ABSTRACT

KEOGH, MATTHEW RYAN. New Insights into Phospholipid Metabolism and Signaling In Plants. (Under the direction of Ralph Earl Dewey.)

This dissertation is the compilation of three projects all relating to phospholipids in plants. The introduction contains a literature review giving a broad overview of the metabolism of phospholipids and phospholipid signaling in plants. Particular attention was given to phosphoinositide signaling and phosphatidylinositol transfer proteins.

Chapter 2 reports on the identification and molecular genetic characterization of the phosphatidylcholine (PtdCho) biosynthetic enzyme, phospholipid N-methyltransferase (PLMT) from Arabidopsis thaliana and soybean. In contrast to their homologs in mammals and yeast which are able to use phosphatidylethanolamine, phosphatidylmonomethylethanolamine (PtdMMEttn) and phosphatidyldimethylethanolamine (PtdDMEtn) as substrates, the plant PLMTs can only catalyze the latter two methylation reactions. This observation has important implications regarding the mechanisms by which plants synthesize PtdCho. A PLMT-null Arabidopsis mutant was found to have 9-fold and 3.5-fold increases in their PtdMMEttn and PtdDMEtn content, respectively. Despite this notable accumulation in PtdCho intermediates (which are normally found in plants in only trace amounts), the mutant failed to demonstrate any obvious growth phenotype and possessed normal levels of PtdCho. These data indicate that other routes of PtdCho metabolism are able to compensate for the loss of PLMT activity.

Chapter 3 presents the study of two closely related Sec14p-type phosphatidylinositol transfer proteins (PITPs) from Arabidopsis, designated AtSec14-1 and AtSec14-5. Despite the fact that genes encoding Sec14p-type PITPs are very prevalent in plant genomes, little is
known concerning the function of this enigmatic class of proteins in higher plants. A double mutant Arabidopsis plant lacking both AtSec14-1 and AtSec14-5 proteins was compromised in its ability to germinate under non-ideal growth conditions (such as hyperosmotic stress) and was also hypersensitive to the hormone abscisic acid (ABA) during this same stage of development. *AtSec14-1* and *AtSec14-5* single mutants germinated at the same frequency as wild-type for all conditions tested. These data suggest that AtSec14-1 and AtSec14-5 function redundantly in their roles in seed germination. Radiolabeling studies and mass spectrometric phospholipid analyses showed that phosphatidylinositol monophosphate (PtdInsP) synthesis and accumulation was reduced in double mutant plants. The results reported in this chapter provide the best evidence to date that Sec14p-like proteins function *in planta* by altering phosphoinositide metabolism, a phenomenon that has been well documented in yeast. This study also adds to a growing body of work connecting ABA-mediated regulation of seed dormancy with phosphoinositide signaling.

In order to obtain the above-described data on total endogenous levels of PtdInsPs in Arabidopsis, a new methodology for the extraction and quantification of these lipids was required. Chapter 4 describes the method that was developed, in collaboration with researchers at the Kansas Lipidomics Center, for the quantification of PtdInsPs from plants. This project required both the optimization of PtdInsP extraction protocols and the development of mass spectrometric-based procedures to quantify these compounds from plant extracts. The availability of this technique should provide a powerful tool for future studies of phosphoinositide metabolism and signaling in higher plants.
New Insights into Phospholipid Metabolism and Signaling in Plants

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Plant Biology

Raleigh, North Carolina

2009

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Rebecca S. Boston      Ralph E. Dewey
Chair of Advisory Committee
DEDICATION

To cancer survivors and those who fought hard.
Matthew Ryan Keogh grew up on the family farm in Morristown, Minnesota. He attended Shattuck-St. Mary’s for middle and high schools. There he had the fortune of having a gifted chemistry teacher who helped pique his interests in the natural sciences and to understand the importance of chemistry in the world. Matt traveled south to attend Rhodes College in Memphis, TN where he majored in biology and acquired an appreciation for good barbeque. There he also met an Alabamian named Jessica Hubbs whom he went on to marry in 2005.

While taking the biology core curriculum, he found that he preferred the botany section more than zoology. At Rhodes, he was introduced to research by Dr. Chuck Stinemetz, a classical plant physiologist. Matt began his graduate work in the fall of 2003 thinking that he wanted to work on phytohormone responses, however his interest in chemistry led to working with phospholipids under the advisement of Drs. Ralph Dewey and Wendy Boss. By chance, one of his projects demonstrated a link between phospholipids and a particular phytohormone, so things have come full-circle. When he is not in the lab, Matt keeps busy on the soccer field, on a run with friends, or trying to tame the backyard. In the future, he hopes to do more fishing.
ACKNOWLEDGEMENTS

There are many people responsible for me being able to complete a graduate degree. I would like to thank Dr. Ralph Dewey for taking me into his lab and teaching me how to approach science practically. Dr. Wendy Boss for being equally supportive and critical. I would also like to thank the other two committee members, Dr. Rebecca Boston and Dr. Jose Alonso for their time and advice on my projects. I am indebted to past and current members of the Dewey lab, particularly Carol Griffin, Dr. Karthik Aghoram, Dr. Steven Bowen, and Brittany Ross. Members of the Boss and Boston labs have been helpful, good neighbors. The administrative staff in both the Plant Biology and Crop Science departments have been big help, with special acknowledgement to Sue Vitello and Kathy Kelly. Dr. Boston and Dr. Gary Payne need to be acknowledged for securing and administering the IFAFS training grant which funded me for four years, helped me to learn a little about agriculture and allowed me to attend some great scientific meetings. The USDA for funding the IFAFS training grant from which I was able to benefit. My collaborators, Dr. Ruth Welty and Dr. Pamela Tamura at Kansas Lipidomics Research facility have been kind and helpful. Together we accomplished something that we couldn’t do alone. Thanks are needed for the classmates and friends that I have had throughout graduate school, and Dr. Phillip Febbo and Duke University Medical Center for his care when I was sick.

The support of my family has been great. Their sacrifices have allowed me to get to the point of even pursuing a graduate education. I am also fortunate to have wonderful in-
laws, who have welcomed me into their family. Most importantly, I need to thank my wife Jessica Hubbs. She has given the support and encouragement that has made it possible at all.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAPT</td>
<td>aminoalcoholphosphotransferase</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
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<td>AtPLMT</td>
<td><em>Arabidopsis thaliana</em> PLMT</td>
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PCCT  choline-phosphate cytidylyltransferase
PCR   polymerase chain reaction
PEAMT phosphoethanolamine N-methyltransferase
PECT  ethanolamine -phosphate cytidylyltransferase
PEMT  phosphatidylethanolamine N-methyltransferase
PI    phosphoinositides
PIS   PtdIns synthases
PITP  phosphatidylinositol transfer protein
PLA   phospholipase A
PLC   phospholipase C
PLD   phospholipase D
PLMT  phospholipid N-methyltransferase
PtdCho phosphatidylcholine
PtdDMEtn phosphatidyl(dimethylethanolamine
PtdEtn phosphatidylethanolamine
PtdGly phosphatidylglycerol
PtdIns phosphatidylinositol
PtdInsK phosphatidylinositol kinases
PtdInsP PtdIns monophosphate
PtdInsP5K phosphatidylinositol phosphate kinases
PtdMMEtn phosphatidylmonomethylethanolamine
PtdOH phosphatidic acid
PtdSer phosphatidylserine
PUFA  polyunsaturated fatty-acids
ROS   reactive oxygen species
RT-PCR reverse transcriptase polymerase chain reaction
Ser   serine
SnRK  SNF1-related family of protein kinases
SSH   soybean Sec14 homolog
TCA   trichloroacetic acid
TLC   thin-layer chromatography
V     volts
CHAPTER 1

LITERATURE REVIEW

Matthew R. Keogh
INTRODUCTION

Phospholipids have long been recognized as major components of biological membranes. While their importance in establishing and maintaining membrane structure is well appreciated, over time, studies have shown that phospholipids serve additional functions as well. Phosphatidylcholine (PtdCho), for example, is not only the predominant structural phospholipid in nonphototrophic membranes in plants, it also functions as an important metabolic intermediate in the synthesis of seed storage lipids (Ohlrogge and Browse, 1995; Bates et al., 2009). Particular emphasis has been placed on investigating the roles that certain phospholipids play as signaling molecules, capable of regulating various aspects of plant growth (Boss et al., 2006; Wang et al., 2006; Thole and Nielsen, 2008a; Heilmann, 2009; Munnik and Testerink, 2009). There are also phospholipids, such as phosphatidic acid (PtdOH), that exist at the intersection of metabolism and signaling, having the potential to serve as precursors to structural lipids, precursors to signaling molecules, or as bioactive molecules in their own right (Cruz-Ramirez et al., 2004; Wang et al., 2006; Li et al., 2009). These bi-functional characteristics of phospholipids make it necessary to consider both signaling and metabolism together, to fully understand their cellular function.

