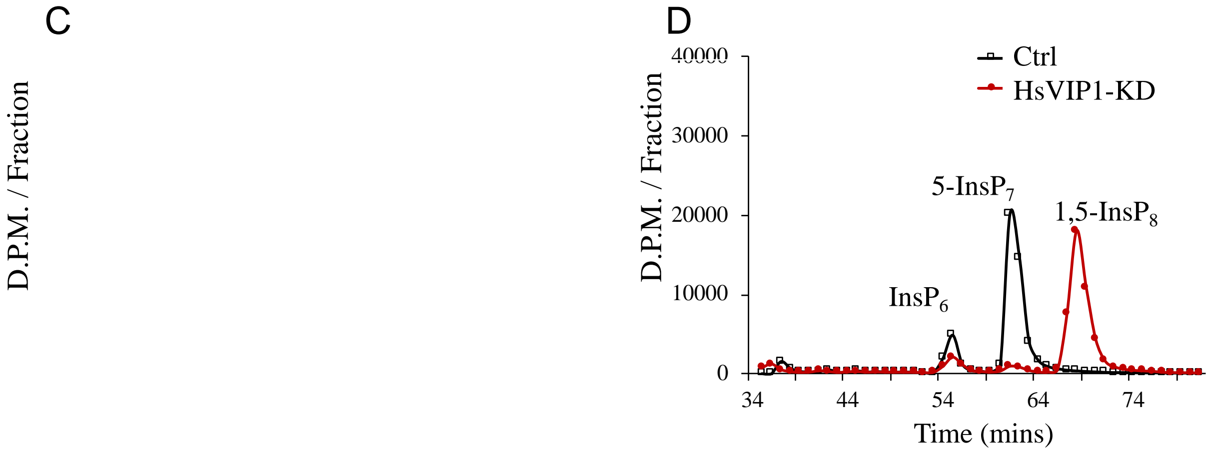
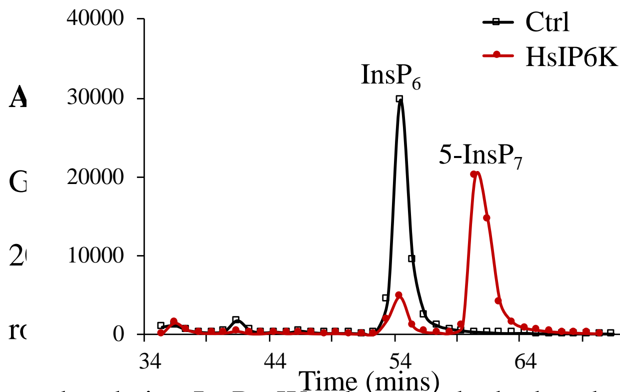


Figure 2. 2. Sequential Synthesis of Substrates for Enzyme Assays.

A, Incubation of 0.13 µM containing 45,000 total CPM of commercial $[^3H]Ins(1,4,5)P_3$ with either buffer (Ctrl; open squares) or 5 µg recombinant fusion AtIPK2α (red circles) in a 100 µL reaction volume at 37°C for 60 minutes. B, Enzymatically synthesized $[^3H]Ins(1,3,4,5,6)P_5$ from panel A containing 44,000 CPM was incubated with either buffer (Ctrl; open squares) or 5 µg recombinant fusion AtIPK1 (red circles) for 60 minutes. C, Incubation of enzymatically synthesized $[^3H]InsP_6$ from panel B containing 37,000 total CPM with buffer (Ctrl, open squares) or 35 µg of recombinant fusion HsIP6K1 (red circles) for 90 minutes. D, Incubation of enzymatically synthesized $[^3H]5\text{-}InsP_7$ containing 37,000 total CPM from panel C with either buffer (Ctrl, open squares) or 7.6 µg of recombinant fusion HsVIP1-KD for 90 minutes. All reactions were terminated by heat-inactivation of enzymes at 90°C for 3 minutes followed by HPLC analysis of products. Data shown are representative of three independent syntheses. Note that small reductions in the total CPM in the product relative to the substrate is due to handling and pipetting errors.



A -KD are Functional VIP/PPIP5Kinases

G thought to act primarily on $5\text{-}InsP_7$ (Choi et al., 2 P1-KD and AtVIP2-KD with $5\text{-}InsP_7$ and found r on time of the product is consistent with the product being $InsP_8$. We also tested whether the AtVIP-KDs could phosphorylate $InsP_6$ as well as $5\text{-}InsP_7$. Under our *in vitro* assay conditions, AtVIP1-KD and AtVIP2-KD show very little

kinase activity towards $InsP_6$, even with an extended, 90 minutes incubation **(Figure 2.4C, D)**. We note that sometimes these extended reactions did result in 3-4% substrate conversion to $InsP_7$ with both AtVIP1-KD and AtVIP2-KD.

We next tested whether 1-$InsP_7$ was a substrate for purified recombinant AtVIP1-KD and AtVIP2-KD. Neither enzyme effectively converted 1-$InsP_7$ to a more phosphorylated form **(Figure 2.5B,C)**, suggesting that AtVIP1-KD and AtVIP2-KD are functional enzymes that phosphorylate the 1-position of 5-$InsP_7$ (5PP-$InsP_5$) yielding an $InsP_8$ product. We also tested whether the AtVIP-KDs could phosphorylate $InsP_5$ or 5PP-$InsP_4$, and found neither of these molecules are phosphorylated by the AtVIPs **(Figures 2.13 and 2.14)**. Together our data on substrate preference of both AtVIP-KDs indicates that their activity is similar to VIP/PPIP5 Kinases characterized from other organisms in that *in vitro*, they robustly phosphorylate 5-$InsP_7$.

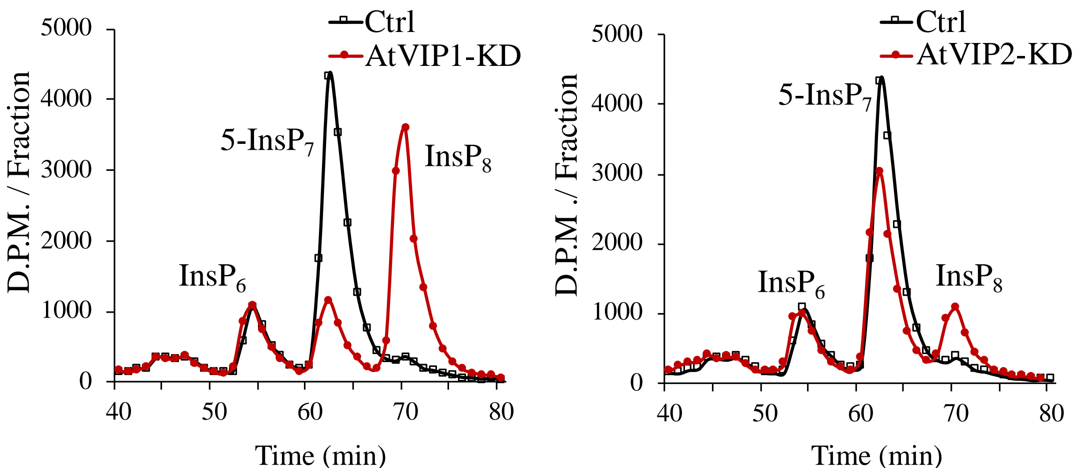


Figure 2. 3. PPIP5 Kinase Activity of AtVIP1-KD and AtVIP2-KD.

Enzymatically synthesized 5-$InsP_7$ containing 18,000 total CPM from commercial $[^3H]InsP_6$ and recombinant HsIP6K was incubated with A, Buffer (Ctrl, open squares) or 7.6 µg of AtVIP1-KD

(red circles). B, Buffer (Ctrl, open squares), or 7.6 µg of AtVIP2-KD (red circles). Reaction was incubated at 37°C for 30 minutes. All reactions were terminated with the addition of 2.5N HCl, and the reaction products were separated on an anion exchange HPLC. Data shown are representative of two independent replicates for each panel.

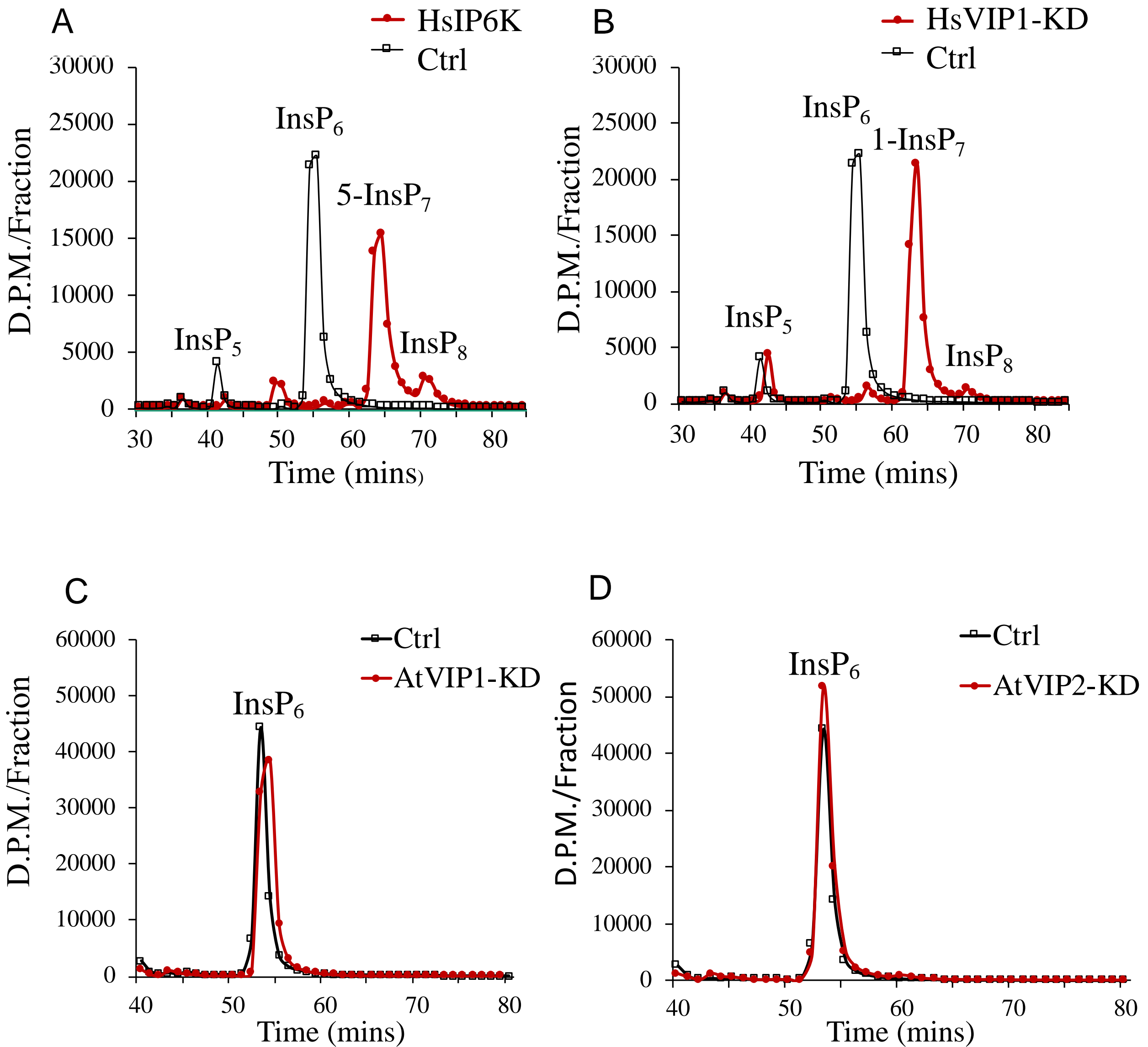


Figure 2. 4. IP6Kinase Activity Assays.

