Molecular Characterization and Loss-of-Function Analysis of an *Arabidopsis thaliana* Gene Encoding a Phospholipid-Specific Inositol Polyphosphate 5-Phosphatase

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ABSTRACT

The phosphatidylinositol signaling pathway utilizes inositol-containing second messengers to mediate signaling events. The enzymes that metabolize phosphoinositides can in some cases serve to terminate the signaling actions of phosphoinositides. The inositol polyphosphate 5-phosphatases (5PTases) comprise a large protein family that hydrolyzes 5-phosphates from a variety of inositol phosphate and phosphoinositide substrates. I have examined the substrate specificity of the At5PTase11 protein from the model plant, *Arabidopsis thaliana*. The At5PTase11 gene (At1g47510) encodes an active 5PTase enzyme that can dephosphorylate the phosphoinositide substrates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2], and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. In addition, the At5PTase11 gene is regulated by abscisic acid, jasmonic acid, and auxin, suggesting a role for phosphoinositide action in these signal transduction pathways.

To further delineate the function of At5PTase11 in *Arabidopsis thaliana*, two independent T-DNA insertion mutant lines were isolated (*At5ptase11-1* and *At5ptase11-2*). Analysis of *At5ptase11* mutant lines revealed that *At5ptase11* mutant seeds germinate slower compared to wild-type seeds. Moreover, *At5ptase11* mutant seedlings

demonstrated less hypocotyl growth when grown in the dark. These results indicate that At5PTase11 is required for the early stages of seed germination and seedling growth.

Since there are 15 predicted 5PTases in *Arabidopsis thaliana*, a group of 5PTases have been analyzed to identify the 5PTases with similar substrate selectivity. At5PTase1 (At1g34120), At5PTase2 (At4g18010) and At5PTase3 (At1g71710) have been found to hydrolyze all four potential substrates, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. At5PTase7 (At2g32010) hydrolyzed PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 which is similar to the substrate selectivity of At5PTase11. In addition, At5PTase4 (At3g63240), and At5PTase9 (At2g01900) hydrolyzed only PtdIns(4,5)P_2. These results indicate that there are different groups of *Arabidopsis thaliana* 5PTases based on the substrate selectivity. These results suggest that *Arabidopsis thaliana* 5PTases with similar substrate selectivity may have overlapping functions.

In summary, the findings that At5PTase11 is a phospholipid-specific 5PTase and At5PTase11 functions in the early stages of seed germination and seedling growth indicate that 5PTases play important roles in plant growth and development.

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LIST OF ABBREVIATIONS

5PTases	*Myo*-inositol polyphosphate 5-phosphatases
ABA	Abscisic acid
AP	Ammonium Phosphate
At	*Arabidopsis thaliana*
CaMV	Cauliflower Mosaic Virus
DAG	1,2-diacylglycerol
GroPIns	Deacylated phosphoinositides
GUS	β-glucuronidase
IAA	Indole-3-acetic acid
IgG hc	Immunoglobulin heavy chain
Ins(1,4,5)P_3	Inositol 1,4,5-trisphosphate
IPPc	Inositol phosphate phosphatase catalytic domain
JA	Jasmonic acid
OCRL	Oculocerebrorenal Lowe
PI	Phosphoinositide
PIPP	Proline-rich inositol-polyphosphate 5-phosphatase
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
pMT	Insect metallothionine promoter
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
Pten	Phosphatase and tensin homolog

RT	Reverse transcription
S2 cells	*Drosophila melanogaster* S2 tissue culture cells
SH2	Src homology 2
SKIP	Skeletal muscle and kidney enriched inositol phosphatase
TLC	Thin-layer chromatography

CHAPTER I

Introduction and Objectives

All organisms have different ways to respond to their environment in order to adapt and survive. In general, plants do not have the ability to move freely like most other organisms, which forces them to have unique ways to respond to the environment. One of the ways that plants respond to extracellular signals is through utilizing inositol-containing compounds. The phosphoinositide (PI) signaling pathway (Persson et al., 2002) (also referred to as the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] signaling pathway) is one example where inositol containing compounds serve as second messengers (Streb et al., 1984). Most of the components of this signaling pathway were characterized in animal systems demonstrating the involvement of this pathway controlling a variety of cellular events (Shears, 1998; Insall and Weiner, 2001). Inositol polyphosphate 5-phosphatases (5PTases, E.C. 3.1.3.36), which have the ability to catalyze hydrolysis of 5-position phosphates from the inositol ring, have been shown to be potential signal terminators for the pathways involving inositol compounds with a 5-position phosphate (Speed et al., 1999; Sanchez and Chua, 2001; Burnette et al., 2003). In *Arabidopsis thaliana* (*Arabidopsis*) there are fifteen 5PTases as predicted by the sequence homology to the known 5PTases from other organisms (Berdy et al., 2001). My work has focused on the smallest *Arabidopsis* 5PTase, At5PTase11 which contains 331 amino acids. Besides being the smallest 5PTase in any organism, the promoter region of

At5PTase11 contained putative regulatory elements, such as a jasmonic acid response element, which intrigued me to focus on this enzyme.

I have chosen these objectives to characterize and determine the physiological function of At5PTase11 gene in *Arabidopsis*.

Objective I: Determine the Substrate Specificity of At5PTase 11

Objective II: Examine the Physiological Consequence of Altering At5PTase11 Function in *Arabidopsis*

Objective III: Examine the Substrate Selectivity of a Group of *Arabidopsis* 5PTases

Literature Review

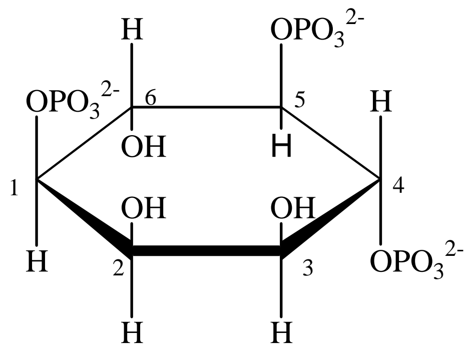
I. Overview of Phosphoinositide Signaling

It was fifty years ago that the outstanding work by Hokin and Hokin detailed the involvement of PIs and enzymes responsible for their synthesis in the regulation of enzyme secretion from the pancreas, by measuring ^{32}P incorporation into phospholipids upon stimulation (Hokin and Hokin, 1953). This brought attention of many researchers to the importance of the PI signaling pathway. Today, it is well accepted that these molecules are involved in many cellular processes such as signal transduction (Hirono et

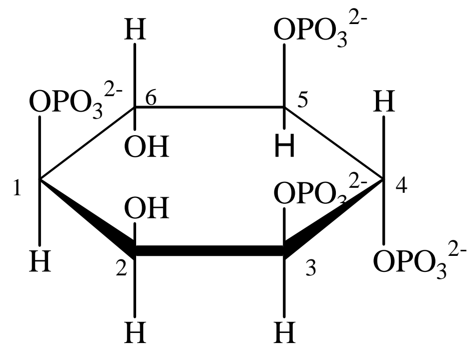
al., 2004), actin and cytoskeleton organization (Meiri, 2004; Papayannopoulos et al., 2005), and vesicular trafficking (Aikawa and Martin, 2003; Kanzaki et al., 2004).

It is now known that the PI signaling pathway is utilized by many organisms to respond to extracellular signals (Berridge et al., 1999). According to one model, membrane bound receptors are coupled to production of second messenger Ins(1,4,5)P_3 (Berridge, 1993; Berridge et al., 1999). Upon stimulation, phospholipase C (PLC, E.C. 3.1.4.3) is activated by these receptors and produces Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG) using phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] as a substrate (Nishizuka, 1992; Berridge, 1993). A rapid increase in Ins(1,4,5)P_3 levels results in Ca^{+2} release from intracellular stores which activates downstream signaling components to mediate biological responses (Berridge et al., 2003). There are number of studies that provide evidence that plants also utilize this pathway (Munnik et al., 1998; Stevenson et al., 2000; Fordham-Skelton and Lindsey, 2001; Meijer and Munnik, 2003). Moreover, transient increases in Ins(1,4,5)P_3 levels have been shown in response to abscisic acid (ABA) (Lee et al., 1996; Sanchez and Chua, 2001; Burnette et al., 2003), gravity (Perera et al., 1999; Perera et al., 2001) and hyperosmotic stress (DeWald et al., 2001; Takahashi et al., 2001).

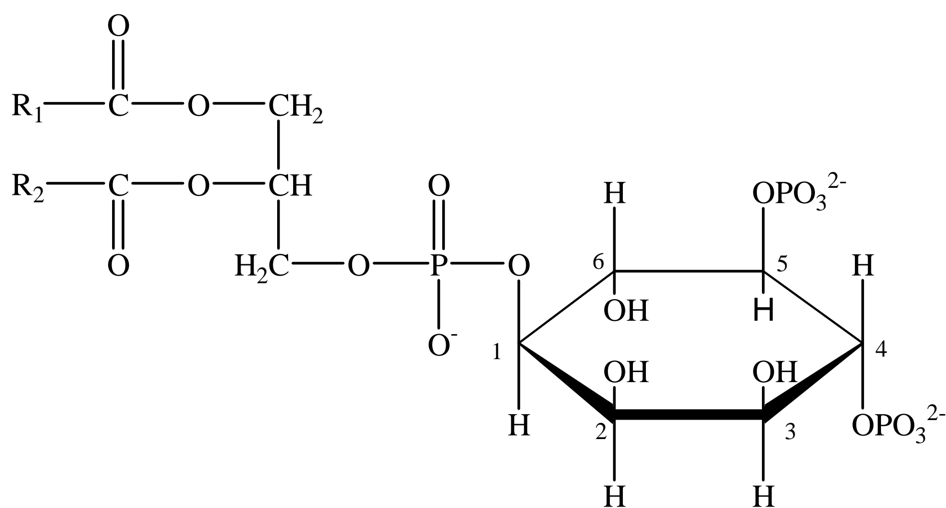
In addition to Ins(1,4,5)P_3, several other inositol-containing second messengers have been identified, such as Ins(1,3,4,5)P_4 (Tsubokawa et al., 1994), PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Insall and Weiner, 2001), indicating the complexity of the PI signaling pathway (Figure 1). Potential regulators of this pathway are the 5PTase enzymes that can dephosphorylate key second messenger molecules like PtdIns$(4,5)_2$, PtdIns$(3,4,5)_3$,



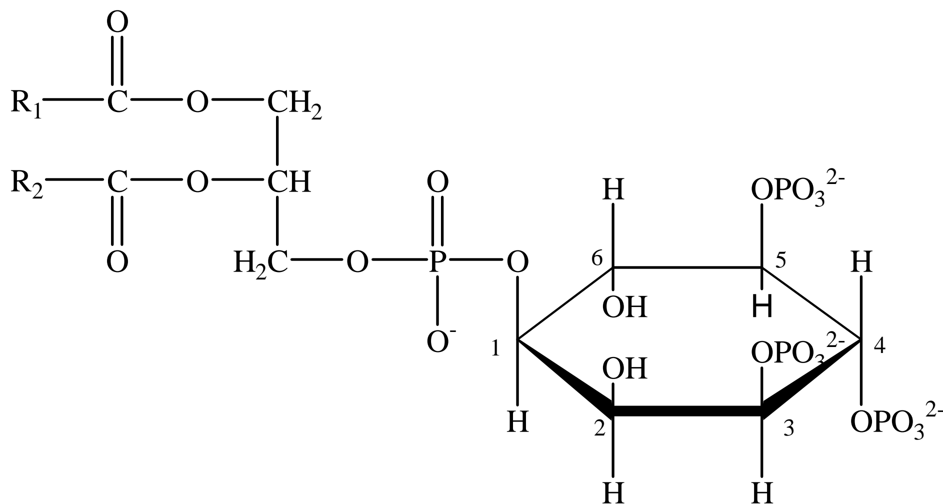
**D-*Myo*-Inositol (1,4,5)-
trisphosphate**



**D-*Myo*-Inositol (1,3,4,5)-
tetrakisphosphate**



**Phosphatidylinositol (4,5)-
bisphosphate**



**Phosphatidylinositol (3,4,5)-
trisphosphate**

Figure 1. Structures of Inositol Phosphates and Phosphoinositides. Fatty acyl chains are designated by R_1 and R_2.

$Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ (Majerus et al., 1999). This makes the 5PTases excellent candidate enzymes for the regulation of the events involving these substrates as second messengers. For example, signals induced by $Ins(1,4,5)P_3$ can be terminated by 5PTases through hydrolysis of $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ (De Smedt et al., 1997; Speed et al., 1999; Sanchez and Chua, 2001; Burnette et al., 2003).

$Ins(1,3,4,5)P_4$ has not been shown to act as a second messenger in plants. However, in animal systems, it has been shown to be involved in several signaling pathways (Tsubokawa et al., 1994; Hermosura et al., 2000; Reiser et al., 2004; Solyakov et al., 2004). In gerbil hippocampal neurons, application of $Ins(1,3,4,5)P_4$ to normal cells resulted in neuronal degeneration through Ca^{+2} accumulation which mimicked ischemic neurons, providing evidence for $Ins(1,3,4,5)P_4$ action as a second messenger (Tsubokawa et al., 1994). In a mast cell line, $Ins(1,3,4,5)P_4$ was shown to inhibit $Ins(1,3,4)P_3$ metabolism by inhibition of 5PTase activity which facilitates Ca^{+2} influx, suggesting multiple functions for $Ins(1,3,4,5)P_4$ (Hermosura et al., 2000). More recently, $Ins(1,3,4,5)P_4$ was shown to stimulate the catalytic activity of a protein kinase, casein kinase 2, providing evidence for the involvement of inositol-containing second messengers in regulation of protein phosphorylation (Solyakov et al., 2004). Another intriguing function for $Ins(1,3,4,5)P_4$ is in chromatin remodeling (Shen et al., 2003). Studies with the yeast SWI/SNF complex, which can regulate transcription through modifying nucleosome structure, have shown that $Ins(1,3,4,5)P_4$ can stimulate SWI/SNF complex mobilization (Shen et al., 2003). Their findings provided a link between transcriptional regulation and inositol phosphate compounds (Shen et al., 2003).

Unlike the soluble second messengers, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, PIs like PtdIns(4,5)P_2 are components of membranes. One important role that PtdIns(4,5)P_2 plays is that, as discussed above, it serves as a precursor for Ins(1,4,5)P_3 production by PLC. In addition, without further modification PtdIns(4,5)P_2 is shown to regulate a number of cellular events such as actin and cytoskeleton organization (Corgan et al., 2004; Kumar et al., 2004), vesicular (Kanzaki et al., 2004) and membrane trafficking (De Camilli et al., 1996). Hence, defects in the synthesis of this molecule result in abnormal actin cytoskeleton organization. For example in *Saccharomyces cerevisiae*, calmodulin, which is a small Ca^{+2} binding protein, was shown to regulate actin cytoskeleton organization via regulating PtdIns(4,5)P_2 synthesis (Desrivieres et al., 2002). Calmodulin mutants were shown to have reduced PtdIns(4,5)P_2 levels and exhibit defects in actin cytoskeleton organization, indicating the importance of PtdIns(4,5)P_2 levels for normal actin cytoskeleton organization in yeast (Desrivieres et al., 2002). In addition, studies with mice lacking a 5PTase, synaptojanin, have indicated that abnormal PtdIns(4,5)P_2 levels resulting from loss-of-5PTase function cause aberrant synaptic vesicle trafficking (Cremona et al., 1999). PtdIns(4,5)P_2 levels were higher in synaptojanin knock-out mice because of the lack of synaptojanin activity which hydrolysis PtdIns(4,5)P_2 (Cremona et al., 1999).

PtdIns(3,4,5)P_3 is another potential substrate in the PI signaling pathway that can be hydrolyzed by 5PTases. In contrast to PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 levels are very low in the resting state of animal cells and increase in response to certain stimuli (Hinchliffe, 2001). PI-3 kinase is the enzyme that catalyzes the synthesis of PtdIns(3,4,5)P_3. Studies

with *Drosophila melanogaster* demonstrated that overexpression of a *Drosophila* PI-3 kinase in certain tissues of the flies correlates with growth, suggesting a role for PtdIns(3,4,5)P_3 in growth (Leevers et al., 1996). Pten (phosphatase and tensin homolog), which is mutated in certain cancers such as glioblastomas, endometrial carcinoma and prostate cancer, uses PtdIns(3,4,5)P_3 as a substrate. Mutation of this gene results in increased PtdIns(3,4,5)P_3 levels and causes tumorigenesis via the protein kinase Akt/PKB (Stambolic et al., 1998; Sun et al., 1999). Akt/PKB, which is a serine/threonine kinase, was shown to mediate downstream events triggered by increased PtdIns(3,4,5)P_3 levels (Stocker et al., 2002). In *Drosophila*, lethality that resulted from loss-of-Pten function was rescued by a mutation in the PtdIns(3,4,5)P_3-interacting domain of Akt/PKB, indicating that Akt/PKB is the key player in mediating the downstream effects of PtdIns(3,4,5)P_3 (Stocker et al., 2002). In plants, PtdIns(3,4,5)P_3 has not been detected (Meijer and Munnik, 2003) , but the *Arabidopsis* homolog of Pten has been shown to be required for pollen development (Gupta et al., 2002). It is possible, as in animal systems, that this molecule can be produced in plants in response to certain stimuli.

II. Substrate Selectivity and Possible Functions of Eukaryotic 5PTases

Substrate selectivity of the 5PTases was first studied in animal model systems. With the completion of the genome sequences of model plants such as *Arabidopsis* and *Oryza sativa*, PI signaling research is rapidly expanding in the plant sciences. In this section, I review the 5PTases from eukaryotic organisms in terms of their substrate selectivity and physiological function.

A. Arabidopsis thaliana 5PTases

After sequencing and annotation of the genome sequence of the first plant, *Arabidopsis*, it was estimated that 25,498 genes are present in the *Arabidopsis* genome (Arabidopsis-Genome-Initiative, 2000). Addition of this complete genome sequence made *Arabidopsis* an invaluable tool for identifying and characterizing gene function. In *Arabidopsis*, 15 putative 5PTases were identified (Berdy et al., 2001) containing two well conserved signature motifs (F/I)W*X*GD*X*N(F/Y)R and (R/N)*X*P(S/A)(W/Y)(C/T)DR(I/V)(L/I) which are present in all 5PTases (Majerus et al., 1999). I will refer to this domain as the inositol phosphate phosphatase catalytic domain (IPPc). In this section, functionally and biochemically characterized *Arabidopsis* 5PTases will be described.

At5PTase1 is the first *Arabidopsis* 5PTase enzyme that was characterized in terms of its substrate selectivity (Berdy et al., 2001). When expressed in *E.coli*, recombinant At5PTase1 was catalytically inactive. Berdy et al., (2001) generated transgenic *Arabidopsis* plants that ectopically express At5PTase1 (Berdy et al., 2001). At5PTase1 transgenic plants that carry the 35S promoter of the cauliflower mosaic virus-At5PTase1 full-length cDNA cassette were generated via *Agrobacterium tumefaciens* mediated transformation (Berdy et al., 2001). Soluble protein extracts from these transgenic plants were used in their assays (Berdy et al., 2001). Potential substrates [Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and PtdIns(4,5)P_2] were tested, and only hydrolysis of the soluble substrates [Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4] was observed (Berdy et al., 2001). In addition, At5PTase1 did not result in increased hydrolysis of Ins(1)P or Ins(1,4)P_2 with transgenic

plant extracts (Berdy et al., 2001). This activity profile was similar to the Type I 5PTases identified in mammalian systems (Majerus et al., 1999).

In order to delineate the function of At5PTase1 in *Arabidopsis*, consequences of At5PTase1 ectopic expression were investigated (Burnette et al., 2003). When stomata of At5PTase1 transgenic plants were exposed to ABA and light, they were less responsive than stomata in non-transgenic controls (Burnette et al., 2003). It was also found that in WT seedlings, Ins(1,4,5)P_3 levels were induced by exposure to ABA and this Ins(1,4,5)P_3 increase was reduced in At5PTase1 transgenic plants, indicating that At5PTase1 overexpression altered the Ins(1,4,5)P_3 levels (Burnette et al., 2003). This provided evidence for the importance of Ins(1,4,5)P_3 levels for ABA signaling that mediates stomatal closure (Burnette et al., 2003), Moreover, At5PTase1 was shown to be regulated by ABA at both the transcriptional and translational level based on RT-PCR and western blot analyses using an anti-rabbit At5PTase1 peptide antibody (Burnette et al., 2003). Together, the phenotypes of these At5PTase1 transgenic plants provided strong evidence for At5PTase1 as a negative regulator downstream of ABA in the signaling pathway (Burnette et al., 2003).

Another At5PTase that was investigated via a dexamethasone inducible expression system is At5PTase2 (Sanchez and Chua, 2001). Although At5PTase1 was inactive when produced in a bacterial system with an N-terminal His-tag, At5PTase2 was reported to be active and to hydrolyze Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, but not PtdIns(4,5)P_2 (Sanchez and Chua, 2001). This substrate selectivity also resembles Type I 5PTases (Majerus et al.,

1999). When At5PTase2 expression was induced in transgenic plants with dexamethasone, ABA-induced Ins(1,4,5)P_3 production was reduced compared to control plants (Sanchez and Chua, 2001). This provides evidence for the involvement of At5PTase2 in regulation of ABA-induced Ins(1,4,5)P_3 levels (Sanchez and Chua, 2001). Thus, At5PTase1 and At5PTase2 might have overlapping functions in regulation of ABA signaling as both gain-of-function transgenic plant lines are defective in ABA signaling. However, At5PTase1 and At5PTase2 transgenic seeds were different in their germination, a process known to be inhibited by ABA. At5PTase2 transgenic seeds were insensitive to exogenous ABA (Sanchez and Chua, 2001). However, At5PTase1 transgenic seeds were not insensitive (Burnette et al., 2003) indicating that ectopic expression of At5PTase1 and At5PTase2 have different effects on seedlings.

Isolation of *cvp2* (At5PTase6) in a genetic screen for mutants with defects in cotyledon vascular pattern formation in *Arabidopsis* demonstrated an unexpected function for a plant 5PTase (Carland and Nelson, 2004). Although, the *cvp2* mutant plants were normal in plant growth and morphology, they demonstrated an increase in open vein endings. Although substrate selectivity of At5PTase6 was not examined in the study, Ins(1,4,5)P_3 levels were found to be higher in *cvp2* plants compared to wild-type plants (Carland and Nelson, 2004). This suggests that the CVP2 protein is required to hydrolyze Ins(1,4,5)P_3. In addition, the expression pattern of At5PTase6 was examined by generation of transgenic plants that carry a At5PTase6 promoter: β-glucuronidase (GUS) reporter cassette (Carland and Nelson, 2004). At5PTase6 expression was not restricted at the early stages of embryogenesis, but became restricted to vascular tissues at later stages of

the embryogenesis (Carland and Nelson, 2004). A similar pattern of expression was observed in leaves where expression became restricted to developing vascular cells (Carland and Nelson, 2004). However, it is still unclear that how alteration of Ins(1,4,5)P_3 signaling results in open vein endings. It is possible that increased Ins(1,4,5)P_3 levels in vascular cells might trigger a programmed cell death pathway resulting in open vein endings. Supporting this idea, a similar role for Ins(1,4,5)P_3 and Ca^{+2} in signaling for the differentiation and programmed cell death of tracheary elements of *Zinnia elegans* L. cells was also suggested (Zhang et al., 2002).

Another unexpected function for an *Arabidopsis* 5PTase was identified through a genetic screen for mutants that are defective in stem strength (Zhong et al., 2004). The *FRA3* gene (At5PTase12) was isolated in these mutant screens and found to be necessary for secondary wall synthesis and normal actin organization in fiber cells (Zhong et al., 2004). FRA3 contains six WD-repeat domains in addition to the IPPc domain and there are three additional 5PTases in *Arabidopsis* that have WD-repeats (Berdy et al., 2001; Zhong et al., 2004). The FRA3 enzyme was reported to hydrolyze PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3 but not Ins(1,3,4,5)P_4 which is an unusual pattern (Zhong et al., 2004). At5PTase14, which is another WD-repeat containing 5PTase, has a similar substrate selectivity pattern whereas other WD-repeat containing 5PTases (At5PTase12 and At5PTase13) were shown to hydrolyze only Ins(1,4,5)P_3 (Zhong and Ye, 2004). Taken together, these data indicate that the At5PTases have diverse and important functions in *Arabidopsis*. Ongoing efforts to characterize the At5PTases with unknown function will further extend the knowledge of these proteins.

B. Mammalian 5PTases

Ten mammalian 5PTases have been characterized and proposed to form four groups depending on their substrate selectivity (Hodgkin et al., 1994; Zhang et al., 1995; Majerus et al., 1999; Kisseleva et al., 2000). Group I contains the HsTypeI 5PTase whose activity was first detected in human erythrocyte membranes (Hodgkin et al., 1994). The HsTypeI 5PTase has the ability to hydrolyze both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 but not PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 substrates (Hodgkin et al., 1994). In later studies, the same enzyme was also purified from other tissues such as brain and platelets (Connolly et al., 1985; De Smedt et al., 1994). Protein kinase C has been shown to phosphorylate and activate the HsTypeI 5PTase (Connolly et al., 1986). Sequence analysis of the HsTypeI 5PTase has shown that it has a C-terminal farnesylation site, CVVQ (Laxminarayan et al., 1994; Verjans et al., 1994). When HsTypeI 5PTase containing a mutation in the farnesylation motif was expressed in Cos-7 cells, it resulted in protein association with the soluble fraction rather than the particulate fraction. This was further supported by immunofluorescence analysis which demonstrated the localization of the mutant HsTypeI 5PTase to the cytosol (De Smedt et al., 1996). An important function for the HsTypeI 5PTase in controlling Ca^{+2} signaling is suggested based on the studies with intact Chinese hamster ovary cells stimulated by ATP (De Smedt et al., 1997). Overexpression of intact HsTypeI 5PTase resulted in total loss of Ca^{+2} oscillations in 87% of the transfected Chinese hamster ovary cells in response to ATP (De Smedt et al., 1997). However, only 1% of the mutant HsTypeI 5PTase (that cannot be farnesylated) transfected cells demonstrated loss-of Ca^{+2} oscillations indicating the importance of the localization signal

(De Smedt et al., 1997). Moreover, a complex regulation of the HsTypeI 5PTase activity has been demonstrated by showing phosphorylation of the HsTypeI 5PTase by a calcium/calmodulin-dependent protein kinase II (Communi et al., 2001).

A separate type of animal 5PTase enzyme can dephosphorylate not only soluble substrates but also lipid substrates such as PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Majerus et al., 1999). These are called Group II 5PTases, and this group contains OCRL, the HsTypeII 5PTase, Synaptojanin-1, and Synaptojanin-2 (Zhang et al., 1995; Cremona et al., 1999; Majerus et al., 1999). The OCRL protein is the 5PTase whose absence results in the Lowe syndrome in humans (Zhang et al., 1995). Cell lines from kidney proximal tubules of Lowe patients have been shown to accumulate PtdIns(4,5)P_2 as compared to cell lines from healthy individuals (Zhang et al., 1998). This suggests that the *in vivo* preferred substrate for OCRL is PtdIns(4,5)P_2 (Zhang et al., 1998). Although in humans OCRL deficiency results in Lowe syndrome, OCRL knock-out mice show no phenotype suggesting that in humans, the OCRL protein has unique functions that cannot be compensated for by other human 5PTase isozymes (Janne et al., 1998). Patients with Lowe syndrome suffer from growth and mental retardation, renal tubular acidosis, and cataracts (Lowe et al., 1952). Studies that attempt to identify the localization site of OCRL have shown that in kidney proximal tubule cells OCRL associates with lysosomes (Zhang et al., 1998) whereas in fibroblasts it localizes to the trans-Golgi network (Suchy et al., 1995). More recently, OCRL has been shown to associate with endosomes suggesting a role in the control of endosomal receptor trafficking (Ungewickell et al., 2004).

Synaptojanin-1 is another member of the Group II 5PTases (Majerus et al., 1999). It was shown to hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2 (McPherson et al., 1996; Chung et al., 1997). Synaptojanin-1 knock-out mice display increased PtdIns(4,5)P_2 levels and clathrin-coated vesicles in nerve endings that result in a short lifespan after birth (Cremona et al., 1999). This indicates that Synaptojanin-1 is important for synaptic vesicle recycling (Cremona et al., 1999). Synaptojanin-2, which is a ubiquitously expressed 5PTase with a similar substrate selectivity compared to Synaptojanin-1 (Nemoto et al., 1997), has been shown to have a distinct function from Synaptojanin-1 based on studies with a lung carcinoma cell line (Rusk et al., 2003). Using a small interfering RNA approach, it was shown that repression of Synaptojanin-2 expression causes aberrations in clathrin-mediated receptor internalization (Rusk et al., 2003). HsTypeII 5PTase, which also belongs Group II, was shown to hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and PtdIns(4,5)P_2 (Jefferson and Majerus, 1995). Functional characterization of HsTypeII 5PTase was done via a knock-out mouse approach (Hellsten et al., 2001). Lack of this enzyme in male mice resulted in disruption of sperm function and infertility (Hellsten et al., 2001).