Phospholipids can be defined broadly as lipids that contain a phosphate functional group. A stricter, and more widely accepted, definition is as follows: a glycerol molecule with fatty-acids bound through ester bonds at the sn-1 and sn-2 positions and a phosphate group on the sn-3 position (Figure 1A). For the sake of simplicity, I will be using the narrower of these two definitions when referring to phospholipids. The array of possibilities
that exists for combining two of several possible fatty-acid chains with the numerous known head-groups found on phospholipids underscores the size and diversity of this complex family of molecules.

A. Generic Phospholipid Structure

B. Head Groups (R₃)

Ethanolamine

Choline

Serine

Glycerol

Inositol

Figure 1: General structure of a phospholipid and structures of major phospholipid headgroups. A) R₁ and R₂ indicate the position of the acyl chains of the fatty acid, in position 1 and position 2 respectively. R₃ indicates the site of additional head-groups added to the phosphate. Squiggled lines indicate the sites of cleavage by the major phospholipases. B) Head-groups of major phospholipids. The dashed line corresponds to the phosphate of the head-group.
Fatty acids in plant phospholipids are typically 16 or 18 carbon species (Browse et al., 1986). Differences in the number of double bonds within the fatty acid chains (commonly 0-3), provides the basis for much of the variability in phospholipid structure. The different structural components, i.e., length of fatty-acids and number of double bonds, confer different properties to the molecule, particularly with respect to their fluidity and melting point. In addition, unsaturated fatty-acids are capable of being oxidized. While some oxidized fatty-acids have been shown to be potential signaling molecules in mammalian systems (recently reviewed by Catala, 2009), this particular class of signaling molecules is outside the scope of this dissertation.

The head-groups of phospholipids vary in both size and charge. PtdOH possesses the simplest head-group, a phosphate moiety. The other common head-groups consist of one of the following small molecules linked to the phosphate through its hydroxyl group: choline (Cho), ethanolamine (Etn), inositol (Ins), serine (Ser) and glycerol (Gly) (Figure 1B). PtdCho and phosphatidylethanolamine (PtdEtn) contain amine functional groups and together with the negative charge of the phosphate group, collectively result in a zwitterionic neutral charge. Phosphatidylinositol (PtdIns) and phosphatidylglycerol (PtdGly) are both negatively charged, as neither the glycerol nor the inositol moieties carry a charge. Phosphatidylserine (PtdSer) has an amine functional group similar to PtdCho and PtdEtn, however it also possesses a carboxylic acid group, resulting in a net negative charge. Interestingly, the zwitterionic phospholipids, PtdEtn and PtdCho, are primarily derived from
the same lipid precursor while the negatively charged phospholipids, PtdIns, PtdGly, and PtdSer share a different common precursor (see below).

I will frame my discussion of the metabolic and signaling aspects of phospholipids by focusing on head-groups, with particular attention given to PtdCho, PtdIns, and their related metabolites as they are topics of the original research presented in this dissertation. Constructing the review in this matter will provide a good context in which to introduce the research findings presented in the later chapters and to elaborate on their implications within this field of study. The metabolic and physiological relevance of PtdOH will also be addressed briefly, as it is of major importance to both phospholipid metabolism and signaling in plants.

PHOSPHOLIPID METABOLISM

Morris Kates was one of the first scientists to study phospholipid biosynthesis in plants. As a graduate student, he was the first to synthesize PtdCho and clearly determine its structure (Baer and Kates, 1949). Dr. Kates was also a pioneer in the characterization of the galactolipids that represent the primary lipid component of chloroplast membranes (Kates, 1953, 1955; Sastry and Kates, 1963, 1964b, 1964a). Around that same time, Harry Beevers published a substantial body of work on plant biochemistry including valuable contributions to our basic understanding of phospholipid metabolism. Among Dr. Beevers's major contributions was the determination of the cellular compartments that are sites of phospholipid biosynthesis (Lord et al., 1973; Moore et al., 1973; Donaldson and Beevers,
Unlike today, scientists working at this time did not have the luxury of highly purified, commercially available radiolabeled compounds or refined analytical techniques. Most of these studies were conducted using in vitro preparations of different cell fractions combined with various potential substrates with subsequent efforts focused on slowly refining these techniques. Nevertheless, early researchers were able to successfully define the seminal aspects of phospholipid metabolism, identify most of the metabolic intermediates and characterize properties of the enzymes involved. The relatively recent advances in molecular biology and genomics have allowed researchers to identify and characterize the genes that code for the enzymes that catalyze the steps of phospholipid metabolism. The findings revealed through both traditional biochemistry and physiology, and molecular biology have shown the synthesis and regulation of phospholipids in plants to be very complex.

Eugene Kennedy, arguably the father of phospholipid biochemistry research, when pressed as to why the biosynthetic mechanism that he presented was "complicated and roundabout" can be quoted as saying “…we are not responsible for how Nature chooses to make these compounds. Yet I think it would be unfortunate if the impression got around that Nature really would do well to study biochemistry rather than vice-versa” (1958). (The mechanism was part of what is now referred to as the Kennedy pathway). Kennedy’s response was a sharp retort, but he hit on the important point that phospholipid biosynthetic pathways are complex and at times can seem inefficient, but it is up to the scientist to dig deeper and make sense of this complexity. Those working in the field of plant phospholipids
have started to put together a picture of the physiological consequences of this complex metabolic network.

One notable characteristic of phospholipid metabolism in plants is redundancy. This redundancy is not solely based upon the presence of multiple genes encoding different isoforms of enzymes that catalyze a given reaction (which is often the case), but is also based on the existence of multiple pathways to synthesize the same molecule. Figure 2A shows a traditional, linear view of phospholipid metabolism. The full complexity of phospholipid metabolism, however, is better illustrated by Figure 2B. Having “back-up systems” to generate essential molecules is clearly beneficial from an evolutionary perspective, and also makes the study of phospholipid function in plants all the more challenging, as the effects of perturbations in one part of the pathway may be difficult to detect due to compensation via a different route of metabolism.
Figure 2: General metabolic pathways of major phospholipid and polyphosphoinositides. The figure is simplified to indicate which phospholipids are of DAG or PtdOH origins. A) A general pathway that indicates the common anabolic biosynthetic step found in plants. B) A comprehensive diagram indicating the metabolic reactions that have been reported in plants.
1) Phospholipid N-methyltransferase
2) Amino-alcohol phosphotransferase
3) Phospholipase C
4) Diacylglycerol kinase
5) Phosphatidic acid phosphatase
6) Phospholipase D
7) Phosphatidylserine synthase
8) Phosphatidylserine decarboxylase
9) phosphatidylethanolamine: l-serine phosphatidyltransferase
10) Phosphatidylinositol : myo-inositol exchange enzyme
11) Phosphatidylinositol synthase
12) Phosphatidylinositol 3-kinase
13) Phosphatidylinositol 3-phosphate phosphatase
14) Phosphatidylinositol 4-kinase
15) Phosphatidylinositol 4-phosphate phosphatase
16) Phosphatidylinositol 3-phosphate 5-kinase
17) Phosphatidylinositol 3,5 bisphosphate 5-phosphatase
18) Phosphatidylinositol 4 phosphate 5-kinase
19) Phosphatidylinositol 4,5 bisphosphate 5-phosphatase
Two fates of PtdOH

PtdOH is the centerpiece of phospholipid metabolism as it is the first phospholipid formed de novo and follows two potential metabolic fates. 1) PtdOH can be dephosphorylated by a PtdOH phosphatase to form diacylglycerol (DAG) which can be channeled toward synthesis of PtdEtn and PtdCho via the nucleotide, or Kennedy pathway. In brief, one of the following aminoalcohol–phosphates: ethanolamine-P (Etn-P), monomethylethanolamine-P (MMEtn-P), dimethylethanolamine-P (DMEtn-P), or choline-P reacts with cytidine-tris-phosphate (CTP) to form a CDP-aminoalcohol. Either an ethanolamine-phosphate cytidylyltransferase (PECT) or a choline-phosphate cytidylyltransferase (PCCT) catalyze this rate-limiting step in the synthesis of aminoalcohol-containing phospholipids (PtdEtn, PtdMMEtn, PtdDMEtn and PtdCho). The final step involves the CDP-aminoalcohol reacting with DAG to produce an aminoalcohol phospholipid, catalyzed by an aminoalcohol phosphotransferase. A mutation that completely abolished PECT activity was embryo lethal, underlining the essential nature of this gene, however a knock-down mutant in PECT was hypersensitive to cold stress and showed a significant decrease in PtdEtn with a “reciprocal increase” in PtdCho (Mizoi et al., 2006). This is one of many examples to demonstrate the interdependent nature of phospholipid metabolism (this aspect of phospholipid metabolism in plants is discussed at further length in Chapter 2).