A, $[^3H]InsP_6$ containing 47,000 total CPM enzymatically synthesized from commercial $[^3H]Ins(1,4,5)P_3$ using purified recombinant AtIPK2α and AtIPK1 was incubated with either buffer (Ctrl, open squares) or 7.6 µg of purified recombinant HsIP6K (red circles). B, Buffer (Ctrl, open squares) or 7.6 µg of purified recombinant HsVIP1-KD (red circles). C, Commercial $InsP_6$ containing 72,000 total CPM was incubated with either buffer (Ctrl, open squares) or 7.6 µg of purified recombinant AtVIP1-KD (red circles). D, Buffer (Ctrl, open squares) or 7.6 µg of purified recombinant AtVIP2-KD (red circles). All reactions were incubated at 37°C for 90 minutes, and reactions were terminated by heat-inactivation of enzyme at 90°C for 3 minutes

followed by HPLC analysis of products. Data shown are representative of three independent replicates.

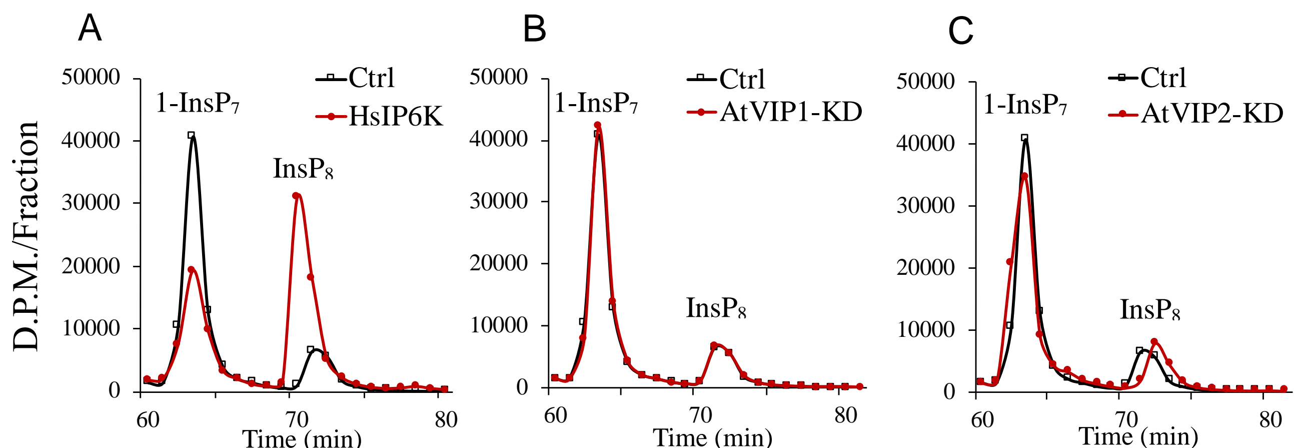


Figure 2. 5. Lack of Kinase Activity of Recombinant AtVIP1-KD and AtVIP2-KD with 1-InsP$_7$.

Enzymatically synthesized 1-InsP$_7$ containing 198,000 total CPM from commercial [^{3}H]InsP$_6$ and 19 µg purified recombinant HsVIP1-KD was incubated with A, Buffer (Ctrl, open squares) or 19 µg purified recombinant HsIP6K1 (red circles). B, buffer (Ctrl, open squares) or 7.6 µg purified recombinant AtVIP1-KD (red circles). C, Buffer (Ctrl, open squares) or recombinant AtVIP2-KD (red circles). All reactions were incubated at 37°C for 90 minutes, terminated by incubation at 90°C for 3 minutes; reaction products were analyzed using an anion exchange HPLC. Data shown are representative of two independent replicates.

Arabidopsis ITPK1 and ITPK2 have IP6K Activity

Since our characterization of the AtVIP-KDs indicates that they are not capable of generating InsP$_7$, this begs the question of how InsP$_7$ is synthesized in plants. Plant genomes only contain VIP homologues and are missing a bonafide InsP6K gene (Desai et al., 2014). Given this, we sought to identify a novel plant enzyme capable of converting InsP$_6$ to InsP$_7$. We reasoned that such a gene would encode a protein with known inositol phosphate 5-kinase homology. This narrowed the spectrum of our focus to the inositol polyphosphate kinase 2 enzymes which are

known 6-/3-/5-kinases (Stevenson-Paulik et al., 2002), and the Arabidopsis inositol (1,3,4) triphosphate 5-/6-kinases (ITPKs) (Wilson and Majerus, 1997, Sweetman et al., 2007). There are four AtITPK genes encoded in the Arabidopsis genome (AtITPK1, AtITPK2, AtITPK3, AtITPK4) (Wilson and Majerus, 1997, Sweetman et al., 2007), and four characterized genes from soybean as well (Stiles et al., 2008). These gene products, along with a potato ITPK (Caddick et al., 2008), encode ATP-grasp domain containing proteins that are very structurally similar to the kinase domain of the human PPIP5K, even though they have limited sequence identity (Wang et al., 2011).

To test whether the AtITPKs can phosphorylate $InsP_6$, we cloned and expressed AtITPK1 and AtITPK2 as recombinant 6X-histidine fusion proteins in *E. coli* **(Figure 2.12A)**. 6X-His-tagged versions of AtITPK1 and AtITPK2 were incubated with $[^3H]InsP_6$, resulting in near complete conversion of substrate to a product more phosphorylated than $InsP_6$ **(Figure 2.6A, B)**. We next sought to test if the AtITPK1-generated $InsP_7$ product could be a substrate for our purified recombinant AtVIP-KDs. We incubated the AtITPK1-generated $InsP_7$ product with AtVIP1-KD, and found that the $InsP_7$ could be phosphorylated to $InsP_8$ **(Figure 2.6C)**. Our results support a role for AtITPK1 and AtITPK2 in phosphorylating $InsP_6$, and suggest that the AtITPKs function in place of the missing IP6K in plants.

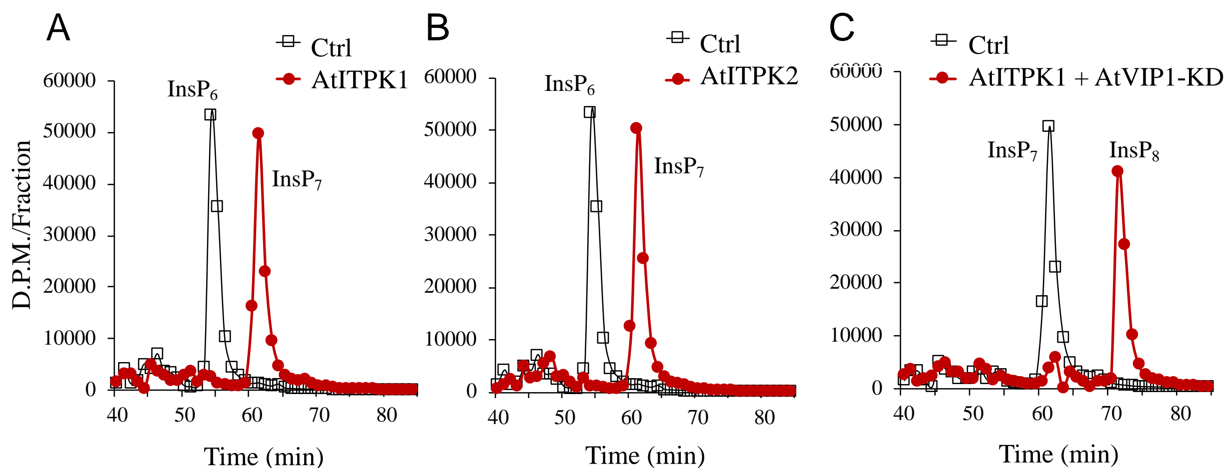


Figure 2. 6. IP6Kinase Activity of AtITPK1 and AtITPK2.

A, Commercial [^{3}H]InsP$_6$ containing 110,000 total CPM was incubated with buffer (Ctrl, open squares) or 7.6 µg recombinant AtITPK1 (red circles). B, Incubation with buffer (Ctrl, open squares) or 7.6 µg recombinant AtITPK2. C, Reaction product of commercial [^{3}H]InsP$_6$ and AtITPK1 was incubated with buffer (Ctrl, open squares) or 7.6 µg recombinant AtVIP1-KD (closed circles) All reactions were incubated at 37°C for 90 minutes followed by termination of reaction by heat inactivation of the enzyme at 90°C for 3 minutes. Reaction products were separated by anion exchange HPLC. Data shown are representative of two independent replicates.

VIP Genes Impact Inositol Pyrophosphate Accumulation

To determine if the plant *VIP* genes impact the levels of inositol pyrophosphates in plants, we isolated multiple genetic mutants in both *vip1* (*vip1-1* GK_204E06; *vip1-2* GK_008H11) and *vip2* (*vip2-1* SALK_094780*; vip2-2* SAIL_175_H09) and crossed parental lines, resulting in three unique *vip* double mutants: *vip1-1/vip2-1*, *vip1-1/vip2-2*, and *vip1-2/vip2-2*. We next performed quantitative Real-Time PCR and determined that *vip1-2/vip2-2* double mutants showed the most significant reduction in expression of *VIP1* and *VIP2* of all our double mutant lines (**Figure 2.15**). We also generated a VIP2-Green Fluorescent Protein (GFP) fusion construct driven by a 35S CaMV constitutive promoter (*VIP2 OX*), and showed that *VIP2 OX* lines

accumulate an app. 146 kDa protein **(Figure 2.15)**. We used [^{3}H]-*myo*-inositol labelling of *vip1-2/vip2-2 and VIP2 OX* seedlings to quantify higher InsPs and PP-InsPs in these plants. HPLC analyses of extracts from WT, *vip1-2/vip2-2* and *VIP2 OX* plants revealed different enantiomers of $InsP_4$ and $InsP_5$, and a single peak corresponding to $InsP_6$, $InsP_7$ and $InsP_8$. We found that *vip1-2/vip2-2* mutants have an elevation in $InsP_7$ as compared to WT seedlings, such that the $InsP_7/InsP_8$ ratio is 140% of the WT value **(Figure 2.7)**. We also determined that *VIP2 OX* plants contain an increase in $InsP_8$ levels relative to *WT,* such that the $InsP_7/InsP_8$ ratio is 69% of the *WT* value **(Figure 2.7A, B)**. We conclude that knock down of expression of both *VIP1* and *VIP2* genes in *vip1-2/vip2-2* mutants reduces VIP-catalyzed conversion of $InsP_7$ to $InsP_8$ *in vivo*. Conversely, overexpression of *AtVIP2* elevates $InsP_8$ levels.

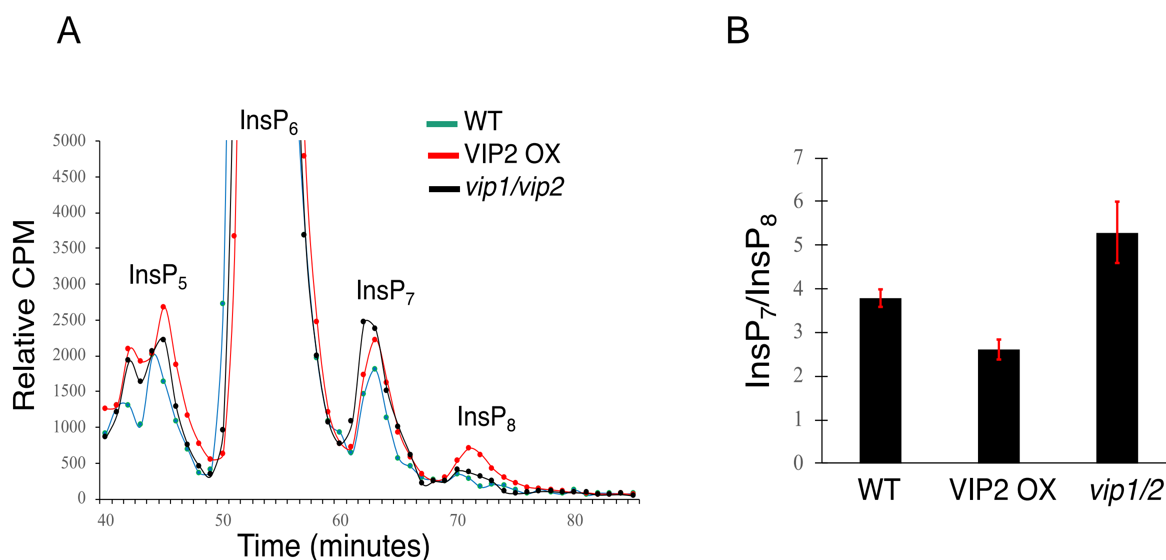


Figure 2. 7. Inositol Phosphate Profiling of WT, VIP2 OX and *vip1-2/2-2* mutants.