SKIP (skeletal muscle and kidney enriched inositol phosphatase) and PIPP (proline-rich inositol-polyphosphate 5-phosphatase) are two enzymes that have catalytic activity similar to Group II 5PTases (Mochizuki and Takenawa, 1999; Ijuin et al., 2000). Both of these enzymes were shown to hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and PtdIns(4,5)P_2 (Mochizuki and Takenawa, 1999; Ijuin et al., 2000). Moreover, SKIP was also shown to hydrolyze PtdIns(3,4,5)P_3 *in vitro* (Ijuin et al., 2000). In addition, both enzymes contain a

novel domain that targets them to membrane ruffles (Gurung et al., 2003) suggesting that targeting of these 5PTases to specific compartments is important for their function.

Group III 5PTases are defined as enzymes that can only hydrolyze substrates containing a 3-position phosphate, i.e. PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4. The first enzyme identified from this group is SHIP-1 that contains a Src homology 2 (SH2) domain that mediates interactions with certain growth factor receptors (Koch et al., 1991; Damen et al., 1996). SHIP-1 knock-out mice have been generated (Helgason et al., 1998). Lack of SHIP-1 shortened the lifespan of the mice, and myeloid cell expansion and lung infiltration occurred, which was consistent with the fact that SHIP-1 expression was restricted to hematopoietic and lymphoid cells (Damen et al., 1996; Helgason et al., 1998). The second characterized member of this group is SHIP-2, which shares biochemical and structural similarities with SHIP-1 (Damen et al., 1996; Pesesse et al., 1997). Because of their ability to hydrolyze PtdIns(3,4,5)P_3, these 5PTases have been investigated for their involvement in the regulation of the PI-3 kinase pathway where PtdIns(3,4,5)P_3 levels are critical to activate downstream signaling pathways (Aman et al., 1998; Hunter and Avalos, 1998). Since it differs from SHIP-1 in its tissue expression pattern, it was proposed that SHIP-2 might be a regulator of the PI-3 kinase pathway by hydrolyzing PtdIns(3,4,5)P_3 (Aman et al., 1998). Initially it was reported that mice lacking SHIP-2 had increased sensitivity to insulin leading to early postnatal lethality (Clement et al., 2001). A caveat of this experiment is the fact that a second gene, *Phox2a*, was also partially deleted in the SHIP-2 knock-out mice (Clement et al., 2004). This may indicate that the phenotype described for SHIP-2 knock-out mice could also result from a loss-of

function in the *Phox2a* gene (Clement et al., 2004). To resolve this problem, a second SHIP-2 knock-out mouse was generated in a recent study which was null for SHIP-2 mRNA and protein (Sleeman et al., 2005). These mice did survive and had normal glucose homeostasis and were normal in their insulin and glucose sensitivity (Sleeman et al., 2005). However, these mice resisted gaining weight when they were fed a high-fat diet, suggesting a role for this enzyme in dietary obesity (Sleeman et al., 2005).

Group IV 5PTases have been described as having activity towards only the lipid substrates [PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3] (Majerus et al., 1999). One such enzyme, which is highly expressed in brain tissue, has been characterized (Kisseleva et al., 2000). Studies with cultured cells have shown that overexpressing the HsTypeIV enzyme leads to apoptosis by inhibiting phosphorylation of Akt/Protein Kinase B which is a downstream mediator of the PI-3 kinase signaling pathway (Kisseleva et al., 2002).

C. Saccharomyces cerevisiae 5PTases

In *Saccharomyces cerevisiae*, there are four 5PTase enzymes, Inp51, Inp52, Inp53 and Inp54 (Stolz et al., 1998a; Stolz et al., 1998b). The IPPc domain of all four enzymes has been shown to dephosphorylate PtdIns(4,5)P_2 at the 5-position (Guo et al., 1999). However, *S. cerevisiae* 5PTases do not demonstrate significant hydrolysis of soluble substrates such as Ins(1,4,5)P_3 (Stolz et al., 1998a; Stolz et al., 1998b). Besides their IPPc domain, Inp51, Inp52 and Inp53 also have a separate domain called Sac1, which is also present in the yeast Sac1 protein (Guo et al., 1999). Mutations in *SAC1* were identified as

suppressors of *sec14-1* temperature sensitive mutants that are defective in phosphatidylinositol/phosphatidylcholine transfer protein (Cleves et al., 1989). When the catalytic activity of the Sac1 domain of Inp52p and Inp53p was examined, it was found that this domain, separate from the 5PTase domain, has the ability to dephosphorylate, PtdIns(3)P, PtdIns(4)P and PtdIns(3,5)P_2 to PtdIns, but not PtdIns(4,5)P_2 (Guo et al., 1999). However, HsSac2 which is a Sac-domain containing protein from humans was shown to hydrolyze PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 suggesting that Sac1 domain catalytic activity is not restricted to PtdIns(3,5)P_2 in humans (Minagawa et al., 2001). Null mutants of all four Sc5PTases individually demonstrated a relatively normal growth phenotype, whereas a *S. cerevisiae* triple mutant lacking Inp51, Inp52 and Inp52 is unviable indicating 5PTases are essential enzymes with overlapping functions (Stolz et al., 1998b). However, reports suggesting unique functions for some of the Sc5PTases are also published (Stolz et al., 1998a). It was shown that *S. cerevisiae* lacking Inp51 demonstrated a cold-resistant phenotype (Stolz et al., 1998a). Moreover, Inp54, which does not have a Sac1 domain, was shown to localize to the endoplasmic reticulum via a C-terminal anchor tail (Wiradjaja et al., 2001). Analysis of Inp54 deficient *S. cerevisiae* provided evidence for its involvement in the regulation of secretion (Wiradjaja et al., 2001).

D. Drosophila melanogaster 5PTases

In *Drosophila melanogaster*, 5PTases have not been characterized in detail (Seeds et al., 2004). In a recent study addressing the pathway for inositol polyphosphate synthesis in

D. melanogaster, it was reported that the *D. melanogaster* genome contains a single putative 5PTase (Dm5PTaseI) with 58% similarity to the HsType I 5PTase, two putative Type II 5PTase isozymes, a putative synaptojanin ortholog and a putative Type IV 5PTase (Seeds et al., 2004). These putative 5PTases have not been characterized for their biochemical activities in detail (Seeds et al., 2004). However, using dsRNA to knockdown Dm5PTaseI expression in *Drosophila* S2 cells, it was shown that extracts prepared from S2 cells with reduced dm5PTaseI expression demonstrated a 10-15% decrease in dephosphorylation of Ins(1,4,5)P_3 compared to control extracts (Seeds et al., 2004).

E. Dictyostelium discoideum 5PTases

In *Dictyostelium discoideum*, four 5PTases have been identified and named Dd5P1, Dd5P2, Dd5P3, and Dd5P4 (Loovers et al., 2003). *In vitro* characterization of the substrate selectivity of these Dd 5PTases was performed using *Escherichia coli* expressed catalytic domains (Loovers et al., 2003). According to these studies, Dd5P2 and Dd5P4 can hydrolyze all four substrates (Loovers et al., 2003). However, Dd5P3 hydrolyzed Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and PtdIns(4,5)P_2 (Loovers et al., 2003). Dd5P2 displayed the highest activity towards Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 which suggests a Group III-like substrate selectivity (Loovers et al., 2003). Moreover, Dd5P3 hydrolyzed Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 better than lipid substrates which indicates a preference towards soluble substrates (Loovers et al., 2003). Because of the low level of expression, Dd5P1 activity could not be characterized (Loovers et al., 2003). Functional

characterization of *D. discoideum* 5PTases were performed through a gene inactivation approach which identified Dd5P4 having role in growth and development (Loovers et al., 2003). However, single and double gene knock-outs for Dd5P1, Dd5P2, and Dd5P3 suggested that these enzymes are functionally redundant (Loovers et al., 2003).

F. Caenorhabditis elegans 5PTases

In *Caenorhabditis elegans*, two enzymes that contain the IPPc domain, have been functionally characterized (Harris et al., 2000; Bui and Sternberg, 2002). Mutations in Unc-26, which encodes a *C. elegans* ortholog of synaptojanin, were shown to result in abnormal synaptic vesicle recycling in *C. elegans* (Harris et al., 2000). IPP-5, which is closely related to HsTypeI 5PTase, was studied in intact *C. elegans* through gene deletion (Bui and Sternberg, 2002). It was reported that IPP-5 negatively regulates ovulation possibly though negative regulation of Ins(1,4,5)P_3 signaling (Bui and Sternberg, 2002). However, biochemical characterization of the Unc-26 and IPP-5 remains to be studied.

III. Phosphoinositide Signaling and Auxin, Abscisic Acid, and Gravity

There is a substantial amount of evidence that plants use the PI signaling pathway (Munnik et al., 1998; Stevenson et al., 2000; Meijer and Munnik, 2003). Over the past fifteen years several signals such as auxin, ABA and gravity were studied and examined for their role in stimulating synthesis of Ins(1,4,5)P_3 in plants.

A. Auxin

Auxin (indole-3-acetic acid, IAA) is a plant growth hormone (Thimann, 1977). It is involved in the regulation of many processes such as cell elongation, cell division and differentiation (Woodward and Bartel, 2005). To examine the effect of auxin on PI metabolism, membranes of carrot suspension cells were labeled with [γ-^{32}P]ATP (Zbell et al., 1989). It was shown that auxin causes a rapid and transient decrease in ^{32}P label in PI extracts of the carrot suspension cells (Zbell et al., 1989). In addition, exogenous Ins(1,4,5)P_3 application resulted in Ca^{+2} release (Zbell et al., 1989). Together, these studies provided evidence for auxin stimulation of PI signaling in carrot suspension cells.

B. Abscisic Acid

The plant hormone ABA regulates seed germination and is involved in plant responses to drought (Evans and Hetherington, 2001). ABA induces closure of stomata (Schroeder et al., 2001; Finkelstein et al., 2002). It has been shown that microinjection of Ins(1,4,5)P_3 into guard cells initiates closure through an increase in [Ca^{+2}] (Gilroy et al., 1990). Lee et al., (1996) demonstrated that endogenous levels of Ins(1,4,5)P_3 increase within 2 min of ABA addition to stomata (Lee et al., 1996). Other supporting evidence for the involvement of Ins(1,4,5)P_3 in the ABA signal transduction pathway(s) comes from a study using U-73122, an inhibitor of PLC (Staxen et al., 1999). In that study, it was shown that this inhibitor resulted in inhibition of ABA-induced cytosolic Ca^{+2} oscillations and reduced stomatal closure (Staxen et al., 1999). More recent experiments

using transgenic plants that ectopically express At5PTase1 (Burnette et al., 2003) and an antisense approach to decrease the level of expression of the PLC gene (AtPLC1) in *Arabidopsis* (Sanchez and Chua, 2001), provided further evidence for the role of Ins(1,4,5)P_3 in ABA signaling. Following an ABA stimulus, antisense AtPLC1 plants showed reduced levels of Ins(1,4,5)P_3 (Sanchez and Chua, 2001). Moreover, antisense AtPLC1 seedlings showed no inhibition of germination or growth in the presence of ABA concentrations that are normally inhibitory to germination (Sanchez and Chua, 2001). Xiong et al., (2001) provided genetic evidence that Ins(1,4,5)P_3 plays a general role in plant responses to ABA, drought, cold and salinity by analyzing *Arabidopsis* plants that contain a mutation in an inositol polyphosphate 1-phosphatase gene called FIERY1 (Xiong et al., 2001). The *fiery1* mutants were more sensitive to ABA than wild-type in seed germination and growth (Xiong et al., 2001). In addition, the *fiery1* mutants had higher Ins(1,4,5)P_3 levels compared to wild-type when exposed to ABA indicating the importance of Ins(1,4,5)P_3 in ABA signaling (Xiong et al., 2001). Together, these studies provide strong evidence that plants utilize PI signaling pathway to mediate ABA signaling.

C. Gravity

Gravity is an environmental signal to which plants need to respond for proper growth control. Perception of gravity in plants is mediated through a cascade of biophysical and biochemical events (Morita and Tasaka, 2004). Studies with maize pulvini demonstrated that gravistimulation results in transient and persistent increases in Ins(1,4,5)P_3 (Perera et

al., 1999). Within 10 s of gravistimulation, a transient 5-fold increase in Ins(1,4,5)P_3 levels were observed in the lower half of the pulvinus (Perera et al., 1999). Moreover, rapid oscillations in Ins(1,4,5)P_3 levels were observed between the lower and upper halves of the pulvini over 30 min and Ins(1,4,5)P_3 levels continued to increase up to 5-fold over basal levels until growth was noticeable (Perera et al., 1999). In addition, after the bending started, Ins(1,4,5)P_3 levels returned to basal levels (Perera et al., 1999). Furthermore, the synthesis of PtdIns(4,5)P_2 was up-regulated as a result of an increase in PtdIns(4)P 5- kinase activity within 10 min of gravistimulation (Perera et al., 1999). Together, these results suggest short and long term roles for Ins(1,4,5)P_3 in the perception of gravity. Consistent results were obtained from studies with oat shoot pulvini which further supported the idea of involvement of Ins(1,4,5)P_3 in the gravity response (Perera et al., 2001).

CHAPTER II

Objective I: To Determine the Substrate Specificity of At5PTase11

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Consent of coauthor G. Gillaspy and the American Society of Plant Biologists is appreciated.

Abstract

Phosphoinositides are important molecules that serve as second messengers and bind to a complex array of proteins modulating their subcellular location and activity. The enzymes that metabolize phosphoinositides can in some cases serve to terminate the signaling actions of phosphoinositides. The inositol polyphosphate 5-phosphatases (5PTases) comprise a large protein family that hydrolyzes 5-phosphates from a variety of inositol phosphate and phosphoinositide substrates. We previously reported the identification of 15 putative 5PTase genes in *Arabidopsis* and have shown that overexpression of the At5PTase1 gene can alter abscisic acid signaling. At5PTase1 and At5PTase2 have been shown to hydrolyze the 5-phosphate from inositol phosphate substrates. We have examined the substrate specificity of the At5PTase11 protein, which is one of the smallest predicted 5PTases found in any organism. We report here that the At5PTase11 gene (At1g47510) encodes an active 5PTase enzyme that can only dephosphorylate phosphoinositide substrates containing a 5-phosphate. In addition to hydrolyzing known substrates of 5PTase enzymes, At5PTase11 also hydrolyzes the 5-phosphate from phosphatidylinositol (3,5) bisphosphate. We also show that the At5PTase11 gene is

regulated by abscisic acid, jasmonic acid, and auxin, suggesting a role for phosphoinositide action in these signal transduction pathways.

Introduction

Phosphoinositides (PIs) play a role in a variety of critical eukaryotic cellular processes (Stevenson et al., 2000; Meijer and Munnik, 2003). For example, phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2] serves as a precursor to second messenger molecules such as inositol (1,4,5) trisphosphate [Ins(1,4,5)P_3], diacylglycerol, and, in animal cells, phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3]. In animal cells, PtdIns(4,5)P_2 is also involved in regulation of the actin cytoskeleton, stress fiber formation, and membrane trafficking (Toker, 1998). In plants, although much less is known, evidence suggests that PtdIns(4,5)P_2 plays an important role in actin cytoskeleton rearrangements via its binding to profilin (Staiger et al., 1994), which has been localized to the growing ends of root hairs (Braun et al., 1999) and pollen tubes (Kost et al., 1999). In fact, either hydrolyzing PtdIns(4,5)P_2 or binding it via a plextrin-homology domain disrupts tip growth in pollen tubes (Kost et al., 1999), suggesting that availability of PtdIns(4,5)P_2 is crucial to growth in these cells.

The enzymes that metabolize PIs such as PtdIns(4,5)P_2 can control the amount of these molecules and, in general, are considered to be important targets for understanding disease and growth control (Pendaries et al., 2003). The inositol polyphosphate 5-phosphatases (5PTases) are a large group of enzymes that have the ability to hydrolyze 5-phosphates from a variety of inositol phosphate and PI substrates (Majerus et al., 1999). Studies on human 5PTases have classified the 5PTases into four groups according to the substrates they hydrolyze *in vitro*. All 5PTases characterized to date contain a conserved

catalytic domain referred to as the inositol polyphosphate phosphatase catalytic (IPPc) domain. In addition, the IPPc domain is often accompanied by other domains that presumably allow for modification of 5PTases or association with specific signal transduction components (Majerus et al., 1999).

We previously reported the identification of 15 putative 5PTase genes in *Arabidopsis* (Berdy et al., 2001). We and others have shown that overexpression of either At5PTase1 or At5PTase2 alters abscisic acid (ABA) signaling (Sanchez and Chua, 2001; Burnette et al., 2003). The At5PTase1 and At5PTase2 gene products have been characterized biochemically and shown to hydrolyze the 5-phosphate from inositol phosphate substrates (Berdy et al., 2001; Sanchez and Chua, 2001). In the current work to be described in this chapter, I have examined the substrate specificity of another At5PTase, the At5PTase11 protein, which is one of the smallest predicted 5PTases found in any organism. I found that the At5PTase11 gene (At1g47510) encodes an active 5PTase enzyme that has a different substrate specificity as compared to other plant 5PTases. I also show that the At5PTase11 gene is regulated by ABA, jasmonic acid (JA), and auxin, suggesting a role for PI action in these signal transduction pathways.

Materials and Methods

Plant Growth and Treatment

Arabidopsis ecotype Columbia plants were used for all experiments. Growth conditions of soil-grown plants have been described previously (Berdy et al., 2001). For ABA (Sigma-Aldrich, St. Louis), JA (95% methyl jasmonate solution, Sigma-Aldrich), and auxin (α-naphthalene acetic acid, Sigma-Aldrich) experiments, seed from wild-type plants was germinated in flasks containing Murashige and Skoog medium (Murashige and Skoog, 1962) containing 1% Suc and grown for 5 d under constant light and shaking (91 rpm). Seedlings were then treated with 100 μM ABA (0.1% ethanol final concentration), 100 μM methyl jasmonate, and 10 μM 1α-naphthalene acetic acid and frozen in liquid nitrogen at the indicated times. Hormone treatment experiments were repeated three times.

Gene Cloning and Sequence Analysis

Full-length At5PTase11 cDNA was generated by RT-PCR using *Arabidopsis* mixed-tissue mRNA and the following oligonucleotide primers: At5PTase11-Nterm (5'ATGGGGAATAAGAATTCGATGT3') and At5PTase11-Cterm (5'TTAACTGTTGACCCACTTCAAGCAAA3'). RT reaction (2 μl), At5PTase11-Nterm (12.5 pmol/μl), At5PTase11-Cterm (12.5 pmol/μl), elongase polymerase and supplied buffer (Invitrogen, Carlsbad, CA), 0.5 mM dNTPs, and 2.5 mM final $MgCl_2$ were mixed

and heated to 94°C for 3 min. PCR amplification consisted of 30 cycles (1 min 94°C, 1 min 58°C, 1.5 min 72°C). The full-length At5PTase11 cDNA product was gel purified (Qiagen gel extraction kit; Qiagen, Valencia, CA), and cloned into the T7/NT TOPO TA expression vector (Invitrogen) according to the manufacturer's instructions. The resulting prokaryotic expression construct (pNTTOPOAt5PTase11) was used as template in a PCR reaction with primers pMTAt5PTase11-Nterm (5'GCCATGGGGAATAAGAATTCGATGTGTGGGT3') and pMTAt5PTase11-Cterm (5'ACTGTTGACCCACTTCAAGCAAA3') to introduce a Kozak sequence for efficient translation initiation and to generate a C-terminal V5-His tag. The modified cDNA was cloned into the pMT/V5-His-TOPO vector (Invitrogen). The resulting construct (pMTAt5PTase11) was sequenced and compared to the genomic and predicted cDNA sequences present in the NCBI database (AC007519 and NM_103644). The At5PTase1 full-length cDNA (Berdy et al., 2001) was similarly expressed as a C-terminal V5-His-tagged protein in S2 cells, resulting in the production of recombinant At5PTase1 protein as judged by SDS-PAGE and western blotting. Purified human OCRL protein was a gift from Drs. Phillip Majerus and Marina Kisseleva (Washington University School of Medicine, St. Louis); its purification and activity have been described (Zhang et al., 1995). 5PTase protein alignments were created with ClustalX and Boxshade software.

Transient Expression of At5PTase11 in Drosophila melanogaster Cells

For transient expression studies, pMTAt5PTase11 was used to transfect *Drosophila melanogaster* S2 cells utilizing an Effectene transfection kit (Qiagen). Briefly, S2 cells

were cultured in Schneider's Drosophila medium (Invitrogen) supplemented with 10% heat-inactivated (60°C for 30 min) fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated in a 28°C incubator with humidity and were routinely passaged every 3 d at a cell density of approximately 5×10^6 cells/ml. For transfections, cells were seeded into 25-cm^2 flasks at a density of 1×10^6 cells/ml in 10 ml of medium; 24 h later cells were spun at 800 g for 3 min and washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2), and the resulting cell pellets were resuspended in 4 ml of fresh medium. Two micrograms of plasmid DNA, 16 μl of enhancer, and 20 μl of Effectene reagent (Qiagen) were mixed according to manufacturer's instructions and added into the cell culture. After 24 h, cells were induced with 500 μM (final concentration) $CuSO_4$ for 2 d. Cells were harvested and washed with phosphate-buffered saline (pH 7.2) and frozen in liquid N_2 at –80°C. For labeling of S2 cells, we have modified a described method for human astrocytoma cells (Stephens et al., 1989). Transfections were performed as described above, except 125 μCi of [1,2 ^{3}H (N)]*myo*-inositol (American Radiolabeled Chemicals, St. Louis; 30 Ci/mM in sterile water) was added after 36 h of induction with $CuSO_4$. Cells were harvested 12 h later and lipids were purified using an acidified Bligh and Dyer extraction (Bird, 1998). Deacylation of the purified lipids was performed (Bird, 1998), and the resulting deacylation products (GroPIns) were analyzed by HPLC.

Immunoprecipitation of At5PTase11 from Transiently Transfected S2 Cells

S2 cells (10 ml) transfected with pMTAt5PTase11 or pMTβ-gal were harvested and cell pellets were dissolved in 300 μl of immunoprecipitation buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100). Cell lysis was achieved by passage through a 25-gauge syringe 10 times. The lysate was spun at 14,000 rpm for 10 min, and the resulting soluble fraction was incubated with protein A sepharose (Sigma-Aldrich) complexed with mouse anti-V5 monoclonal antibody (Invitrogen) for 2 h at 4°C with end-over-end rotating. The protein A sepharose beads were pelleted and washed three times with immunoprecipitation buffer followed by one wash with 5PTase activity assay buffer. A portion of this immunoprecipitate was analyzed by SDS-PAGE and western blotting to estimate the amount of immunoprecipitated protein.

Western-Blot Analysis

Protein extracts and immunoprecipitates prepared from pMTAt5PTase11-transfected, pMTβ-gal-transfected, and mock-transfected S2 cells were separated on 10% SDS-PAGE gels and either stained with Coomassie dye or transferred to nitrocellulose. Western-blot conditions have been previously described (Burnette et al., 2003). For detection of the V5 epitope tag, a 1:5,000 dilution of the mouse anti-V5 monoclonal antibody, followed by a 1:5,000 dilution of goat anti-mouse HRP conjugated antibody (Amersham, Piscataway, NJ), was used. Purified Positope V5-tag protein (175 ng; Invitrogen) was loaded on the same gel to estimate the amount of recombinant V5-tagged protein.

5PTase Activity Assays

Fluorescent substrates (Echelon Research Laboratories, Salt Lake City) were used in a modified version of a previously described phosphatase assay (Taylor and Dixon, 2001). Briefly, immunoprecipitated At5PTase11 was incubated for 1 h at room temperature with 1.5 µg of di-C_6-NBD6-phosphatidylinositol 4,5-bisphosphate, di-C_6-NBD6-phosphatidylinositol 3,5-bisphosphate, di-C_6-NBD6-phosphatidylinositol 3,4,5-trisphosphate, or di-C_6-NBD6-phosphatidylinositol 5-monophosphate, in lipid assay buffer containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 50 mM KCl with agitation every 15 min. The reaction was stopped by addition of 100 µl acetone and was dried under low heat vacuum. After dissolving in 10 µl of methanol:2-propanol:glacial acetic acid; 5:5:2), samples were loaded on a TLC plate (silica gel 60, Merck, Gibbstown, NJ) that had been pretreated in 1.2% potassium oxalate in 60:40 MeOH:water and dried at 100°C for 30 min. The plates were developed in 180 ml of chloroform:methanol:acetone:glacial acetic acid:water (70:50:20:20:20). To visualize fluorescence lipids, the TLC plate was dried and analyzed using a Storm 860 Blue Fluorescence Chemifluorescence detector and Scanner Control Software version 4.1 (Molecular Dynamics, Piscataway, NJ). For densitometric analysis of the images produced via Storm 860, we have utilized AlphaEaseFC software version 3.1.2 (Alpha Innotech, San Leandro, CA).

For the analysis of inositol phosphate substrates, activity assays were performed using $[^3H]$*myo*-inositol (1,4,5)P_3 (10 µCi/ml; 22 Ci/mmol; NEN, Shelton, CT) and $[^3H]$*myo*-

inositol (1,3,4,5)P_4 (10 µCi/ml; 22 Ci/mmol; NEN). Immunoprecipitated 5PTases were incubated with 30 nCi of [^{3}H]*myo*-inositol (1,4,5)P_3 and [^{3}H]*myo*-inositol (1,3,4,5)P_4 in reaction buffer (250 mM KCl, 3 mM $MgCl_2$, 50 mM Tris, pH 7.5) in a total volume of 300 µl at room temperature for 1 h. The resulting products were stored at –20°C and analyzed by HPLC (Beckman System Gold; Beckman Instruments, Fullerton, CA) using a Waters Spherisorb S5 SAX 4 x 125-mm analytical column (Milford, MA) equilibrated with a 10 mM AP (Ammonium phosphate) buffer at pH 3.8. Samples were applied to column using an autosampler, and products were eluted with a linear gradient from 10 mM AP to 340 mM AP over 30 min; 340 mM AP to 1.02 M AP over 15 min; and constant 1.02 M AP over 5 min (Stolz et al., 1998a). For the analysis of deacylated phosphatidylinositols, a gradient described by Stephens et al. (1989) was used. Radioactivity was counted with an in-line Beckman 171 radioisotope detector and the data file was converted to an asci file format by 32 Karat software (Beckman Coulter, Fullerton, CA) and analyzed by Excel (Microsoft, Seattle).

RT-PCR

Conditions for semiquantitative RT-PCR have been described previously (Berdy et al., 2001). Total RNA was extracted from 100 mg of frozen tissue using the Qiagen Plant RNeasy kit according to manufacturer's specifications. RNA (1 μg) was analyzed by two-step RT-PCR utilizing a Qiagen Omniscript reverse transcriptase kit and the manufacturer's instructions. Conditions for actin amplification have been described and generate a 428-bp product (An et al., 1996; Berdy et al., 2001). For At5PTase11-specific

amplification, forward primer (5'GAGCCGTGGCTATTCGTATT3') and reverse primer (5'AGTCGCTGCTTCCTACATTG3') were used, resulting in a 363-bp product. Amplification with a cloned At5PTase11 cDNA and *Arabidopsis* genomic DNA was used as a control for all PCR experiments to verify the sizes of bona fide reaction products from RNA and contaminating genomic DNA. Molecular weight markers were used to estimate product sizes. Each RT-PCR experiment was independently repeated three times to verify that the observed changes in expression were reproducible.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AC007519.