PtdOH’s other fate is to react with CTP to synthesize CDP-DAG. Bessoule and Moreau (2004) suggested that it be more appropriately referred to as CMP-PtdOH to better clarify the metabolic precursors. The CDP-DAG molecule is the PtdOH donor for the
synthesis of PtdIns, PtdSer and PtdGly. PtdIns is synthesized by the enzymatic reaction of CDP-DAG with inositol. The Arabidopsis genome encodes two PtdIns synthases (PIS), PIS1 and PIS2, respectively, which are both localized to the endoplasmic reticulum (ER). It was found that over-expression of each of these enzymes resulted in significant changes in the levels of PtdIns as anticipated, but intriguingly over-expression of PIS1 also resulted in increased DAG and PtdEtn while PIS2 enhancement led to increases in the polyphosphoinositides PtdIns(4)P and PtdIns(4,5)P2 (Lofke et al., 2008). This finding suggested that the fate of some lipids can be determined by the specific enzymatic isoform that synthesized the lipid. A likely explanation of this finding is that PIS2 shows greater activity toward CDP-DAG species containing polyunsaturated fatty-acids (PUFA). PtdIns can then be further modified by phosphorylation to form a series of polyphosphoinositides, which will be discussed at length later in this chapter (see Phospholipid Signaling).

A similar reaction occurs between free serine and CDP-DAG, catalyzed by a PtdSer synthase. This reaction differs in plants in comparison to animals, as the latter synthesizes PtdSer by a head-group exchange reaction that switches a free serine with the Etn moiety of PtdEtn, or the Cho moiety of PtdCho (Tomohiro et al., 2009). Classical biochemical studies conducted in castor bean demonstrated that plants also have this activity, but there has been no further work repeated in this area (Shin and Moore, 1990). Over-expression of a wheat PtdSer synthase gene in tobacco led to increased PtdSer and decreases in PtdIns and PtdGly. This indicates that PtdSer synthase is controlled transcriptionally and competes with PIS and PtdGly phosphate synthase for the same pools of CDP-DAG (Delhaize et al., 1999). PtdSer
 synthase over-expressers also developed necrotic lesions on their leaves, suggesting that PtdSer may play a role in programmed cell death in plants, similar to what has been shown in animal systems (Fadok et al., 1992).

In yeast, PtdSer has an important role as a metabolic intermediate, as it is the major substrate for the production of ethanolamine moieties de novo (Trotter et al., 1995). PtdEtn is synthesized from PtdSer via a single decarboxylation reaction. While plants are able to produce PtdEtn through this mechanism as well, in Arabidopsis, a triple knockout mutant in all three PtdSer decarboxylase isoforms showed no overall reduction in PtdEtn. However, the triple mutant had a slight but significant decrease in PtdEtn in the mitochondrial membrane that resulted in no overall growth phenotype (Nerlich et al., 2007). This result was not particularly surprising given that it had been shown previously that main source of ethanolamine moieties in plants originates through the decarboxylation of free serine to form free ethanolamine (Rontein et al., 2003). The resultant free ethanolamine may then be channeled through the nucleotide pathway to make PtdEtn or it may be channeled into the hybrid methylation/nucleotide pathway for PtdCho biosynthesis.

Unlike PtdIns and PtdSer, PtdGly is synthesized in two steps. CDP-DAG and glycerol-phosphate react to form PtdGly-phosphate, which is subsequently dephosphorylated to form PtdGly (Andrews and Mudd, 1985). Unlike the other phospholipids discussed, PtdGly is primarily a chloroplast lipid and will not be discussed in further detail.
Phospholipases

In addition to the anabolic pathways described above, there are three classes of phospholipases whose functions are described in brief below (the bonds that they cleave are indicated in Figure 1A). Phospholipase A (PLA) hydrolyzes a fatty-acid from a phospholipid substrate leaving a free fatty-acid and phospholipid with only one acyl-chain remaining (referred to as lyso-phospholipids). There are two types of PLA enzymes; one that hydrolyzes the acyl group from the sn-1 position of the glycerol (PLA₁ enzymes), and others that cleave the acyl group on sn-2 (PLA₂ enzymes). Phospholipase C (PLC) enzymes cleave phospho-ester bonds between the glycerol molecule and the phosphate group, yielding DAG and a phosphorylated head-group moiety. Phospholipase D (PLD) enzymes break the phospho-diester bonds on the opposite side, generating PtdOH and a free head-group as the reaction products (Fig. 1A). All three classes of lipases have been studied extensively in plants. All are comprised of multi-gene families with specific members of each families having been implicated in physiological signaling processes.

PLCs and PLDs have great capacities to remodel membrane phospholipids. Upon cellular perturbation, such as an abiotic or biotic stress, their activation can be rapid and robust, so much so that most laboratory procedures involved in the analysis of phospholipids require a treatment to specifically abolish their activities (Hama et al., 2000; Bonangelino et al., 2002; Welte et al., 2002). Some of the activities of PLCs and PLDs in stress response scenarios are to synthesize the signaling molecules inositol 1,4,5 tri-phosphate (InsP₃) or PtdOH. While the production of these bioactive molecules is surely important, phospholipase
activities also allow for a remodeling of the cell’s membranes and thus alter the characteristics of a given membrane through directed “re-synthesis” of phospholipids from the DAG and PtdOH produced by PLC and PLD reactions. Changes that may be imposed on membrane structure from altering the relative proportions of the various phospholipids can be based on differences in overall charge, curvature, or rigidity as different phospholipids interact with other molecules (and each other) in the plane of the membrane to better adapt to the stress imposed.

It should be noted that DAG may also be phosphorylated directly by DAG kinase enzymes as an additional route for PtdOH synthesis. The dynamic balance between DAG and PtdOH pools has the potential to dictate whether PtdEtn and PtdCho, or PtdIns, PtdGly, and PtdSer are preferentially synthesized. The enzymes that regulate this balancing act have yet to be studied in depth. Instead, research in this area has been primarily focused on the study of PtdOH as a signaling molecule rather than as the hub of phospholipid metabolism (recently reviewed by Li et al., 2009).

In Chapter 2, I report the identification and characterization of a phospholipid N-methyltransferase (PLMT) involved in the phosphatidylcholine biosynthetic pathway (see Fig. 2). This was the first report of the cloning and characterization of a plant PLMT, responsible for the synthesis of the phosphoaminoalcholol species PtdMMETn and PtdDMEtn. The findings strongly support the conclusion that plants cannot synthesize PtdCho through the direct methylation of PtdEtn. While other researchers have suggested this previously, the results in Chapter 2 represent the strongest evidence to date that this is indeed the case. The
understanding that plants lack the ability to methylate PtdEtn is a critical finding in the field of phospholipid metabolism in plants. It indicates that unlike yeast and animals, neither PtdEtn or PtdSer (a PtdOH derived lipid) can be used as precursors for the synthesis of PtdCho. This apparent lack of continuity between the phospholipids of DAG and PtdOH origins suggests that restructuring membrane composition likely will require PLC and/or PLD activities, rather than occurring solely through the use of anabolic machinery. This study also allows for further conjecture regarding the enzymes that truly dictate the flow of metabolites through the alternate branches of the PtdCho biosynthetic pathway.

A null mutant of the sole Arabidopsis PLMT (AtPLMT) did not display an obvious growth phenotype. Furthermore, the mutant displayed no significant change in overall PtdCho levels (despite a significant increase in metabolic intermediates). The fact that the AtPLMT mutant possesses wild-type levels of PtdCho indicates other branches biochemical pathways are sufficient to compensate for the loss of AtPLMT in Arabidopsis. This provides a good example of the biochemical redundancy discussed above, but is not likely to be universal in plants because of the expectation that similar perturbations in soybean would be lethal (see Chapter 2).
PHOSPHOLIPID SIGNALING

By definition, signaling involves a bio-active molecule or ion that acts on a specific target. The availability of the bio-active molecule to the receptor must be transient. The bio-active molecule present at low levels or absent, or sequestered from its target in the non-activated state. A classic example of a sequestration mechanism would be the release of stored Ca^{2+} in the ER by InsP_{3}. Examples where the bio-active molecule is metabolized at the time of the sensing to stimulate the response are plentiful, and many of the bioactive phospholipids fall in this category. The following sections discuss aspects of phosphoinositide signaling in plants, with a specific emphasis on the phosphatidylinositol monophosphates (PtdInsPs), as they are the species of greatest relevance to Chapters 3 and 4. Other phosphoinositides will only be mentioned in brief.

Phosphoinositides: What do we know?