A, WT *Arabidopsis thaliana*, *AtVIP2-OE* and *vip1-2/vip2-2 (X3)* double knock-out mutant seeds were grown on semi-solid 0.5X-MS media containing 0.2% agar for 14 days and 100 μCi [^{3}H]-*myo*-inositol was added for 4 days. Inositol phosphates were extracted and analyzed on an anion exchange HPLC. B, Graph showing ratio of $InsP_7$ to $InsP_8$ in the different genetic constructs. The experiment was performed two times; standard error is shown. Note that the total counts in the fractions representing $InsP_8$ in *vip1-2/vip2-2* double mutant is higher than WT.

Localization of PP-InsP Pathway Enzymes

To determine which cellular compartments accumulate the enzymes required for PP-InsP synthesis, we constructed 35S CaMV promoter-GFP fusion proteins for each enzyme in the pathway shown in **Figure 2.1**. We transiently expressed these constructs in *N. benthamiana* leaf epidermis and assessed fusion protein integrity **(Figure 2.16)**, followed by confocal imaging to localize the fusion proteins **(Figures 2.8-2.11)**. A time course of IPK2α-GFP, IPK2β-GFP, and IPK1-GFP expression revealed these fusion proteins accumulate in the nucleus and cytoplasm **(Figure 2.8)**. All three of these fusion proteins were excluded from the nucleolus **(Figure 2.8)**. The pattern of nuclear localization is consistent over a time course of 24-72 hours post infiltration, suggesting that sequential phosphorylation of Ins(1,4,5)P_3 resulting in newly synthesized $InsP_6$ could occur in the nucleus.

The subcellular localization of AtITPK1, AtVIP1, and AtVIP2 was assessed by transiently expressing C-terminal GFP-tag fusion proteins in tobacco leaves and confocal microscopy. Given the bipartite nature of catalytic domains in the AtVIPs, we also expressed the kinase domain (KD) with a C-terminal GFP tag, and the phosphatase domain (PD) with an N-terminal GFP tag. Cells were imaged at 12, 36, 24, 48 and 72 hours post infiltration to ensure observed patterns were not a consequence of overexpression or off-target accumulation (example time courses are shown in **Figures 2.17 and 2.18**. GFP fluorescence is not detectable at 12 hours post

infiltration under our conditions, and at the 72-hour time point the signal from many of the GFP constructs is beginning to decrease **(Figure 2.18)**. To visualize the cytoplasm or the endoplasmic reticulum (ER), the GFP constructs were co-infiltrated with plasmids encoding unconjugated mCherry or ER-mCherry (PMID 17666025).

For all the GFP constructs investigated here, we observed extensive colocalization with mCherry and partial colocalization with ER-mCherry **(Figures 2.9-2.11)**, indicating a major presence in the cytoplasm, and a smaller amount in the ER. Similarly, we observed localization in the nucleus for AtITPK1-GFP, AtVIP1-KD and AtVIP2-KD, but not for AtVIP1, AtVIP1-PD, AtVIP2, and AtVIP2-PD **(Figures 2.9-2.11)**.

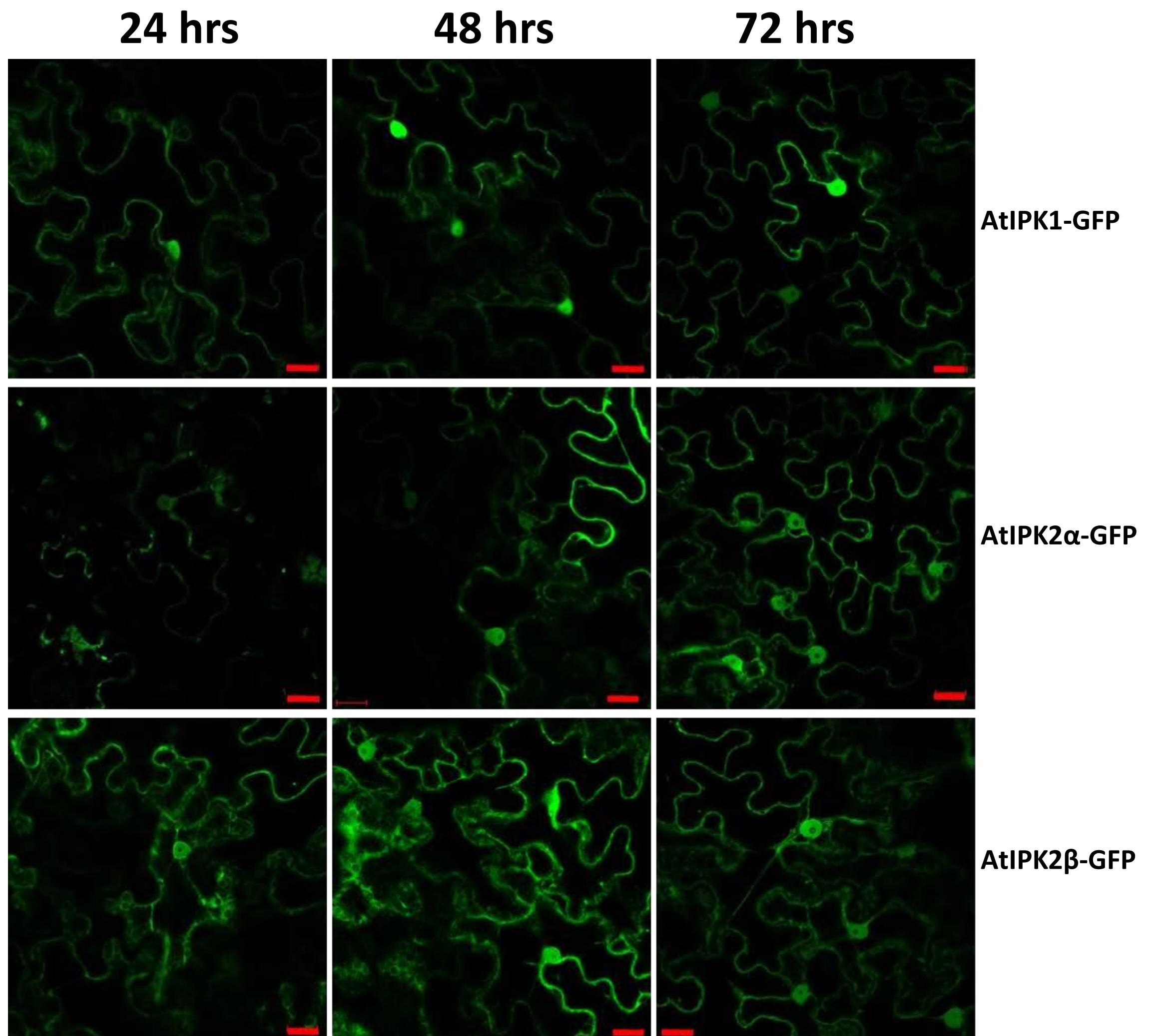


Figure 2. 8. Time Course of IPK1, IPK2α, IPK2β-GFP Expression.

IPK1-GFP, IPK2α-GFP, and IPK2β-GFP fusion proteins were transiently expressed in *N. benthamiana*. Leaves were imaged at 24, 48, and 72 hours post infiltration using confocal microscopy. No signal was detected at 12 hours post infiltration. Scale bar = 20 µm.

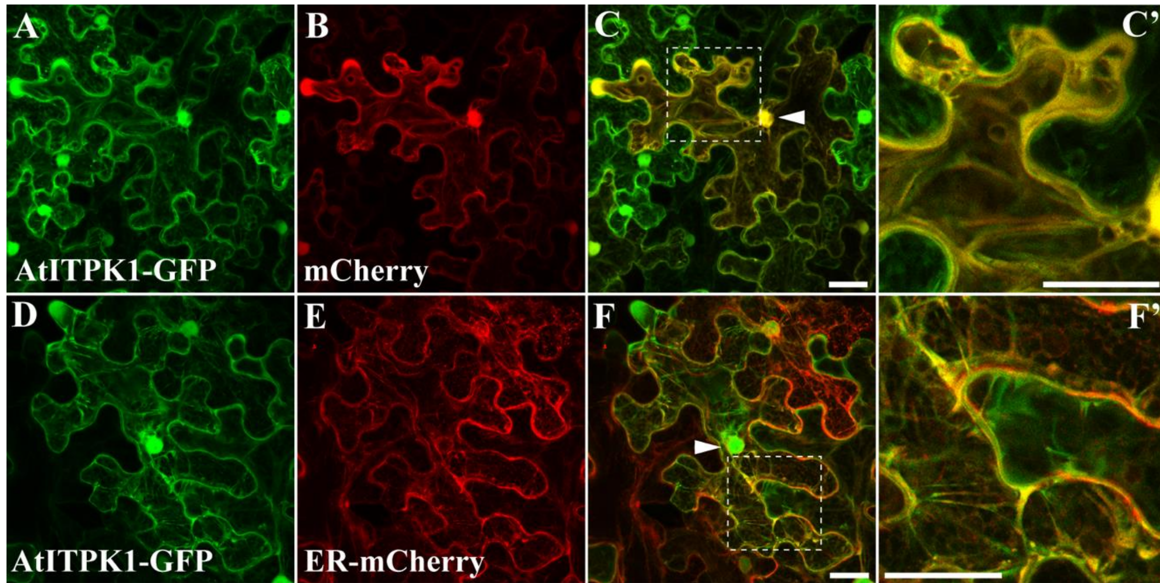


Figure 2. 9. AtITPK1-GFP localization in *N. benthamiana.*

N. benthamiana leaves were co-infiltrated with AtITPK1-GFP and mCherry (A-C') or AtITPK1-GFP and ER-mCherry (D-F'). Z-stacks of confocal optical sections are presented as maximum intensity projections. (C') and (F') are 3X enlargements of the regions outlined in (C) and (F), respectively. Arrowheads mark nuclei. Scale bars = 30 μm.