Results

Identification of a Unique At5PTase Gene

We previously identified two classes of plant 5PTases based on their phylogenetic relationships: the Group A 5PTases, such as At5PTase1 (At1g34120) and At5PTase2 (At4g18010), which contain only the IPPc domain; and the Group B 5PTases, such as At5PTase12 (At1g65580), which contain the IPPc domain and multiple N-terminal WD40 repeats (Berdy et al., 2001); Figure 1). The At5PTase11 gene (At1g47510) is predicted to encode the smallest Group A 5PTase (331 amino acids, identified as AAD46036 in Berdy et al., 2001). At5PTase11 contains the conserved IPPc domain (amino acids 53–324; see supplemental data available at www.plantphysiol.org), and differs from the previously characterized Group A 5PTases in that it is not interrupted by a stretch of nonconserved residues (compare At5PTase1 and At5PTase11 in Figure 1; supplemental data). By using BLASTp searches of the nonredundant protein database at the National Center for Biotechnology Information (NCBI), we determined the most closely related protein to At5PTase11 is the Os3541 protein (AAR01658 from rice (*Oryza sativa*). The Os3541gene is predicted to encode a protein of 301 amino acids, and has 50% amino acid identity to At5PTase11. The next most closely related protein to At5PTase11 is the yeast (*Saccharomyces cerevisiae*) 5PTase, Inp54 (CAA90520, which has 20.5% identity. This is similar to the 19.4% identity At5PTase11 shares with At5PTase1. Thus, At5PTase11 is more closely related to the rice Os3541 protein than it is

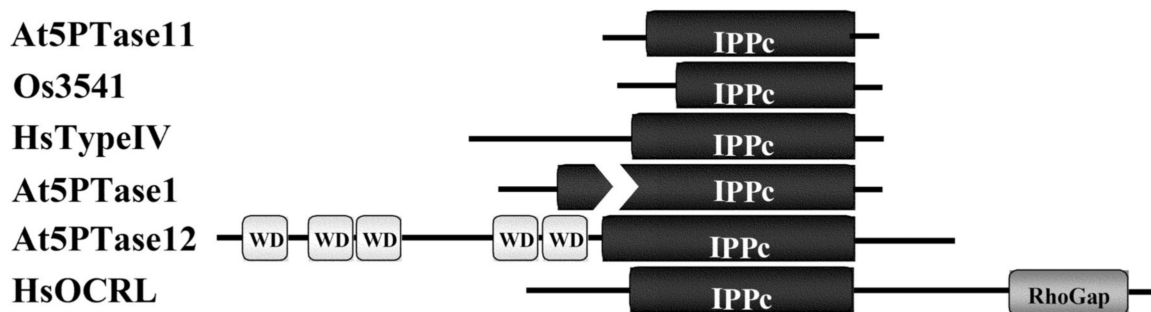

Figure 1. Domain Structure of At5PTase11 and Representative 5PTases. Amino acid sequences corresponding to At5PTase11 (AAD46036), the closely related rice gene Os3541 (ARR01658), At5PTase1 (At1g34120), At5PTase12 (At1g65580), the human Type IV 5PTase (NP_063945), and the human Type II 5PTase OCRL (Q01968) were analyzed with Pfam and SMART database tools, and the resulting domain structures are graphically presented. IPPc is the conserved 5PTase catalytic domain; WD corresponds to WD40 repeat regions.

to other *Arabidopsis* or yeast and animal 5PTases, indicating the possibility that At5PTase11 and Os3541 may be homologous genes.

We used reverse transcription (RT)-PCR to verify that the At5PTase11 gene is expressed in *Arabidopsis* tissues. Oligonucleotide primers were designed using the predicted cDNA sequence surrounding the start and stop codons. RNA from mixed *Arabidopsis* tissues, including seedling, roots, flowers, stems, and leaves, was used as template in an RT reaction. The products of this reaction were amplified with PCR using the At5PTase11-specific primers. The resulting At5PTase11 cDNA was sequenced and shown to be identical with the predicted cDNA (NM_103644) contained in the NCBI database. We conclude that the At5PTase11 gene is an expressed gene encoding a small and unique 5PTase.

At5PTase11 Encodes an Active 5PTase Enzyme

To obtain protein for 5PTase activity assays, the At5PTase11 protein containing a C-terminal His tag was expressed under control of the insect metallothionine promoter (pMT) in *Drosophila melanogaster* S2 tissue culture cells (S2 cells). We chose this strategy because previous efforts to obtain active, recombinant At5PTases in prokaryotic expression systems had failed, suggesting that a eukaryotic modification may be necessary for At5PTase activity. The At5PTase11 expression construct (pMTAt5PTase11) also contains a C-terminal V5 epitope tag, which we used for immunoprecipitation. We transfected S2 cells with either pMTAt5PTase11 DNA, a

pMTβ-galactosidase (pMTβ-gal) control construct, or no DNA (i.e. mock transfection). After the transfection, we stimulated expression for 2 d with the addition of $CuSO_4$. Protein extracts were made, and immunoprecipitation with an anti-V5 antibody was performed. Analysis of the soluble extracts prior to the immunoprecipitation revealed that the anti-V5 antibody did not react with any native Drosophila proteins (Figure 2, lane 4). S2 cells transfected with the pMTAt5PTase11 construct, however, contained a recombinant protein of approximately 47 kDathat reacted with the anti-V5 antibody. At5PTase11 is predicted to encode a 36.4-kD protein, and the addition of the C-terminal V5 and His tags is expected to increase the molecular mass by 4.4 kDa. The apparent molecular mass of the recombinant At5PTase11 is slightly higher than expected.

To isolate the recombinant At5PTase11 protein, we used the anti-V5 antibody to immunoprecipitate At5PTase11 and analyzed the resulting complex with western blotting (Figure 2). As expected, immunoprecipitated complexes from mock transfections (lane 6) or transfections with pMTβ-gal revealed the presence of the immunoglobulin heavy chain (IgG hc) from the anti-V5 antibody. Analysis of an immunoprecipitated pMTAt5PTase11 extract reveals the presence of an additional protein that is the same size as recombinant At5PTase11 protein (Figure 2). To examine the content of our immunoprecipitations, we analyzed immunoprecipitated proteins from pMTAt5PTase11-transfected and mock-transfected S2 cells (Figure 2, lanes 1 and 2, respectively). The immunoprecipitation from mock-transfected cells reveals the presence of the IgG hc and IgG light chain migrating at

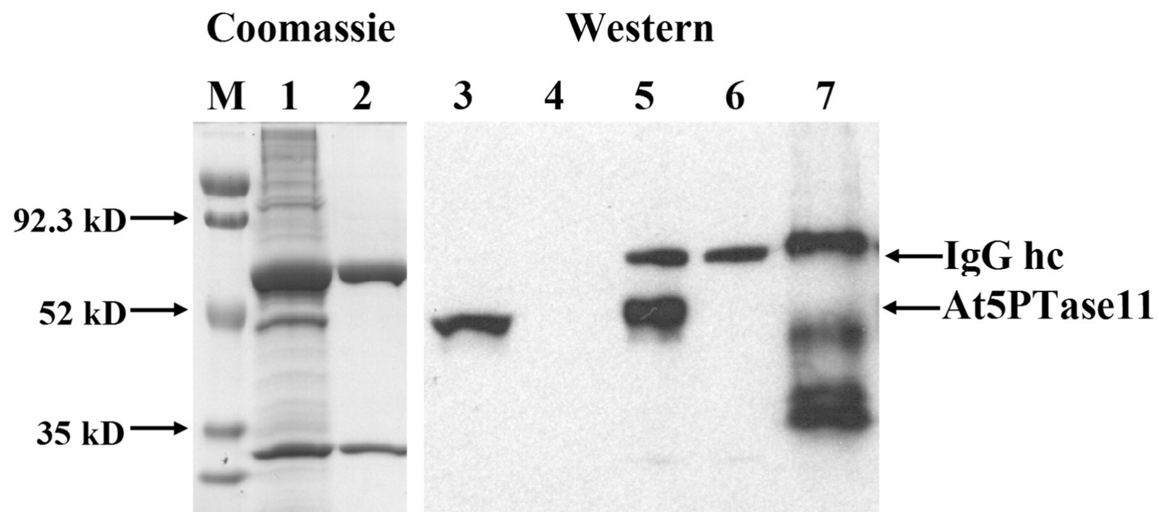

Figure 2. Expression and Immunoprecipitation of Recombinant At5PTase11 in Drosophila S2 Cells. The pMTAt5PTase11 construct was transfected into S2 cells, and soluble extracts were prepared and immunoprecipitated with a monoclonal anti-V5 antibody as described in "Materials and Methods." Immunoprecipitates from S2 cells transfected with pMTAt5PTase11 (lanes 1 and 5) and mock-transfected S2 cells (lanes 2 and 6) were stained with Coomassie dye after SDS-PAGE separation or were analyzed by western blotting with the monoclonal anti-V5 antibody. Lanes 3 and 4 contain a portion of the soluble protein extract from pMTAt5PTase11-transfected (lane 3) or mock-transfected (lane 4) S2 cells. Lane 7 contains purified Positope (Invitrogen) protein and was used to estimate the amount of recombinant At5PTase11 immunoprecipitated. Arrows on the left indicate the sizes of molecular mass markers (M); arrows on the right indicate the positions of At5PTase11 recombinant protein and the IgG hc.

55 and 25 kD, respectively. By contrast, an overloaded lane from an immunoprecipitation of pMTAt5PTase11-transfected cells contains one other major band, corresponding in size to recombinant At5PTase11. Since other background bands are not present in stoichiometric amounts with At5PTase11, we conclude that recombinant At5PTase can be expressed in S2 cells and immunoprecipitated in a fairly pure form.

To determine whether At5PTase11 encodes an active 5PTase, we assayed for 5PTase activity directly following immunoprecipitation (Taylor and Dixon, 2001). We controlled for the amount of At5PTase11 added to activity assays by visual quantification of immunoprecipitates on SDS-PAGE gels, as shown in Figure 2, and by using a known amount of standard protein containing the V5 epitope tag (Figure 2, lane 7). Figure 3 shows the results of incubating immunoprecipitates with a fluorescent PtdIns(4,5)P_2 substrate. When PtdIns(5)P, PtdIns(4)P, or PtdIns(4,5)P_2 is incubated under assay conditions in the absence of enzyme and analyzed by thin-layer chromatography (TLC), we obtain excellent separation between the mono- and bis-phosphorylated compounds. We obtained less separation of PtdIns(5)P and PtdIns(4)P. When 0.12 μg of At5PTase11 is incubated with PtdIns(4,5)P_2, approximately 41% of the fluorescent PtdIns(4,5)P_2 is converted to a product migrating in a similar position as PtdIns(4)P (Figure 3, lane 4). When the amount of At5PTase11 is increased to 1 μg, the conversion of substrate to product is increased to approximately 90% (Figure 3, lane 7). Immunoprecipitates from pMTβ-gal- or mock-transfected cells resulted in no conversion of substrate to product (Figure 4; data not shown). This indicates that the At5PTase11 gene encodes an active 5PTase that dephosphorylates PtdIns(4,5)P_2 *in vitro*.

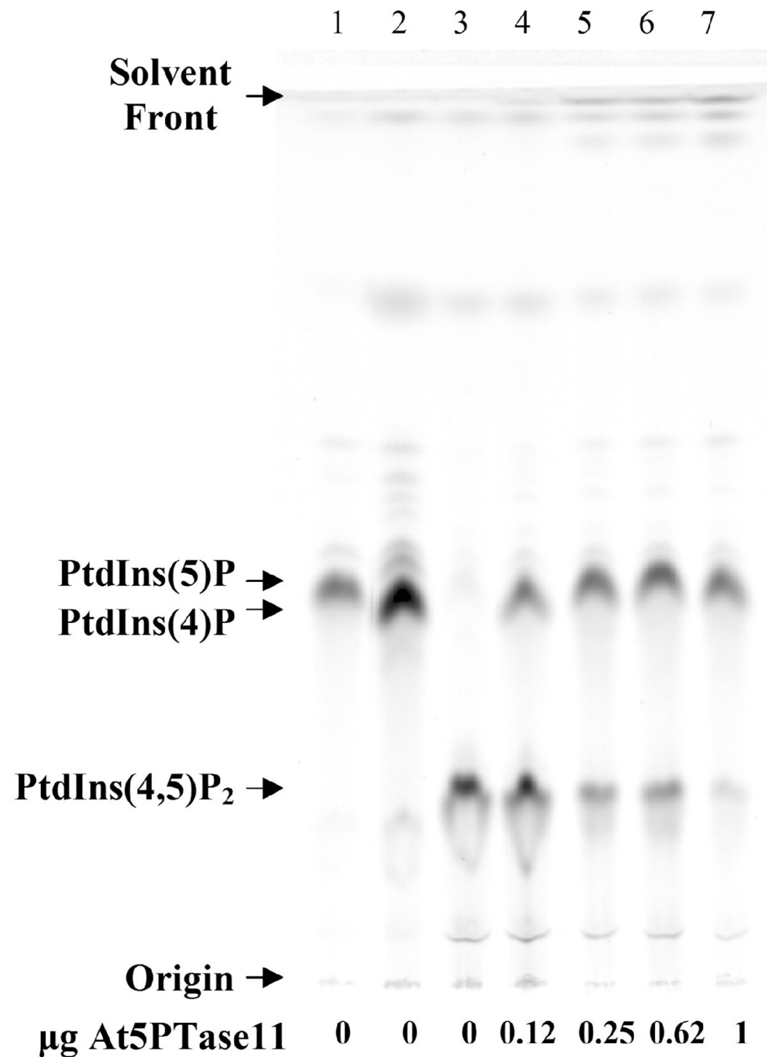

Figure 3. Effect of Increasing At5PTase11 Concentration on Fluorescent PtdIns(4,5)P_2 Dephosphorylation. PtdIns(4,5)P_2 fluorescent substrate (1.5 μg), or PtdIns(5)P and PtdIns(4)P standards (1.5 μg each), were incubated with 0 to 1.0 μg of At5PTase11 for 1 h at room temperature. Supernatants were processed and separated by TLC as described in "Materials and Methods." Lane 1, PtdIns(5)P alone; lane 2, PtdIns(4)P alone; lane 3, PtdIns(4,5)P_2 alone; lanes 4 to 7, PtdIns(4,5)P_2 incubated with the indicated amount of At5PTase11 immunoprecipitated protein. To visualize fluorescent lipids, the TLC plate was analyzed with a Storm 860 (Molecular Dynamics).

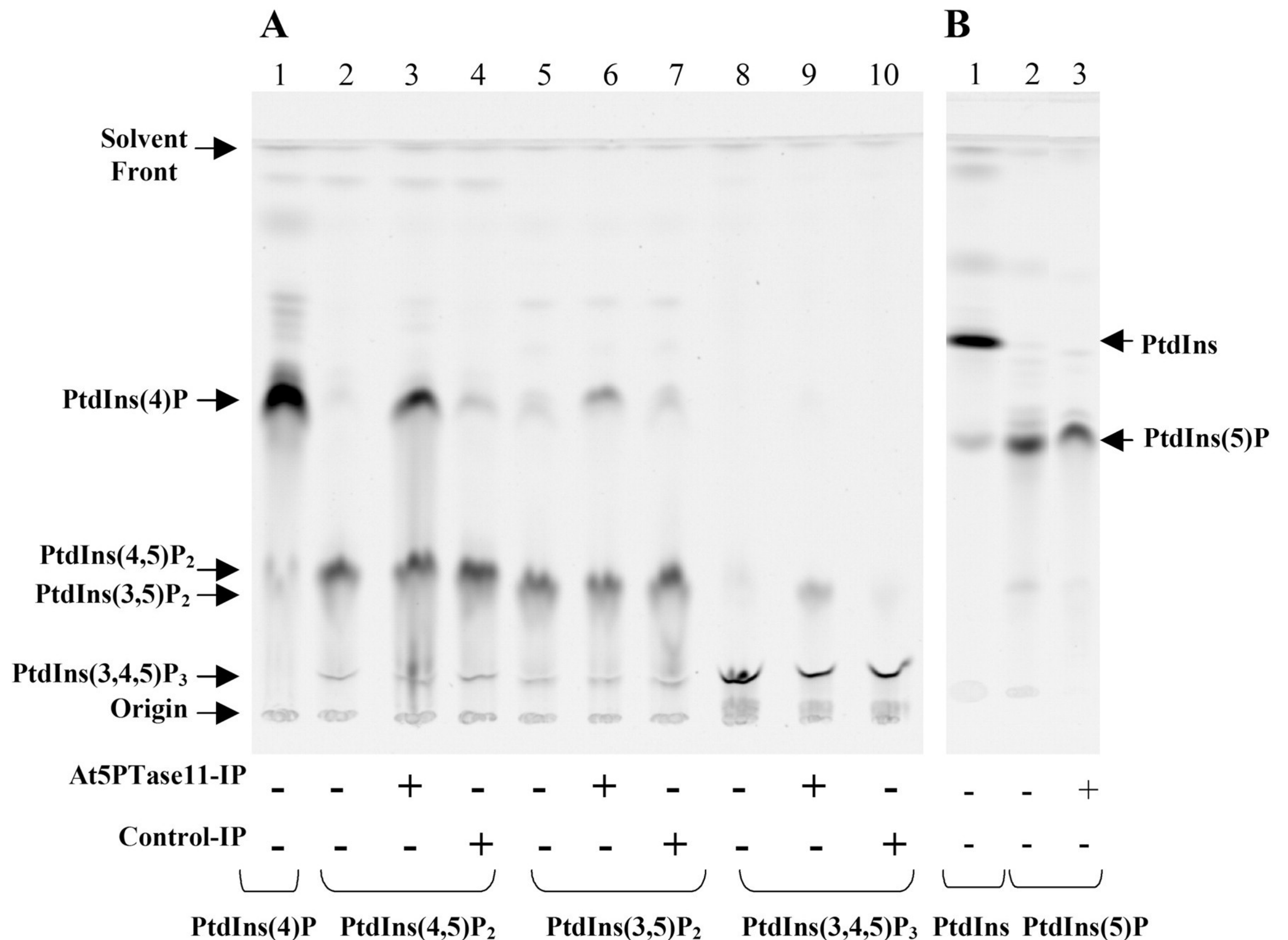

Figure 4. Determination of At5PTase11 Substrate Specificity Using Fluorescent Phosphoinositide Substrates. A and B, Phosphatase reactions containing 1.5 μg of the indicated fluorescent phosphoinositide substrates were incubated in the same reaction conditions as described for Figure 3. A, Lanes 1, 2, 5, and 8 contain the substrate indicated at the bottom incubated in the presence of buffer only. Lanes 3, 6, and 9 contain the indicated substrate, buffer, and immunoprecipitated At5PTase11. Lanes 4, 7, and 10 contain the indicated substrate, buffer, and immunoprecipitates of mock-transfected S2 cells (Control-IP). B, Lanes 1 and 2 contain the PtdIns and PtdIns(5)P substrates alone. Lane 3 contains PtdIns(5)P incubated in the presence of immunoprecipitated At5PTase11. The migration of fluorescent standards and the origin are indicated (at left and right).

At5PTase11 Hydrolyzes Various 5-Phosphate-Containing Phosphoinositide Substrates

Studies of 5PTases from a variety of organisms have shown that the IPPc domain is capable of removing the 5-phosphate from both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 substrates. To determine whether the At5PTase11 enzyme hydrolyzes PI substrates other than PtdIns(4,5)P_2, we examined hydrolysis of PtdIns(5)P, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 by immunoprecipitates of pMTAt5PTase11- and mock-transfected S2 cells (Figure 4). In all experiments, we incubated standards of fluorescent substrates and products in assay conditions and observed their migration on TLC plates within each experiment. As expected, the order of migration is PtdIns(5)P (fastest) > PtdIns(4)P > PtdIns(4,5)P_2 > PtdIns(3,5)P_2 > PtdIns(3,4,5)P_3 (slowest). When immunoprecipitates from mock-transfected cells are incubated with each substrate, a small amount of product is observed, which corresponds to background hydrolysis (Figure 4, Control-IP lanes). As expected from the results in Figure 3, when 0.25 μg of At5PTase11 is incubated with PtdIns(4,5)P_2, conversion to product migrating at the same position as PtdIns(4)P is observed (Figure 4A, lane 3). Using the same amount of At5PTase11 protein, we tested whether At5PTase11 would dephosphorylate PtdIns(3,5)P_2. Figure 4A (lane 6) indicates that PtdIns(3,5)P_2 is also dephosphorylated by the At5PTase11 enzyme to a greater degree than the background hydrolysis. Incubation of At5PTase11 with PtdIns(3,4,5)P_3 also reveals conversion of this substrate, and the conversion is greater than what occurs in the absence of the enzyme (Figure 4A, lane 9). The product of PtdIns(3,4,5)P_3 dephosphorylation by a 5PTase is expected to be PtdIns(3,4)P_2, which will migrate slower than PtdIns(4,5)P_2 and PtdIns(3,5)P_2. Since the observed product in lane 9 of Figure 4A

migrates slower than the PtdIns(4,5)P_2 and PtdIns(3,5)P_2 standards, it suggests that removal of the 5-phosphate has occurred. Last, we tested whether PtdIns(5)P would be dephosphorylated by At5PTase11 and observed no increase in hydrolysis over background (Figure 4B, compare lanes 2 and 3). From these experiments we conclude that At5PTase11 can dephosphorylate tris- and bis- but not mono-phosphorylated PI substrates that contain a 5-phosphate.

At5PTase11 Does Not Hydrolyze Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 Substrates

Since many 5PTase enzymes also hydrolyze inositol tris- and tetrakis-phosphate substrates containing a 5-phosphate, we tested the ability of At5PTase11 to dephosphorylate Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. For these experiments, we incubated immunoprecipitates from mock-transfected or pMTAt5PTase11-transfected S2 cells with [^{3}H]*myo*-inositol (1,4,5)P_3 or [^{3}H]*myo*-inositol (1,3,4,5)P_4. To analyze the products of these reactions, a small amount was injected onto a Spherisorb S5 SAX HPLC column, and separation of inositol phosphates was performed with an ammonium phosphate (AP) gradient (Stolz et al., 1998a). Radiolabeled standards incubated under assay conditions were used to assign peak positions. When either At5PTase11 or no enzyme is incubated with [^{3}H]*myo*-inositol (1,4,5)P_3 substrate, no conversion to product [^{3}H]*myo*-inositol (1,4)P_2 takes place (Figure 5, A and C, respectively). We saw a similar lack of hydrolysis when [^{3}H]*myo*-inositol (1,3,4,5)P_4 was incubated with At5PTase11 (data not shown). In these experiments, we used up to 10 times the amount of At5PTase11 protein as in the experiments testing PI substrates and still found no hydrolysis. As a

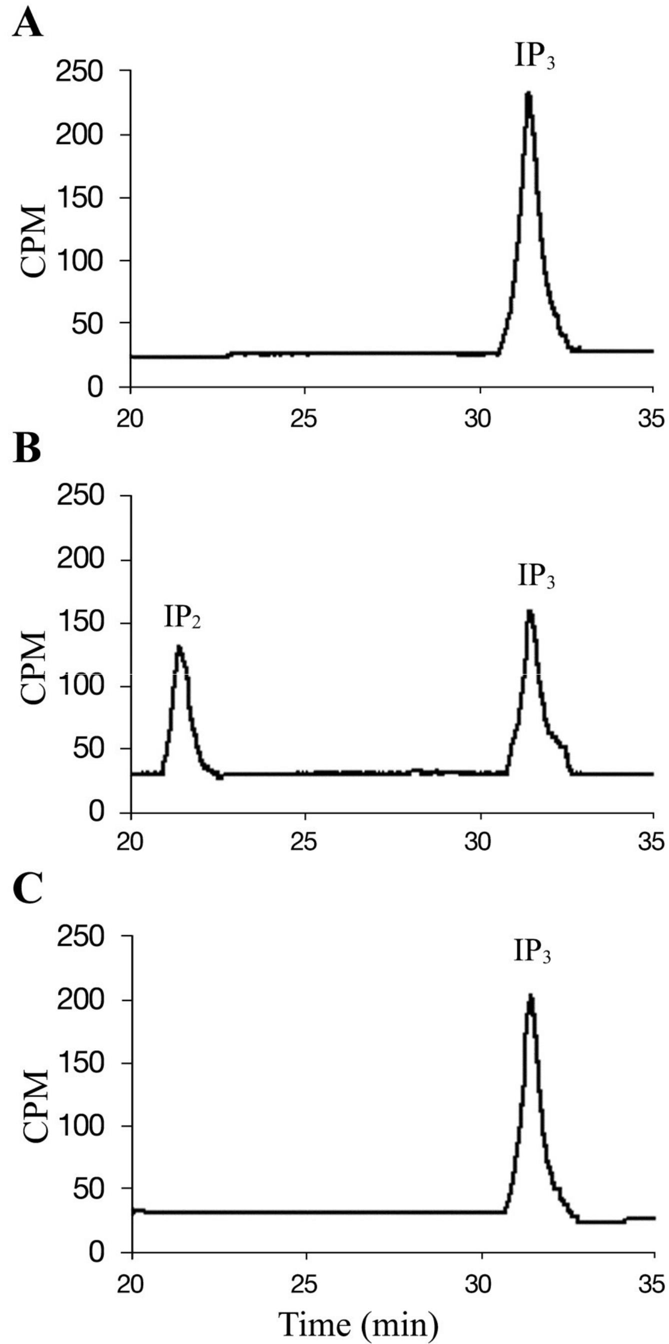

Figure 5. At5PTase11 Does Not Hydrolyze [^{3}H]Ins(1,4,5)P_3. Immunoprecipitated At5PTase11 (0.25 μg) (A), At5PTase1 (0.25 μg) (B), or buffer (C) was incubated with [^{3}H]Ins(1,4,5)P_3. Products were separated using HPLC as described.

control for our assay conditions, we incubated 0.12 and 0.25 μg immunoprecipitated V5-tagged At5PTase1 with both [^{3}H]*myo*-inositol (1,4,5)P_3 and [^{3}H]*myo*-inositol (1,3,4,5)P_4. In each case, conversion to [^{3}H]*myo*-inositol (1,4)P_2 and [^{3}H]*myo*-inositol (1,3,4)P_3 took place as expected (Figure 4B; data not shown). From these data, we conclude that, under our *in vitro* assay conditions, the substrate specificity of At5PTase11 differs from previously characterized At5PTase enzymes in that it does not hydrolyze the water-soluble inositol phosphate substrates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4.