In a recent review, Heilmann listed the three major questions remaining for those studying phosphoinositides (PIs) in plants as follows: “(i) where, how and under what circumstances are certain PIs generated?; (ii) what are the target proteins ?; and (iii) what are the effects of PI binding on the targets at the molecular and physiological levels?” (Heilmann, 2009). His delineation of these open questions leaves one to wonder what if anything is actually known about PIs in plants.

The completion of the Arabidopsis genome sequencing project allowed for identification of many of the likely candidate genes related to PI signaling and metabolism.
One notable characteristic of some of the PI related genes is the size and diversity of their respective gene families. Enzymatic steps represented by large gene families include phosphatidylinositol kinases (PtdInsKs), phosphatidylinositol phosphate 5 kinases (PtdInsP5K), SAC-like phosphoinositide phosphatases, PLCs, and P LDs. The natural question to ask is, “why so many?” To date it appears that at least part of the explanation behind these large gene families can be attributed to the following: (1) some of the gene isoforms function redundantly; (2) some isoforms are expressed specifically in certain tissues; and (3) some genes function during a specific developmental stage or in response to a specific environmental stimulus. An example of such redundancy, or tissue specificity, includes the fact that two PtdInsP5K genes were shown to be necessary for normal pollen tube germination (Ischebeck et al., 2008). Also, plants lacking both of the PtdIns4Kβs have morphologically abnormal root hairs (Preuss et al., 2006).

In addition to the genomics data available, the collective work in the field shows that the PI machinery is involved in certain cellular and whole-plant physiological processes. Common themes found throughout the literature include the relationship of PI to the metabolism of other phospholipids, vesicle trafficking, polar growth, hyperosmotic stress responses, and phytohormone responses (Recently reviewed by: Boss et al., 2006; Thole and Nielsen, 2008a, Heilmann, 2009).
The study of PtdIns(3)P in plants has been relatively minimal, especially when compared to the heavy focus on PtdIns(3)P function in mammalian, and to a lesser extent yeast, studies. The interest in PtdIns(3)P in mammalian systems has been based upon its relation to cancer biology and diabetes via its role in insulin signaling (Kok et al., 2009; Skwarek and Boulianne, 2009). Animals possess three different classes of kinases that catalyze the phosphorylation of PtdIns on the 3 position of the inositol ring. In contrast, the yeast and Arabidopsis genomes only encode class III types of PtdIns 3 kinases. Much of the work in this area has relied upon non-specific inhibitors, and often suffer from a lack of sufficient controls. A good review on the pharmacological inhibitors of PI pathway is presented by Balla, (2001).

Expression of anti-sense transcripts directed against the PtdIns 3 kinase-encoding VPS34 gene in Arabidopsis resulted in severe developmental and morphological defects (Welters et al., 1994). This is consistent with the reported results of others. They found a true vsp34 knock-out to be lethal, unable to form viable pollen (Lee et al., 2008a). The majority of studies implicate PtdIns(3)P as having a role in vesicular trafficking. One study in particular showed that PtdIns(3)P was important for trafficking from the endosome to the vacuole (Kim et al., 2001). Plants that are compromised in PtdIns(3)P signaling have been shown to have reduced root hair elongation, indicating a role in polar cell growth, presumably through PtdIns(3)P’s regulation of vesicle trafficking (Lee et al., 2008b).
Some of the most interesting information on the function of PtdIns(3)P came from work by Leshem et al. (2007), who showed that PtdIns(3)P is critical for endocytosis during hyperosmotic salt stress through mediating the production of reactive oxygen species (ROS). This investigation relied upon the somewhat non-specific inhibitor wortmannin, along with the study of T-DNA insertion mutants that were Arabidopsis vps34 knock-downs, which proved to be salt hypersensitive. The endocytic impairment in the knock-down plants was rescued with the addition of exogenous PtdIns(3)P and partially restored with the addition of PtdIns(4)P. Adding PtdInsPs exogenously to live plant tissue has the drawback that it is unclear whether the lipid is taken up by the cell and/or is intact. These issues, coupled with the fact that both PtdIns(3)P and PtdIns(4)P could function to restore the mutant phenotype are a bit puzzling, given that PtdIns(3)P and PtdIns(4)P are known to have distinct roles in vesicular trafficking. Nonetheless, the suggestion that endocytosis is critical in a plant’s ability to tolerate salt stress is intriguing.

_PtdIns(4)P_

PtdIns(4)P is the most abundant of the polyphosphoinositides in plants. As was the case in yeast and animal systems, PtdIns(4)P in plants was originally considered as simply a precursor to PtdIns(4,5)P2. More recently, however, it has gained attention for its role in some fundamental cell processes _per se_, most of which are related to vesicle trafficking (Balla and Balla, 2006; D'Angelo et al., 2008). PtdIns(4)P is synthesized by a PtdIns 4-kinase. Arabidopsis has open reading frames (ORFs) representing two of the 3 subgroups of PtdIns
4-kinases defined in animal systems. The Type II or PtdIns4KIIγ genes are the largest group, comprised of eight putative PtdIns4Ks (Mueller-Roeber and Pical, 2002). There have been no reports of any of these possessing lipid kinase activity, however, and a recent study suggests that these enzymes may actually function as protein kinases (Galvao et al., 2008).

The lack of lipid kinase activity in type II kinases means that the class III kinases may be the sole source of PtdIns-4 kinase activity in plants. There are four ORFs corresponding to two PtdIns4KIIIα and two PtdIns4KIIIβ isoforms. In Arabidopsis, there are no ESTs corresponding to PtdIns4KIIIα2. This suggests that there may be only three functional PtdIns-4 kinases. The Arabidopsis PtdIns4KIIIα1 enzyme has been well characterized biochemically. PtdIns4KIIIα1 is a very large protein consisting of a lipid kinase domain and a pleckstrin homology domain that associates with filamentous actin and binds PtdIns(4)P and PtdIns(4,5P)2 (Stevenson et al., 1998). PtdIns4KIIIα1 is inhibited by its end product, PtdIns(4)P, whereas PtdIns4KIIIβ1 is stimulated by PtdIns(4)P (Stevenson-Paulik et al., 2003). As mentioned above, loss of both of the PtdIns4KIIIβs resulted in abnormal root hair development and overall reduced biomass. Somewhat paradoxically, the loss of a Sac-1 phosphatase that preferentially hydrolyzes PtdIns(4)P also resulted in deformed root hairs. Microscopic analysis of the Sac1 deficient root hairs expressing a fluorescent PtdIns(4)P binding protein showed increased PtdIns(4)P in the internal membranes compared to wild-type roots (Thole et al., 2008b). These results highlight the importance of PtdIns(4)P turnover in polar growth and vesicle trafficking.
Not all PtdIns(4)P is created equal. Several studies have shown that there are different pools of PtdIns(4)P within the cell. König et al. (2007) showed that in response to hyperosmotic stress, the PtdInsPs synthesized are enriched in polyunsaturated fatty acids. This finding indicates that there are specific metabolic pools of PtdIns. This phenomenon is not confined only to plants. In mammalian systems, it was found that renewal of plasma membranes pools of PtdIns(4,5)P only occurred after hormone stimulation through a specific PtdIns 4-kinase (PtdIns 4-kinase IIIα), (Balla et al 2008).

**PtdIns(4,5)P₂**

PtdIns(4,5)P₂ is synthesized by the phosphorylation of PtdIns(4)P on position 5 of the inositol ring. In animal systems, PtdIns(4,5)P₂ is hydrolyzed by PLC, generating IP₃ that in turn can signal Ca²⁺ release from the endoplasmic reticulum (ER) upon binding to the IP₃ receptor (Miyazaki, 1995). In animals, the DAG product also functions as a signaling molecule, activating Protein Kinase C. PtdIns(4,5)P₂ mediated signal transduction appears to differ substantially in plants. To date, no InsP₃ receptor has been identified in plants. Furthermore, plants do not possess Protein Kinase C homologs, nor has DAG been shown to function as a signaling molecule (Meijer and Munnik, 2003).

Similar to the canonical InsP₃ pathway in mammals, however, plants do hydrolyze PtdIns(4,5)P₂ via PLC during development, and in response to external stimuli. The resultant InsP₃ has been shown to correspond with Ca²⁺ oscillations in plants as well. There are interesting questions whether InsP₃ itself is the signaling molecule, as opposed to being
converted to InsP₆ (phytate) which in turn may represent the actual signaling molecule (Lemtiri-Chlieh et al., 2003). There are also questions regarding the subcellular location of the Ca²⁺ prior to its release into the cytosol. Ca²⁺ has been shown to be stored in the ER, the vacuole, the chloroplast, the mitochondrion and even extracellularly within the cell wall.