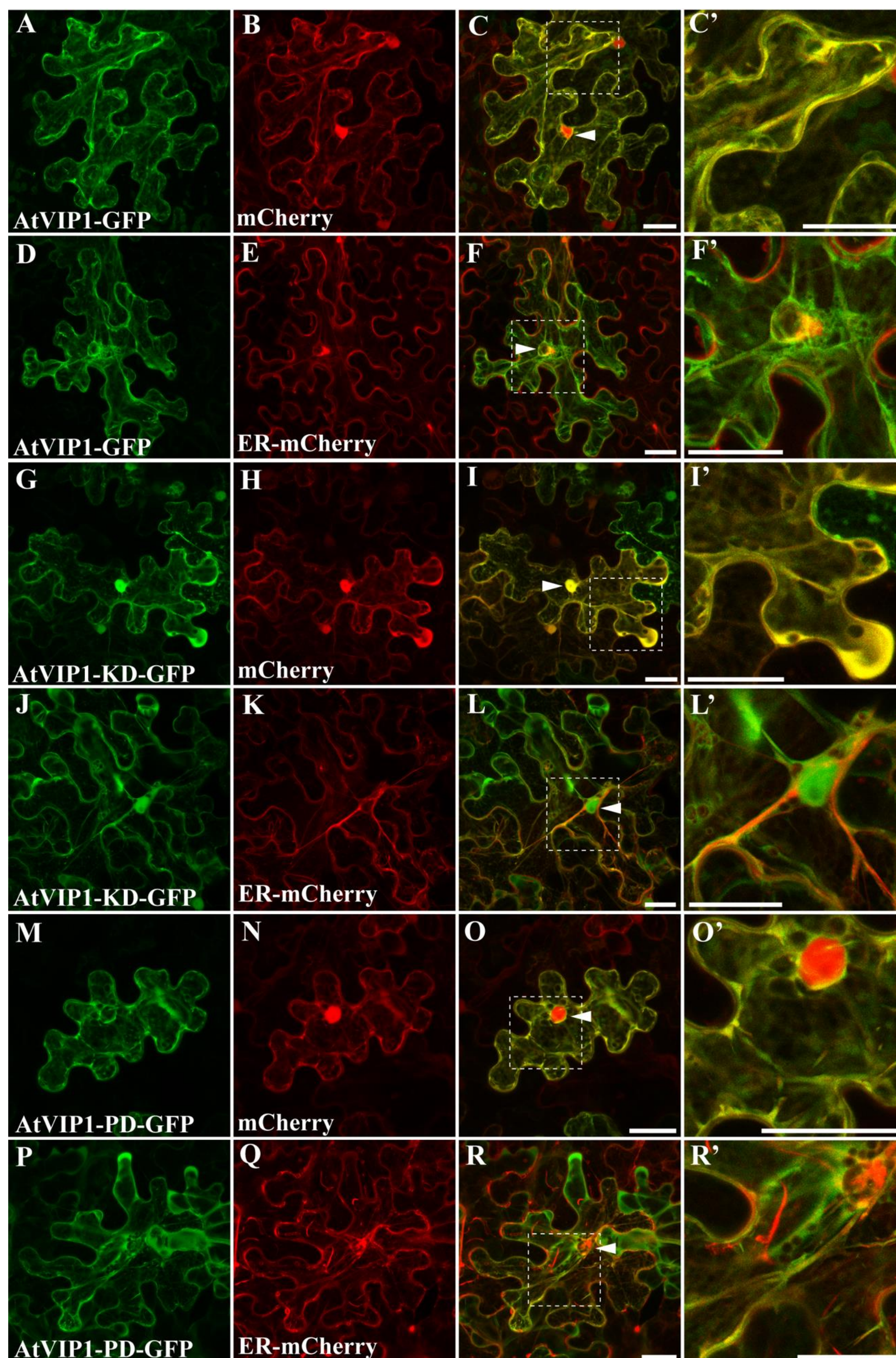

A
AtVIP1-GFP
B
mCherry
C
C'
D
AtVIP1-GFP
E
ER-mCherry
F
F'
G
AtVIP1-KD-GFP
H
mCherry
I
I'
J
AtVIP1-KD-GFP
K
ER-mCherry
L
L'
M
AtVIP1-PD-GFP
N
mCherry
O
O'
P
AtVIP1-PD-GFP
Q
ER-mCherry
R
R'

Figure 2. 10. AtVIP1-GFP localization in *N. benthamiana.*

N. benthamiana leaves were co-infiltrated with the indicated VIP1 constructs and mCherry or ER-mCherry. Z-stacks of confocal optical sections are presented as maximum intensity projections. The far-right panels are 3X enlargements of the regions outlined in the merged images. Arrowheads mark nuclei. Scale bars = 30 µm.

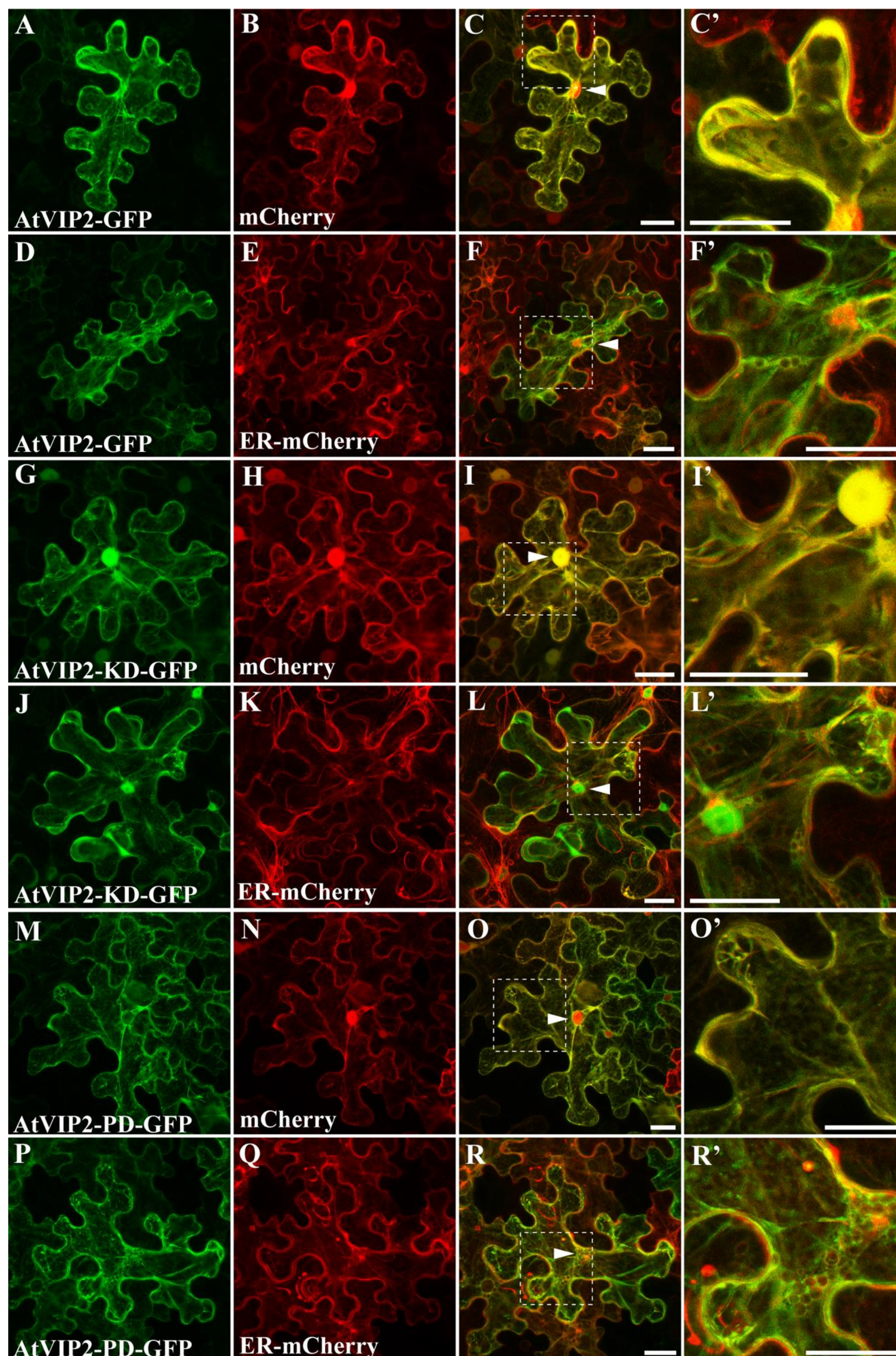

A
B
C
C'
AtVIP2-GFP
mCherry
D
E
F
F'
AtVIP2-GFP
ER-mCherry
G
H
I
I'
AtVIP2-KD-GFP
mCherry
J
K
L
L'
AtVIP2-KD-GFP
ER-mCherry
M
N
O
O'
AtVIP2-PD-GFP
mCherry
P
Q
R
R'
AtVIP2-PD-GFP
ER-mCherry

Figure 2. 11. AtVIP2-GFP localization in *N. benthamiana.*

N. benthamiana leaves were co-infiltrated with the indicated VIP2-GFP constructs and mCherry or ER-mCherry. Z-stacks of confocal optical sections are presented as maximum intensity projections. The far-right panels are 3X enlargements of the regions outlined in the merged images. Arrowheads mark nuclei. Scale bars = 30 μm.

DISCUSSION

InsP signaling molecules have been of great interest to plant scientists since the late 1980s, starting with Ruth Satter's work on leaflet movement and production of Ins(1,4,5)P_3 in pulvini (Morse et al., 1987), to current day studies linking $InsP_6$ and the PP-InsPs to P*i* sensing and homeostasis (Stevenson-Paulik et al., 2005, Kuo et al., 2014, Wild et al., 2016, Kuo et al., 2018). We have sought to fill in the missing steps in the PP-InsP synthesis pathway, and in doing this, we have defined a molecular basis for both $InsP_7$ and $InsP_8$ synthesis in plants **(Figures 3-6)**. Further, we have reconstituted the synthesis pathway from Ins(1,4,5)P_3 to $InsP_8$ with plant enzymes *in vitro* **(Figures 2-6)** and shown that knock-down or overexpression of the AtVIPs *in vivo* results in the expected changes to $InsP_7$ accumulation **(Figure 7)**.

The ability to synthesize and separate radiolabeled substrates *in vitro* was key to work, as most of these molecules are not commercially available **(Figure 2)**. These substrates allowed us to analyze the substrate preference of the Arabidopsis VIP kinase domains, which we show can catalyze the conversion of $InsP_7$ to $InsP_8$ *in vitro* **(Figure 3)**. Our data indicates that these plant enzymes are similar in their substrate preference as compared to previously characterized yeast and mammalian enzymes, acting preferentially to phosphorylate 5-$InsP_7$ substrates (Choi et al., 2007, Fridy et al., 2007, Weaver et al., 2013). Given that all known VIPs are 1-kinases, the most likely enantiomer of $InsP_8$ in plants cells is 1,5PP_2-$InsP_4$, which awaits further analysis by NMR for elucidation.

We coupled our *in vitro* biochemistry with an *in vivo* analysis of Arabidopsis *vip1/vip2* double mutants and AtVIP2 OX lines. While others have reported measurement of $InsP_8$ in plants can be problematic (Kuo et al., 2018), our [^{3}H]-*myo*-inositol labeling experiments allowed us to reproducibly measure accumulation of lower InsPs, along with $InsP_6$, $InsP_7$ and $InsP_8$ in both this current study and previously (Desai et al., 2014). We have shown that a loss of both *VIP* genes causes a statistically significant elevation of the $InsP_7/InsP_8$ ratio, which results from elevated $InsP_7$, while overexpression of *VIP2* results in a lower $InsP_7/InsP_8$ ratio **(Figure 7)**. Together, these results support that $InsP_8$ levels are maintained by the action of the *VIP* genes. Knock-out of the two human PPIP5K genes results in 2-3-fold higher 5-$InsP_7$ levels in a tissue culture cell line (Gu et al., 2017). Thus, while our *vip1/vip2* double mutants show elevation of $InsP_7$, the degree of the change is smaller and is consistent with the fact that our *vip1/vip2* double mutants are not complete nulls.

Our work has also delineated the AtITPKs as the missing enzyme in the PP-InsP pathway. Previously, two separate studies showed that the *AtVip* genes can restore $InsP_7$ accumulation to yeast mutants devoid of both the IP6K and VIP enzymes (Desai et al., 2014, Laha et al., 2015). This, along with the absence of any gene encoding an IP6K homologue in plants, suggested that the AtVIP enzymes might catalyze two steps in the conversion of $InsP_6$ to $InsP_8$. Our biochemical analysis of the AtVIP-KDs made this seem unlikely, so we tested other Arabidopsis inositol phosphate kinases for the ability to phosphorylate $InsP_6$. We determined that the Arabidopsis ITPKs can convert $InsP_6$ to a more phosphorylated product **(Figure 6)**. The ITPK family of enzymes possess an ATP-grasp fold and have been previously described as

multifunctional inositol phosphate kinases capable of phosphorylating different isomers of $InsP_3$ and $Ins(3,4,5,6)P_4$ in plants (Wilson and Majerus, 1997, Sweetman et al., 2007). To our knowledge, this is the first reported *in vitro* IP6Kinase activity for any member of this family of enzymes and represents a critical piece in the puzzle of how PP-InsPs are made in plants. We suggest here that plants may have co-opted the ITPKs to perform a novel function in PP-InsP synthesis. Our observation that the reaction product of both AtITPK1 and AtITPK2 was subsequently phosphorylated by the AtVIP-KDs also suggests that the AtITPKs synthesize the 5-$InsP_7$ isomer from $InsP_6$, and shows that we can reconstitute the entire PP-InsP synthesis pathway *in vitro* using recombinant enzymes from plants. Given the recently suggested roles of PP-InsPs in binding to the SPX proteins that control P*i* sensing and homeostasis (Wild et al., 2016), as well as the finding that *itpk1* mutants are deficient in sensing P*i* (Kuo et al., 2018), our work suggests that lack of $InsP_7$ synthesis in *itpk1* mutants may be an important factor in this mutant's defect in P*i* sensing.