At5PTase11 Hydrolyzes PtdIns(4,5)P_2 In Vivo in Drosophila Cells

Our *in vitro* experiments with fluorescent PI substrates have shown that At5PTase11 has the ability to hydrolyze PtdIns(4,5)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3. To determine whether At5PTase11 can hydrolyze PIs produced within the cell (*in vivo*), we used [^{3}H]*myo*-inositol labeling of S2 cells that were transiently expressing recombinant At5PTase11. S2 cells were transfected with pMTAt5PTase11 or pMTβ-gal, and 36 h postinduction, [^{3}H]*myo*-inositol was added for 12 h. Deacylated PIs were then analyzed by HPLC and, in each case, data were normalized to the total [^{3}H] counts in each run (Figure 6). To identify the peak position of GroPIns(4,5)P_2, we used a radiolabeled standard. To identify the peak position of GroPIns(4)P, we incubated extracts from labeled cells with a previously characterized, purified human 5PTase and examined the peak position of the resulting GroPIns(4)P product. Comparison of HPLC traces in Figure 6, A and B, indicates that GroPIns(4,5)P_2 is reduced and GroPIns(4)P is elevated in cells transiently expressing At5PTase11. We have

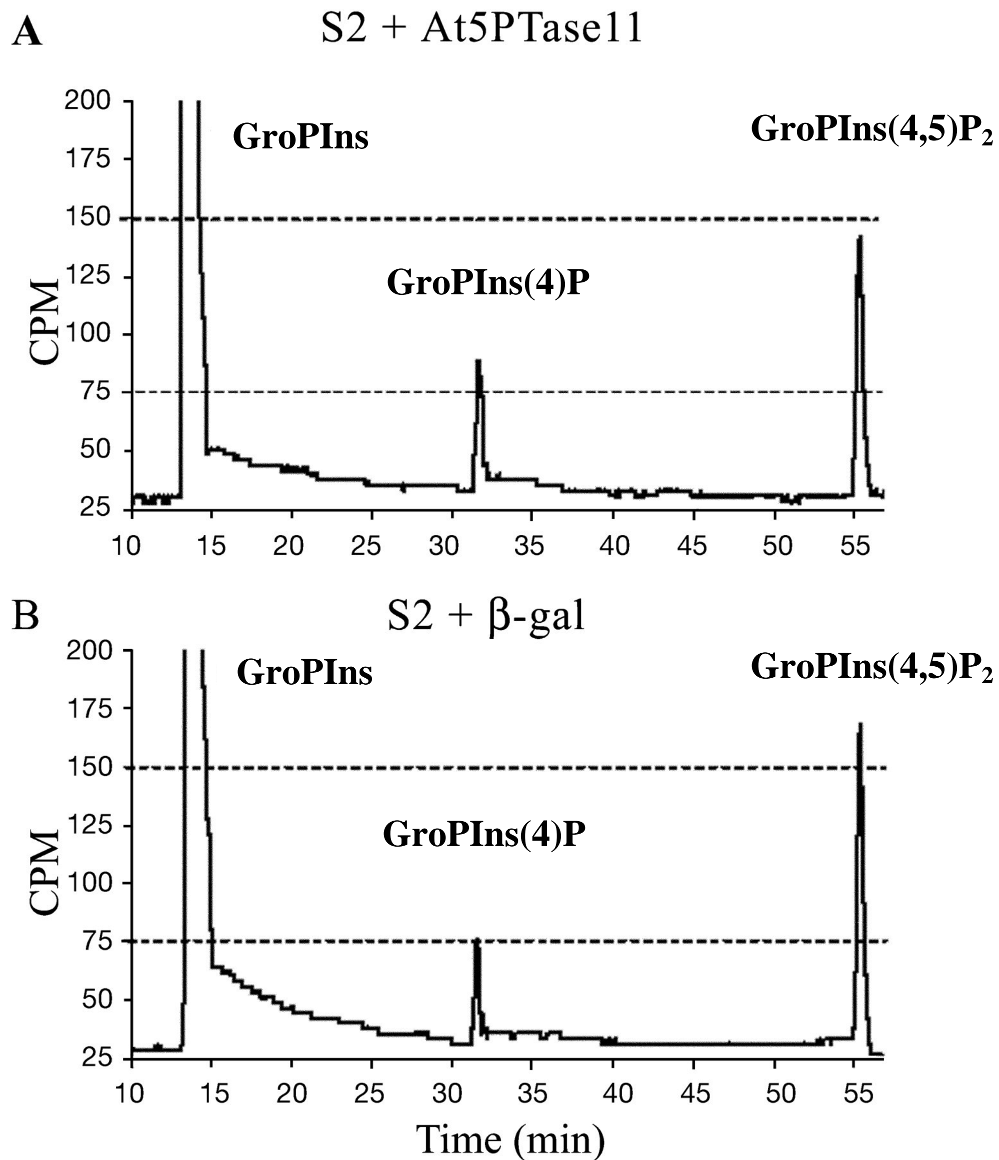

Figure 6. PtdIns(4,5)P_2 Is an *In Vivo* Substrate for At5PTase11. HPLC analysis of deacylated [^{3}H]*myo*-inositol-labeled lipids from S2 cells that were transfected with pMTAt5PTase11 (A) or pMTβ-gal (B). The positions of GroPIns standards are indicated. Equal amounts of total counts were analyzed for each sample.

repeated this experiment multiple times and found that the PtdIns(4)P and PtdIns(4,5)P_2 levels are always altered in cells transiently expressing At5PTase11. Specifically, levels of PtdIns(4)P are increased 15% ± 0.3% on average in our experiments, indicating that, *in vivo*, At5PTase11 hydrolyzes PtdIns(4,5)P_2. We were not able to detect any PtdIns(3,4,5)P_3 in either transfected or control cells. This is not surprising since PtdIns(3,4,5)P_3 is present in very low amounts in most eukaryotic cells (Czech, 2000).

Analysis of At5PTase11 Expression

To examine expression of At5PTase11, we utilized semiquantitative RT-PCR. Oligonucleotide primers that amplify the 3' ends of At5PTase11 and an actin gene were used to amplify cDNA products synthesized from RNAs of different tissues (Figure 7). We have shown that the At5PTase11 primers are specific for the At5PTase11 gene by using the primer sequences as queries in a search of the BLAST short, nearly exact match database, and by comparing them to similar regions of the other At5PTase genes. The expected product from At5PTase11 cDNA using these primers is 363 bp. Contaminating At5PTase11 genomic DNA present would yield a 1,071-bp PCR product. We found that At5PTase11 is expressed in several *Arabidopsis* tissues, including flowers, rosette leaves, cauline leaves, roots, siliques, bolts, and seedlings (Figure 7A).

Examination of the promoter of At5PTase11 indicated the presence of putative regulatory elements for JA and auxin. To test if At5PTase11 is regulated by JA or auxin, we

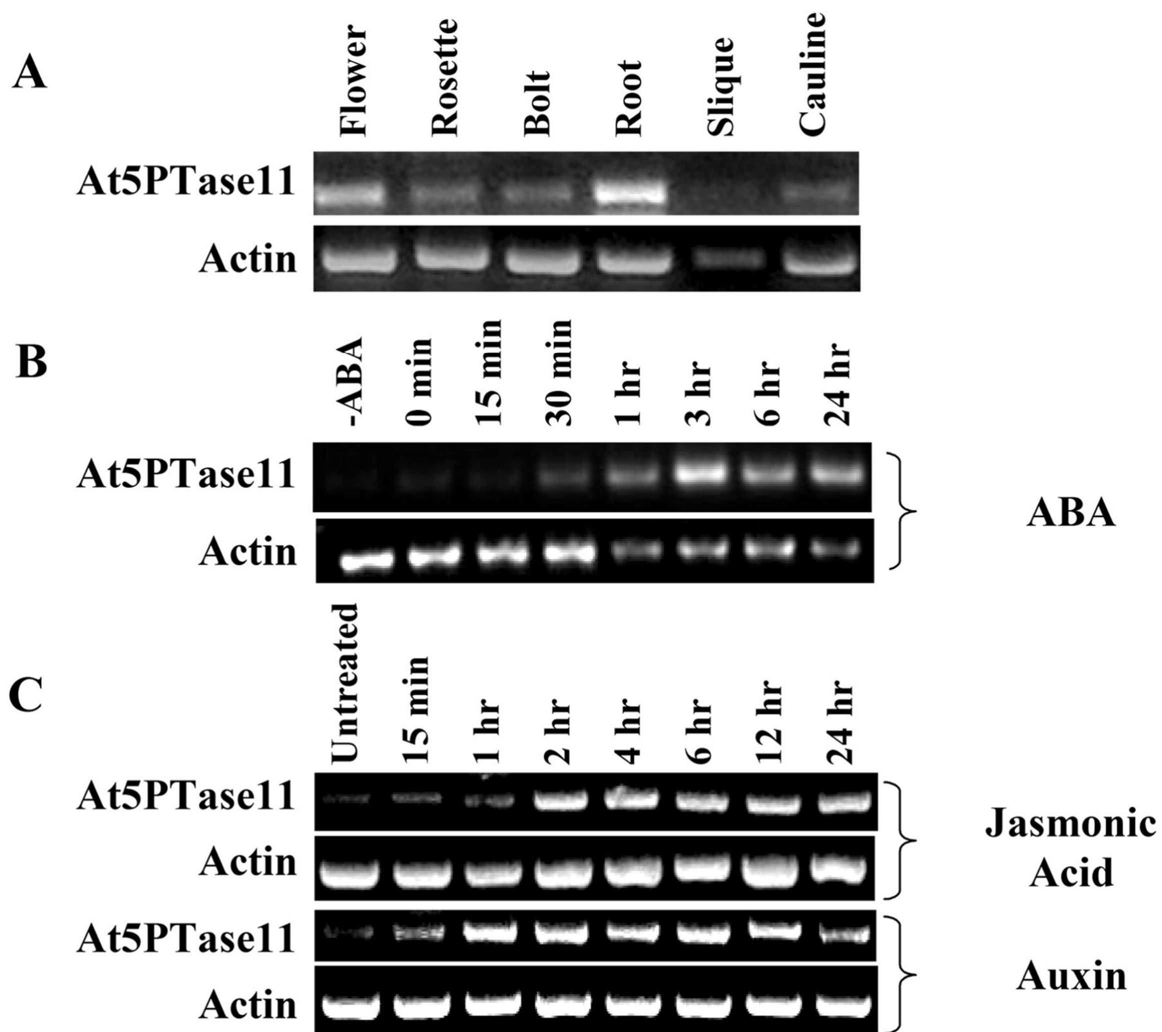

Figure 7. ABA, JA, and Auxin Treatment Alters At5PTase11 Expression in Seedlings. Five- to 7-d old seedlings were treated with either of 100 μM ABA, 10 μM auxin, or 100 μM JA and harvested at the indicated times. Total RNA was harvested and used in RT-PCR experiments with At5PTase11- and actin-specific primers as described in "Materials and Methods."

measured At5PTase11 mRNA levels during JA and auxin stimulation. Since this approach was previously used to show that the At5PTase1 gene is regulated by ABA, we included seedlings stimulated by ABA in our analysis. Figure 7B shows that At5PTase11 mRNA levels increase during the first 3 h of ABA treatment and decrease slightly after this (Figure 7B). This pattern is different from At5PTase1 regulation by ABA in which rapid induction is followed by an oscillatory behavior (Burnette et al., 2003). Seedlings treated with solvent alone (0.1% ethanol) and analyzed at various time points indicated no induction of the At5PTase11 gene (data not shown). We have also tested regulation of At5PTase11 mRNA levels in response to JA and auxin. At5PTase11 mRNA levels increased within 2 h of JA treatment and remained at induced levels for 24 h (Figure 7C). Auxin also stimulated At5PTase11 expression (Figure 7C). In contrast to ABA and JA treatments, auxin-stimulated At5PTase11 expression was transient, decreasing after 12 h. We conclude that ABA, JA, and auxin regulate At5PTase11 RNA expression.

Discussion

We previously identified the *Arabidopsis* 5PTase protein family, which contains 15 putative proteins (Berdy et al., 2001). At5PTase1 and At5PTase2 have been characterized and shown to remove a 5-phosphate from the water-soluble substrates, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Berdy et al., 2001; Sanchez and Chua, 2001). In these studies, there was no evidence that these proteins could remove a 5-phosphate from radiolabeled PtdIns(4,5)P_2 as measured by TLC separation of products (Berdy et al., 2001; Sanchez and Chua, 2001). In our studies on At5PTase11, we utilized fluorescent PI substrates that have been used by others (Giuriato et al., 2002; Laporte et al., 2002) and allows for more sensitivity in detecting reaction products (Taylor and Dixon, 2001). We show here that At5PTase11 hydrolyzes 5-phosphates from a group of PI substrates including PtdIns(4,5)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3. By comparing the activity of At5PTase1 and At5PTase11, we have shown that, unlike At5PTase1 and At5PTase2, At5PTase11 does not act on the water-soluble substrates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in our *in vitro* assay conditions. For comparison purposes, we also tested the ability of At5PTase1 and At5PTase2 to hydrolyze fluorescent PtdIns(4,5)P_2 and found that, in contrast to previous reports (Berdy et al., 2001; Sanchez and Chua, 2001), these enzymes do hydrolyze PI substrates (M.E. Ercetin and G.E. Gillaspy, unpublished data, see also Chapter IV). This discrepancy is most likely due to the increased sensitivity of our assay. These data suggest that At5PTase11 has a more restricted substrate specificity than At5PTase1 and At5PTase2.

Under controlled conditions, At5PTase11 dephosphorylated more PtdIns(4,5)P_2 than either of the other two substrates, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3, indicating a possible preference for this substrate (Figure 4). Besides its involvement in cytoskeletal rearrangements and potential roles in vesicle trafficking and ion transport, PtdIns(4,5)P_2 is known to act as a docking site for proteins via binding to plextrin-homology and Tubby protein domains (Toker, 1998). In some cases, binding to these domains can mediate the subcellular location and activity of the bound proteins. Thus, regulated PtdIns(4,5)P_2 hydrolysis would lead to the loss of such docking sites and could influence several processes.

The *in vitro* substrate specificity of At5PTase11 is somewhat similar to that of the human Type IV 5PTase, although the Type IV enzyme has a lower K_{app} for PtdIns(3,4,5)P_3 than it does for PtdIns(4,5)P_2 (Kisseleva et al., 2000). Because the molecular basis for 5PTase substrate specificity is of critical interest, several investigators have mutated various amino acid residues of 5PTase proteins in attempts to discern required sequence elements for specificity (Tsujishita et al., 2001). As both At5PTase11 and the human Type IV enzyme fail to act on inositol phosphate substrates *in vitro*, a comparison of these two protein sequences with other 5PTases that act on both PI and inositol phosphate substrates might be expected to reveal critical residues for substrate specificity. Our sequence alignments (supplemental data) did not reveal any amino acid differences in At5PTase11 and the human Type IV enzyme as compared to other characterized 5PTases.

A unique feature of At5PTase11 is its ability to hydrolyze PtdIns(3,5)P_2 *in vitro*. PtdIns(3,5)P_2 is a recently identified PI that was first found in hyperosmotic-stressed yeast (Dove et al., 1999) and some lower plants like *Chlamydomonas moewusii* (Meijer et al., 1999). However, since PtdIns(3,5)P_2 levels were found to be very low in *Arabidopsis* and do not change after osmotic or salt stress (Pical et al., 1999; DeWald et al., 2001), the role of this PI is unclear at this time. It is also interesting to note that, under our conditions, At5PTase11 did not hydrolyze PtdIns(5)P, which has been found to be up-regulated in response to osmotic stress in plants (Meijer et al., 2001).

We have also shown that At5PTase11 can hydrolyze PtdIns(3,4,5)P_3 *in vitro*, and the potential role of At5PTase11 with regard to this substrate is also unclear. PtdIns(3,4,5)P_3 synthesis has not been shown in any plant to date. In animal cells, PtdIns(3,4,5)P_3 is present at very low levels, and synthesis is stimulated by specific signal transduction pathways. Thus, it is possible that PtdIns(3,4,5)P_3 has escaped our attention in plant cells because conditions for maximal synthesis have not been examined. In support of this, it is important to note that a recombinant *Arabidopsis* phosphatidylinositol-4-phosphate 5-kinase (AtPK51) protein produced in insect cells can phosphorylate PtdIns(3,4)P_2 producing PtdIns(3,4,5)P_3 (Elge et al., 2001). In addition, an *Arabidopsis* homolog of a human tumor suppressor (Pten) has been shown to remove a 3-phosphate from PtdIns(3,4,5)P_3 *in vitro* and is essential for pollen development (Gupta et al., 2002). It is possible that each of these proteins, At5PTase11, AtPten1, and AtPK51, maintains the ability to interact with PtdIns(3,4,5)P_3 as a consequence of another function and that they do not encounter PtdIns(3,4,5)P_3 in the plant cell. Examination of knockout mutations in

these genes combined with careful labeling studies utilizing [^{3}H]*myo*-inositol or [^{32}P]orthophosphate under the appropriate signaling or growth conditions may resolve this issue.

The regulation of At5PTase11 by the three different signaling conditions tested is intriguing, and may point to the involvement of PIs in these pathways. There are many published data regarding ABA signal transduction and PIs. It is known that ABA stimulates phospholipase C activity, resulting in new Ins(1,4,5)P_3 synthesis (for review, see (Meijer and Munnik, 2003). Genetic studies have shown the necessity of the *fiery1* gene encoding an inositol polyphosphate 1-phosphatase for ABA signaling (Xiong et al., 2001). This suggests that inositol phosphate breakdown is critical for ABA signaling. It has also been shown that ABA stimulates production of At5PTase1, which hydrolyzes Ins(1,4,5)P_3, and that this terminates ABA signaling (Burnette et al., 2003). Our present data indicate that ABA-stimulated expression of At5PTase11 may also be involved in ABA signal termination. Reduction of PtdIns(4,5)P_2 by At5PTase11 could reduce the pool of PtdIns(4,5)P_2 available for Ins(1,4,5)P_3 synthesis, imparting another level of control over Ins(1,4,5)P_3 production. Reduction of PtdIns(4,5)P_2 could also reduce phospholipase D activity, as some isoforms of this enzyme require PtdIns(4,5)P_2 for activity (Pappan et al., 1997; Qin et al., 1997). With respect to At5PTase11 induction after auxin and JA addition, much less is known about whether these signaling pathways utilize PIs. Our data are consistent with information in the MPSS database, which reports very low levels of At5PTase11 expression except in callus tissue that was grown on auxin-containing media and in salicylic acid-treated plants (http://mpss.udel.edu/).

In summary, the cloning and identification of a novel inositol 5-phosphatase with a substrate specificity toward PtdIns(4,5)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 expand our knowledge of the eukaryotic 5PTase protein family and offer a unique tool to examine the importance of these PIs in plant physiological processes.

CHAPTER III

Objective II: To Examine the Physiological Consequence of Altering At5PTase11 Function in *Arabidopsis*

Introduction

The phosphoinositide (PI) signaling pathway has been shown to be involved in many crucial cellular functions (Stevenson et al., 2000; Meijer and Munnik, 2003). In animal cells, phosphatidylinositol-4,5 bisphosphate [PtdIns(4,5)P_2] is involved in regulation of the actin cytoskeleton, stress fiber formation, and membrane trafficking (Toker, 1998). In addition to PtdIns(4,5)P_2, PI signaling pathway utilizes other inositol-containing second messengers such as inositol-1,4,5 trisphosphate [Ins(1,4,5)P_3], inositol-1,3,4,5 tetrakisphosphate [Ins(1,3,4,5)P_4] and phosphatidylinositol-3,4,5 trisphosphate [PtdIns(3,4,5)P_3].

Enzymes capable of catalyzing the hydrolysis of the phosphate positioned at carbon 5 of the inositol ring are called *myo*-inositol polyphosphate 5-phosphatases (5PTases, E.C. 3.1.3.36). In *Arabidopsis* there are 15 predicted 5PTases, based on the sequence homology to known 5PTases from other organisms (Berdy et al., 2001). The *Arabidopsis* 5PTases (At5PTases) have been shown to be important regulatory components of the plant PI signaling pathway by selectively utilizing inositol-containing second messengers (Sanchez and Chua, 2001; Burnette et al., 2003). At5PTase1 (At1g34120) was the first At5PTase characterized biochemically; it was found that At5PTase1 hydrolyzes

$Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ (Berdy et al., 2001). I have published the substrate specificity of At5PTase11 which is a phospholipid-specific 5PTase that hydrolyzes $PtdIns(4,5)P_2$, $PtdIns(3,5)P_2$, and $PtdIns(3,4,5)P_3$ (Ercetin and Gillaspy, 2004). More recently, the substrate specificities of WD repeat-containing At5PTases have been published (Zhong et al., 2004; Zhong and Ye, 2004). These studies indicate that *Arabidopsis* has 5PTases with distinct substrate selectivities.

The function of At5PTase1 and At5PTase2 in *Arabidopsis* was investigated by ectopic expression of these genes in *Arabidopsis* (Sanchez and Chua, 2001; Burnette et al., 2003). Both At5PTase1 and At5PTase2 have been shown to alter abscisic acid (ABA) signaling in *Arabidopsis* (Sanchez and Chua, 2001; Burnette et al., 2003). ABA signaling is known to utilize $Ins(1,4,5)P_3$ as a second messenger to regulate stomatal closure (Gilroy et al., 1990; Lee et al., 1996; Staxen et al., 1999). Studies aimed at examining the potential role(s) of 5PTases in ABA signal transduction have shown that stomata from transgenic plants overexpressing At5PTase1 are ABA-insensitive and expression of ABA-regulated genes is delayed (Burnette et al., 2003). Moreover, reduction in ABA-induced $Ins(1,4,5)P_3$ levels suggests that At5PTase1 might act as a negative regulator of ABA signaling through alteration of $Ins(1,4,5)P_3$ levels (Burnette et al., 2003). However, seed germination, which is regulated by ABA, was not affected by At5PTase1 overexpression suggesting that At5PTase1 does not play a role in regulation of seed germination (Burnette et al., 2003). In contrast, At5PTase2 ectopic expression was shown to result in insensitivity to exogenous ABA in seed germination when overexpressed via a dexametasone inducible promoter system (Sanchez and Chua, 2001). However, under

no-hormone treatment conditions, the germination rate of At5PTase2 transgenic seeds was similar to that of wild-type seeds (Sanchez and Chua, 2001).

The *cvp2* and *fra3* mutants were identified as having deficiency in At5PTase6 (At1g05470) and At5PTase12 (At1g65580), respectively (Carland and Nelson, 2004; Zhong et al., 2004). The Cvp2 gene has been shown to be involved in vascular patterning (Carland and Nelson, 2004) and Fra3 has been shown to be involved in secondary wall synthesis and actin organization in fiber cells (Zhong et al., 2004). Moreover, *cvp2* seeds were shown to be hypersensitive to exogenous ABA in seed germination suggesting that 5PTase function in the regulation of seed germination is a complex process that involves multiple 5PTases (Carland and Nelson, 2004).These findings strengthen the connection between 5PTase function and growth and development of *Arabidopsis* plants.

To delineate the function of At5PTase11 in *Arabidopsis*, T-DNA insertion mutant lines were utilized. Two independent lines containing T-DNA insertions in the coding region of At5PTase11 have been identified. Both *At5ptase11* mutant lines exhibited slower germination and shorter hypocotyl growth in the dark as compared to wild-type. These results indicate that At5PTase11 plays an important role for the early stages of seed germination and seedling growth.

Materials and Methods

Isolation of At5ptase11-1 and At5ptase11-2 Mutant Lines

By searching available T-DNA insertional mutant collections of *Arabidopsis* for putative At5PTase11 gene knock-outs, *At5PTase11-1* (Garlic_784_G03.b.1a.Lb3Fa) and *At5PTase11-2* (SALK_108673) T-DNA insertional mutant lines were identified. Seeds for each mutant line were obtained from the SAIL collection of TMRI (Syngenta, Research Triangle Park, NC) and the SALK T-DNA insertion mutant collection (Genomic Analysis Laboratory, The Salk Institute for Biological Studies, La Jolla, CA). To map the T-DNA insertion sites, genomic DNA was isolated from the mutant lines using a Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. PCR reactions were performed using the T-DNA left border primer (for *At5ptase11-1*: 5'TTCATAACCAATCTCGATACAC3', for *At5ptase11-2*: 5'GCGTGGACCGCTTGCTGCAACT3'), the At5PTase11 gene-specific primer (At5PTase11-Cterm primer: 5'TTAACTGTTGACCCACTTCAAGCAAA3'or At5PTase11-Nterm primer: 5'ATGGGGAATAAGAATTCGATGT3') and 25 ng of genomic DNA. Components of the PCR reactions have been described (Ercetin and Gillaspy, 2004). PCR amplifications consisted of 30 cycles (1 min 94ºC, 1 min 55ºC for *At5ptase11-1* or 60ºC for *At5PTase11-2*, 2 min 72ºC). The PCR reaction with *At5PTase11-1* genomic DNA, left border and At5PTase11-Nterm primer resulted in an amplification of a 1200 bp fragment. In contrast, the PCR reaction with *At5PTase11-1* genomic DNA, left border and At5PTase11-Cterm primer resulted in an amplification of

a 1300 bp fragment. In addition, the PCR reaction with *At5PTase11-2* genomic DNA, left border and At5PTase11-Cterm primer resulted in amplification of a 300 bp fragment. The resulting PCR products were sequenced and compared to the wild-type At5PTase11 genomic sequence to verify the position of the T-DNA insertion.

Southern Blot Analysis

Genomic DNA was isolated from wild-type, homozygous and heterozygous *At5PTase11-1 Arabidopsis* plants using a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. Ten microgram of each genomic DNA sample was digested with *Eco*RI (Promega). After separation of the fragments on a 1% agarose gel, the DNA fragments were transferred to a Qiagen nylon membrane according to the manufacturer's recommended alkaline transfer protocol. Using a Bioslink UV-linker (BIOS Corp, New Haven, CT), DNA was cross-linked to the nylon membrane. An At5PTase11-specific probe was produced with 25 ng of an At5PTase11 genomic PCR fragment using a Stratagene Prime-It Random Primer Labeling kit (Stratagene, La Jolla, Ca) and [α-^{32}P]-dATP according to the manufacturer's protocol. Overnight hybridization of the probe with the membrane was performed at 65ºC. Low stringency washes were performed at 42ºC in 0.1x SSC and membrane was exposed to a Storm PhosphoImager cassette for two days. The card was scanned using the Storm PhosphoImager (Molecular Dynamics, Piscataway, NJ).

RT-PCR

Conditions for semiquantitative RT-PCR have been described previously (Berdy et al., 2001). Total RNA was extracted from 100 mg of frozen tissue using the Qiagen Plant RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. RNA (1 μg) was analyzed by a two-step RT-PCR utilizing a Qiagen Omniscript reverse transcriptase kit. Conditions for actin amplification have been described and generated a 428-bp product (An et al., 1996; Berdy et al., 2001). For At5PTase11 gene-specific amplification, the At5PTase11-Nterm primer and At5PTase11-Cterm primer were used in 30 cycles of PCR amplification (1 min 94ºC, 1 min 58ºC, 1.5 min 72ºC) resulting in a 996-bp product.

Plant Growth and Treatment

Arabidopsis (ecotype Columbia) plants were used for all experiments. Growth conditions of soil-grown plants have been described previously (Berdy et al., 2001). For hypocotyl elongation experiments, wild-type and mutant seeds were surface sterilized for 10 min in 30% (v/v) bleach and rinsed with five times with sterile, distilled water. After sterilization seeds were placed in round 9-cm petri dishes containing 20 ml of ½ x Murashige and Skoog medium (Murashige and Skoog, 1962)and 1% sucrose. Plates were wrapped with aluminum foil and stratified for 3 days at 4ºC. Hypocotyl length was measured after 54 and 66 hours of growth in the dark at 20.5ºC using a Zeiss SV 11 microscope equipped with a JVC digital camera KY-F70 (Zeiss, Thornwood, NY) and

Auto-montage Pro software version 5.01.0005 (Synoptics, Frederick, MD). For the analysis of germination, the same procedure was followed except that separate plates were prepared for each time point. For consistency, wild-type and mutant seeds were grown on the same plate. Root radical emergence was used as a criterion for scoring germination. For ABA treatments, plates were prepared in a similar way except that 1, 2 and 2.5 μM ABA (final concentration) was included in the media. After stratifying seeds for 3 days at 4ºC, plates were transferred to 25ºC under continuous light. Seeds were scored for germination over a 10 day period. For auxin experiments, after 3 days of stratification at 4ºC, wild-type and mutant seeds were grown vertically for three days on ½ x Murashige and Skoog medium and 10 seedlings with approximately 1.5 cm root length were transferred to plates containing 0, 0.5, 1 and 10 μM auxin (IAA) (Sigma, St. Louis, MO). Root tip images were taken using the same microscope system used for hypocotyl elongation experiments.

Labeling of Seedlings with [^{3}H]Myo-inositol

Wild-type and *At5PTase11-1* seed were stratified for 3 days at 4ºC, germinated and grown under constant light and shaking (91 rpm) in 50 ml aqueous medium [1/2 x Murashige and Skoog medium containing nicotinic acid (1 μg/ml), thiamine HCl (10 μg/ml), pyrodoxin HCl (1 μg/ml) and *myo*-inositol (100 μg/ml)] for 10 days. Seedlings were transferred to 4 ml medium containing reduced *myo*-inositol (10 μg/ml) and [2-^{3}H]-*myo*-inositol (37.5 μCi/ml) and grown for 20 h under constant light and shaking (91 rpm) (DeWald et al., 2001). For the salt stress treatment, 0.2 M (final concentration) NaCl was

added concomitantly with the [2-^{3}H]-*myo*-inositol and seedlings were treated for 30 min before harvesting.

Extraction of Phosphoinositides and Inositol Phosphates

Methods for the extraction of PIs and inositol phosphates have been described before (DeWald et al., 2001). Briefly, after labeling, the seedlings were treated with 5 ml of 5% (w/v) TCA on ice for 1 hour. Seedlings were washed with 10 ml of ddH_2O for five times and homogenized in 1.5 ml of 1M hydrochloric acid:chloroform:methanol solvent system (0.5:1:1, v/v). To separate organic (bottom) and aqueous phase, samples were centrifuged at 5000g for 5 min. After drying the organic and aqueous phases under nitrogen gas, aqueous phase was re-extracted with methanol:chloroform solvent and dried again under nitrogen gas. Samples containing aqueous phase were resuspended in 100 µl of ddH2O and 5 µl of each sample was counted with a liquid scintillation counter (LS 5801, Beckman Instruments). The samples were stored at -80ºC and later analyzed by HPLC as described below. The organic phases were deacylated as described below.

Phosphoinositide Deacylation

Deacylation of the PIs (named as GroPIns after deacylation) has been described (DeWald et al., 2001). The dried organic phases obtained from extraction of PIs were resuspended in 0.5 ml of methylamine solution [25% (w/v) methylamine:methanol:*n*-butanol, 3.75:4:1 (v/v)]. After incubation at 53 ºC for 30 min, samples were dried under nitrogen gas. The

resulting deacylation products were resuspended in 0.75 ml of ddH_2O and extracted four times with 0.75 ml of *n*-butanol:petroleum ether:ethyl formate (20:4:1, v/v). After drying under nitrogen gas, samples were resuspended in 100 µl ddH_2O and 5 µl of each sample was counted with a liquid scintillation counter.