There are 11 ORFs encoding PtdIns(4)P 5-kinases in Arabidopsis (Mueller-Roeber and Pical, 2002). This final phosphorylation on position 5 is generally regarded as the rate limiting step in PtdIns(4,5)P₂ synthesis in plants (Im et al., 2007). PtdIns(4)P 5-kinase activities also appears to be important in controlling the increases in PtdIns(4,5)P₂ synthesis that are induced by hyperosmotic stress (DeWald et al., 2001; Konig et al., 2008; Darwish et al., 2009). A T-DNA insertion mutation in the Arabidopsis Sac9 phosphatase led to increased PtdInsP₂ and InsP₃ levels that were associated with a stress phenotype, including the up-regulation of several stress-related transcripts (Williams et al., 2005).

In addition to serving as the direct precursor of InsP₃, PtdIns(4,5)P₂ is a signaling molecule in its own right. The signaling function of PtdIns(4,5)P₂ that is of particular relevance to this discussion, is its demonstrated role as the activator of specific PLD isoforms (Qin et al., 2002; Zheng et al., 2002). PtdIns(4,5)P₂-mediated activation of PLD implicates this phosphoinositide as a regulator of membrane remodeling.

**PtdIns(5)P and Ptd(3,5)P₂**

PtdIns(3,5)P₂ was originally discovered in yeast, with its synthesis being enhanced during severe hyperosmotic stress treatment (Dove et al., 1997). PtdIns(3,5)P₂ is synthesized by a
PtdIns(3)P 5-kinase called Fab1 in yeast, an enzyme that is critical in regulating vacuolar morphology (reviewed by Efe et al., 2005). Yeast Fab1 appears to be highly regulated, associating with several other protein that comprise the "Fab1 complex" (Jin et al., 2008).

Though in low abundance, PtdIns(3,5)P$_2$ has been shown to occur in carrot cell cultures and *Chlamydomonas* (Dove et al., 1997; Meijer et al., 1999). There are 5 loci in the Arabidopsis genome that encode predicted proteins that share homology to yeast Fab1. Very little work has been done in characterizing these genes, so their function and significance remains an open area in plant biology. The exceptional size of the predicted Fab1 homologs (~170-200 kDa) and the low abundance of endogenous PtdIns(3)P substrate may be factors contributing to the lack of attention paid to their study in plants.

PtdIns(5)P is an extremely low abundance lipid species in plants as well. The route to its synthesis remains somewhat unclear, as plants do not contain proteins with homology to the characterized PtdIns(4,5)P$_2$ 4-phosphatase responsible for its production in animals. One hypothesis is that plants may possess a PtdIns(3,5)P$_2$ 3-phosphatase. Peterman et al. (2004) demonstrated that Pattelin1, an integral membrane protein with a Sec14-like lipid binding domain, binds PtdIns(5)P *in vitro* and localizes to the phragmoplast, prompting the authors to speculate that PtdIns(5)P may have a role in cell division. Another group showed that the binding to PtdIns(5)P represses the function of the ATX1 histone methyltransferase. This finding implies that PtdIns(5)P could be involved in the epigenetic regulation of gene expression (Alvarez-Venegas et al., 2006).
Phosphatidylinositol transfer proteins and phosphoinositide signaling

Phosphatidylinositol transfer proteins (PITPs) were originally discovered by their ability to transfer PtdIns and PtdCho between separate biological membranes \textit{in vitro}. This activity, while interesting, may now be viewed as somewhat misleading given our current understanding of PITP function. A review by Ile et al. (2006) argued that PITPs are functioning in the cell as “nanoreactors” that regulate the enzymes of lipid metabolism, as opposed to serving as lipid transporters. Nanoreactor is a better descriptor for PITPs based on the current knowledge. The term refers to the fact that these proteins are regulating, or organizing, discrete pools of phospholipids within specific locations in the cell. Examples of PITPs as regulators of specific lipid pools are discussed below.

PITPs are divided into two major classes, metazoan PITPs and Sec14p-like proteins. Unlike most other examples of proteins belonging to the same general family, these two classes share no primary sequence homology (Phillips et al., 2006). Despite this lack of homology, there is a great deal of functional redundancy between the classes. For example, PITPs from both classes possess \textit{in vitro} PtdIns and PtdCho transfer activity and are able to complement a yeast strain with a temperature-sensitive \textit{sec14} mutation. Their role in regulating polyphosphoinositide metabolism is the most meaningful feature shared with regard to \textit{in vivo} function, yet PITPs are often omitted from reviews on phosphoinositide metabolism. This omission is likely based upon the fact that their functions have only
recently been recognized, especially in plants. All plant PITPs characterized to date belong to the Sec14-like class of PITPs.

Sec14p-like proteins in yeast have been shown to regulate the synthesis of the different phospholipid pools, including PtdIns, PtdCho and PtdIns(4)P. The original Sec14p was discovered in a genetic screen for yeast that was defective in its secretion of invertase (Bankaitis et al., 1989). It was shown to have a role in the regulation of PtdCho metabolism, but more recently its function in regulating PtdIns(4)P metabolism has been a major focus of research in this field. In yeast, the sec14 mutant allele can be rescued by over-expression of Pik1p, a Golgi localized PtdIns 4-kinase. Over-expression of Stt4p, another PtdIns 4-kinase, failed to rescue the secretion defect (Hama et al., 1999).

Complicated synthetic lethality screens involving the temperature-sensitive sec14 mutant have yielded considerable data regarding the cellular processes in which Sec14 is implicated (Howe et al., 2007; Mousley et al., 2008; Curwin et al. 2009). One such study further extended our knowledge of the mechanism underlying Sec14p-mediated regulation of Pik1p. It was discovered that the Kes1p oxysterol binding protein, which is also a PtdIns(4)P binding protein, acts antagonistically to Sec14p in modulating Pik1p-based synthesis of PtdIns(4)P in the Golgi (Fairn et al., 2007). Most current models propose that Sec14p stimulates Pik1p activity by presenting the PtdIns substrate to Pik1p, making the substrate more accessible for phosphorylation by the kinase (Schaaf et al., 2008).

Sec14p is not the only ITP in yeast that appears to function by modulating PtdIns(4)P synthesis. A yeast Sec14p homolog, designated Sfh5p, was found to stimulate the
Stt4p PtdIns 4 kinase to produce PtdIns(4)P pools specific to the plasma membrane (Routt et al., 2005). It was further demonstrated that the Sfh5p/Stt4p association also functions in concert with the PtdIns(4)P 5-kinase Mss4p. A mutation that renders Sfh5p unable to bind PtdIns could not restore the secretory defects (Yakir-Tamang and Gerst, 2009). This indicates that Sfh5p stimulation of Stt4p is essential for normal secretion in yeast.

The Arabidopsis genome contains 31 genes that encode Sec14p-like proteins. A list of these genes, including alternative names and known functional characteristics is shown in Table 1. Although some of the Sec14-like genes are predicted to encode relatively small soluble proteins similar to the original yeast Sec14p, most of the homologs contain additional domains. There have been three reports in plants characterizing small, soluble Sec14p homologs. Two Sec14p homologs from soybean were identified by screening for complementation of the temperature-sensitive sec14 yeast mutant. The two proteins discovered in this screen were designated Ssh1p and Ssh2p (Kearns et al., 1998). Curiously, Ssh1p appeared as a doublet on immunoblots. The larger species was later found to be a phosphorylated form of Ssh1p, which was also found to be rapidly enhanced during exposure of plant cells to severe hyperosmotic stress (Monks et al., 2001). The soybean Ssh1p protein is phosphorylated by a soybean SnRK2b type kinase, a family of kinases for which almost all members characterized have been implicated in hyperosmotic drought stress (Boudsocq et al., 2004). Compelling evidence linking Ssh1p with phosphoinositide signaling was obtained by showing that purified recombinant Sh1p could stimulate the synthesis of PtdIns(4)P and PtdIns(3)P from plant extracts and recombinant enzyme preparations, respectively. In a n
independent study, Joune et al. (1998) identified an Arabidopsis Sec14 homolog that displays high sequence homology with soybean Ssh1p. This Arabidopsis Sec14 homolog, named AtSec14-1, was isolated through complementation of the same temperature sensitive sec14 yeast strain that was used to identify Ssh1p.
Table 1
Sec14 proteins in Arabidopsis thaliana, associated publications and summary of their known characteristics.