Our subcellular localization results of enzymes involved in $InsP_6$, $InsP_7$ and $InsP_8$ synthesis suggest the presence of both nuclear and cytoplasmic locations. Specifically, we saw that the three enzymes involved in $InsP_6$ synthesis have a nuclear and cytoplasmic location, as described previously by others (Xia et al., 2003, Kuo et al., 2018). Similarly, AtITPK1 and all the various AtVIP constructs displayed substantial colocalization with unconjugated mCherry, indicating localization in the cytoplasm. We also observed, to a lesser extent, partial colocalization with ER-mCherry. AtITPK1-GFP, and the kinase domains (KD) of AtVIP1 and AtVIP2, were also observed in the nucleus, but the full-length and phosphatase domains (PD) of AtVIP1 and

AtVIP2 were seen to be excluded from the nucleus. This suggests that the AtVIP PD might contain a nuclear export signal (NES), since the AtVIP-KD constructs were able to access the nucleus in the absence of the PD. We note that a nuclear localization sequence (NLS) has been identified near the C-terminus of the human PPIP5K2, and mutation of this NLS increases the portion of PPIP5K2 that accumulates in the cytoplasm of mammalian cells (Yong et al., 2015). Similarly, the human IPK2 paralogue (IMPK) contains an NLS, along with an NES, and together these allow for nucleocytoplasmic shuttling of the enzyme in human cells (Meyer et al., 2012). It will be interesting to further dissect the mechanisms regulating AtVIP localization, and it is tempting to speculate that AtVIP access to the nucleus could be dynamically regulated during plant development and/or nutrient demand, resulting in a nuclear pool of $InsP_8$.

This work furthers our understanding of critical biochemical and cell biological aspects of the PP-InsP synthesis pathway in plants. Given the importance of seed $InsP_6$ and P*i* in agriculture, delineation of the PP-InsP synthesis pathway has important ramifications for future approaches to control P*i* sensing in plants.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]-*myo*-Inositol (20 Ci/mmol), [^{3}H]Ins(1,4,5)P_3 (17.1 Ci/mmol) and [^{3}H]InsP_6 (19.3Ci/mmol) were purchased from American Radiolabeled Chemicals (ARC), St. Louis, MO, USA. [^{3}H]Ins(1,3,4,5,6)P_5, and all other radiolabeled PP-InsPs (5PP-InsP_4, 1PP-InsP_5, 5PP-InsP_5 and 1,5(PP)$_2$-InsP_4) were synthesized enzymatically with the appropriate purified recombinant enzymes.

Plant Materials and Growth Conditions

Seeds of T-DNA mutants of *Arabidopsis thaliana* (ecotype Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). The T-DNA lines used in this study are as follows: *vip1-1* (GK_204E06), *vip1-2* (GK_008H11), *vip2-1* (SALK_094780) and *vip2-2* (SAIL_175_H09). Homozygous lines were identified by PCR using T-DNA left and right border primers and gene-specific sense or antisense primers (Supplemental Table 1). Double loss-of function mutant lines were generated by crossing *vip1-1* with *vip2-1*, *vip1-1* crossed with *vip2-1*, and *vip1-2* was crossed with *vip2-2*. All lines analyzed in this study, including Col-0 plants, were grown in parallel under identical conditions on soil (16 h light and 8 h dark, day/night temperature 23/21°C and 120 μE light intensity), and seeds of the respective last progenies were used for all analyses described. For growth in sterile conditions, seeds were sterilized in 30% (v/v) Clorox for 5 min and washed three times in ddH_2O. Sterilized seeds were

plated onto 0.5X MS media supplemented with 0.2% agar and stratified for 3 days at 4°C, grown under conditions of 16 h light (23°C) and 8 h dark (21°C) and 120 μE light intensity.

Synthesis of InsPs and PP-InsPs

[^{3}H]Ins(1,3,4,5,6)P_5 was prepared by incubating [^{3}H]Ins(1,4,5)P_3 with recombinant AtIPK2α at 37°C for 90 minutes. Enzymatically synthesized [^{3}H]Ins(1,2,3,4,5,6)P_6 was prepared by incubating [^{3}H]Ins(1,3,4,5,6)P_5 with recombinant AtIPK1 at 30°C for 60 minutes. [^{3}H]5PP-Ins(1,2,3,4,6)P_5 and [^{3}H]1PP-Ins(2,3,4,5,6)P_5 were generated by incubating [^{3}H]Ins(1,2,3,4,5,6)P_6 at 37°C for 60 minutes and 240 minutes with recombinant human $InsP_6$ Kinase (HsIP6K) and the human VIP1 Kinase Domain (HsVIP1-KD) respectively. [^{3}H]5PP_2-Ins(1,3,4,6)P_4 was synthesized by incubating [^{3}H]Ins(1,3,4,5,6)P_5 with recombinant HsIP6K at 37°C for 60 minutes. Reaction products were separated on an anion exchange HPLC, fractions were taken and analyzed by scintillation counting, and identity of the products was determined based on their elution profile, and elution of standards in separate runs.

Construction of Plasmids

Expression constructs and oligonucleotide primers used are summarized in **Supplemental Table 1**. Additional expression constructs were provided by Ryan Irving and John York (Vanderbilt University); His-AtIPK2(JYB897), GST-AtIPK1 (JYB308), GST-HsIP6K (JYB 1629) and GST-HsVIP1-KD (JYB1556). Recombinant AtVIP1-KD, AtVIP2-KD, AtITPK1 and AtITPK2 were generated as follows: Total RNA was extracted from young *Arabidopsis thaliana* seedlings using

the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). The RNA was reverse transcribed using the Bio-Rad iScripts reverse transcriptase (Hercules, CA, USA) to generate cDNA and primers were designed against the coding sequence of the kinase domains of AtVIP1 (At3G01310), AtVIP2 (At5G15070), and full length AtITPK1 (At5G16760) and AtITPK2 (At4G33770) with the addition of a 5'-CACC sequence for site-specific cloning into the pENTR Gateway entry vector (Thermo Fisher Scientific, Hampton, NH, USA). The PCR amplified products were gel purified using a QIAGEN DNA gel purification kit (QIAGEN, Hilden, Germany), and the purified products were cloned into the Gateway pENTR/D Topo cloning vector (Thermo Fisher Scientific, Hampton, NH, USA). and transformed into TOP10-competent *E. coli* cells (Fisher Scientific, Hampton, NH, USA). Positive entry clones were sub-cloned into a Gateway-compatible destination vector. pDEST-17 carrying an N-terminal 6X-His purification tag, or pDEST-24 carrying a C-terminal GST purification tag (Thermo Fisher Scientific, Hampton, NH, USA).

Expression and Purification of Recombinant GST-tagged Proteins

Recombinant protein for all GST-tagged constructs (HsVIP1-KD, HsIP6K, AtIPK1, AtVIP1-KD and AtVIP2-KD) were expressed in BL21 DE3 Turbo competent *E. coli* cells (Gelantis Biotechnology, San Diego, CA, USA) through auto-induction by culturing cells in a highly-enriched terrific broth (TB) media at 18°C for 16 hours. Cells were harvested by centrifugation at 4000 x g for 20 minutes and resuspended for 30 minutes at 4° C in lysis buffer (25 mM Tris (pH 8.0), 350 mM NaCl, 1mM DTT) supplemented with 100 µg/mL lysozyme and 1 tablet of EDTA-free complete protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Resuspended cells were

lysed by sonication, centrifuged at 17,500 RPM for 60 minutes and the clarified lysate loaded onto a 5 mL GSTrap HP (GE Healthcare, Chicago, IL, USA). The GSTrap HP column was washed with wash buffer (25 mM Tris (pH 8.0), 350 mM NaCl, 1 mM DTT, 0.1 mM reduced glutathione), and protein was eluted with elution buffer (25 mM Tris (pH 8.0), 350 mM NaCl, 1 mM DTT, 20 mM reduced glutathione) at room temperature. Three elutions were combined, and proteins were concentrated using Amicon ultracentrifugal 30-kDa cut-off filters for 30 min at 4000 X g at 4°C (Eppendorf 5810 R centrifuge). The purified recombinant protein was stored in storage buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM DTT, and 25% glycerol) at 80°C. Aliquots were examined on a 10% SDS-PAGE followed by Coomassie Blue staining or Western Blot analysis with rabbit α-GST-HRP antibody (Life Technologies, Carlsbad, CA) at a 1:50,000 dilution in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl), 2.5% non-fat milk and 0.1% Tween 20, and the proteins were compared with a Low range Prestained protein standard (Bio-Rad Laboratories, Hercules, CA).

Expression and Purification of Recombinant 6X-His-tagged Proteins

All 6X-His-tagged plasmid constructs (AtIPK2α, AtITPK1 and AtITPK2) were expressed in BL21 DE3 Turbo competent *E. coli* cells (Gelantis Biotechnology, San Diego, CA, USA) through auto-induction by culturing cells in a highly-enriched terrific broth (TB) media at 18°C for 16 hours. Cells were harvested by centrifugation at 4000 x g for 20 minutes and resuspended for 30 minutes at 4 °C in lysis buffer (25mM HEPES (pH 7.5), 350 mM NaCl, 1 mM DTT, 1 mM PMSF) supplemented with 100 µg/mL lysozyme and 1 tablet of EDTA-free complete protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Resuspended cells were lysed by

sonication, centrifuged at 17,500 RPM for 60 minutes and the clarified lysate loaded onto a 5 mL HisTrap HP (GE Healthcare, Chicago, IL, USA). The HisTrap HP column was washed with wash buffer (25 mM HEPES (pH 7.5), 350 mM NaCl, 1 mM DTT, 20 mM imidazole), and eluted with elution buffer (25 mM HEPES (pH 7.5), 350 mM NaCl, 1 mM DTT, 250 mM imidazole) at room temperature. Three elutions were combined, and proteins were concentrated using Amicon ultracentrifugal 30-kDa cut-off filters for 30 min at 4000 x g at 4°C (Eppendorf 5810 R centrifuge). The purified recombinant protein was stored in storage buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1mM DTT, and 25% glycerol) at 80°C. Aliquots were analyzed on a 10% SDS-PAGE followed by Coomassie Blue staining or Western blot analysis with goat α-6X-His-HRP antibody (Life Technologies, Carlsbad, CA) at a 1:5,000 dilution in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl), 2.5% non-fat milk and 0.1% Tween 20, and the proteins were compared with a Low range Pre-stained protein standard (Bio-Rad Laboratories, Hercules, CA). The purified recombinant protein was stored in storage buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1mM DTT, and 25% glycerol) at 80°C.

Inositol Phosphate Kinase Enzyme Assays

VIP kinase activity assays were performed according to (Volgmaier et al., 1996) with the following modifications: 100 L reactions containing 20 mM HEPES pH 6.2, 50 mM NaCl, 5 mM Na_2ATP, 1 mM DTT, 6 mM MgSO4, 6 mM phosphocreatine, 24 Units/mL phosphocreatine kinase (Calbiochem, Burlington, MA, USA) and reactions were incubated at 37°C for the times indicated. Both AtVIP1-KD and AtVIP2-KD acted on 5-$InsP_7$ and had linear accumulation of $InsP_8$ product over the first 10 minutes of the reaction. In some cases, incubation times of up to

90 minutes were used to assess whether molecules other than 5-$InsP_7$ could be acted upon by these enzymes. ITPK assays were performed in a 100 uL reaction buffer containing 20 mM HEPES pH 6.2, 50 mM NaCl, 5 mM Na_2ATP, 1 mM DTT, 6 mM $MgSO_4$, 6 mM phosphocreatine, 24 Units/mL phosphocreatine kinase (Calbiochem, Burlington, MA, USA). Reactions were incubated at 37°C for 90 minutes unless otherwise noted, and enzyme reactions were heat-inactivated at 90°C for 3 minutes. Reaction products from enzyme assays were analyzed by HPLC using a 125 X 4.6 mm Partisphere SAX-column (Sigma-Aldrich, St. Louis, MO, USA), and a previously described elution gradient (Azevedo et al., 2006).