HPLC Analysis

For the analysis of deacylated PIs, HPLC (Beckman System Gold; Beckman Instruments, Fullerton, CA) equipped with a Partisil 10 SAX (4.6 x 250 mm) column (Whatman, Clifton, NJ) and 1 cm guard column (Waters) was used. An aliquot, 0.45×10^6 cpm (for GroPIns) and 1.0×10^6 cpm (for inositol phosphates) of each sample was applied to the column. Gradients for the separation of the GroPIns and inositol phosphates has been described (DeWald et al., 2001). For the analysis of GroPIns, samples were eluted with a constant 10 mM ammonium phosphate (AP; pH 3.8) for 5 min, followed by a linear gradient from 10 mM AP to 0.7 M AP over 40 min at a flow rate of 1 ml/min. For the analysis of inositol phosphates, samples were eluted with a constant flow of 10 mM AP (pH 3.8) for 5 min, followed by a linear gradient from 10 mM AP to 0.8 M AP over 60 min at a flow rate of 1 ml/min. Radioactivity was counted with an in-line Beckman 171 radioisotope detector (Beckman Coulter, Fullerton, CA) and the data file was converted to an asci file format with 32 Karat software (Beckman Coulter, Fullerton, CA) and analyzed by Excel (Microsoft, Seattle).

[^{3}H]-PtdIns(4,5)P_2, [^{3}H]-PtdIns, [^{3}H]*myo*-inositol (1,4,5)P_3, and [^{3}H]*myo*-inositol

(1,3,4,5)P_4 standards were obtained from American Radiolabeled Chemicals (St. Louis, MO).

Generation of Transgenic Plants That Overexpress At5PTase11

Generation of the pNTTOPOAt5PTase11 clone was described before (Ercetin and Gillaspy, 2004). To generate constructs for transformation of *Arabidopsis* to overexpress At5PTase11, a His-Xpress-At5PTase11-cDNA cassette from pNTTOPOAt5PTase11 plasmid was first cloned into the *Sma*I site of the pBluescript 316 shuttle vector between the 35SCaMV promoter and Nos 3' sequences, resulting in the clone pBS316At5PTase11. A 35SCaMV-His-Xpress-At5PTase11-cDNA-Nos fragment was subcloned into the binary vector pCambia3300 between the *Sac*I and *Bam*HI sites, resulting in the pCambiaAt5PTase11 construct, which was transformed into *A. tumefaciens*. To generate the pSLJAt5PTase11 construct, a 35SCaMV-His-Xpress-At5PTase11-cDNA-Nos fragment from pCambiaAt5PTase11 clone was subcloned into the binary vector pSLJ7292 between the *Sac*I and *Bam*HI sites. The resulting pSLJAt5PTase11 construct was transformed into *A. tumefaciens* by triparental mating. *A. tumefaciens* carrying either the pSLJAt5PTase11 or the pCambiaAt5PTase11 construct was used in vacuum infiltration transformation of *Arabidopsis* as described (Clough and Bent, 1998). For stable transformation of pCambiaAt5PTase11, seeds were grown on soil and transformants were selected by Basta herbicide application. For transient expression studies, *Arabidopsis* plants were grown for 24 hours post vacuum infiltration. Crude protein extracts were generated by grinding plant tissue in liquid nitrogen. 50 mM Tris

(pH 8.5), 150 mM KCl, 0.5 mM EDTA, and plant protease inhibitor cocktail (Sigma, St. Louis) was added and samples were Dounce homogenized and centrifuged at 14,000 rpm at 4°C for 10 min. Proteins in the supernatant were quantified with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, California). Protein samples from untreated wild-type plants were prepared and used as a control in western blot analysis. Equal amounts of protein samples were analyzed by western blot analysis using anti-His and anti-Xpress monoclonal antibodies (Invitrogen).

Results

Isolation and Characterization of At5ptase11 Mutant Lines

To investigate the role of the At5PTase11 gene in *Arabidopsis*, I conducted a search for potential At5PTase11 knock-out mutants in available T-DNA insertion mutant collections. Two independent mutant lines were obtained, and named *At5ptase11-1* and *At5ptase11-2*. T-DNA insertions were confirmed by PCR using a T-DNA left border primer and At5PTase11 gene-specific primers (At5PTase11-Nterm and At5PTase11-Cterm). Sequencing of the amplified fragments showed that the T-DNA insertion was within the fifth exon for *At5ptase11-1* and seventh exon for *At5ptase11-2* (Figure 1A). In addition, when a left border primer and an At5PTase11-Cterm primer were used in a PCR reaction with genomic DNA isolated from *At5ptase11-1* mutants, a 1200 bp band was amplified, indicating the potential for a second T-DNA insertion. Sequencing of this fragment suggest that two tandem T-DNA insertions were present in *At5ptase11-1*. Similar tandem insertion of the T-DNAs has been observed by others as well (Schneider et al., 2002). In contrast, similar PCR experiments examining the At5PTase11 locus from the *At5ptase11-2* mutant indicated only one T-DNA insertion in the At5PTase11 gene.

To further characterize the T-DNA insertion and determine the homozygosity of mutants, Southern blot analysis was performed. When a radioactive labeled At5PTase11 genomic fragment was used as a probe on a genomic Southern blot, an approximately 2.2 kb band was detected in wild-type plant genomic DNA digested with *Eco*RI (Figure 1B, lane 1).

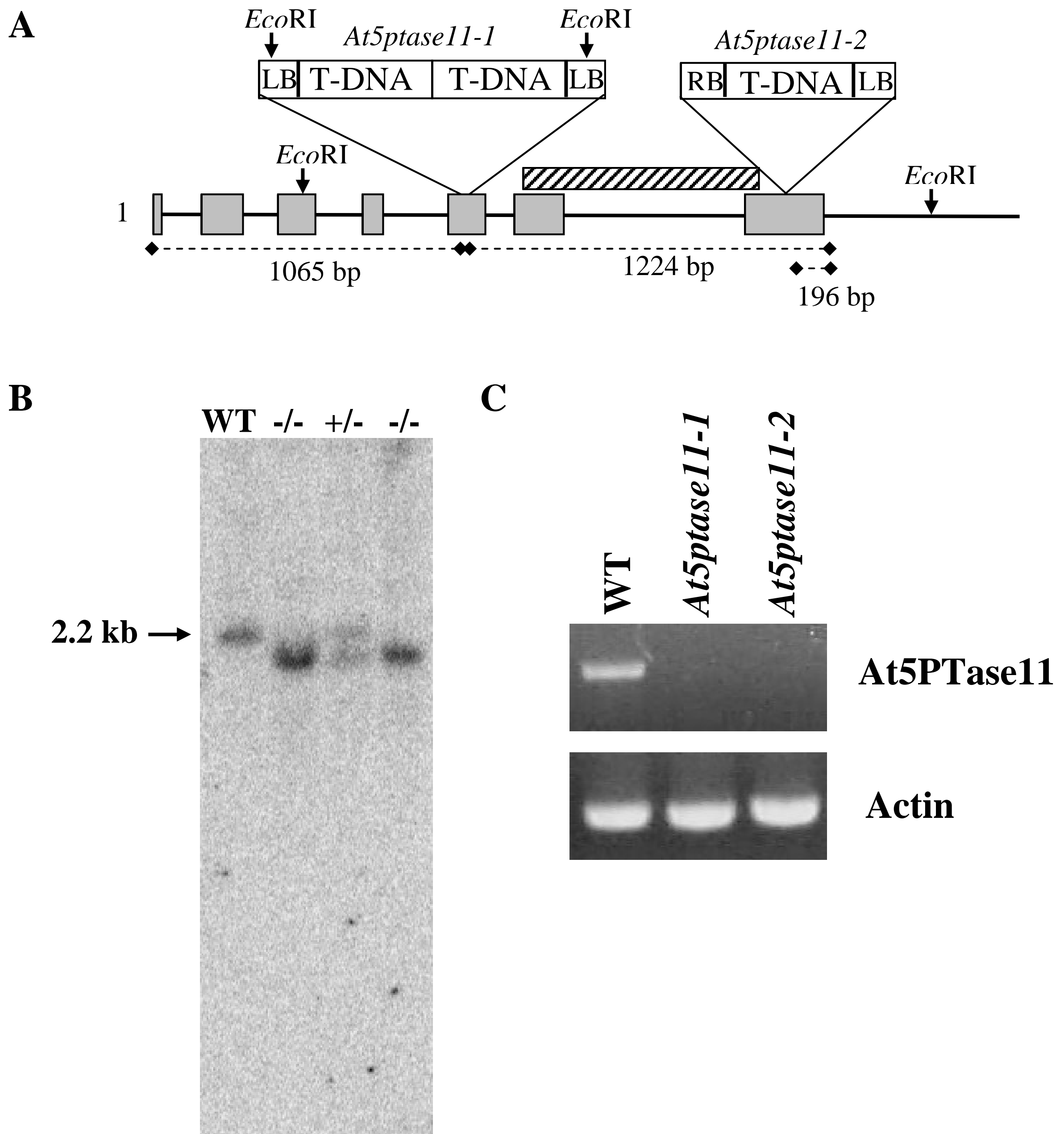

Figure 1. Characterization of *At5ptase11* T-DNA Insertion Mutants. A) T-DNA insertion sites in *At5ptase11*. Gray boxes represent exons. Hatched box represents the At5PTase probe used in the Southern blot. LB: T-DNA Left border, RB: T-DNA Right border. *Eco*RI digestion sites are indicated by arrows. The regions of At5PTase11 gene amplified by PCR using LB and At5PTase11 gene-specific primers are indicated with dashed lines. The first base in the At5PTase11 gene is indicated by "1". The size of T-DNA is not to scale. B) Southern blot analysis with At5PTase11 probe. The indicated lanes contain 10 µg *Eco*RI digested genomic DNA from wild-type seedlings (WT), and seedlings homozygous for the *At5ptase11-1* (-/-), and heterozygous for the *At5ptase11-1* (+/-). C) RT-PCR analysis of At5PTase11 transcript in wild-type and *At5ptase11* (-/-) mutant seedlings. Analysis of actin gene was included as a control. See "Materials and Methods" for details.

This corresponds to the predicted size of the genomic At5PTase11 locus when digested with *Eco*RI. In contrast, the At5PTase11 probe hybridized to a 1.9 kb fragment in homozygous *At5ptase11-1* genomic DNA digested with *Eco*RI (Figure 1B, lanes 2 and 4) indicating a genomic alteration in both copies of At5PTase11. This shift was due to the T-DNA insertion which generated a new restriction site for *Eco*RI which resulted in the formation of a smaller fragment. As expected, both bands were detected in the *At5ptase11* genomic DNA heterozygous for the T-DNA insertion (Figure 1B, lane 3).

To verify the absence of the At5PTase11 transcript in homozygous mutant lines, semi-quantitative reverse transcription-PCR was performed using RNA isolated from seedlings (Figure 1C). Both homozygous mutant lines (Figure 1C, lanes 2 and 3) contained no detectable cDNA corresponding to At5PTase11 (Figure 1C, lane 1) showing that the insertion of the T-DNA abolished At5PTase11 transcription. The amplification of the actin gene was included as a positive control for RT reactions (Figure 1C). Taken together, these results show that *At5ptase11-1* and *At5ptase11-2* are two independent mutant lines that do not express the At5PTase11 gene.

Comparison of Seedling Growth and Germination of Wild-type and At5ptase11

In order to determine if At5PTase11 functions in seedling growth in *Arabidopsis*, *At5ptase11* mutant lines were compared to wild-type. At5PTase11 is expressed in seedlings (Figure 1C), as well as in flowers, rosette leaves, bolts, roots, sliques, and cauline leaves suggesting that At5PTase11 functions in these tissues (Ercetin and

Gillaspy, 2004). *At5ptase11-1* and *At5ptase11-2* mutant lines homozygous for the T-DNA insertion did not show apparent defects in growth and development. This is similar to the findings of other laboratories investigating 5PTase mutants (Carland and Nelson, 2004; Zhong et al., 2004). When *At5ptase11-1* and *At5ptase11-2* mutant seeds and wild-type *Arabidopsis* seeds were germinated in the dark for three days, both *At5PTase11* mutant lines demonstrated greater hypocotyl growth as compared to wild-type. However, when the timing of germination of the mutant and wild-type seed was measured under the same conditions, it was found that the rate of germination of wild-type was lower than the mutant lines. This finding raised the possibility that the seed lots used for the hypocotyl elongation experiments might have differences in their harvesting time which may be the reason for difference in the germination time.

To eliminate the differences resulting from harvesting seed at different times, wild-type and mutant lines were grown under identical conditions and seed was harvested at the same time to be used in germination experiments. Age-matched wild-type and mutant seeds were compared for their germination timing when grown in the dark for 3 days. In contrast to previous results, it was found that both mutant lines germinated slightly slower than the wild-type (Figure 2A). Similarly, under continuous light conditions, wild-type seeds germinated slightly faster than the mutant seeds (Figure 2B). This indicates that loss-of-At5PTase11 function results in slower germination.

To further investigate the loss-of-At5PTase11 function, wild-type and *At5ptase11* seeds were germinated in the dark and hypocotyls were measured at 54 and 66 h (Figure 3). At

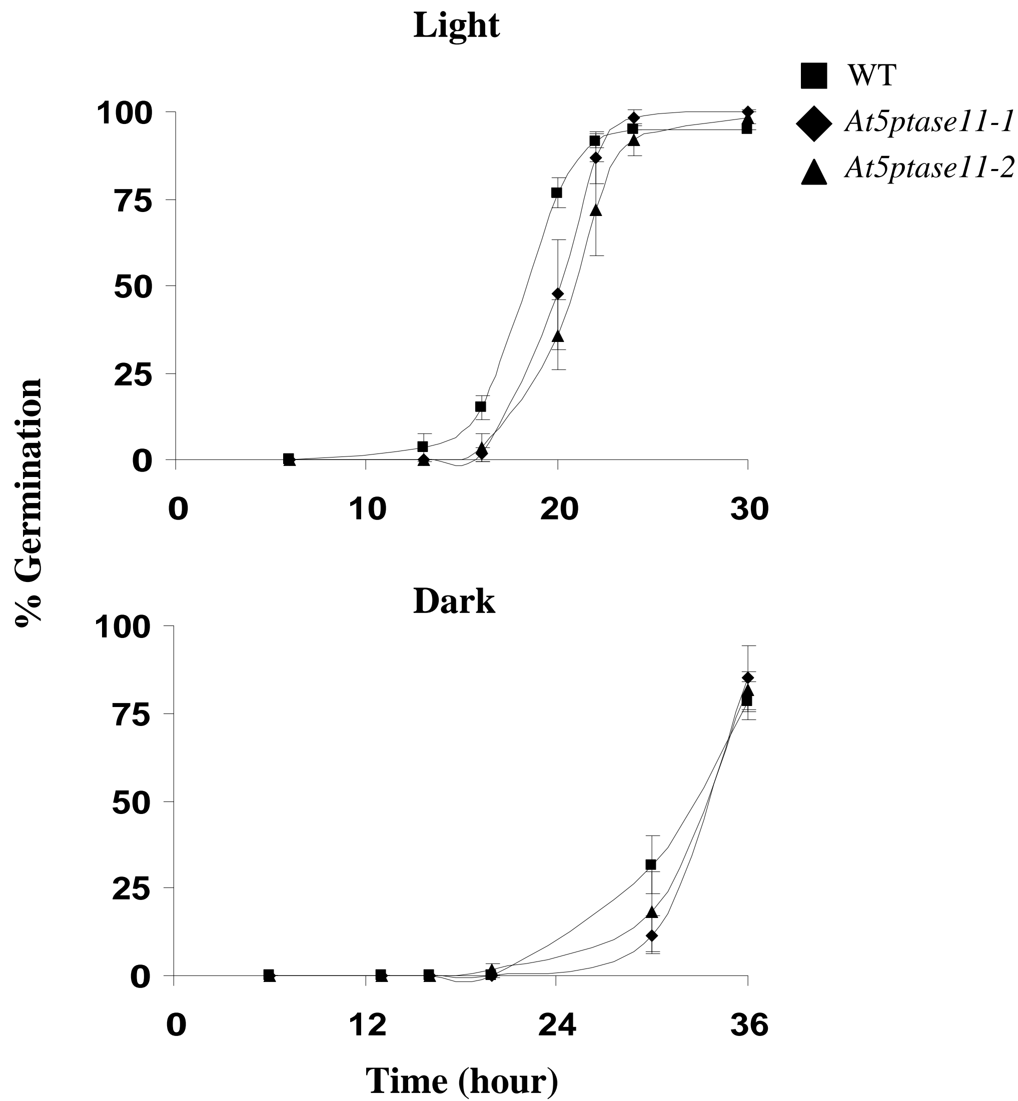

Figure 2. Comparison of Germination of WT and *At5ptase11* Seed. WT (closed squares), *At5ptase11-1* (closed diamonds), and *At5ptase11-2* seed (closed triangles) were germinated under continuous light (top panel) or dark (bottom panel) and scored for germination at the indicated time points. Germination was scored as root radical emergence. The mean and standard error for three independent experiments are presented. Twenty WT and *At5ptase11* seeds were analyzed during each experiment.

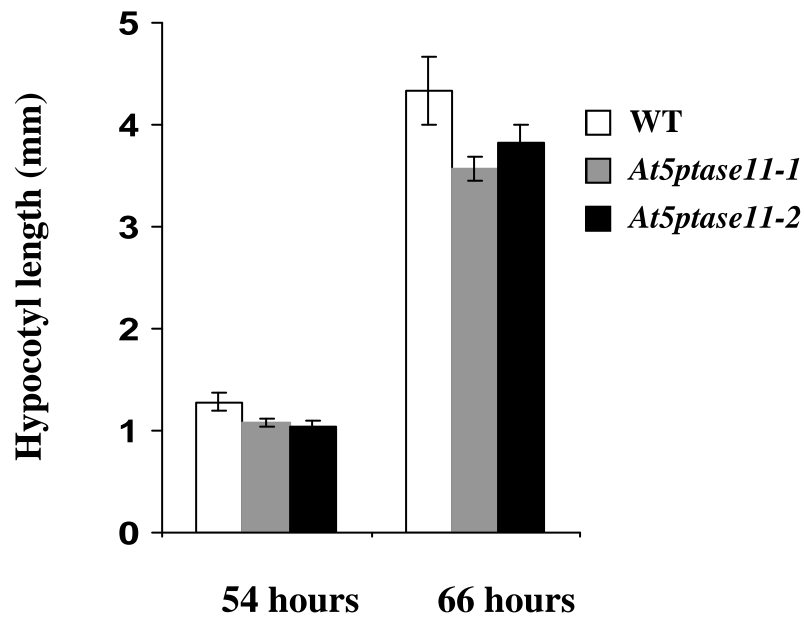

Figure 3. Comparison of Hypocotyl Elongation of WT and *At5ptase11* Mutants in the Dark. WT (white bars), *At5ptase11-1* (gray bars), and *At5ptase11-2* seed (black bars) were germinated in the dark and hypocotyl length was measured after 54 and 66 hours. The top shows representative seedlings at the 66 hour time point. The mean and standard error for three independent experiments are presented. Twenty WT and *At5ptase11* seedlings were analyzed during each experiment.

both time points, the hypocotyl length of *At5ptase11-1* and *At5ptase11-2* mutants was shorter than wild-type. Together, these results indicate that *At5ptase11* mutant seeds germinate slower and exhibit reduced hypocotyl growth compared to wild-type.

Determination PtdIns(4,5)P_2 Levels in At5ptase11 Mutant Seedlings

I have previously shown that At5PTase11 is a phospholipid-specific inositol polyphosphate 5-phosphatase (Ercetin and Gillaspy, 2004). In order to examine the effect of loss-of At5PTase11 function on *in vivo* substrate levels, wild-type and *At5PTase11-1* seedlings were germinated, grown in liquid culture and labeled with [^{3}H]-*myo* inositol (37.5 µCi/ml) for 20 hours. Lipids were extracted from the seedlings and deacylated as described in the "Materials and Methods." Equal amounts of total cpm for each sample were analyzed by HPLC to separate the deacylated lipids (GroPIns). [^{3}H]-PtdIns(4,5)P_2 and [^{3}H]-PtdIns standards were obtained from American Radiolabeled Chemicals, Inc. (ARC) and preparation of the [^{3}H]-PtdIns(4)P standard was described (Ercetin and Gillaspy, 2004). Standards were deacylated and used to identify the elution time of the GroPIns. Comparison of the HPLC traces in Figure 4A indicates that basal PtdIns(4,5)P_2 levels were not different in *At5PTase11-1* mutants and wild-type seedlings. Levels of PtdIns(4)P were also similar in *At5ptase11-1* mutants and wild-type seedlings indicating that basal levels of PtdIns(4)P and PtdIns(4,5)P_2 are not affected by loss-of-At5PTase11 function.

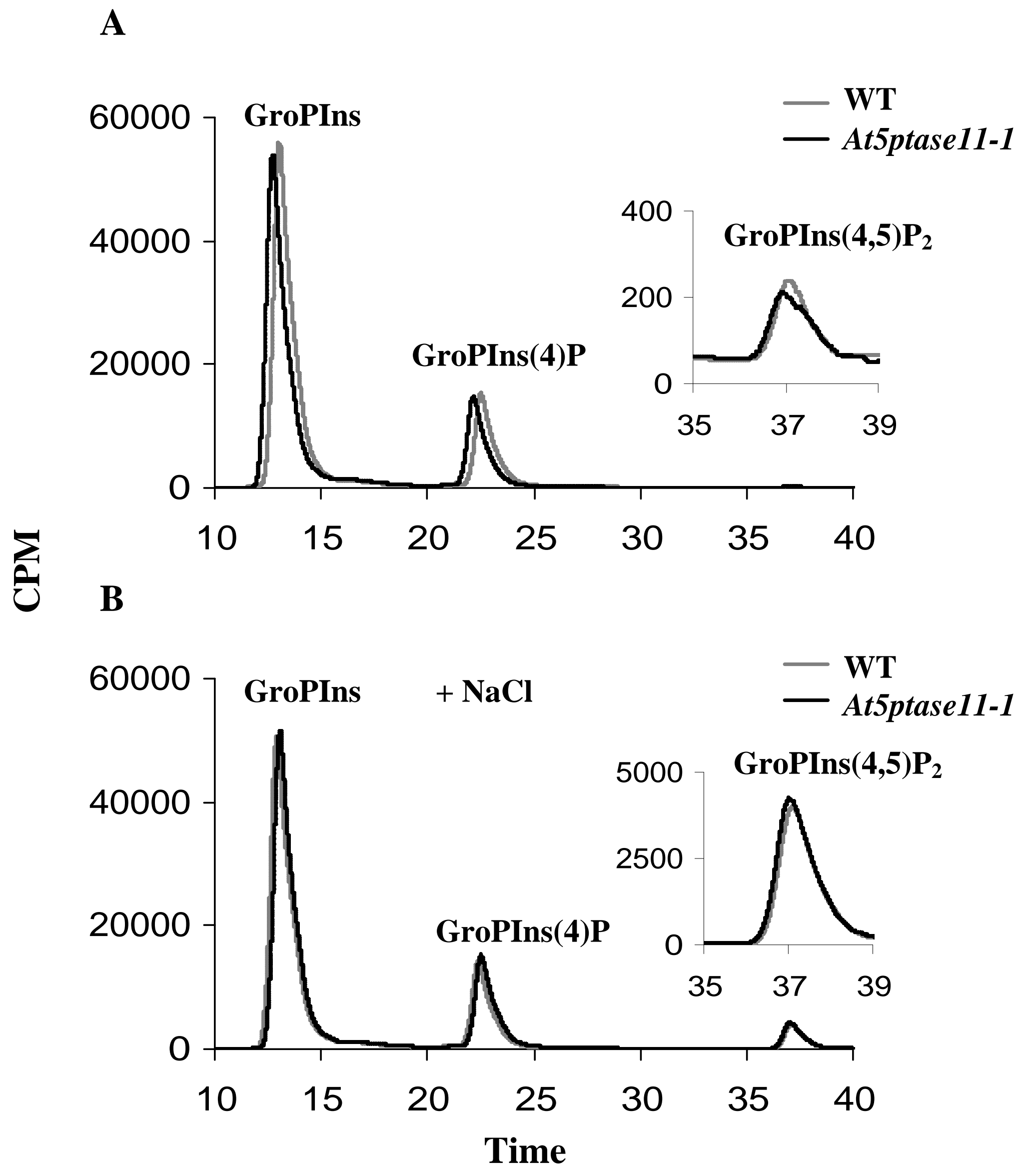

Figure 4. Analysis of *in vivo* PtdIns(4,5)P_2 Levels in *At5ptase11-1* and WT Seedlings. HPLC analysis of deacylated [^{3}H]*myo*-inositol-labeled lipids from WT (gray lines) and *At5ptase11-1* (black lines). Top panel (A) Unstimulated seedlings. Bottom panel (B) 30 min after 0.2 M NaCl stimulation. The positions of the standards derived from the indicated phosphoinositides are shown. Equal amounts of total counts were analyzed for each sample. Labeling experiments were performed twice and similar results were obtained.

Previously it was shown that treatment with 0.25 M NaCl results in an approximately 20-fold increase in PtdIns(4,5)P_2 levels, providing evidence for stimulation of PI signaling in response to NaCl treatment (DeWald et al., 2001). I decided to examine the effect of salt treatment on *At5ptase11-1* seedlings as described in Materials and Methods. When the PtdIns(4,5)P_2 levels of NaCl treated *At5ptase11-1* seedlings were compared to non-treated *At5ptase11-1* seedlings, an approximately 20-fold increase was observed (Figure 4A, B). However, a similar increase in PtdIns(4,5)P_2 levels were observed in wild-type seedlings when treated with NaCl (Figure 4A, B). These labeling experiments indicate that under basal conditions and salt stimulation conditions, PtdIns(4,5)P_2 levels in seedlings were not affected significantly by the loss-of At5PTase11 gene function.