<table>
<thead>
<tr>
<th>Gene Locus</th>
<th>Names</th>
<th>Publications</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g55840</td>
<td>AtSec14-1/AtSec14-1</td>
<td>Jouannic et al. 1998, Chapter 3</td>
<td>Involved in seed germination, PtdInsP synthesis, PtdIns binding</td>
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<tr>
<td>At5g47730</td>
<td>AtSec14-5</td>
<td>Chapter 3</td>
<td>Involved in seed germination, PtdInsP synthesis</td>
</tr>
<tr>
<td>At4g34580</td>
<td>AtSFH1/COW1</td>
<td>Böhme et al 2004, Vincent et al., 2005</td>
<td>Root hair development, PtdIns4,5P2 binding</td>
</tr>
<tr>
<td>At4g39180</td>
<td>AtSFH2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>At2g21540</td>
<td>AtSFH3</td>
<td>Mo et al. 2007</td>
<td>Highly expressed in flowers</td>
</tr>
<tr>
<td>At1g19650</td>
<td>AtSFH4</td>
<td>-</td>
<td>-</td>
</tr>
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<td>AtSFH5</td>
<td>-</td>
<td>-</td>
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<td>AtSFH12</td>
<td>Mo et al. 2007</td>
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<td>AtSFH13</td>
<td>-</td>
<td>-</td>
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<td>At5g56160</td>
<td>AtSFH14</td>
<td>-</td>
<td>-</td>
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<tr>
<td>At1g72150</td>
<td>PATL1</td>
<td>Peterman et al. 2004, Deng et al. 2007</td>
<td>Localizes to cell plate, PtdIns5P binding, brassinosteriod induced expression</td>
</tr>
<tr>
<td>At1g22530</td>
<td>PATL2</td>
<td>Deng et al. 2007</td>
<td>Brassinosteriod induced</td>
</tr>
<tr>
<td>At1g72160</td>
<td>PATL3</td>
<td>-</td>
<td>-</td>
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<tr>
<td>At1g30690</td>
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<tr>
<td>At3g46450</td>
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</table>
Chapter 3 puts forth evidence to suggest that AtSec14-1 and AtSec14-2, the two Arabidopsis Sec14-like proteins that are most closely related to soybean Ssh1p, function in vivo through altering PtdInsP metabolism. The data gathered indicate that the two proteins are functionally redundant, and serve to maintain normal germination and promote early seedling growth under non-ideal conditions such as hyperosmotic stress. This work provides the best evidence to date that plant Sec14 proteins affect phosphoinositide metabolism in planta. Furthermore, the results of this study are the first to implicate Sec14-like proteins in ABA signaling and seed germination.

In order to fully characterize the consequences of knocking out AtSec14-1 and AtSec14-2 gene function on PtdInsP accumulation in Arabidopsis, accurate quantification of the total endogenous levels of these lipid species was required. Unfortunately, techniques to accurately measure and quantify PtdInsP in plants had yet to be developed. In collaboration with Drs. Ruth Welti and Pamela Tamura at the Kansas Lipidomics Research Center (Manhattan, KS), we developed the first method to quantify PtdInsP lipids from plants using mass spectrometry. The protocol describing this new technique is presented in Chapter 4. This work represented a unique team effort, combining of our knowledge of extraction and handling of phosphoinositides at North Carolina State University (Raleigh, North Carolina), with the expertise in phospholipid mass spectrometry found at the Kansas Lipidomics Center. The technique developed will be beneficial for future researchers to not only quantify total endogenous levels of PtdInsP, but also gain information on the individual fatty-acid species and combinations associated with specific pools of PtdInsPs. Furthermore, because this
method was developed in collaboration with a widely used service facility, this analytical tool will become available to many researchers.

CONCLUSIONS

The work presented in this dissertation makes new contributions to the larger field of plant phospholipid research. The contributions are made at the levels of metabolism with the characterization of plant phospholipid N-methyltransferases, and both signaling and metabolism in the study of the in vivo function of two closely related Sec14 proteins in Arabidopsis. Additionally, a contribution was made to methodology available to phospholipid researchers through the development of a method to quantify PtdInsPs from plants using mass spectrometric analysis.
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CHAPTER 2

FUNCTIONAL CHARACTERIZATION OF PHOSPHOLIPID N-METHYLTRANSFERASES FROM ARABIDOPSIS AND SOYBEAN

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This manuscript was accepted for publication in the Journal of Biological Chemistry, April 14, 2009 (PMID: 19366698).
ABSTRACT

Phospholipid \(N\)-methyltransferase (PLMT) enzymes catalyze the \(S\)-adenosylmethionine-dependent methylation of ethanolamine-containing phospholipids to produce the abundant membrane lipid phosphatidylcholine (PtdCho). In mammals and yeast, PLMT activities are required for the \textit{de novo} synthesis of the choline headgroup found in PtdCho. PLMT enzyme activities have also been reported in plants, yet their roles in PtdCho biosynthesis are less clear because most plants can produce the choline headgroup entirely via soluble substrates, initiated by the methylation of free ethanolamine-phosphate. To gain further insights into the function of PLMT enzymes in plants, we isolated PLMT cDNAs from Arabidopsis and soybean (\textit{Glycine max}) based upon primary amino acid sequence homology to the rat PLMT, phosphatidylethanolamine \(N\)-methyltransferase. Using a heterologous yeast expression system, it was shown that plant PLMTs methylate phosphatidylmonomethylethanolamine and phosphatidyltrimethylethanolamine but cannot utilize phosphatidylethanolamine as a substrate. Identification of an Arabidopsis line containing a knock-out dissociator transposon insertion within the single copy \textit{AtPLMT} gene allowed us to investigate the consequences of loss of PLMT function. Although the accumulation of the PLMT substrates phosphatidylmonomethylethanolamine and phosphatidyltrimethylethanolamine was considerably elevated in the \textit{atplmt} knock-out line, PtdCho levels remained normal, and no obvious differences were observed in plant morphology or development under standard growth conditions. However, because the metabolic routes through which PtdCho is synthesized in plants vary greatly among differing species, it is predicted that the degree
with which PtdCho synthesis is dependent upon P LMT activities will also vary widely throughout the plant kingdom.
INTRODUCTION

Phosphatidylcholine (PtdCho) is the most abundant phospholipid in most non-plastid membranes of eukaryotes. PtdCho biosynthesis has been studied intensively in plants not only because of its importance as a structural membrane lipid, but also because of its role as a precursor to important lipid-based signaling molecules, such as phosphatidic acid, and phospholipase A₂-derived free fatty acids (Meijer and Munnik, 2003). The choline headgroup of PtdCho serves multiple functions as well. In addition to being an essential human nutrient (Zeisel, 2007), in many plant species choline can be oxidized to produce the potent osmoprotectant glycine betaine (Hitz et al., 1981; Rontein et al., 2002).

For over 2 decades it has been apparent that here are fundamental differences between the manner in which PtdCho is produced in plants versus how it is synthesized in mammals and fungi. In the latter two systems, PtdCho can be formed through two distinct pathways as follows: (a) the "nucleotide pathway" in which free choline is incorporated in PtdCho using CDP-choline as an intermediate, and (b) the "methylation pathway" whereby PtdCho is produced directly from phosphatidylethanolamine (PtdEtn) via three sequential methylation reactions using S-adenosylmethionine (AdoMet) as the methyl donor (Carman and Henry, 1989; Vance, 1996). In contrast, PtdCho biosynthesis in plants occurs through a branched pathway that utilizes components of both the nucleotide and methylation pathways (Kinney, 1993). The greatest distinction between the contrasting mechanisms of PtdCho biosynthesis can be attributed to the presence of plant enzymes that are capable of converting ethanolamine headgroups to choline at the phospho-base level,
activities that are absent in mammals and yeast. Conversely, mammals and fungi possess methylation enzymes that act directly on PtdEtn, a reaction that cannot be detected in most plant systems investigated [reviewed in (Kinney, 1993)].