Cloning of GFP constructs

The coding regions of IPK1, IPK2α, IPK2β, VIP1-FL, VIP1-KD, VIP1-PD, VIP2-FL, VIP2-KD, VIP2-PD, ITPK1 and ITPK2 were amplified from plasmids, WT Arabidopsis genomic or cDNA using the primers indicated in Supplemental Table 2. The PCR product corresponding to the full length coding sequence without the stop codon was cloned into pENTR™\D-TOPO® (Invitrogen Corp., Carlsbad, CA) and sequenced before recombining either VIP2PD into pK7WGF2 (N-terminus GFP) or all others into pK7FWG2 (C-Terminus GFP) (Karimi et al. 2002) containing the 35S Cauliflower Mosaic virus promoter and eGFP gene using the Gateway® LR Clonase™ II kit (Invitrogen Corp., Carlsbad, CA). A single colony was used to amplify the [Gene-pK7FW2/pK7WGF2] plasmid and it was sequenced before transforming Agrobacterium (strain GV3101) for transient expression in *N. benthamiana*. Cloning and expression plasmids were amplified in *E. coli* One Shot®Top10 cells (Invitrogen

Corp., Carlsbad, CA) and purified from an overnight culture using DNA Mini prep kit (Qiagen Co., Valencia Ca).

GFP Localization and Imaging

N. benthamiana plants were agro-infiltrated as previously described (Kapila et al., 1997). Briefly, Agrobacterium cultures were grown over night in liquid media. Cells were pelleted and suspended in MMA (1x MS, 10 mM MES, 200 µM acetosyringone) to an optical density of 1.0, A600nm. Cultures were allowed to incubate at room temperature 2-4 hours before infiltration. *N. benthamiana* plants were grown under long day conditions (16 hours light) and 150 µE light. Leaf sections were imaged 12, 24, 36, 48, or 72 hours post infiltration as indicated using a Zeiss LSM 880 (Carl Zeiss). Slides were examined with a 40x C-Apochromat water immersion lens. A set of mCherry tagged organelle markers were used for co-localization experiments (Nelson et al., 2003). GFP was excited using a 488-nm argon laser and its fluorescence was detected at 500- to 550-nm. mCherry was imaged using excitation with a 594-nm laser and fluorescence was detected at 600- 650-nm. Chlorophyll signal was collected using a 594-nm laser and emission above 650 nm was collected.

Protein Blot Analyses of GFP-Fusion Proteins

Conditions have been previously reported (Burnette et al., 2003). Briefly, tissues were ground in liquid nitrogen, homogenized, cellular debris was pelleted, and SDS-bromophenol blue loading

dye added to the supernatant. The supernatant was boiled, centrifuged, and the subsequent supernatant was loaded onto a polyacrylamide gel for separation. Equal amounts of protein were loaded onto gels. SDS-PAGE was followed by western blotting with a 1:10,000 dilution of rabbit anti-GFP antibody (Invitrogen Molecular Probes, Eugene, OR). A secondary goat anti-rabbit horseradish peroxidase antibody (Bio-Rad Laboratories, Hercules, CA) was used at a 1:2,500 dilution For **Supplemental Figure S4**, proteins were transferred to PVDF membrane and blotted proteins were incubated in a 1:10,000 dilution of mouse monoclonal anti-GFP antibody (Living Colors JL-8, Clontech) followed by 1:5000 horseradish peroxidase conjugated sheep anti-mouse IgG antibody (Amersham, Buckinghamshire, UK). Immunoreactive bands were detected using an ECL™ Prime Western Blotting Detection Reagent (Amersham, Buckinghamshire, UK). Ponceau S staining of blots was performed to ensure that equal amounts of protein within extracts were analyzed.

RNA preparation and qRT-PCR

RNA was isolated from 2 week-old seedlings grown on MS medium with 1% sucrose. RNA was isolated using the Plant RNeasy kit (Qiagen) and was DNAse treated with DNA-free Turbo (Invitrogen). Reverse transcription was carried out using 2 µg of total RNA Multiscribe Transcriptase (Applied Biosystems). The equivalent of 20 ng of cDNA was used for quantitative PCR using gene specific primers for *AtVip1* and *AtVip2* and SYBRgreen as described in Desai et al., 2014. Reactions were carried out in triplicate with PP2A as the reference gene. Relative expression was calculated by the ΔΔCT method.

ACCESSION NUMBERS

AtIPK2: At5G07370; AtIPK2β: At5G61760; AtIPK1: At5G42810; AtITPK2: At4G33770; AtVIP1: At3G01310; AtVIP2: At5G15070.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GG and IP conceived of the work and GG, IP and PS provided day to day guidance. OA and DM performed enzyme analyses. SPW and BC performed cell biological experiments. OA and EL characterized mutant and transgenic plants. OA, BC, PW and GG wrote the manuscript. All authors read the manuscript and provided input.

SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure 2.12. Supplemental Data: SDS-PAGE and Western Blot of Purified Recombinant Fusion Proteins Used in the Study.

Figure 2.13. Supplemental Data: $InsP_5$ is not a Substrate for Recombinant AtVIP1-KD or AtVIP2-KD.

Figure 2.14. Supplemental Data: 5PP-$InsP_4$ is not a Substrate for Recombinant AtVIP1-KD or AtVIP2-KD.

Figure 2.15. **Supplemental Data:** Characterization of VIP2 OX and *vip1/vip2* double knock-out mutants.

Figure 2.16. Supplemental Data: Immunoblot of GFP fusion proteins in infiltrated *N. benthamiani* leaves.

Figure 2.17. Supplemental Data: Time course of ITPK1-GFP expression.

Figure 2.18. **Supplemental Data:** Time Course of VIP2FL-, VIP2KD-, VIP2PD-GFP Expression.

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APPENDIX TO CHAPTER II

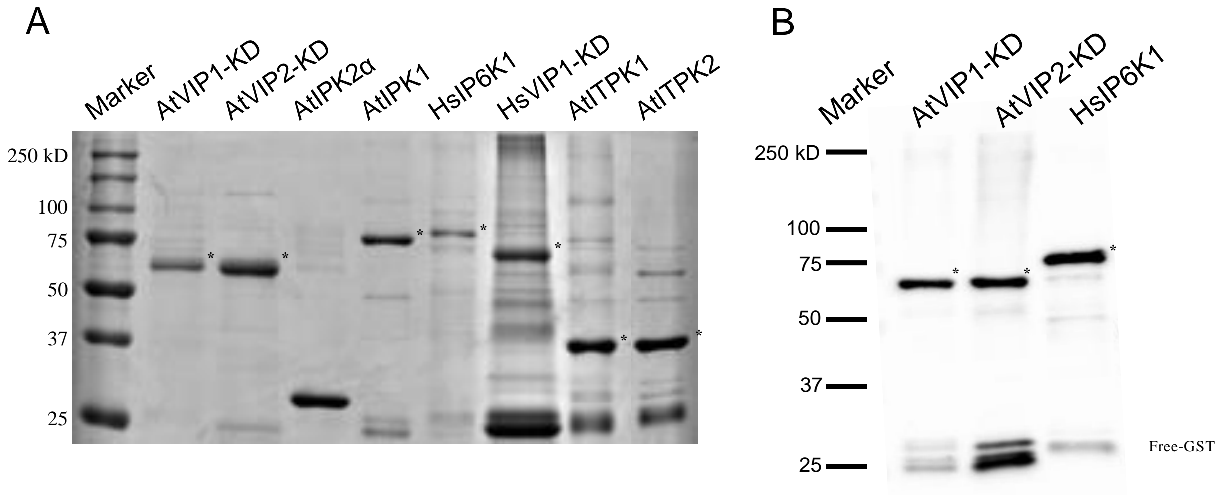

Figure 2. 12. Supplemental Data: SDS-PAGE and Western Blot of Purified Recombinant Fusion Proteins Used in the Study.

A, Recombinant fusion proteins were analyzed by SDS-PAGE, and stained with Coomassie blue. All target recombinant fusion proteins used in study are marked with asterisks. Recombinant GST-tagged AtVIP1-KD and AtVIP2-KD ran slightly below their expected molecular weight of 73kD, 6His-tagged AtIPK2α ran slightly below its expected molecular weight of 33kD, recombinant GST-tagged AtIPK1 was seen around 76kD, recombinant GST-tagged HsIP6K ran at the expected 75kD, recombinant GST-tagged HsVIP1-KD migrated at the expected 70kD and recombinant 6His-tagged AtITPK1 and AtITPK2 both ran slightly below their expected 36kD and 41kD respectively. B, Immunoblot analyses showed that the contaminating band around 25kD on the GST-tagged proteins is free GST as it immunoreacted with α-GST antibody.

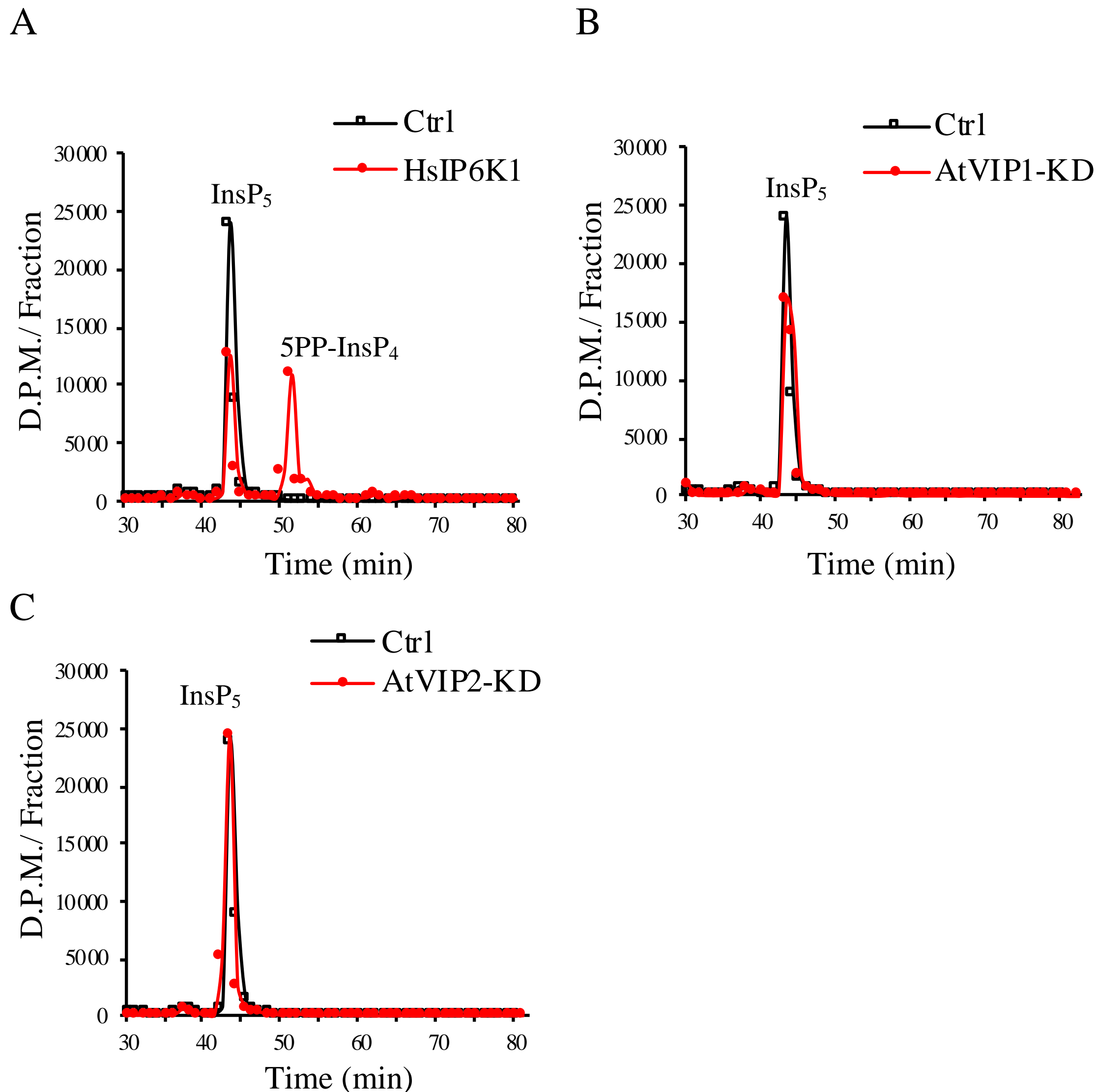

Figure 2. 13. Supplemental Data: InsP5 is not a Substrate for Recombinant AtVIP1-KD or AtVIP2-KD.