ABA and auxin treatment of At5ptase11 mutants

Prior to this study I have shown that At5PTase11 was transcriptionally regulated by ABA, JA and auxin (Ercetin and Gillaspy, 2004). I have conducted a series of experiments to analyze the consequence of At5PTase11 loss-of function in response to these hormones. In order to examine the effect of ABA, I have tested the effect of different ABA concentrations on germination. Wild-type and mutant seeds were germinated on ½ X MS agar plates containing 1, 2, and 2.5 µM ABA and scored for germination through the course of 10 days under constant light. Wild-type and *At5ptase11* lines responded similarly to ABA (Figure 5). In the presence of 1 µM ABA, both wild-type and *At5ptase11* reached ~100% germination on day 10. In the presence of 2 µM ABA after 10 days, germination was only ~50% and 2.5 µM ABA caused

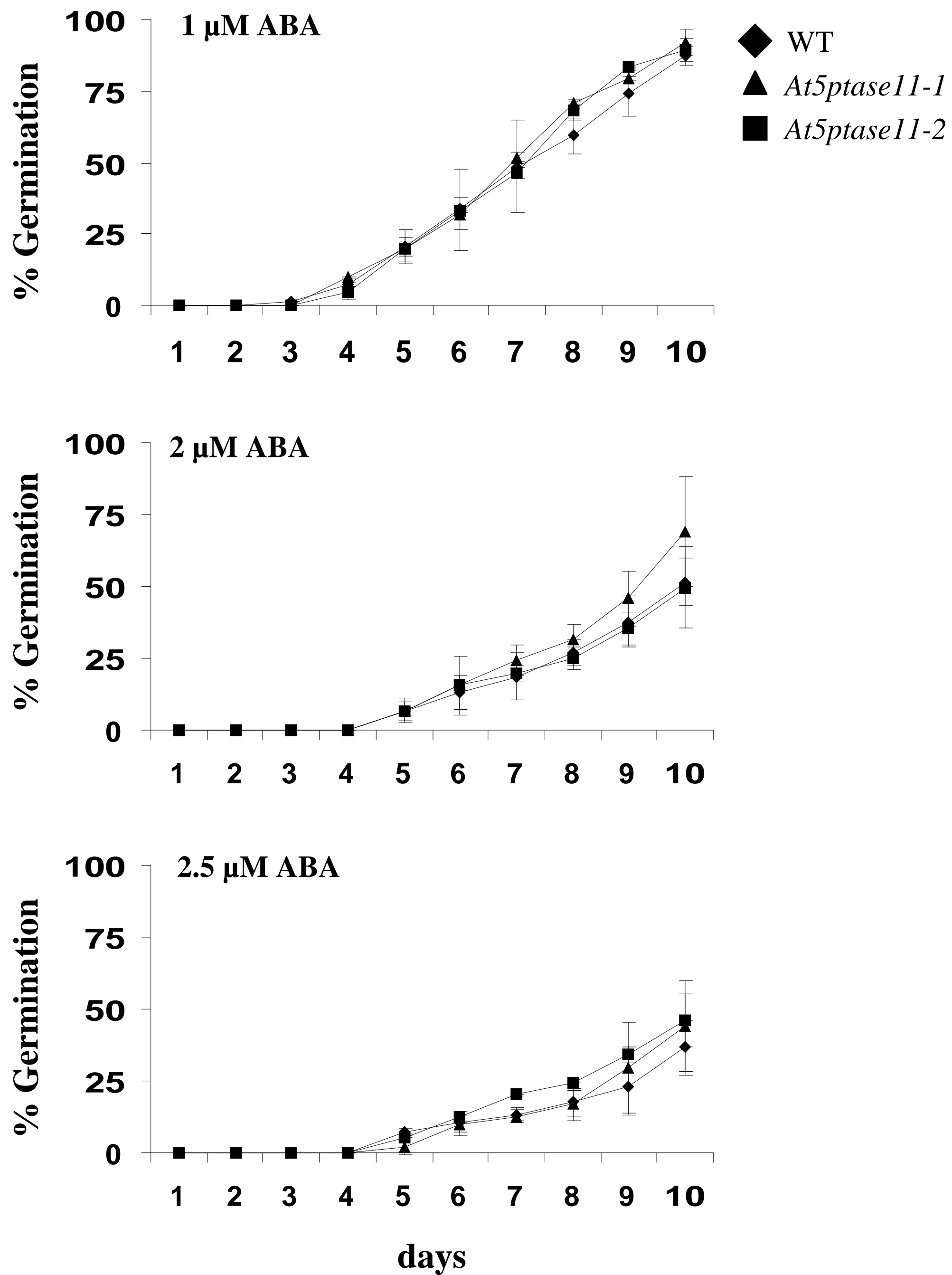

Figure 5. Germination of *At5ptase11* Seed in the Presence of ABA. WT seed (diamond), *At5PTase11-1* seed (triangle) and *At5ptase11-2* seed (square) germinated in the presence of 1 µM (top graph), 2 µM (middle graph) and 2.5 µM (bottom graph) ABA. The mean and standard error for two independent experiments are presented. At least 50 WT and *At5ptase11* seeds were analyzed during each experiment.

inhibition of germination similarly in both wild-type and *At5ptase11* seeds (~40% germination on day 10). These results indicate that *At5ptase11* mutants are not altered in their seed germination response to ABA.

To investigate the effect of auxin on *At5ptase11*mutants, wild-type and *At5ptase11* seedlings were grown vertically on plates containing 0.5-10 μM IAA. When *At5ptase11* mutant plants were examined for differences in response to auxin, a normal wild-type response was observed (primary root growth was inhibited and the root tip was thickened and lateral root growth was induced) (Figure 6). Together these results suggest that loss-of At5PTase11 function did not alter auxin response pathways.

Overexpression of At5PTase11 in Arabidopsis

Previously it has been shown that ectopic expression of At5PTase1 in *Arabidopsis* resulted in alteration of the ABA signal transduction pathway (Burnette et al., 2003). Using a similar approach to explore the consequences of ectopic expression of At5PTase11, I have generated two different clones that carry the His-Xpress-At5PTase11 cDNA cassette under the control of the 35S CaMV (Cauliflower Mosaic Virus) promoter (Figure 7). The pSLJAt5PTase11 and pCambiaAt5PTase11 constructs were transformed into *A. tumefaciens*, which was used in vacuum infiltration transformation of *Arabidopsis* plants. For the pCambiaAt5PTase11 construct, the expression of recombinant At5PTase11 protein carrying both His and Express epitope tags was analyzed in both

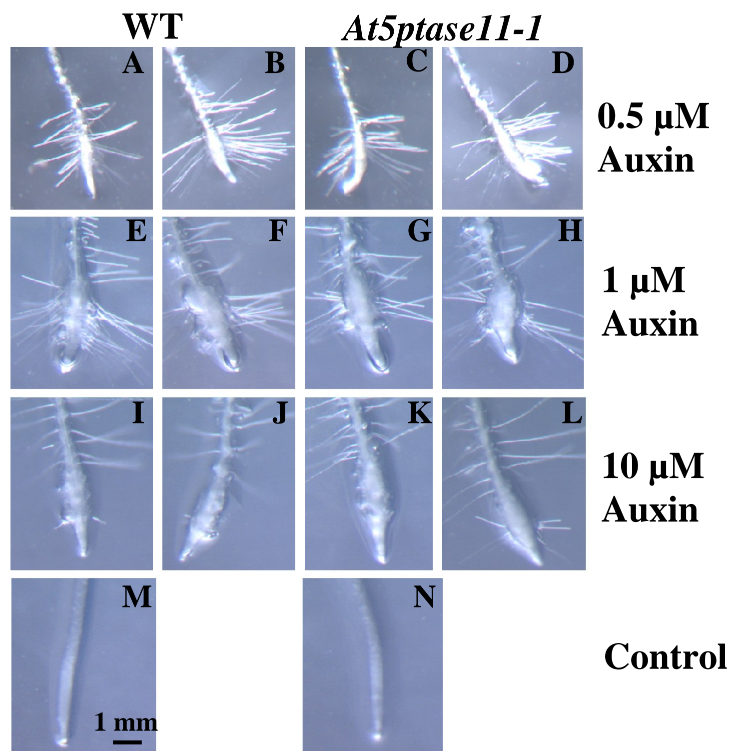

Figure 6. Auxin Treatment of *At5ptase11* Mutants. 3-day old WT (A, B, E, F, I, J, M) and *At5ptase11-1* (C, D, G, H, K, L, N) seedlings were exposed to 0.5 µM (A to D), 1 µM(E to H), and 10 µM(I to L) auxin. Root tips images were taken after 24 h. Representative images are presented. Scale bar = 1 mm.

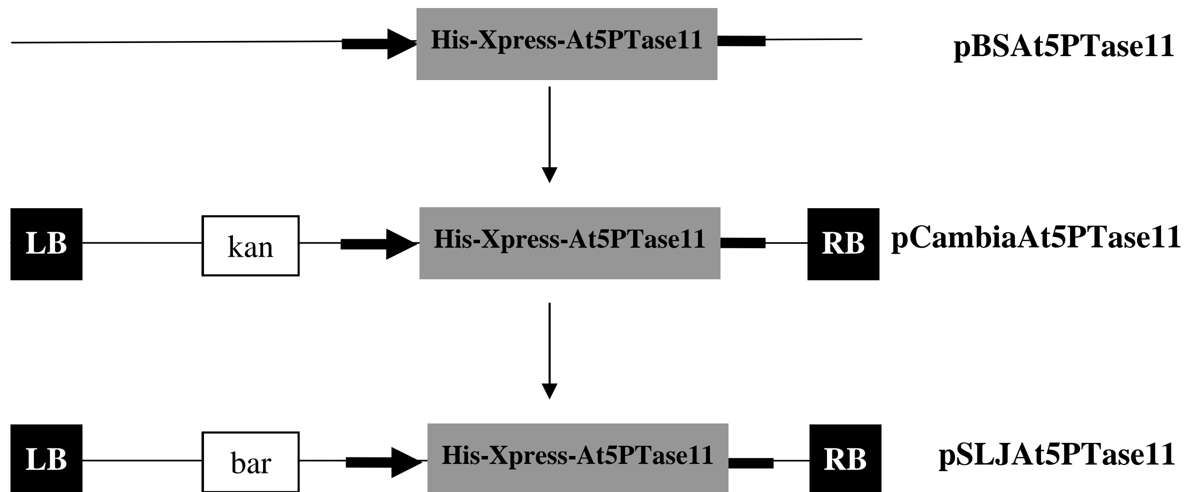

Figure 7. Cloning of At5PTase11 into pCambia3300 and SLJ7292. The His-Xpress-At5PTase11 cDNA cassette was first cloned into the pBS316 shuttle vector resulting in the pBSAt5PTase11 clone. The 35SCaMV promoter (black arrows), His-Xpress-At5PTase11 and nos terminator (black bars) sequences were mobilized into a pCambia3300 vector resulting in pCambiaAt5PTase11 clone. To generate the pSLJAt5PTase11 clone, the 35SCaMV promoter, His-Xpress-At5PTase11 and nos terminator sequences from pCambiaAt5PTase11 were mobilized into a pSLJ7292 vector. LB: left border, RB: right border, bar: Basta resistance gene, kan: kanamycin resistance gene.

transiently and stably transformed plants. Fourteen transgenic lines that were resistant to Basta herbicide were analyzed. For the pSLJAt5PTase11 construct, only transient expression of recombinant At5PTase11 was pursued as described in "Materials and Methods." In both approaches, recombinant At5PTase11 expression could not be detected using either anti-his or anti-express monoclonal antibodies in western blot analysis (data not shown). Since the monoclonal antibodies were very specific in detecting the recombinant tagged protein, I concluded from these experiments that transgenic plants transformed with the pSLJAt5PTase11 or pCAMBIAAt5PTase11 constructs did not produce recombinant At5PTase11 protein.

Discussion

Previously I have shown that At5PTase11 has a distinct substrate selectivity in that it hydrolyzes the phospholipid PtdIns(4,5)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 (Ercetin and Gillaspy, 2004). In this section I report that a loss-of At5PTase11 gene function results in slower germination and seedling growth as compared to wild-type seeds.

The finding that a loss-of At5PTase11 gene function results in slower germination is very important and unique to this 5PTase. The impact of altered 5PTase function to seed germination was also investigated for other 5PTases such as At5PTase1 and At5PTase2, Cvp2 (At5PTase6) and Fra3 (Sanchez and Chua, 2001; Burnette et al., 2003; Carland and Nelson, 2004). When overexpressed in *Arabidopsis*, At5PTase2 was shown to result in reduced Ins(1,4,5)P_3 levels and insensitivity to exogenous ABA (Sanchez and Chua, 2001). In contrast, Cvp2 loss-of-function caused elevated Ins(1,4,5)P_3 levels and hypersensitivity to exogenous ABA (Carland and Nelson, 2004). These studies with At5PTase2 and At5PTase6 genes suggest that altering Ins(1,4,5)P_3 levels impacts seed germination in the presence of exogenous ABA. On the other hand, studies with At5PTase1 overexpressing transgenic plants have shown that ectopic expression of At5PTase1 results in reduced Ins(1,4,5)P_3 (Burnette et al., 2003), and yet At5PTase1 transgenic plants were not ABA-insensitive. This suggests that changing Ins(1,4,5)P_3 levels is not sufficient to alter ABA-sensitivity of seed germination (Burnette et al., 2003). Furthermore, effect of overexpressing At5PTase1 and At5PTase2, and loss-of-Cvp2 and Fra3 function on seed germination were studied without exogenous ABA

application (Sanchez and Chua, 2001; Burnette et al., 2003; Carland and Nelson, 2004; Zhong et al., 2004). Unlike *At5ptase11*mutant seeds which germinate slower than wild-type seeds, germination of transgenic seeds with altered At5PTase1, At5PTase2, Cvp2 and Fra3 function was similar to germination of wild-type seeds (Sanchez and Chua, 2001; Burnette et al., 2003; Carland and Nelson, 2004; Zhong et al., 2004). This indicates that loss-of At5PTase11 gene function has a distinct effect on seed germination.

In wild-type *Arabidopsis*, hypocotyl growth is affected mainly by two factors: the number of hypocotyl cells and cell elongation (Tsuge et al., 1996). Typically, 20 hypocotyl cells are established during embroyogenesis (Ullah et al., 2001) and elongation of these cells results in hypocotyl growth after germination (Tsuge et al., 1996). Thus, differences in hypocotyl growth might be the result of change in cell number or in elongation. Since *At5ptase11* mutants have shorter hypocotyls compared to wild-type, this suggests an alteration in the cell division or elongation.

In order to understand the effect of At5PTase11 loss-of-function on *in vivo* substrate levels, I labeled *At5PTase11* mutant and wild-type seedlings with [^{3}H]*myo*-inositol. A similar approach was taken by DeWald et al. to analyze the effect of osmotic stress on *Arabidopsis* PI levels (DeWald et al., 2001). Our findings indicate that levels of PtdIns(4,5)P_2 were not different in 10-day old wild-type and *At5PTase11* seedlings. In addition, salt stimulation caused similar increase in PtdIns(4,5)P_2 levels. It is important to note that in labeling experiments, the seedlings were grown for 10 days but in the hypocotyl elongation experiments, seedlings were grown for only 2-3 days. Since the

medium did not contain sucrose in the labeling experiments, seedling growth was slower compared to the experiments documenting seedling growth in the dark. These differences may have resulted in growth differences between the two different experiments. These results suggest that loss-of-At5PTase11 function did not alter PtdIns(4,5)P_2 levels significantly. However, one limitation of this approach is that in these labeling experiments, one could measure the amount of PIs labeled with [^{3}H]*myo*-inositol which is different than measuring the total amount of PIs. On the other hand, this method has an advantage over measuring mass PI levels in that one can measure the pool of PIs utilized for signaling.

These findings indicate that *At5PTase11* mutant seed demonstrated similar responses as compared to wild-type when exposed to ABA and auxin. It is not known yet whether there are other 5PTases in addition to At5PTase11 whose expression is regulated by auxin. However, the normal response of *At5ptase11* mutants to auxin suggests that other 5PTases may compensate for the absence of At5PTase11 function. Supporting this idea, expression of multiple 5PTases in the seedlings has been shown (Gunesekera and Gillaspy, unpublished data). Thus, it is necessary to characterize all *Arabidopsis* 5PTases in terms of substrate selectivity in order to predict biochemical redundancy. Based on the information obtained from the substrate selectivity of 5PTases, generation of double or even triple mutants might be necessary to observe severe phenotypes that may result from a loss-of-5PTase function.

CHAPTER IV

Objective III: To Examine the Substrate Selectivity of a Group of *Arabidopsis* 5PTases

Introduction

The PI signaling pathway, which utilizes inositol-containing compounds to mediate signaling events, has been shown to be involved in many critical cellular events in organisms as diverse as yeast or humans (Zhang et al., 1995; Stolz et al., 1998a; Toker, 2002; Burnette et al., 2003). Depending on the position and number of phosphates on the inositol ring, phosphatidylinositol and inositol phosphates can carry different cellular information. PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are the most studied examples of these molecules that are the key players of this pathway (Tsubokawa et al., 1994; Insall and Weiner, 2001; Berridge et al., 2003). Thus, regulation of the synthesis and break-down of these molecules by kinases and phosphatases becomes very important for controlling the signaling events mediated by these molecules (Majerus et al., 1999).

5PTases are enzymes that can dephosphorylate the 5-position phosphate from the inositol ring (Majerus et al., 1999). Because of their potential to dephosphorylate important signaling molecules such as PtdIns(4,5)P_2 and Ins(1,4,5)P_3, 5PTases are excellent candidate enzymes for the regulation of the termination of the signaling events that are mediated through these molecules. Hence, transcriptional, translational and/or post-

translational control of the 5PTase activity might be a way of controlling this pathway. In combination to these control mechanisms, selectivity of the substrate by 5PTases might be another way of controlling this pathway as evident from the studies with mammalian 5PTases (Majerus et al., 1999). Based on their substrate selectivity, mammalian 5PTases are categorized into four groups supporting the idea of a control mechanism due to substrate preference (Majerus et al., 1999).

All 5PTases contain a catalytic motif which is well conserved in all eukaryotes (Majerus et al., 1999).The identification of critical residues that are important for substrate selectivity was addressed through bioinformatics, point mutation and crystal structure approaches (Whisstock et al., 2000; Tsujishita et al., 2001). A bioinformatics approach suggested a common mechanism of catalysis by 5PTases and apurinic/apyrimidinic base excision repair endonucleases, and this idea is supported by the crystal structure of the 5-phosphatase domain of synaptojanin (a 5PTase from *Schizosaccharomyces pombe*) complexed with Ca^{+2} and the product Ins(1,4)P_2 (Whisstock et al., 2000; Tsujishita et al., 2001). However, it is still unclear how catalytic activity is restricted to certain substrates.

In *Arabidopsis*, 15 5PTases that contain the conserved 5PTase catalytic domain have been identified (Berdy et al., 2001). At5PTase1 and At5PTase2 have been shown to hydrolyze water soluble substrates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Berdy et al., 2001; Sanchez and Chua, 2001). In addition, overexpressing At5PTase1 and At5PTase2 in transgenic plants has been shown to alter ABA signaling (Sanchez and Chua, 2001; Burnette et al., 2003). I have shown that At5PTase11 hydrolyzes only lipid substrates,

PtdIns(4,5)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 (Ercetin and Gillaspy, 2004). Substrate selectivities of 5PTases that contain WD-repeat domains were reported. At5PTase12 and At5PTase13 only have activity towards Ins(1,4,5)P_3, and At5PTase13 and Fra3 have activity towards PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and Ins(1,,4,5)P_3 (Zhong et al., 2004; Zhong and Ye, 2004). Fra3 was also shown to be required for secondary wall synthesis and actin organization in fiber cells (Zhong et al., 2004). These studies demonstrated that *Arabidopsis* 5PTases have distinct substrate selectivities and play important roles in signaling, growth and development.

In this section, I aimed to characterize the substrate selectivity of the remaining 5PTases in *Arabidopsis*. In order to obtain recombinant proteins, *Drosophila* S2 cells were utilized based on the fact that active 5PTases can be produced with this system (Ercetin and Gillaspy, 2004). Results indicate that At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7, and At5PTase9 hydrolyze PtdIns(4,5)P_2. In addition At5PTase1, At5PTase2, At5PTase3 and At5PTase7 have demonstrated activity towards PtdIns(3,4,5)P_3. Moreover, At5PTase1, At5PTase2, and At5PTase3 were the most robust enzymes and hydrolyze Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4.

Materials and Methods

Plant Growth

Arabidopsis ecotype Columbia plants were used for all experiments. Growth conditions of soil-grown plants have been described previously (Berdy et al., 2001).

Cloning of At5PTases

Cloning of At5PTase1 and At5PTase11 was described previously (Ercetin and Gillaspy, 2004). Similarly, to clone other At5PTases, full-length cDNAs were generated by RT-PCR using 1 µg of *Arabidopsis* seedling mRNA which was purified using a Qiagen RNeasy kit (Qiagen) and the following primer combinations: At5PTase1 (At5PTase1-Nterm: 5'GTACGCTCGAGCATGGCGGAAGTACGATCACGA3', and At5PTase1-Cterm: 5'CATCGGGGATCCTCAGGCGTCAAGGCCTTGAAT3'), At5PTase2 (At5PTase2-Nterm: 5'GAGATGGAAACAAGACGTGGGAAACGTC3', and At5PTase2-Cterm: 5'TCCAAAAGAAGGTTCAGGATGAAC3'), At5PTase3 (At5PTase3-Nterm: 5'GCCATGGCTCCAGTTGAACCCGCCG3', and At5PTase3-Cterm: 5'AACGAGAACGGCTACAAGTCCCTC3'), At5PTase4 (At5PTase4-Nterm: 5'GCCATGGGTGATGGTAACCTCAAG3', and At5PTase4-Cterm: 5'GTACGGGTTAAGCTCAGAATATC3'), At5PTase7 (At5PTase7-Nterm: 5'GCCATGGTGGTGATTCTTGAGAAC3', and At5PTase7-Cterm: 5'GAAAAATGTTAGCTCGGTGTATC3'), At5PTase9 (At5PTase9-Nterm:

5'GCCATGGGGCCAAGACTTGTG3', and At5PTase9-Cterm:

5'TGTAGATATCCACGAGTAGTC3'), At5PTase13 (At5PTase13-Nterm:

5'GCCATGGATTCGCTAATTATC3', and At5PTase13-Cterm:

5'CCGGCTTTTACCTCGTCT3'). RT reaction (2 µl of 20 µl total), At5PTase-Nterm gene-specific primer (12.5 pmol/µl), At5PTase-Cterm gene-specific primer (12.5 pmol/µl), elongase polymerase and supplied buffer (Invitrogen, Carlsbad, CA), 0.5 mM dNTPs, and 2.5 mM final $MgCl_2$ were mixed and heated to 94°C for 3 min. PCR amplification for At5PTase2, 3, 4, 7, and 9 consisted of 30 cycles (1 min 94°C, 1 min 55°C, 3 min 72°C). For At5PTase13, PCR amplification consisted of 30 cycles (1 min 94°C, 1 min 55°C, 3.5 min 72°C). The full-length cDNA products were gel purified (Qiagen gel extraction kit; Qiagen, Valencia, CA), and cloned into the pMT/V5-His-TOPO vector (Invitrogen) according to the manufacturers instructions and are herein called the pMTAt5PTase constructs.

Expression and Immunoprecipitation of At5PTases from S2 cells

Expression and immunoprecipitation of At5PTases from *Drosophila* S2 cells were performed as described (Ercetin and Gillaspy, 2004). Briefly, S2 cells were transfected with 2 µg of pMTAt5PTase constructs using an Effectene transfection kit (Qiagen). After 2 d of induction with 500 µM $CuSO_4$, cells were harvested. Immunoprecipitation of the At5PTases and analysis of the resulting complexes were performed as described (Ercetin and Gillaspy, 2004).

Activity Assays with Fluorescent and Radiolabeled Substrates

Activity assay conditions for fluorescent di-C_6-NBD6-phosphatidylinositol 4,5-bisphosphate, di-C_6-NBD6-phosphatidylinositol 3,4,5-trisphosphate and [^{3}H]-labeled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were described before (Ercetin and Gillaspy, 2004). Recombinant At5PTases (15-80 ng) were incubated with 1.5 μg of fluorescent substrate in assay buffer containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 50 mM KCl. All activity assays were performed for 1 hour at room temperature except for comparison of the At5PTase1, At5PTase2, and At5PTase3 using fluorescent lipid substrates, where reactions were performed for 15 min. Reaction products were separated by TLC and analyzed as described before (Ercetin and Gillaspy, 2004). The amount of product formation was quantified by densitometric analysis of the scanned images of the TLC plates utilizing AlphaEaseFC software version 3.1.2 (Alpha Innotech, San Leandro, CA).

For the analysis of inositol phosphate substrate catalysis, activity assays were performed using [^{3}H]*myo*-inositol (1,4,5)P_3 (10 μCi/ml; 22 Ci/mmol; NEN, Shelton, CT) and [^{3}H]*myo*-inositol (1,3,4,5)P_4 (10 μCi/ml; 22 Ci/mmol; NEN). Immunoprecipitated 5PTases were incubated with 30 nCi of [^{3}H]*myo*-inositol (1,4,5)P_3 and [^{3}H]*myo*-inositol (1,3,4,5)P_4 in reaction buffer (250 mM KCl, 3 mM $MgCl_2$, 50 mM Tris, pH 7.5) in a total volume of 50 μl at room temperature for 1 h. The resulting products were stored at –20°C and analyzed by HPLC as described (Ercetin and Gillaspy, 2004). Amount of product formation was quantified utilizing 32 Karat software (Beckman Coulter). The area under each peak was calculated for each chromatogram generated via HPLC analysis to

determine the amount of product formed for each reaction. Enzyme activities are presented as amount of product formation per time per microgram protein.

Expression and Immunoprecipitation of At5PTases from yeast

To clone At5PTase genes into the pYes2/CT yeast expression vector (Invitrogen), the pMTAt5PTase constructs were digested with *Pme*I (New England Biolabs) and *Kpn*I (Promega) and the resulting fragments (At5PTase-V5-his) were ligated into the pYes2/CT vector pre-digested with *Pme*I and *Kpn*I. Yeast transformation and induction of the recombinant proteins were performed according to the manufacturer's instructions (Invitrogen). Expression of the recombinant proteins was induced by 2% (w/v) galactose for 24 hours. Yeast cells were broken via glass beads by vigorous vortexing for 1 min and then cooling on ice for 1 min for a total of 8 times. The resulting extracts were transferred to 2 ml eppendorf tubes. To obtain soluble extracts, samples were spun at 14,000 rpm for 15 min and soluble portions were analyzed by western blotting using an anti-V5 antibody (Invitrogen).

To immunoprecipitate At5PTases from yeast extracts, the same procedure that was used for immunoprecipitation from S2 cells was applied (Ercetin and Gillaspy, 2004). Yeast cultures (50 ml) containing recombinant At5PTases were harvested and broken with glass beads in immunoprecipitation buffer [50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100, and 1mM phenylmethylsulfonyl fluoride (PMSF)]. Soluble extracts were prepared by centrifugation at 14,000 rpm for 15 min and the soluble portions of the

extracts were immediately incubated with anti-V5 monoclonal antibody (Invitrogen) coupled to Protein A Sepharose beads (Sigma-Aldrich) for 2 h at 4ºC with end-over-end rotating. The Protein A Sepharose beads were pelleted and washed three times with immunoprecipitation buffer followed by one wash with 5PTase activity assay buffer. A portion of this immunoprecipitate was analyzed by SDS-PAGE and western blotting using an anti-V5 antibody.

Purification of Recombinant At5PTases from Yeast Cells Transformed with pYes2/CTAt5PTases

I used Talon resin which uses cobalt ions for purification of His-tagged proteins (BD Biosciences) to purify His-tagged recombinant At5PTase1, At5PTase2, At5PTase11, and β-galactosidase produced in yeast cells. For a background control extract, the same purification steps were applied to InvSc1 (the host yeast strain used for expression studies) cell extracts. Cell pellets from 100 ml yeast cultures containing recombinant At5PTases were resuspended in 5 ml Yeast Lysis Buffer (YLB) containing 50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100, and 1mM PMSF, and protease inhibitors Complete-EDTA (one tablet for 50 ml extract was used as recommended by manufacturer) (Roche Applied Science, Indianapolis, IN). The resulting yeast cell suspensions were divided into two 15 ml conical tubes which contained 2 ml acid washed glass beads (Sigma-Aldrich). The yeast cells were broken by vigorous vortexing for 1 min for a total of 8 times and kept on ice for 1 min between each vortexing. Cell extracts were transferred into 2 ml eppendorf tubes and centrifuged at

14,000 rpm to obtain a soluble fraction. In a 15 ml conical tube, 0.5 ml of Talon resin for each sample was equilibrated in YLB and then soluble fractions were added. To allow binding of recombinant proteins to the Talon resin, the samples were incubated at 22ºC for 30 min end-over-end rotating. After centrifuging at 2000g for 1 min, the supernatant was discarded and resin was washed with 10 ml YLB for 10 min at 22ºC with end-over-end rotating. Following the first wash step, resin was transferred to a column (BioRad) and washed with 1 ml YLB for 10 times. Finally, the recombinant At5PTases were eluted with 2 ml of 150 mM immidazole, 50 mM Tris, pH 7.0, 5 mM $MgCl_2$, 1 mM PMSF, 300 mM NaCl, and protease inhibitors Complete-EDTA (Roche). The resulting elutes were transferred into Slide-A-Lyzer dialysis cassettes (Pierce Biotechnology, Rockford, IL) and dialyzed against 1 L 50 mM Tris, pH 7.0, 1.5 mM $MgCl_2$. After dialysis, elutes containing recombinant 5TPases were concentrated using Millipore spin-columns with a 30 kDa molecular weight cut-off (Millipore, Billerica, Massachusetts). The final volume was adjusted by adding 50 mM Tris, pH 7.0, 1.5 mM $MgCl_2$ to 301 µl. To determine the presence of recombinant proteins, a portion of the elutes was analyzed by gel electrophoresis followed by Coomassie staining and western blotting using an anti-V5 antibody.

Phosphate Release Assays with Cold Substrates

Immunoprecipitated At5PTases or 43 µl of the purified At5PTases were incubated for 30 min at 22ºC with 200 µM cold substrates Ins(1,4,5)P_3 (Sigma-Aldrich) and Ins(1,3,4,5)P_4 (Echelon)in a reaction buffer containing 50 mM Tris-HCl, pH 7.0, 0.25% β-D-

octylglucoside, and 1 mM PMSF. For the analysis of lipid substrate hydrolysis, unlabeled PtdIns(4,5)P_2 (American Radiolabeled Chemicals) was first dissolved in reaction buffer with the aid of sonication before addition of the enzyme. The amount of inorganic phosphate released was measured using a malachite green assay (Lanzetta et al., 1979). The malachite green solution (3:1;0.045% malachite green oxalate:4.2% ammonium molybdate in 4 N HCl) was prepared 20 min prior to usage for maximum stability and 800 µl was added into 50 µl reaction mixtures. To stabilize the phosphate in the reaction, 100 µl 34% sodium citrate was added immediately. The color was developed for 20 min and absorbance at 660 nm was compared to a standard curve.