A diagram of the most widely accepted model of phosphoamino alcohol biosynthesis in plants is shown in Fig. 1. Similar to animals and yeast, free choline can be directly incorporated into PtdCho via nucleotide pathway enzymes in plants. In the absence of choline, however, the methylation of Etn-phosphate represents the first committed step in PtdCho biosynthesis. The resulting monomethylethanolamine-phosphate (MMEtn-P) metabolite can be further methylated at the phospho-base level to produce Cho-P. Alternatively, MMEtn-P can be incorporated into phatidylmonomethylethanolamine (PtdMMEtn) via the cytidylyltransferase and amino alcohol phosphotransferase activities of the nucleotide pathway and then methylated at the phosphatidyl-base level to complete the synthesis of PtdCho (Fig. 1). The extent with which PtdCho is formed by the flow of metabolites through phospho-bases as opposed to phosphatidyl-bases varies greatly among different plant species. In most higher plants, it is likely that the methylation of the phospoamino alcohol headgroups involves the flow of metabolites through both branches of the pathway, as has been shown in species such as barley, carrot, and tobacco (Hitz et al., 1981; Datko and Mudd, 1988a; McNeil et al., 2000). Nevertheless, examples have also been reported where only one of the branches appears to be utilized. In Lemna paucicostata, for example, the methylation steps in PtdCho biosynthesis were shown to occur almost exclusively at the phospho-base level (Mudd and Datko, 1986). At the other end of the spectrum is soybean,
where all methylations beyond the initial formation of MMEtn-P were reported to occur on phosphatidyl-bases (Datko and Mudd, 1988a, 1988b). The tremendous variability observed among plants with regard to PtdCho formation is also exemplified by a study conducted by Williams and Harwood (Williams and Harwood, 1994) where it was shown that the predominant route of PtdCho synthesis in olive culture cells involved the first two methylation reactions taking place at the phospho-base level (producing dimethylethanolamine phosphate) and the final methylation occurring on a phosphatidyldimethylethanolamine (PtdDMEtn) substrate.
Our understanding on the mechanisms by which plants synthesize PtdCho and regulate its accumulation has been further enhanced as the genes encoding the various steps of the phosphoamino alcohol pathway have been isolated and characterized. For example, molecular characterizations led to the conclusion that all of the amino alcohol phosphotransferase reactions depicted in Fig. 1 can be mediated by the product of a single gene (designated \textit{AAPTI}) that displays a broad substrate specificity (Dewey et al., 1994; Goode and Dewey, 1999). Similarly, it was the isolation of the phosphoethanolamine methyltransferase (PEAMT) genes from Arabidopsis and spinach that led to the discovery that all three phospho-base methylation reactions could be catalyzed by a single
enzyme (Bolognese and McGraw, 2000; Nuccio et al., 2000). Inhibition of PEAMT gene function in Arabidopsis through T-DNA insertion or co-suppression revealed unexpected associations between the phosphoamino alcohol pathway and root development, salt hypersensitivity, and male sterility (Mou et al., 2002; Cruz-Ramirez et al., 2004).

Although most of the reactions depicted in Fig. 1 have been characterized at the molecular genetic level, conspicuously absent is information on the plant genes/enzymes responsible for the methylation reactions conducted at the phosphatidyl-base level. In contrast, these reactions are among the most well characterized in animals and yeast, catalyzed by enzymes commonly referred to as phospholipid $N$-methyltransferases (PLMTs). In mammals, the 18-kDa integral membrane protein phosphatidylethanolamine $N$-methyltransferase (PEMT) is a PLMT that is expressed primarily in the liver (Ridgway and Vance, 1987). PEMT catalyzes all three of the methylation reactions needed to convert PtdEtn to PtdCho. Yeast uses two distinct PLMT enzymes to catalyze the three methylation reactions as follows: Cho2p/Pem1p that mediates the direct methylation of PtdEtn to produce PtdMMEtn (Kodaki and Yamashita, 1987; Summers et al., 1988), and Opi3p/Pem2p, an enzyme homologous to the mammalian PEMT, that primarily catalyzes the methylation of PtdMMEtn to PtdDMEtn and PtdDMEtn to PtdCho, the final two steps of the methylation pathway (Kodaki and Yamashita, 1987; McGraw and Henry, 1989). PLMT activities are critical in both of these systems. Mice possessing pemt knock-out mutations are completely dependent on dietary choline for survival, and they display abnormal levels of choline metabolites within the liver and develop hepatic steatosis even when fed diets supplemented with choline (Zhu
et al., 2003). Yeast lacking PLMT activities (cho2/opi3 double mutants) are obligate choline auxotrophs, unable to synthesize PtdCho de novo in the absence of exogenous choline.

To gain a greater understanding of the specific function of PLMT reactions in higher plants, and their contribution toward PtdCho biosynthesis, we cloned and characterized PLMT homologs from Arabidopsis and soybean. By expressing the candidate DNAs in yeast, we were able to confirm that they encoded functional PLMT activities as well as to establish their substrate specificities. We also identified a mutant Arabidopsis line containing a knock-out allele in the single copy PLMT gene found in the Arabidopsis genome, allowing us to characterize the consequences of loss of gene function in this model species.

**EXPERIMENTAL PROCEDURES**

**Yeast Growth and Strains**

Yeast were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) (w/v) media for complex media or yeast nitrogen base without amino acids for defined media (supplemented as needed with amino acids). Yeast strain CDS100 was grown on minimal media with 1 mM choline unless otherwise stated. Yeast strains used included CTY182 (MAT a, ura3-52, his3-200, lys2-801), CTY410 (MAT a his3-200, leu2Δ9, cho2::LEU2), CTY411 (MATa, ade2-101, his3-200, leu2, trp1, can1, opi3::URA3), and C SD100 (his3-200, opi3::URA3, cho2::LEU2). Strain CSD100 was obtained by crossing strains CTY410 and CTY411 using the procedure described by Guthrie and Fink (Guthrie and Fink, 1991).
Cloning of Plant PLMTs for Expression in Yeast

To place the plant PLMT cDNAs under the regulatory control of a strong constitutive yeast promoter, HindIII restriction sites were initially engineered into both the 5’- and 3’-untranslated regions of the AtPLMT and GmPLMT cDNAs via polymerase chain reaction (PCR). Subsequent digestion with HindIII enabled the insertion of the plant cDNAs between the promoter and terminator regions of the yeast ADH1 gene in yeast expression vector pDB20 (Becker et al., 1991). A BamHI digest of the resulting plasmids yielded a 3-kb band containing the yeast ADH promoter/plant PLMT/ADH terminator (partial digests were required for AtPLMT to avoid an internal BamHI site), which was gel-extracted and ligated into BamHI-digested yeast expression vector pRS313 (Sikorski and Hieter, 1989). pRS313 contains a HIS3 selectable marker that facilitated the subsequent transformation of the expression constructs into strains CTY411 (Δhis3-200) and CSD100 (Δhis3-200). Yeast transformation was conducted using the PEG/lithium acetate transformation protocol described by Gietz and Schiestl (Gietz and Schiestl, 2007).

Phospholipid N-Methyltransferase Assays

Yeast cultures were grown overnight to stationary phase, and microsomes were isolated as described by Tang et al. (Tang et al., 2005). In vitro methylation assays were performed following the protocol of Kodaki and Yamashita (Kodaki and Yamashita, 1987). Reactions included 50 µg of microsomal protein, 40 mM Tris-HCl, pH 8.8, 3 mM MgSO4, and 4 µCi of
[methyl-3H]AdoMet (0.72 µM) (60 Ci/mmol; American Radiolabeled Chemicals Inc.). A total reaction volume of 400 µl was achieved by the addition of dH2O. Samples were incubated at 30 °C for 15 min in a tube and terminated by the addition of 400 µl of 2:1 chloroform/methanol. The bottom phase was transferred to a new tube, and the remaining aqueous phase was extracted again using 400 µl of 2:1 chloroform/methanol. The pooled organic phases were washed using dH2O (40% final volume), and the extracted organic phase was evaporated to dryness and resuspended in 100 µl of 2:1 chloroform/methanol. Reaction products were spotted on silica gel 60 plates (Whatman) and resolved using chloroform/methanol/acetic acid (65:35:5, v/v/v). Labeled compounds corresponding to PtdMMEtn, PtdDMEtn, and PtdCho were identified by co-migration with authentic standards (Avanti Polar Lipids, Alabaster, AL) in adjacent lanes and quantitated using scintillation spectroscopy.

**Analysis of Arabidopsis Ds-transposon Insertion Line GT9768**

All plants used in this study were grown under 16-h/8-h light/dark cycle at 22 °C. Genomic DNA was extracted from young leaves according to Edwards *et al.* (Edwards et al., 1991). Plants were genotyped by PCR using the following three primers: forward (5'-CGTAATAAGTCCCAGCTTCACCTAACA-3') and reverse (5'-AAACAGAACTTTAAGGAGCGATTGC-3') corresponding to genomic *AtPLMT* sequences flanking the predicted insertion site, and a primer specific to the Ds element (5'-GAAACGGTCGGGAAACTAGCTCTAC-3'). The PCR conditions were as follows: 94 °C
incubation for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min in a final 7-min extension at 72 °C. For transcript analysis of line G T9763, total RNA was isolated with TRIzol™ reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized using the First Strand synthesis kit (Invitrogen). The primers used to determine AtPLMT expression were 5′-CATCGACGGTAATGGCACGTGTTT-3′ (forward) and 5′-ACCCAAGAGGCATGCCAATAGA-3′ (reverse). To amplify transcripts corresponding to the actin-8 gene control, 5′-CTTTCCGGTTACAGCGTTTG-3′ and 5′-GAAACGCGGATTAGTGCCT-3′ were used as forward and reverse primers, respectively. The PCR conditions were as follows: 94 °C incubation for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final 7-min extension at 72 °C.