[^{3}H]Ins(1,3,4,5,6)P5 containing 45,000 total CPM enzymatically synthesized from commercial [^{3}H]Ins(1,4,5)P3 using purified recombinant AtIPK2α was incubated with A, either buffer (Ctrl, open squares) or 7.6 µg of purified recombinant HsIP6K1 (red circles). B, Buffer (Ctrl, open squares) or 7.6 µg of purified recombinant AtVIP1-KD (closed circles). C, Buffer (Ctrl, open squares) or 7.6 µg of purified recombinant AtVIP2-KD (closed circles). All reactions were incubated at 37°C for 90 minutes, and reactions were terminated by heat-inactivation of enzyme at 90°C for 3 minutes followed by HPLC analysis of products. Data shown are representative of two replicate experiments.

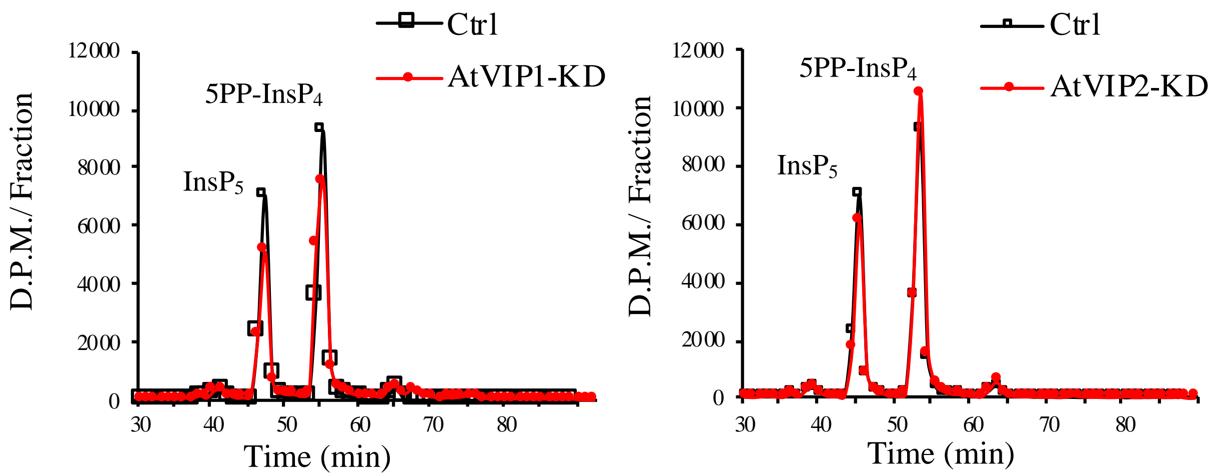

Figure 2. 14. Supplemental Data: 5PP-InsP$_4$ is not a Substrate for Recombinant AtVIP1-KD or AtVIP2-KD.

Enzymatically synthesized 5PP-InsP$_4$ containing 20,000 total CPM from [^{3}H]Ins (1,3,4,5,6)P$_5$ and 7.6 µg purified recombinant HsIP6K was incubated with buffer (Ctrl, open squares) or 7.6 µg purified recombinant AtVIP1-KD or AtVIP2-KD (red circles). Reactions were incubated at 37°C for 90 minutes, reactions were terminated by incubation at 90°C for 3 minutes, and reaction products were analyzed using anion exchange HPLC. Data shown are representative of two replicate experiments.

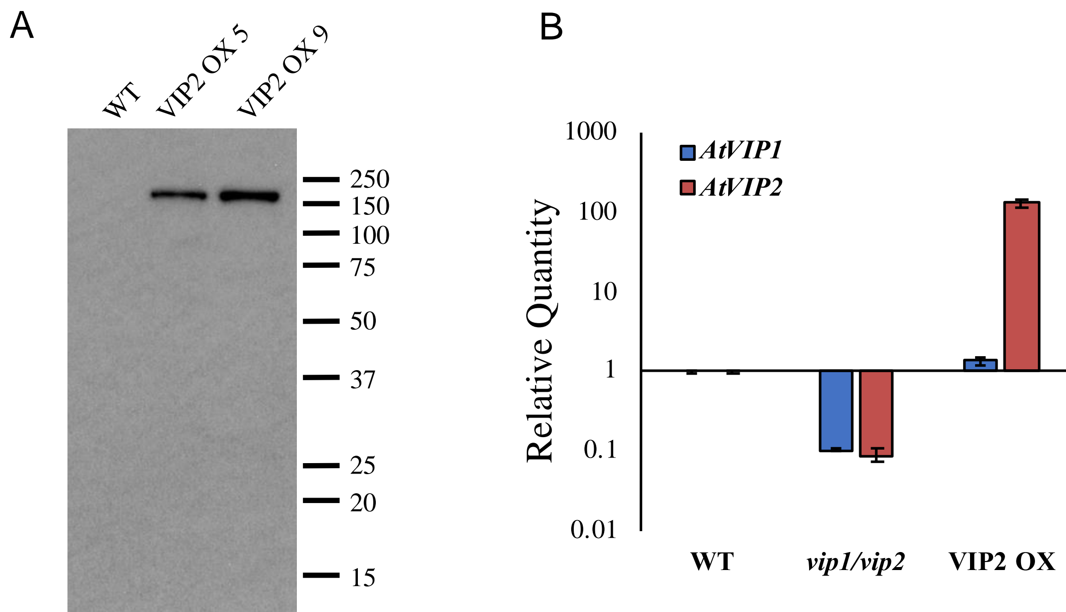

Figure 2. 15. Supplemental Data: Characterization of VIP2 OX and *vip1/vip2* double knock-out mutants.

A, Immunoblot of GFP-Fusion Proteins in VIP2 overexpression (VIP2 OX) transgenic plant lines. 2 week-old seedlings from two different VIP2 OX lines were analyzed by immunoblotting using a mouse monoclonal anti-GFP antibody (Living Colors JL-8, Clontech). B, RNA preparation and qRT-PCR. RNA was isolated from 2 week-old seedlings of the indicated genotype and analyzed by quantitative RT-PCR using gene specific primers for *AtVip1* and *AtVip2* and SYBRgreen as described in Desai et al., 2014. Reactions were carried out in triplicate with PP2A as the reference gene. Relative expression was calculated by the ΔΔCT method for wildtype (WT), *vip1/vip2* double mutants and the VIP2 OX9 line.

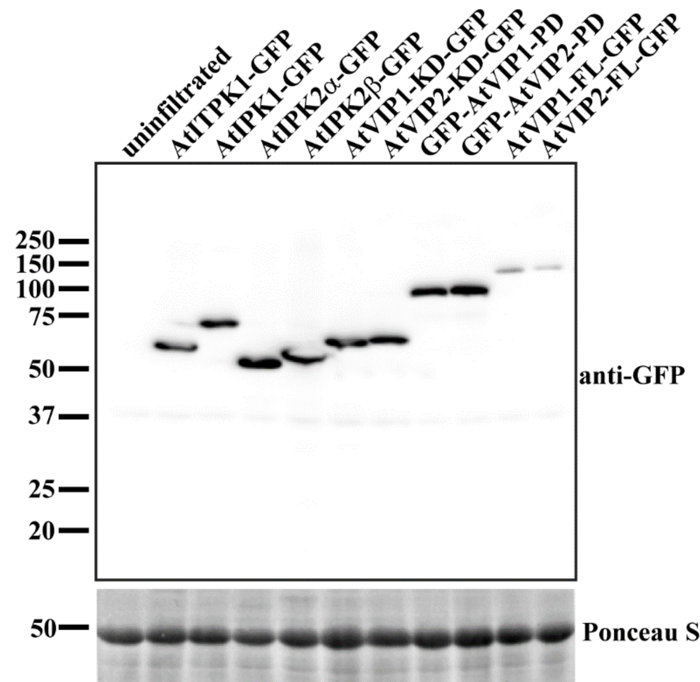

Figure 2. 16. Supplemental Data: Immunoblot of GFP fusion proteins in infiltrated *N. benthamiani* leaves.

N. benthamiani leaves were infiltrated with the indicated GFP fusion constructs, and 48 hours post-infiltration the leaf tissue was harvested and processed for immunoblot with anti-GFP. Ponceau S staining was used as a loading control.

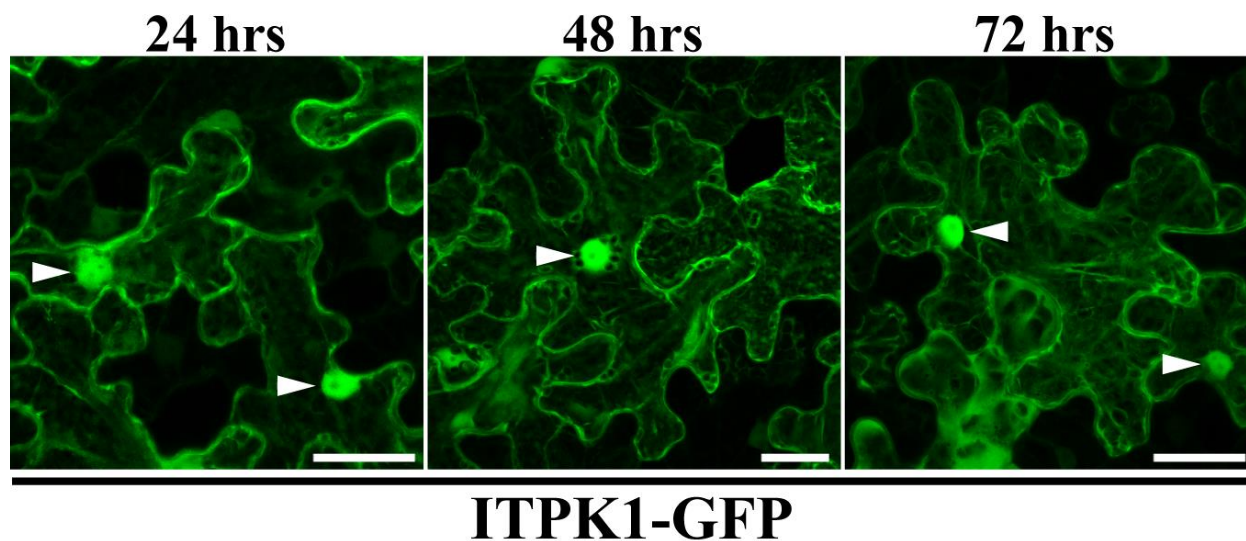

Figure 2. 17. Supplemental Data: Time course of ITPK1-GFP expression.

ITPK1-GFP was transiently expressed in infiltrated *N. benthamiana* leaves, and confocal Z-stacks were acquired at the indicated time points post-infiltration. Z-stacks are presented as maximum intensity projections. Arrowheads mark nuclei. Scale bars = 30 μm.

24 hrs 48 hrs 72 hrs

AtVIP2-GFP

AtVIP2-KD-GFP

AtVIP2-PD-GFP

Figure 2. 18. Supplemental Data: Time Course of VIP2FL-, VIP2KD-, VIP2PD-GFP Expression.

VIP2FL-, VIP2KD- and VIP2-PD-GFP fusion proteins were transiently expressed in *N. benthamiana*. Leaves were imaged at 24, 48, and 72 hours post infiltration using confocal microscopy. No signal was detected at 12 hours post infiltration. Scale bar = 20 µm

Table 2. 1. Supplemental Data: Oligonucleotide Primers Used in this Study.