Results

Cloning of a Group of Arabidopsis 5PTases

In *Arabidopsis*, 15 putative 5PTases were previously identified (Berdy et al., 2001). In order to understand the roles of these 5PTases in signaling, characterization of their substrate selectivity is crucial. At5PTase1 and At5PTase2 have been shown to hydrolyze Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Berdy et al., 2001; Sanchez and Chua, 2001). I have shown that At5PTase11 hydrolyzes PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2 but not Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 indicating that *Arabidopsis* 5PTases have distinct substrate selectivities (Ercetin and Gillaspy, 2004). More recently, substrate selectivities of the WD40-repeat containing 5PTases have been reported (Zhong et al., 2004; Zhong and Ye, 2004).

In order to extend the knowledge on the substrate selectivity of the 5PTases, I generated cDNAs for At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7, At5PTase9 and At5PTase13 through RT-PCR. Seedling mRNA was used as a template in the RT reactions. Gene-specific primers for each 5PTase gene that span the entire coding region from start to stop codon were used to generate cDNAs (Table 1). The stop codons were excluded from the C-terminal primer sequences in order to generate in-frame fusions to a C-terminal V5-His tag. Moreover, for efficient translation initiation, a Kozak sequence was also introduced into the N-terminal gene-specific primers. The sizes of the cDNA fragments that were amplified using gene-specific primers are as follows: 1770 bp

Table 1. Predicted and Apparent Molecular Masses (MM) of At5PTases and Oligonucleotide Sequences.

At5PTase	Gene No.	Oligonucleotide Sequences Used for Cloning	Predicted MM (kDa)	Apparent MM (kDa)
1	At1g34120	At5PTase1-Nterm: 5'GTACGCTCGAGCATGGCGGAAGTACGATCACGA3' At5PTase1-Cterm: 5'CATCGGGGATCCTCAGGCGTCAAGGCCTTGAAT3'	64.9	94.5
2	At4g18010	At5PTase2-Nterm: 5'GAGATGGAAACAAGACGTGGGAAACGTC3' At5PTase2-Cterm: 5'TCCAAAAGAAGGTTCAGGATGAAC3'	71.0	97.3
3	At1g71710	At5PTase3-Nterm: 5'GCCATGGCTCCAGTTGAACCCGCCG3' At5PTase3-Cterm: 5'AACGAGAACGGCTACAAGTCCCTC3'	73.0	100.2
4	At3g63240	At5PTase4-Nterm: 5'GCCATGGGTGATGGTAACCTCAAG3' At5PTase4-Cterm: 5'GTACGGGTTAAGCTCAGAATATC3'	63.1	80.6
7	At2g32010	At5PTase7-Nterm: 5'GCCATGGTGGTGATTCTTGAGAAC3' At5PTase7-Cterm: 5'GAAAAATGTTAGCTCGGTGTATC3'	55.1	80
9	At2g01900	At5PTase9-Nterm: 5'GCCATGGGGCCAAGACTTGTG3' At5PTase9-Cterm: 5'TGTAGATATCCACGAGTAGTC3'	45.9	63.8
13	At1g05630	At5PTase13-Nterm: 5'GCCATGGATTCGCTAATTATC3' At5PTase13-Cterm: 5'CCGGCTTTTACCTCGTCT3'	124.9	133

(At5PTase1), 1938 bp (At5PTase2), 1992 bp (At5PTase3), 1722 bp (At5PTase4), 1503 bp (At5PTase7), 1251 bp (At5PTase9) and 3408 bp (At5PTase13). The resulting fragments were gel-purified and cloned into the pMT/V5-His-TOPO vector to express recombinant 5PTases in S2 cells. Since this set of genes was amplifiable by RT-PCR, I concluded that these 5PTases are expressed in *Arabidopsis*.

Expression and Immunoprecipitation of Recombinant 5PTases in Drosophila S2 Cells

To obtain protein for 5PTase activity assays, the At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7, At5PTase9, At5PTase13 genes were expressed as a C-terminal V5-His fusion under control of the insect metallothionine promoter (pMT) in *Drosophila melanogaster* S2 tissue culture cells (S2 cells). I chose a eukaryotic expression system because previous efforts to obtain active, recombinant At5PTase1 and At5PTase11 in prokaryotic expression systems had failed, suggesting that a eukaryotic modification may be necessary for At5PTase activity. The pMT expression constructs (pMTAt5PTase1, pMTAt5PTase2, pMTAt5PTase3, pMTAt5PTase4, pMTAt5PTase7, pMTAt5PTase9, pMTAt5PTase13) also contain a C-terminal V5 epitope tag which was utilized for immunoprecipitation. S2 cells were transfected with either 2 µg of pMTAt5PTase DNA, or no DNA (i.e. mock transfection) using an Effectene transfection kit (Qiagen). After the transfection, I added $CuSO_4$ to induce expression of the recombinant proteins. Cells were harvested after 2 d and protein extracts were made.

Soluble portions of the S2 cell extracts transfected with the pMTAt5PTase constructs were analyzed by western blotting using anti-V5 antibody (Figure 1). Analysis of pMTAt5PTase1 transfected S2 cell extracts revealed cross-reactivity with a recombinant protein that migrated with an apparent molecular mass of approximately 95 kDa (Figure1, lane 1). At5PTase1 is predicted to encode a 64.9 kDaprotein (Table 1) and the addition of the C-terminal V5 tag is expected to increase the molecular mass. A similar increase in molecular mass was observed for At5PTase11 with the addition of the C-terminal V5 tag [Chapter 2, Figure 2, (Ercetin and Gillaspy, 2004)]. Minor bands pertaining to smaller proteins seen in these extracts are most likely due to breakdown products of At5PTase1, which retain the V5 epitope (Figure 1, lane 1). These smaller size proteins can not be premature translation products because the V5 tag is at the C-terminus. Lane 2 of Figure 1 contains S2 cell extract transfected with pMTAt5PTase2, a recombinant protein that migrated with an apparent molecular mass of approximately 97 kD. At5PTase2 is predicted to encode a 71 kDaprotein (Table 1), and the increase in molecular mass of At5PTase2 is similar to the increase in molecular mass of At5PTase1. At5PTase3 is predicted to have a molecular mass of 73 kDa(Table 1). S2 cell extract transfected with pMTAt5PTase3 results in a recombinant protein that migrated with an apparent molecular mass of 100.2 kDa(Figure 1, lane 3). This increase in molecular mass of At5PTase3 correlates with the increases in molecular masses seen in At5PTase2 and At5PTase3. Smaller size bands which are most likely due to breakdown products of At5PTase3 were also detected (Figure 1, lane 3). Differences in the number and intensity of these smaller bands indicate that At5PTase1 and At5PTase3 are more susceptible to

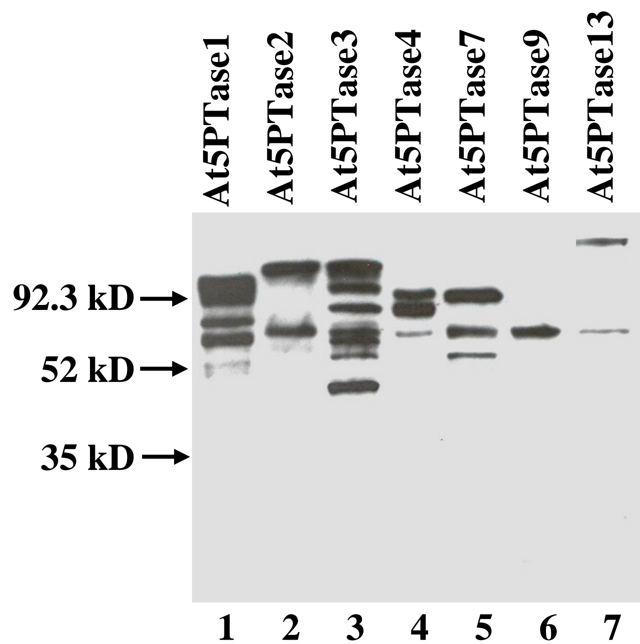

Figure 1. Expression of Recombinant At5PTases in *Drosophila* S2 Cells. The pMTAt5PTase constructs were transfected into S2 cells, and soluble extracts were prepared and analyzed by western blotting with the monoclonal anti-V5 antibody as described in "Materials and Methods." Lane 1: pMTAt5PTase1. Lane 2: pMTAt5PTase2. Lane 3: pMTAt5PTase3. Lane 4: pMTAt5PTase4. Lane 5: pMTAt5PTase7. Lane 6: pMTAt5PTase9. Lane 7: pMTAt5PTase13. Arrows on the left indicate the sizes of molecular mass markers (BioRad).

breakdown when expressed in S2 cells (Figure 1, compare lanes 1 and 3 to lanes 2, 4, 5 and 6).

Analysis of the S2 cell extract transfected with pMTAt5PTase4 revealed cross-reactivity with a recombinant protein that migrated with an apparent molecular mass of ~81 kDa (Figure1, lane 4). Addition of the C-terminal V5 tag is most likely the reason for increase in the molecular mass of At5PTase4 which is predicted to be 63.1 without the tag (Table1). When S2 cell extract transfected with pMTAt5PTase7 was analyzed, a recombinant protein with an apparent molecular mass of 80 kDawas detected (Figure 1, lane 5). Although, the predicted molecular mass of At5PTase7 is 55.1 kDa(Table 1), addition of the C-terminal V5 tag is most likely the reason for the increase in the molecular mass. Similarly, analyses of the S2 cell extract transfected with the pMTAt5PTase9 revealed cross-reactivity with a recombinant protein that migrated with an apparent molecular mass of 64 kDa (Figure 1, lane 6). This is higher than the predicted molecular mass of At5PTase9 which is 45.9 kDa (Table 1) and is most likely due to the addition of the C-terminal V5 tag. When S2 cell extract transfected with pMTAt5PTase13 was analyzed, a recombinant protein that migrated with an apparent molecular mass of 133 kDawas detected. A smaller size band which is most likely a breakdown product of At5PTase13 was also detected. The intensity of the band corresponding to recombinant At5PTase13 is relatively low compared to other At5PTases indicating that expression level of At5PTase13 was relatively low. Together, these results indicate that recombinant At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7, At5PTase9 and At5PTase13 can be expressed in S2 cells.

To isolate the recombinant At5PTase proteins, I applied the same strategy using an anti-V5 antibody to immunoprecipitate At5PTase proteins as used previously in studies on At5PTase11 (Ercetin and Gillaspy, 2004). At5PTases produced in S2 cells were immunoprecipitated using the anti-V5 antibody coupled to Protein A Sepharose beads and the resulting complexes were analyzed with western blotting using anti-V5 antibody (Figure 2). As expected, immunoprecipitated complexes from mock transfection (Figure 2, lane 9) or transfections with pMTAt5PTases (Figure 2, lanes 1-7) revealed the presence of the immunoglobulin heavy chain (IgG hc) from the anti-V5 antibody. Analysis of the At5PTase1 immunoprecipitation (Figure 2, lane 1) revealed that a recombinant band corresponding to the size of recombinant At5PTase1 was detected in addition to the band corresponding to immunoglobulin heavy chain (IgG hc). The putative breakdown products in the At5PTase1 immunoprecipitation correspond to the same extra bands in crude extracts (Figure 1). When At5PTase2 immunoprecipitation was analyzed (Figure 2, lane 2), a slightly higher molecular mass protein compared to the At5PTase1 immunoprecipitation was detected and its size corresponds to the recombinant At5PTase2. Similarly, At5PTase3 immunoprecipitation contained a recombinant protein that corresponds to the size of recombinant At5PTase3 (Figure2, lane 3). In addition, analysis of At5PTase4 immunoprecipitation demonstrated the presence of a recombinant protein corresponding to the size of At5PTase4 (Figure 2, lane 4). Moreover, examination of the At5PTase7 immunoprecipitation indicates that a recombinant protein corresponding to the size of recombinant At5PTase7 is present (Figure 2, lane 5). When, At5PTase9 immunoprecipitation was analyzed, only putative breakdown products were

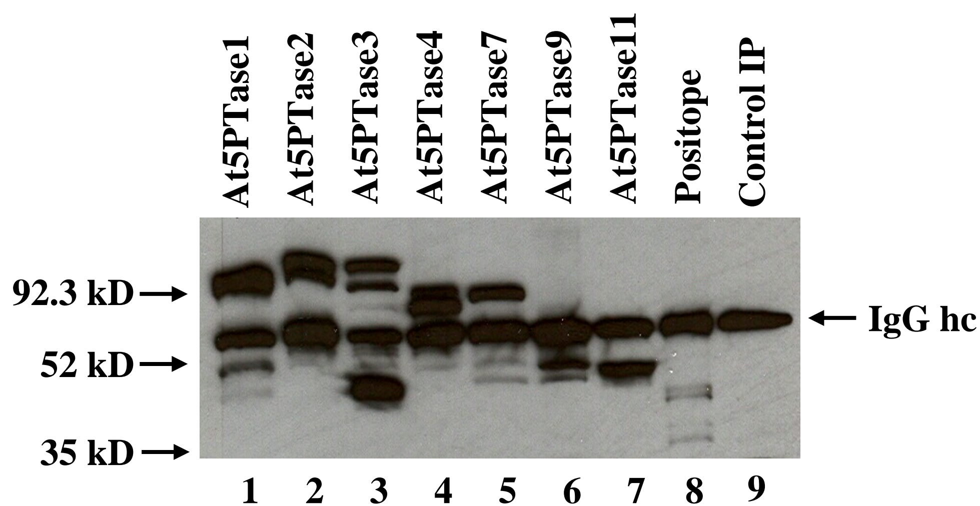

Figure 2. Immunoprecipitation of Recombinant At5PTases in *Drosophila* S2 Cells. The pMTAt5PTase constructs were transfected into S2 cells, and soluble extracts were prepared and immunoprecipitated with a monoclonal anti-V5 antibody as described in "Materials and Methods." Lane 1: At5PTase1-IP. Lane 2: At5PTase2-IP. Lane 3: At5PTase3-IP. Lane 4: At5PTase4-IP. Lane 5: At5PTase7-IP. Lane 6: At5PTase9-IP. Lane 7: At5PTase11-IP. Lane 8 contains 50 ng of purified Positope (Invitrogen) protein and was used to estimate the amount of recombinant At5PTases immunoprecipitated. Lane 9: IP from mock-transfected S2 cells. Arrows on the left indicate the sizes of molecular mass markers; arrow on the right indicates the position the immunoglobulin heavy chain (IgG hc).

detected in addition to the IgG hc. The At5PTase9 band may be obscured because recombinant At5PTase9 and IgG hc migrate similarly. Since the same amount of IgG hc was used for each immunoprecipitation, comparison of the intensities of the IgG hc bands in the At5PTase11 immunoprecipitation (Figure2, lane 7), the control immunoprecipitation from mock-transfected S2 cells (Figure 2, lane 9) and the At5PTase9 immunoprecipitation (Figure2, lane 6) indicates that in addition to IgG hc, there is another recombinant protein that migrates at the same position. I have also included At5PTase11 as a control and 50 ng of purified Positope (Invitrogen) protein to estimate the amount of recombinant At5PTases immunoprecipitated (Figure 2, lanes 7 and 8). Because of the low expression levels and difficulties in immunoprecipitation of recombinant At5PTase13, I did not pursue this protein. Based on the results of immunoprecipitation experiments, I concluded that At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7 and At5PTase9 can be immunoprecipitated using an anti-V5 antibody coupled to Protein A Sepharose beads.

Analysis of Enzyme Activities of At5PTases with Fluorescent PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3

To examine whether recombinant At5PTases produced in S2 cells are active enzymes, I assayed for 5PTase activity directly after immunoprecipitation. The same method was used to characterize S2 cells transfected with a pMTAt5PTase11 construct, which was shown to produce active At5PTases11 enzyme with a phospholipid-specific substrate selectivity [Chapter 2, (Ercetin and Gillaspy, 2004)]. Recombinant At5PTases

immunoprecipitated from S2 cells were tested for their activity on fluorescent lipid substrates PtdIns(4,5)P_2 (di-C_6-NBD6-phosphatidylinositol 4,5-bisphosphate) and PtdIns(3,4,5)P_3 (di-C_6-NBD6-phosphatidylinositol 3,4,5-trisphosphate). A representative TLC plate showing the activities of various At5PTases with fluorescent PtdIns(4,5)P_2 is presented in Figure 3. I had good separation of PtdIns(4)P (Figure 3, lane 1) and PtdIns(4,5)P_2 (Figure 3, lane 2). Reaction products from assays containing immunoprecipitated recombinant At5PTases (Figure 3, lanes 3-9) were compared to the control immunoprecipitate (Figure 3, lane 10). As expected, when At5PTase11 immunoprecipitate is incubated with PtdIns(4,5)P_2, conversion to product migrating at the same position as PtdIns(4)P is observed (Figure 3, lane 3). Formation of PtdIns(4)P is also observed in reactions containing recombinant At5PTase1 (lane 4), At5PTase2 (lane 5), At5PTase3 (lane 6), At5PTase4 (lane 7), At5PTase7 (lane 8), and At5PTase9 (Figure 3, lane 9). I concluded from these experiments that At5PTase1, At5PTase2, At5PTase3, At5PTase4, At5PTase7, and At5PTase9 can dephosphorylate PtdIns(4,5)P_2.

PtdIns(3,4,5)P_3 is another substrate which can be hydrolyzed by 5PTases. Previously I have shown that At5PTase11 can hydrolyze this substrate (Ercetin and Gillaspy, 2004). Thus, I have tested whether other 5PTases can also hydrolyze this substrate. Immunoprecipitated 5PTases were incubated with fluorescent PtdIns(3,4,5)P_3 and reaction products were separated by TLC. A representative TLC plate showing the activities of various At5PTases on fluorescent PtdIns(3,4,5)P_3 is presented in Figure 4. Migration of the PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 standards were indicated by left arrows (Figure 4, lanes 1 and 2). At5PTase1, At5PTase2, At5PTase3, At5PTase7, and

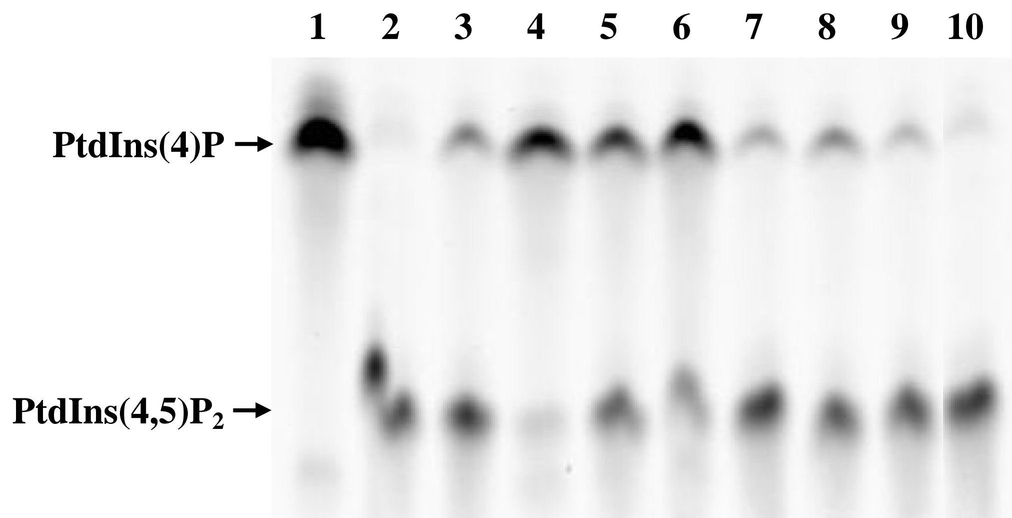

Figure 3. Activity of At5PTases with PtdIns(4,5)P_2. Phosphatase reactions (Lanes 3 to 10) containing 1.5 μg of the fluorescent PtdIns(4,5)P_2 substrate and 15-80 ng of recombinant At5PTases were incubated 1 hour at room temperature. Lane 1: PtdIns(4)P substrate incubated with reaction buffer only. Lane 2: PtdIns(4,5)P_2 substrate incubated with reaction buffer only. Other lanes contain reactions with the following IPs: Lane 3: At5PTase11- IP. Lane 4: At5PTase1-IP. Lane 5: At5PTase2-IP. Lane 6: At5PTase3-IP. Lane 7: At5PTase4-IP. Lane 8: At5PTase7-IP. Lane 9: At5PTase9-IP. Lane 10: Control IP from mock-transfected S2 cells. The migration of fluorescent standards is indicated. Experiments were repeated at least three times.

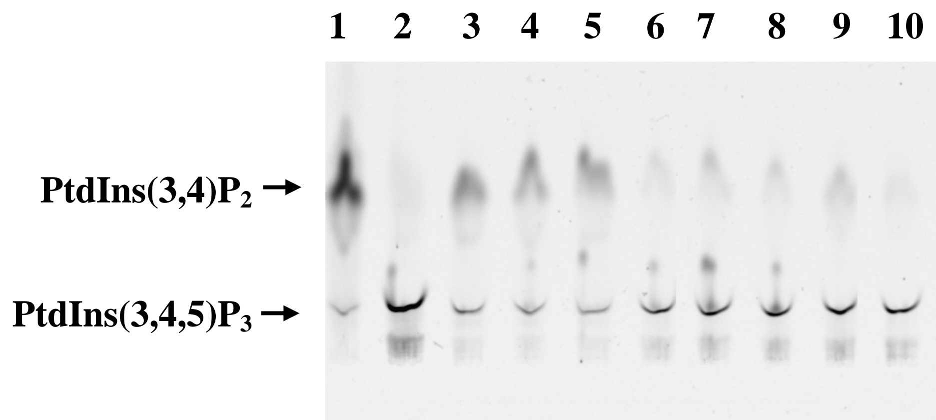

Figure 4. Activity of At5PTases with PtdIns(3,4,5)P_3. Phosphatase reactions (Lanes 3 to 10) containing 1.5 μg of the fluorescent PtdIns(3,4,5)P_3 substrate and 15-80 ng of recombinant At5PTases were incubated 1 hour at room temperature. Lane 1: PtdIns(3,4)P_2 substrate incubated with reaction buffer only. Lane 2: PtdIns(3,4,5)P_3 substrate incubated with reaction buffer only. Other lanes contain reactions with following IPs. Lane 3: At5PTase1- IP. Lane 4: At5PTase2-IP. Lane 5: At5PTase3-IP. Lane 6: At5PTase4-IP. Lane 7: At5PTase7-IP. Lane 8: At5PTase9-IP. Lane 9: At5PTase11-IP. Lane 10: Control IP from mock-transfected S2 cells. The migration of fluorescent standards is indicated. Experiments were repeated two times.

At5PTase11 enzymes resulted in hydrolysis of PtdIns(3,4,5)P_3 when compared to a control reaction where an immunoprecipitate from mock-transfected S2 cells was used (Figure 4). At5PTase4 and At5PTase9 activity were similar to background activity (Figure 4, compare lanes 6, 8 and 10). I concluded from these experiments that At5PTase1, At5PTase2, At5PTase3, At5PTase7 and At5PTase11 can dephosphorylate PtdIns(3,4,5)P_3.

Comparison of Enzyme Activities of At5PTase1, At5PTase2 and At5PTase3 with Soluble and Lipid Substrates

The activity assay results utilizing the fluorescent substrates PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 have shown that At5PTase1, At5PTase2 and At5PTase3 were the most robust enzymes. To determine whether these enzymes have differences in their activities toward four possible substrates, immunoprecipitated At5PTases were incubated with [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-Ins(1,3,4,5)P_4 and fluorescent PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. The activity of each 5PTase is compared based on the amount of product formation per time per microgram protein. The results are summarized in Figure 5 and 6. The activity of At5PTase1 and At5PTase2 towards PtdIns(4,5)P_2 is similar but less than the activity of At5PTase3 (Figure 5). In contrast, At5PTase1, At5PTase2 and At5PTase3 have similar activities towards PtdIns(3,4,5)P_3 (Figure 5).

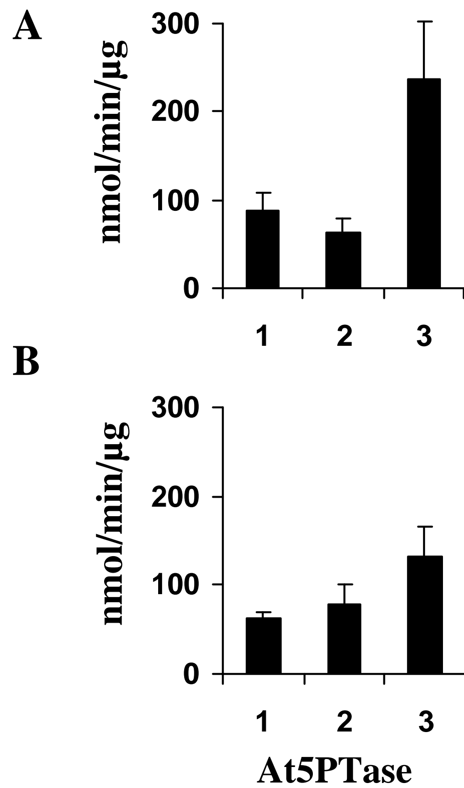

Figure 5. Comparison of Activities of At5PTase1, At5PTase2 and At5PTase3 with PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. 15 – 55 ng of immunoprecipitated At5PTase1, At5PTase2 and At5PTase3 were incubated with 1.5 µg fluorescent PtdIns(4,5)P_2 (A) or PtdIns(3,4,5)P_3 (B). Enzyme activities expressed as nanomoles product formed per min per microgram of protein. The mean and standard error for three experiments are presented.

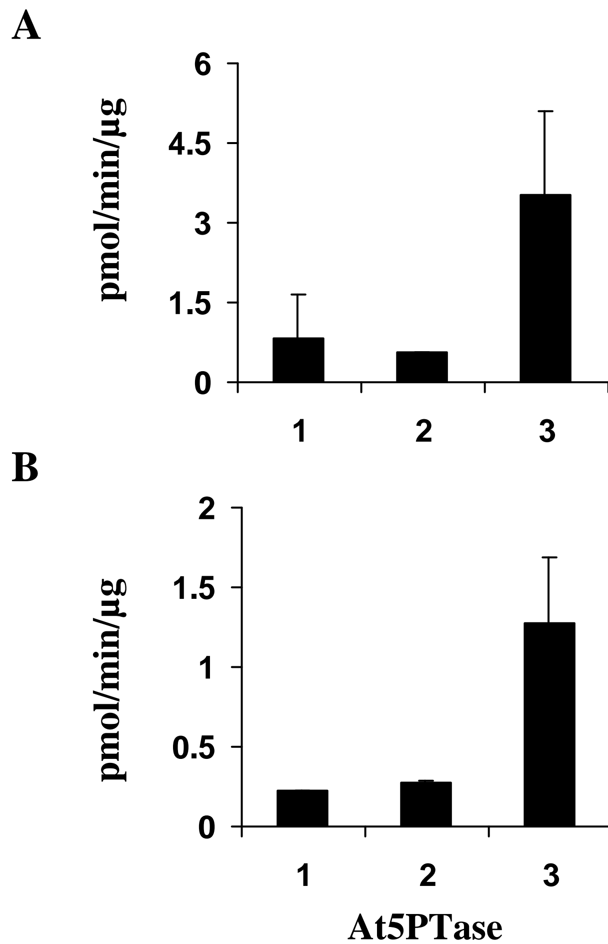

Figure 6. Comparison of Activities of At5PTase1, At5PTase2 and At5PTase3 with [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-Ins(1,3,4,5)P_4. Top panel (A) shows the results of activity assays with Ins(1,4,5)P_3 and bottom panel (B) shows the results of activity assays with Ins(1,3,4,5)P_4. 85 – 200 ng of immunoprecipitated At5PTase1, At5PTase2 and At5PTase3 were incubated with [^{3}H]-labeled substrates. Enzyme activity is expressed as picomoles product formed per min per microgram of protein. The mean and standard error for two independent experiments are presented.