Type of construct	Gene ID	Primer Name	Sequence
pENTR-AtIPK2α	At5g07370	pE-IPK2αFW	CACCATGCAGCTCAAAGCTA
		pE-IPK2αR	CTAAGAATCTGCAGACTCATCTGGG
pENTR-AtIPK2β	At5g61760	pE-IPK2βFW	CACCATGCTCAAGGTCCCTGAACACC
		pE-IPK2βR	GCGCCCGTTCTCAAGTAG
pENTR-AtIPK1	At5g42810	pE-IPK1FW	CACCATGGAGATGATTTTGGAGGAGAAA
		pE-IPK1R	GCTGTGGGAAGGTTTTGAGTTGCC
pENTR-AtITPK1	At5g16760	pE-ITPK1FW	CACCATGTCAGATTCAATCCAG
		pEITPK1R	TCAGACATGATTCTTCTTAGTGACCAT
pENTR-AtITPK2	At4g33770	pE-ITPK2FW	CACCATGTTTGGGACTCTTGCT
		pEITK2R	TCATTTACAATGTTGTCTCTTCTTATATTTGAC CTG
pENTR-AtVIP1	At3g01310	pE-VIP1FW	CACCATGGAGATGGAAGAAGGAGCAAG
		pE-VIP1R	TATGCTCCTTCCATTAGAAGAAGAGTTC
		pE-VIP1KDR	TTCTGACTGCCCAAAAGTGC
		pE-VIP1PDFW	CACCTATTATGACGATGCTGCTTGTG
		pE-VIP1PDR	TTAGCTCCTTCCATTAGAAGAAGAGTTA
pENTR-AtVIP2	At5g15070	pE-VIP2FW	CACCATGGGGGTGGAAGAAGGAGCTG
		pE-VIP2R	GCTTTTGCCATTAGAGGTGTTCTTGTGG
		pE-VIP2KDR	CTCTGACTGCCCAAAAGTTCC
		pE-VIP2PDFW	CACCTATTACGACGATGCTGCTTGTG
		pE-VIP2PDR	TTAGCTTTTGCCATTAGAGGTGTTCTTGTG G

Primers for mutant characterization			
Gene Name / Allele	**Locus / T-DNA Lines**	**Primer Name**	**Sequence**
vip 1-1	GK_204E06	LB / FW	AACAAGCAATGGAGCAAAGAG
		RB / R	TGGATTTTAGCGATGCTTCTC
vip 1-2	GK_008H11	LB / FW	GGAAGGGCTATGGTTTCAGTATC
		RB / R	CAACTGGACGGCACTCTTAAG
vip 2-1	SALK_094780	LB / FW	AGAAAGATGAGGAGCCTTTGC
		RB / R	TTTTTCCATGAACAATGGAGC
vip 2-2	SAIL_175_H09	LB / FW	GAACGAAACGGAAAGAGGTTC
		RB / R	TTTGAACTCAAAAGCCAAACTC

Primers for RT-qPCR			
qRT-PCR	At3g01310	qRTAtVip1F	CACCATGGAGATGGAAGAAGGAGCAAGTG
qRT-PCR	At3g01310	qRTAtVip1R	GCAGCATATGCCTGAGCCTTCTC
qRT-PCR	At5g15070	qRTAtVip2F	CAAGAGGTTGGTTCATGTTTGACCCTC
qRT-PCR	At5g15070	qRTAtVip2R	GTGGAAGGGCCAAAGTTTAACAAGACG
qRT-PCR		qRTPP2AF	TAACGTGGCCAAAATGATGC
qRT-PCR		qRTPP2AR	GTTCTCCACAACCGCTTGGT

CHAPTER III

Summary and Future Directions

PP-InsPs are small signaling molecules that are emerging as key players in many aspects of cell signaling and metabolism in eukaryotes. To this end, a lot of interest has been generated in the scientific community around this unique class of signaling molecules, which has culminated in an increased knowledge and understanding of the cellular roles played by PP-InsPs. Through several research efforts, some of the physico-chemical properties, mechanisms of action, as well as biosynthesis and metabolism of PP-InsPs, have been established. Despite the recent identification of this class of molecules in plants, our understanding of their biosynthesis and physiological function in plants is still further behind. As a result, this dissertation project was designed to gain more understanding of the biosynthesis of PP-InsPs in the model plant *Arabidopsis thaliana* through the biochemical characterization of two AtVIP and AtITPK enzymes, involved in PP-InsP biosynthesis. I also performed the biochemical profiling of PP-InsP levels in AtVIP1 and AtVIP2 double loss-of-function mutant plant lines, as well as AtVIP2-overexpressing plants. I also collaborated with others who investigated the subcellular localization of enzymes involved in InsP and PP-InsP biosynthesis to better understand cellular distribution.

An important contribution of this work to the InsP signaling research community is the elucidation of the enzymatic steps for the biosynthesis of PP-Ins in Arabidopsis. I have demonstrated that the cytosolic and nuclear localized ITPKs have a novel IP6Kinase activity. The purified recombinant ITPKs will catalyze the *in vitro* conversion of $InsP_6$ to $InsP_7$, which is

likely 5PP-$InsP_5$. NMR confirmation to delineate the position of pyrophosphate bond is needed. I have also shown that the cytosolic and nuclear localized kinase domains of the two Arabidopsis VIP enzymes are functional PPIP5Kinases that specifically convert 5PP-$InsP_5$ to 1,5$(PP)_2$-$InsP_4$ *in vitro*. The VIPs will also convert the $InsP_7$ product of AtITPKs to $InsP_8$, which is speculated to be 1,5$(PP)_2$-$InsP_4$. Elucidation of the structure of this molecule also requires confirmation by NMR. Lastly, I showed that the *AtVIP* genes encoding these enzymes have biological relevance, as perturbation of PP-InsP levels was observed in plants whose *VIP* genes have been disrupted or modified. Taken together, I have shown through this work that AtITPKs and AtVIPs are sufficient for PP-InsP biosynthesis in Arabidopsis. We can also strongly suggest based on our subcellular localization results, that PP-InsPs are present in the cytosol and nucleus, however an ER presence cannot be completely ruled out.

$InsP_6$ (phytic acid) which is storage form of phosphorus in plant seeds, chelates important minerals, and the salts (phytate) that is formed is largely excreted. This makes $InsP_6$ an antinutrient and a pollutant. A major significance of understanding how plants biosynthesize PP-InsPs from $InsP_6$, which I have described in this dissertation, is that efforts into the generation of “reduced phytate” or “phytate-free” crops can be expanded into new directions that include the enzymes described in this study. Current genetic and breeding strategies for addressing the nutritional and environmental problems associated with seed-derived dietary phytic acid, has focused primarily on a suite of genes encoding enzymes involved in the biosynthesis of $InsP_6$ (Stevenson-Paulik et al., 2005; for review, see Raboy, 2002). This work has further expanded our repertoire of target genes for manipulation, and enzymes that can be engineered for the goal of

reducing phytic acid content of crop plants. Such expansion can accelerate the pace at which we generate "low phytate" crops, which will be significant in addressing environmental phosphate pollution, as well as the anti-nutritional effects of heavy-seed diet.

One role that has been reported for PP-InsPs, is in plant defense against herbivores (Laha et al., 2015). Specifically, it was shown that $InsP_8$ activates JA-triggered defense response against herbivore-induced plant wounding. Given the findings reported in this dissertation, which has expanded our understanding of the biosynthesis of $InsP_8$, one potential application of this work could be the generation of crops with increased $InsP_8$ levels, through the manipulation of genes encoding enzymes involved in $InsP_8$ biosynthesis. This strategy could result in increased plant fitness through the generation of crops primed for defense against herbivory.

Lastly, the findings of this work could have potential application in phosphate use efficiency in crop plants. It has been reported that PP-InsPs play a key role in P*i*-sensing and homeostasis by modulating the interaction between proteins containing an N-terminal SPX-domain and transcription factors (Wild et al., 2016). The elucidation of the biosynthesis route for PP-InsP production in plants offer avenues for increasing our understanding of this interaction. Manipulation of genes encoding enzymes described in this work can help perturb levels in PPx-InsPs in plants, which can result in changes in sensing, uptake, transport and storage of P*i*. Given the importance of P*i* in plant growth and crop productivity, an increased efficiency in the use of this plant nutrient can have a significant impact on agriculture.

One aspect of this work that requires immediate closure is the NMR determination of the identities of our reaction products. This is critical in order to make logical comparisons with orthologous enzymes in yeast and mammals, and to better understand functions specific to different isomers of PP-InsP. Equally important is the determination of the kinetic parameters of the kinase domains of the two VIP enzymes. This will enable a direct comparison of the catalytic activities of AtVIP1 and AtVIP2, as well as comparison with the yeast and human enzymes. The determination of a novel IP6Kinase activity for the Arabidopsis ITPK1 and ITPK2 requires further studies, as Arabidopsis has four ITPK genes. It is worth investigating AtITPK3 and AtITPK4, as well as the single human ITPK enzyme for this novel catalytic activity. This information will be vital for better understanding the regulation of these unique class of signaling molecules, as there could be potential new players involved in their biosynthesis. Determination of kinetic parameters for the AtITPKs will shed light on their novel IP6Kinase activity, as it can help determine if this is a physiologically relevant activity relative to their previously known catalytic activity.

Our biochemical characterization of the kinase domains of AtVIP1 and AtVIP2 demands that we determine the role of the phosphatase domain of these enzymes. It is unknown if the kinase domain occurs independently in nature, and studying the kinase domain in isolation only tells part of a potentially bigger story. To address this question, future studies aimed at biochemical characterization of the phosphatase domain, and the full-length AtVIPs will give a more thorough understanding of these enzymes. A challenging aspect of this future investigation is the difficulty in stably expressing and purifying the large full-length AtVIP enzymes. Strategies

aimed at stabilizing the intrinsically disordered region of the phosphatase domain of AtVIPs needs to be developed, for successful expression and purification of the recombinant protein. Additional loss-of-function mutant lines of *AtVIPs* are currently being generated through transformation of novel T-DNA insertions and expression of RNAi constructs. Biochemical radiolabeling experiments of these mutants will further our understanding of the impact of these genes on PP-InsP biosynthesis *in vivo*. Also, as we have yet to identify any robust phenotypic differences between wild-type and mutant Arabidopsis plants under our current growth conditions, other "environmentally challenging" conditions needs to be tested in the future. Ongoing work focused on the effect of varying concentrations of P*i* on *VIP* mutant lines will further our knowledge on the connections between PP-InsP and P*i* sensing and homeostasis. Results from such studies have potential agricultural application as increased understanding of P*i* use-efficiency could positively impact crop production and bioremediation. In order to fully understand the regulation of PP-InsPs in plants, other putative PP-InsP phosphohydrolases capable of hydrolyzing these molecules needs to be investigated and characterized. Future work to develop antibodies specific for the different AtVIPs, as well as potential inhibitors will help further our understanding of the differences between these enzymes.

The study of InsP signaling research remains quite challenging for several reasons. PPx-Ins are low abundance molecules, which requires very sensitive analytical equipment for their detection and quantification. The inability of inositol to absorb or emit light in the visible or UV wavelength also limits the detection techniques applicable for PP-InsPs. The standard techniques in the field currently are limited in sensitivity and robustness. Radiolabeling studies of intact

cells are quite expensive, time consuming and cumbersome. Also, extensive biochemical studies that involves the use of radiolabeled substrates becomes quite challenging due to the unavailability of some commercial substrates. Separation of these highly charged molecules, also requires specialized strong anion exchange high pressure liquid chromatography (SAX-HPLC), as well as the use of strong acidic buffers. These molecules form complexes with cations which affects elution profile and makes the separation of different isomers of $InsP_7$ (5PP-$InsP_5$ and 1PP-$InsP_5$) quite challenging. The precipitation of salt from the 1.3M ammonium phosphate buffer used for this separation is also harsh on HPLC, and results in high maintenance and extended down time in operation. All these challenges make the development of newer, more sophisticated and sensitive technology for InsP signaling research a necessity. Also, continuous improvements in method development for the optimal separation of different isomers of PP-InsPs is needed.

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