Analysis of activities of At5PTase1, At5PTase2, and At5PTase3 with $[^3H]$-Ins(1,4,5)P_3 and $[^3H]$-Ins(1,3,4,5)P_4 revealed that the activity of At5PTase1 and At5PTase2 is similar with both substrates (Figure 6). Moreover, the activity of At5PTase3 is higher than At5PTase1 and At5PTase2 for both substrates (Figure 6). At5PTase4, At5PTase7 and At5PTase9 did not show detectable activity with soluble substrates (data not shown). Since the assay conditions for fluorescent and radiolabeled substrates were different, it is not appropriate to make direct comparison of activities towards lipid and soluble substrates.

Expression of At5PTases in Yeast

For the analysis of At5PTase activities, as described earlier, I have used immunoprecipitated recombinant proteins. For some of the 5PTases like At5PTase13, yield was very low. To determine the kinetic parameters for At5PTases, I decided to purify recombinant 5PTases. Thus, I switched to another system where active At5PTases can be produced in large quantities and be purified via metal affinity chromatography. The yeast system was my choice because Zhong et al., (2004) reported that the WD-containing *Arabidopsis* 5PTases can be expressed in yeast, and purified with activity intact (Zhong et al., 2004). To clone At5PTase genes into the pYes2/CT yeast expression vector (Invitrogen), pMTAt5PTase constructs were digested with *Pme*I and *Kpn*I. The resulting fragments (At5PTase-V5-his) were ligated into the pYes2/CT vector pre-digested with *Pme*I and *Kpn*I. Hence, pYes2/CT constructs are also expected to encode recombinant 5PTases with the C-terminal V5 tag. I was able to clone At5PTase1, 2, 4, 6,

7, 9, 11 genes into the pYes2/CT vector successfully. Despite several attempts, the At5PTase3 and 13 fragments could not be cloned into the pYes2/CT vector. The resulting pYes2/CTAt5PTase constructs were transformed into yeast strain InvSc1. The transformed yeast cells were grown to an OD_{600} 0.4 and galactose was added to induce expression of the recombinant At5PTases. Yeast cells were harvested and broken by glass beads after 24 h of induction. Soluble extracts were prepared and analyzed for the expression of the recombinant proteins via western blot using an anti-V5 monoclonal antibody (Figure 7). All constructs resulted in expression of the similar size recombinant proteins as compared to S2 cells (compare Figures 1 and 7). Results of immunoprecipitation of the recombinant proteins from soluble yeast extracts using an Anti-V5 antibody coupled to Protein A Sepharose beads are shown in Figure 8. As expected IgG hc was detected in all lanes containing immunoprecipitations (Figure 8, lanes 2-8). In addition to IgG hc, recombinant proteins corresponding to the At5PTase1 (lane 2), At5PTase2 (lane 3), At5PTase4 (lane 4), At5PTase6 (lane 5), At5PTase7 (lane 6), At5PTase9 (lane 7), At5PTase11 (lane 8, Figure 8) were detected. The amount of immunoprecipitated At5PTase4 and 6 was relatively lower than the At5PTase1, 2, 7 and 11 (Figure 8, compare lanes 4 and 5 to lanes 2, 3, 6, and 8). In lanes 2 and 3 of Figure 8, smaller size bands were also detected. These are most likely the breakdown products of At5PTase1 and At5PTase2 (Figure 8, lanes 2 and 3). Together, I concluded from these experiments that recombinant At5PTase1, 2, 4, 6, 7, 9, 11 with a C-terminal V5-his tag can be produced in yeast and immunoprecipitated using an anti-V5 antibody.

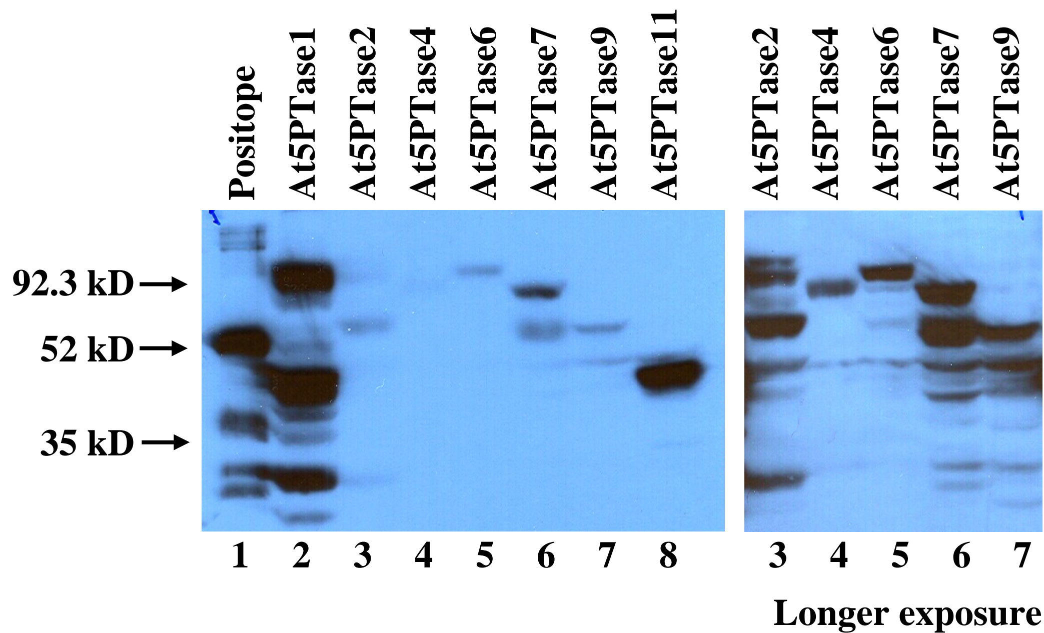

Figure 7. Expression of At5PTases in Yeast. At5PTase1, At5PTase2, At5PTase4, At5PTase6, At5PTase7, At5PTase9 and At5PTase11 were cloned into pYES2/CT vector and transformed into the yeast strain InvSc1. Soluble extracts of galactose-induced cultures were prepared and analyzed for the expression of the recombinant proteins via western blot using an anti-V5 monoclonal antibody. Lane 1, Positope; lane 2, At5PTase1; lane 3, At5PTase2; lane 4, At5PTase4; lane 5, At5PTase6; lane 6, At5PTase7; lane 7, At5PTase9; lane 8, At5PTase11. Longer exposure for lanes 3-7 is also shown on the right panel. Arrows on the left indicate the sizes of molecular mass markers (BioRad).

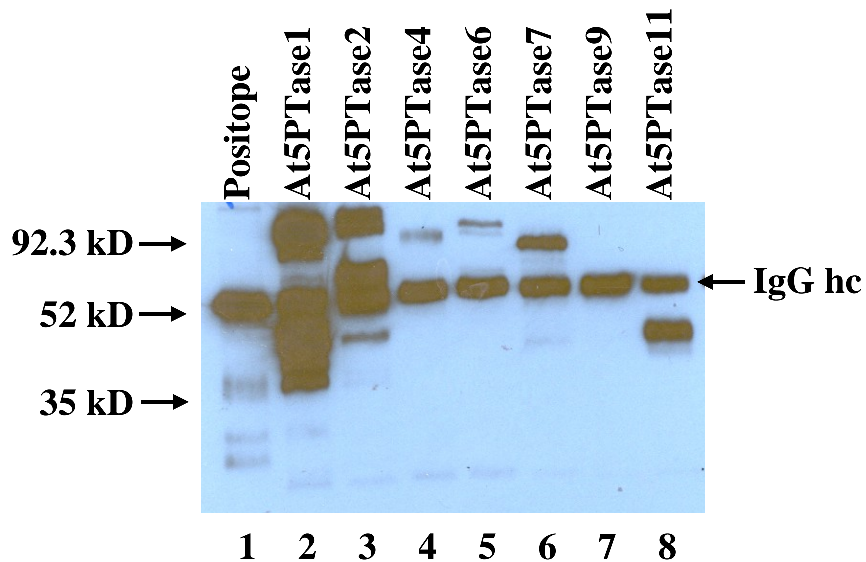

Figure 8. Immunoprecipitation of Recombinant At5PTases in Yeast. Soluble extracts were prepared from yeast cells expressing At5PTases and recombinant proteins were immunoprecipitated with a monoclonal anti-V5 antibody as described in “Materials and Methods.” Lane 1, Positope; lane 2, At5PTase1-IP; lane 3, At5PTase2-IP; lane 4, At5PTase4-IP; lane 5, At5PTase6-IP; lane 6, At5PTase7-IP; lane 7, At5PTase9-IP; lane 8, At5PTase11-IP. Arrows on the left indicate the sizes of molecular mass markers.

Testing the Activities of Immunoprecipitated Recombinant At5PTases from Yeast with Cold Ins(1,4,5)P_3

To examine whether recombinant At5PTases produced in yeast cells are active enzymes, I assayed for 5PTase activity directly after immunoprecipitation. Previously it was shown that recombinant 5PTases can be produced in yeast and 5PTase activity can be measured by using cold substrates and malachite green assays (Zhong et al., 2004; Zhong and Ye, 2004). Thus, I have tested the activities of At5PTase1 and At5PTase2 with cold Ins(1,4,5)P_3 since these At5PTases, when produced in S2 cells, were shown to be active on Ins(1,4,5)P_3 substrate. Immunoprecipitated At5PTase1 and At5PTase2 were incubated with 200 μM Ins(1,4,5)P_3 for 30 min at 22ºC and the amount of inorganic phosphate released was measured using the malachite green assay. Immunoprecipitated recombinant β-galactosidase was used as a control in the assays showing background activity (Figure 9, lane 3). In contrast, At5PTase1 and At5PTase2 resulted in the hydrolysis of 2.6 and 1.5 nmol Ins(1,4,5)P_3 respectively (Figure 9, lanes 1 and 2). These results indicated that the immunoprecipitated recombinant At5PTases from yeast cells are active enzymes. In addition, when immunoprecipitated recombinant 5PTases produced from yeast cells were used in activity assays using radiolabeled Ins(1,4,5)P_3, they demonstrated similar activities as compared to results obtained with recombinant proteins produced from S2 cells (data not shown).

To determine whether activities of recombinant At5PTases can be measured using cold PtdIns(4,5)P_2 and the malachite green assays, I have tested the activity of At5PTase1,

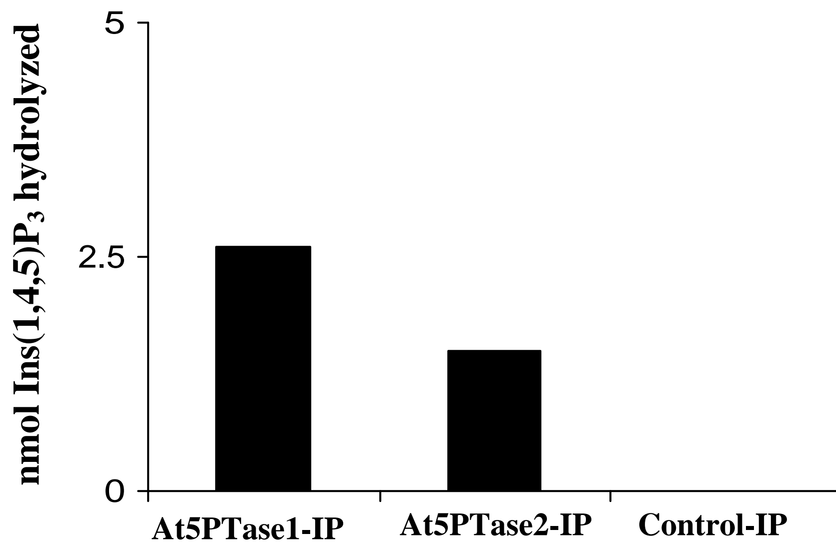

Figure 9. Hydrolysis of Cold Ins(1,4,5)P_3 by Immunoprecipitated At5PTase1 and At5PTase2. At5PTase1 (At5PTase1-IP), At5PTase2 (At5PTase2-IP) and β-galactosidase (Control-IP) were immunoprecipitated from yeast cells containing recombinant proteins by antiV5-antibody coupled to Protein A Sepharose beads. Activity assays were performed with cold Ins(1,4,5)P_3 and the amount of phosphate released was measured by malachite green assays.

At5PTase2 and At5PTase11 with cold PtdIns(4,5)P_2 substrate. Immunoprecipitated recombinant At5PTases were incubated with 200 μM cold PtdIns(4,5)P_2 substrate for 30 min at 22°C. The amount of inorganic phosphate released was quantified using the malachite green assay. Immunoprecipitated recombinant β-galactosidase was used as a negative control and recombinant At5PTase11 served as a positive control. At5PTase1, and At5PTase2 and At5PTase11 did not result in hydrolysis of cold PtdIns(4,5)P_2 compared to background. Since At5PTase11 was shown to hydrolyze PtdIns(4,5)P_2 in S2 cells (Ercetin and Gillaspy, 2004), I concluded that, coupling recombinant At5PTases to anti-V5-Protein A Sepharose beads might interfere with 5PTase activity with cold PtdIns(4,5)P_2 (data not shown).

Purification of Recombinant At5PTases from Yeast Using Metal Affinity Chromatography

Activity assay results of immunoprecipitated recombinant At5PTases with cold PtdIns(4,5)P_2 suggested that coupling of recombinant At5PTases to anti-V5-Protein A Sepharose beads might interfere with the activity assays using cold PtdIns(4,5)P_2. In order to address this, I decided to purify At5PTases from yeast cells transfected with pYes2/CTAt5PTase constructs utilizing Talon resin which uses cobalt ions for purification of His-tagged proteins (BD Biosciences, Franklin Lakes, NJ). At5PTase1, At5PTase2 and At5PTase11 were purified as described in “Materials and Methods.” Recombinant β-galactosidase was purified as a control and to determine the contaminant protein bands that copurify with the recombinant At5PTases, the same purification

procedure was applied to untransformed yeast cells. Equal amount of cells were used as a starting material for each At5PTase and control samples. Analysis of the purified recombinant proteins by western blot indicated the presence of recombinant At5PTase1 (lane 2), At5PTase2 (lane 3), At5PTase11 (Figure 10, lane 4). However analysis of samples by Coomassie staining after separation on a SDS-PAGE gel has shown that the samples contain many contaminanting protein bands (Figure 10).

Although recombinant 5PTases were not in pure form, based on the western blot analysis recombinant At5PTases were present in the samples (Figure 10). I decided to use these enriched protein extracts in activity assays with the appropriate controls, β-galactosidase and the purified samples from untransformed yeast cells. Equal amounts of the enriched protein extracts containing 50-100 ng of recombinant proteins were used in activity assays with 200 μM cold substrates Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2. The amount of inorganic phosphate released was quantified by malachite green assay. The background activity was very similar to activity that was seen with At5PTase1 in all three substrates (compare activities of At5PTase1 and InvSc1, Figure 11). This indicates that the activity that was seen with At5PTase1, At5PTase2 and At5PTase11 cannot be distinguished from control background activity. As a positive control, I used a bacterially purified recombinant 5PTase synaptojanin which has demonstrated activity with all three substrates [(Synaptojanin, Figure 11), (Tsujishita et al., 2001)]. I concluded from these experiments that purification of recombinant At5PTases from yeast needs improvement to eliminate the background activity.

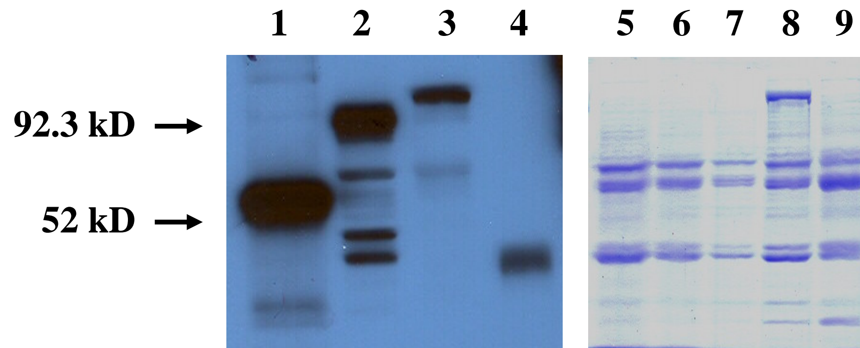

Figure 10. Western Blot Analysis of the Recombinant At5PTases after Purification by Talon Resin. At5PTase1 (Lane 2, 5), At5PTase2 (Lane 3, 6), At5PTase11 (Lane 4, 7) β-galactosidase (Lane 8), and control sample from untransformed yeast cells (Lane 9) were purified using Talon resin as described in "Materials and Methods." A small portion of each sample was analyzed by western blotting using anti-V5 antibody. Positope (50 ng) was included as a control (Lane 1). Right panel shows the Coomassie staining of the samples.

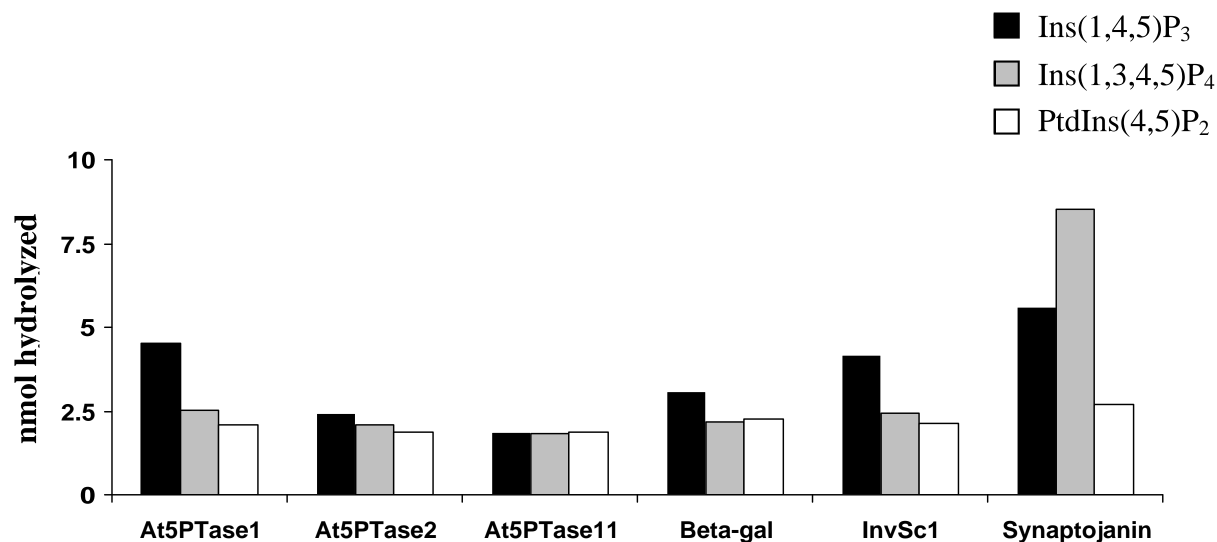

Figure 11. Testing Activity of At5PTase Enriched Protein Preparations. Proteins samples for At5PTase1 (200 ng), At5PTase2 (50 ng), At5PTase11 (40 ng), β-galactosidase (Beta-gal), untransformed yeast cells (InvSc1) were prepared and incubated with cold substrates Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2 as described in "Materials and Methods." Amount of substrate hydrolyzed was calculated by measuring the amount of phosphate released via malachite green assay. Activity assay results with Ins(1,4,5)P_3 (black bars), Ins(1,3,4,5)P_4 (gray bars), PtdIns(4,5)P_2 (white bars) were presented as nanomoles substrate hydrolyzed. Purified synaptojanin was included as positive control.

Discussion

I have previously characterized the substrate selectivity of At5PTase11 showing that it can hydrolyze PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Ercetin and Gillaspy, 2004). In this study, using the same approach, a group of 5PTases was analyzed to characterize their substrate selectivity. At5PTase1, At5PTase2, At5PTase3 enzymes are shown to hydrolyze both the soluble substrates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, and fluorescent lipid substrates PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. In contrast, At5PTase4, At5PTase7, and At5PTase9 hydrolyze PtdIns(4,5)P_2. Moreover, PtdIns(3,4,5)P_3 is hydrolyzed by At5PTase1, At5PTase2, At5PTase3 and At5PTase7. These results suggest that At5PTase1, At5PTase2 and At5PTase3 enzymes have similar substrate selectivity and might have overlapping functions based on their substrate selectivity. This is consistent with the previous reports showing involvement of both At5PTase1 and At5PTase2 in the regulation of ABA signaling (Sanchez and Chua, 2001; Burnette et al., 2003). At5PTase3 has not been characterized in terms of function. However, based on the results of *in vitro* assays, At5PTase3 can be predicted to have overlapping functions with At5PTase1 and At5PTase2. In order to fully understand the functions of these 5PTases, analysis of the T-DNA insertion mutants is very important. Since these 5PTases might have overlapping functions based on substrate selectivity, generation of double and even triple mutants might be necessary to observe an obvious phenotype.

PtdIns(4,5,)P_2 is an important molecule which can be hydrolyzed by 5PTases. In addition to At5PTase1, At5PTase2, and At5PTase3, results have shown that At5PTase4,

At5PTase7, At5PTase9, and At5PTase11 can also hydrolyze PtdIns(4,5)P_2. At5PTase7 was also shown to hydrolyze PtdIns(3,4,5)P_3 indicating that At5PTase11 and At5PTase7 have similar substrate selectivity. However, At5PTase4 and At5PTase9 form a unique group that can only hydrolyze PtdIns(4,5)P_2. Based on these results there are multiple 5PTases with similar substrate selectivity. It can be speculated that these enzymes with similar substrate selectivity may have overlapping functions in *Arabidopsis*. This is supported by the findings that *Arabidopsis* 5PTases do not have tissue specific expression patterns (Berdy et al., 2001; Ercetin and Gillaspy, 2004). However, recent reports on other 5PTases such as At5PTase6 and FRA3 have shown that at different stages of development At5PTase expression might become abundant in certain cell types (Carland and Nelson, 2004; Zhong et al., 2004). Hence, more detailed analysis of 5PTase expression utilizing a β-glucuronidase (GUS) reporter gene might be necessary to identify cell types where certain 5PTase expression might be abundant. This may also help predict phenotypes of 5PTase mutants.

A recent study aimed at characterization of WD40-repeat containing 5PTases from *Arabidopsis* have shown that cold substrates can be used in phosphate release assays to measure 5PTase activity (Zhong and Ye, 2004). Unlike the assays utilizing radiolabeled or fluorescent substrates, the identity of the products formed can not be known directly using phosphate release assays However, the advantage of this approach is one can compare 5PTase activity with Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 under the same assay conditions where free inorganic phosphate released can be measured. I have coupled the immunoprecipitated 5PTases to a phosphate release

assay to compare activities of 5PTases with soluble and lipid substrates. At5PTase1 and At5PTase2 enzymes resulted in hydrolysis of cold Ins(1,4,5)P_3, which is consistent with the results of activity assays using radiolabeled Ins(1,4,5)P_3. However, immunoprecipitated At5PTase1, At5PTase2 and At5PTase11 did not hydrolyze cold PtdIns(4,5)P_2. This is not consistent with the previous finding utilizing fluorescent substrates that At5PTase1, At5PTase2 and At5PTase11 can hydrolyze PtdIns(4,5)P_2. It is possible that coupling recombinant 5PTase to antibody-Protein A Sepharose beads limits the enzyme access to lipid substrate. This is supported by the fact that fluorescent substrates are more soluble because of the shorter fatty acyl chain which may make them more accessible to enzyme. However, when cold substrate PtdIns(4,5)P_2 is used, it forms micelles in the presence of detergent that may restrict access to 5PTase enzyme when coupled to antibody-Protein A Sepharose.

Assay results using immunoprecipitated 5PTases suggest that coupling 5PTase enzyme to the antibody-Protein A Sepharose complex might generate a physical constraint for enzyme to access lipid substrates that are in the micelle form. To address this, I have tried to purify 5PTases produced using the yeast expression system. Initial attempts to purify At5PTase1 using Ni^+-agarose beads resulted in loss of activity (data not shown). Similar reduction of 5PTase activity was observed with other mammalian 5PTases such as OCRL when purified using Ni^+-agarose resin (personal communication, Marina Kisseleva ,Washington University School of Medicine, St. Louis). Talon resin which utilizes cobalt ions to purify His-tagged proteins was chosen. Analysis of purified At5PTase samples indicated the presence of many contaminant protein bands. Using these enriched protein

extracts in activity assays instead of immunoprecipitates should have eliminated the problem of having enzyme coupled to Protein A Sepharose beads. Preliminary experiments using the equal amounts of At5PTase1 protein extracts and control extracts in activity assays with cold Ins(1,4,5)P_3 and PtdIns(4,5)P_2 resulted in similar hydrolyses (Figure 12). These results suggest that At5PTases should be purified to homogeneity to determine the activity with cold lipid substrates.

Previously, both At5PTase1 and At5PTase2 have been reported to not hydrolyze PtdIns(4,5)P_2 (Berdy et al., 2001; Sanchez and Chua, 2001). However, studies with fluorescent PtdIns(4,5)P_2 have shown that At5PTase1 and At5PTase2 can hydrolyze this substrate (Figure3). It is important to note that these fluorescent substrates are more soluble than the lipid substrates and do not require sonication. This suggests that fluorescent PtdIns(4,5)P_2 might have easier access to the 5PTase catalytic pocket as compared to native PtdIns(4,5)P_2 because of the difference in solubility. In addition, these activity assays were performed *in vitro* so they may not necessarily represent the *in vivo* situation. In order to find out whether the *in vitro* data reflects the substrate selectivity *in vivo* labeling experiments with [^{3}H]-*myo*-inositol might be performed. Previously, At5PTase11 was shown to hydrolyze PtdIns(4,5)P_2 in *Drosophila* S2 cells (Ercetin and Gillaspy, 2004). Similarly, yeast cells transformed with At5PTase expression constructs can be labeled with [^{3}H]-*myo*-inositol to address the *in vivo* substrate selectivity of At5PTases.

CHAPTER V

Summary and Future Directions

Many important cellular events that are mediated by PI signaling pathway have been described and will be the focus of many research efforts in the near future. Although its components were first defined in animal models, with the sequencing and annotations of plant model organism genomes, *Arabidopsis* and *Oryza sativa*, research on PI signaling pathways is rapidly expanding.

The PI signaling pathway involves inositol-containing second messengers such as Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. The 5PTases are the enzymes which can hydrolyze the 5-position phosphate from the inositol ring of these second messengers. Thus, 5PTases have the potential to terminate signaling events mediated by inositol-containing second messengers. As it was described in Chapter II, At5PTase11 hydrolyzes only lipid substrates, PtdIns(4,5)$_2$, PtdIns(3,4,5)P_3, and PtdIns(3,5)P_2 indicating that At5PTase11 is a phospholipid-specific 5PTase. In addition, At5PTase11 is shown to be regulated by ABA, auxin, and jasmonic acid suggesting a role for PI action in these signaling pathways.

To further investigate the function of At5PTase11 in *Arabidopsis*, T-DNA insertion mutants were analyzed. Analysis of two independent *At5ptase11* mutant lines revealed that *At5ptase11* mutant seeds germinate slower compared to wild-type seeds. In addition, hypocotyl growth of *At5ptase11* mutant seedlings is slower compared to wild-type

seedlings when grown in the dark. These results indicate that loss-of At5PTase11 function altered seed germination and seedling growth in the dark. It was shown in Chapter II that At5PTase11 does not have a tissue specific expression pattern (Ercetin and Gillaspy, 2004). However, studies with other 5PTases, the Cvp2 and Fra3, have shown that expression of 5PTases might be abundant in certain cell types (Carland and Nelson, 2004; Zhong et al., 2004). The generation of transgenic plants carrying At5PTase11 promoter:GUS cassette may help identifying whether At5PTase11 expression is abundant in certain cell types. This may lead to identification of new functions for At5PTase11 in *Arabidopsis*.

The analysis of substrate selectivity of At5PTase1, At5PTase2 and At5PTase3 has revealed that these 5PTases can hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. In contrast, At5PTase4, At5PTase7, and At5PTase9 have shown to hydrolyze PtdIns(4,5)P_2. In addition, At5PTase7 were also shown to hydrolyze PtdIns(3,4,5)P_3. These results indicate that At5PTase1, At5PTase2, and At5PTase3 have distinct substrate selectivity with the ability to hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. These activity assays were performed *in vitro*, and fluorescent lipid substrates were used for the analysis of activity towards PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. In order to determine whether *in vitro* assay results reflects the substrate selectivity *in vivo*, yeast cells expressing recombinant At5PTases can be labeled with [^{3}H]-*myo*-inositol and resulting [^{3}H]-labeled PIs can be analyzed by HPLC.

The above studies on At5PTase11 and other 5PTases extended the knowledge on *Arabidopsis* 5PTases. Understanding the role of each 5PTase in *Arabidopsis* requires further research.

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