**DNA and RNA Gel Analyses**

Genomic DNA was isolated from young leaf tissue according to Murray and Thompson (Murray and Thompson, 1980). Total RNA was extracted using the TRIzol™ reagent as described above. Nucleic acid blot hybridizations were conducted using either the protocols described by Sambrook and Russell (Sambrook and Russell, 2001) or using the Perfect-Hyb Plus hybridization buffer according to the manufacturer's protocol (Sigma). 32P-Labeled hybridization probes were generated using the Random Prime Labeling kit according to the method provided by the manufacturer (Roche Diagnostics). After overnight incubation with labeled probe, blots were washed as follows: two washes with 2x SSPE 0.1% SDS (1x
SSPE = 150 mM NaCl, 10 mM NaHPO₄, 1 mM EDTA, pH 7.4) at room temperature for 15 min, two washes using 1x SSPE 0.1% SDS at 65 °C for 15 min, two washes with 0.5x SSPE 0.1% SDS at 65 °C for 15 min, and a final wash using 0.1x SSPE 0.1% SDS at 65 °C for 10 min. Autoradiograms were generated by exposing the washed blots to Kodak Biomax XAR film.

$[^{14}C]$Formate Radiolabeling of Arabidopsis Seedlings

The protocol for labeling whole Arabidopsis plantlets with $[^{14}C]$formate was adapted from Hanson and Wyse (Hanson and Wyse, 1982). Arabidopsis seeds were germinated on 1% phytoagar plates with Murashige and Skoog (MS) media and 1% sucrose. Eight to 10 days after germination, plantlets were transferred aseptically to 1 ml of liquid medium containing MS salts, 1% sucrose, and 10 mM MES, pH 5.6. After equilibration in the liquid medium by growth for 24 h on a rotary shaker (120 rpm) using a 16-h light/8-h dark cycle, 20 µCi of $[^{14}C]$formate (56 mCi/mmol; Sigma) was added to the 1-ml volumes, and the seedlings were shaken in the dark for up to 24 h. Labeling was terminated by the addition of 5% trichloroacetic acid (final concentration), followed by a 20-min incubation on ice. Seedlings were rinsed with dH₂O, then ground with a micropestal in 200 µl of extraction solvent (dH₂O/ethanol/diethyl ether/pyridine at 15:15:5:1, v/v), followed by incubation at 65 °C for 20 min. One ml of chloroform/methanol (2:1, v/v) was added to each sample, vortexed thoroughly, and incubated overnight at 4 °C. To promote phase separation, 400 µl of dH₂O was added, vortexed, and centrifuged at 2 min at 13,000 x g. The bottom phase was
extracted, dried under a vacuum, and resuspended in 50 µl of chloroform/methanol (2:1, v/v). Twenty five microliters of each extract were separated on a LK5D silica gel 60- Å TLC plate and developed in a tank containing chloroform/methanol/glacial acetic acid (65:35:5, v/v/v). Regions corresponding to unlabeled standards (visualized with iodine vapor) were scraped from the plate, and radioactivity was quantified by liquid scintillation spectroscopy.

**Lipidomics Analysis**

Arabidopsis seedlings were grown on 1% phytoagar plates containing MS salts and 1% sucrose. Phospholipids were extracted from 10-day-old plants as described previously (Welti et al., 2002). Plant tissue remaining after lipid extraction was placed in a drying oven for 18 h at 80 °C and then weighed to allow for normalization according to dry weight. Lipidomics analysis was conducted using ESI/MS/MS by the Kansas State Lipidomics Research group.

**RESULTS**

**Identification of PLMT Homologs from Arabidopsis and Soybean**

Using the rat PEMT protein and yeast Opi3p as a query sequences, tblastn searches of the expressed sequence tag (EST) databases of GenBank™ revealed an Arabidopsis EST whose predicted protein product shared homology with the rat and yeast sequences. No Arabidopsis sequences with homology to the yeast Cho2p sequence were observed. A partial cDNA lacking part of its 5′ sequence was obtained from the Arabidopsis Biological Resource Center, and a full-length clone was obtained using 5′-rapid amplification of cDNA ends PCR.
A homologous sequence from soybean was obtained by screening a soybean cDNA library using the Arabidopsis cDNA as a probe. As shown in Fig. 2, the predicted Arabidopsis and soybean proteins are 164 and 167 amino acids in size, respectively. The plant proteins share 74% sequence identity to each other, but only about 25% identity to either the yeast Opi3p or rat PEMT enzymes. The presence of three predicted membrane-spanning domains suggests that the plant proteins function as integral membrane proteins.
FIGURE 2: Predicted protein sequence and structure of Arabidopsis and soybean PLMT genes. 

A, amino acid alignment of PLMT proteins from Arabidopsis (GenBank access number BT043493), soybean (GenBank access number FJ858262), yeast, and rat. Residues shown to function in AdoMet binding in the rat PEMT are indicated by asterisks. Amino acids identical to those found in either plant species are shaded black; conservative substitutions are shaded gray. Predicted transmembrane domains for the plant PLMTs are indicated by dotted lines. 

Analysis of the complete Arabidopsis genome sequence showed that the PLMT-like sequence exists as a single copy within the genome (GenBank access number At1g80860). Investigation of the soybean genome revealed two distinct chromosomal segments possessing PLMT-like genes (data not shown). Chromosome 8 contains the gene that corresponds to the soybean cDNA isolated from the library screen. The other soybean
PLMT-like sequence (found on chromosome 7) is likely to be a pseudogene, as described below. DNA blotting results were consistent with the interpretation that soybean possesses two loci with homology to this sequence (supplemental Fig. 1).

The genomic structures of the Arabidopsis and soybean (chromosome 8) PLMT-like genes are shown in Fig. 2B. Both genes contain a single intron midway between two protein-encoding exons. Comparison of Arabidopsis EST sequences with the At1g80860 genomic sequence reveals that two transcript variants originate from this gene. A 73-bp intron within the 3'-untranslated region of the gene is excised in about half of the Arabidopsis ESTs corresponding to At1g80860 (data not shown). Comparison of the soybean PLMT-like gene on chromosome 8 with that on chromosome 7, together with their corresponding ESTs, showed that a series of small deletions and a splice junction mutation in the intron results in incomplete, frame-shifted transcripts from the latter gene that would not be likely to produce a functional protein (data not shown). It is therefore probable that the gene residing on chromosome 8 is the only viable copy within the soybean genome.

**Biochemical Function and Substrate Specificity**

Because of the low primary sequence identity shared between the rat and yeast PLMT protein sequences and the predicted products of the Arabidopsis and soybean PLMT-like sequences (~25%), it was incumbent to test whether the putative plant proteins were indeed functional homologs of the animal and yeast enzymes. According to the most widely accepted model of PtdCho biosynthesis in plants (Fig. 1), PtdMMETn and PtdDMEtn would be the most likely substrates for a plant PLMT enzyme. Because of the integral membrane nature of PLMTs, we
took advantage of mutant yeast strains to test the function and substrate specificities of the candidate plant PLMTs. Yeast strains possessing an *opi3* knock-out allele display negligible PtdMMEtn and PtdDMEtn methylation activities and have been shown to accumulate high levels of the Cho2p product PtdMMEtn (McGraw and Henry, 1989). Full-length cDNAs of both the Arabidopsis and soybean *PLMT*-like sequences were cloned into a yeast transformation vector and introduced into the *opi3* knock-out strain CTY411. Microsomal preparations were incubated with [3H]AdoMet and assayed for their ability to methylate the endogenous phospholipids within this membrane fraction.

The results of the *in vitro* methylation assays are shown in Fig. 3. All possible PtdEtn derivatives (PtdMMEtn, PtdDMEtn, and PtdCho) were readily observed using microsomal membranes from a wild-type control yeast strain (CTY182). As expected, PtdMMEtn was the only product observed from microsomes of CTY411 transformed with the control vector alone. In CTY411 cells expressing either the Arabidopsis or soybean cDNAs, however, radiolabeled PtdDMEtn and PtdCho species were observed in addition to PtdMMEtn (Fig. 3A), demonstrating that the plant enzymes were capable of catalyzing the final two steps in the methylation pathway of PtdCho synthesis in yeast. Because of their abilities to N-methylate phosphatidylamino alcohol substrates *in vitro*, the Arabidopsis and soybean genes were designated *AtPLMT* and *GmPLMT*, respectively. Although both plant enzymes gave the same reaction products, under our assay conditions, microsomal fractions recovered from yeast expressing *AtPLMT* consistently displayed greater activity than those observed from yeast expressing *GmPLMT*.
FIGURE 3. Plant PLMTs methylate PtdMMEtn and PtdDMEtn. A, in vitro phospholipid N-methylation assay utilizing microsomes from wild-type yeast strain CTY182 and opi3 mutant strain CTY411 expressing AtPLMT, GmPLMT, and vector control. Error bars represent S.E. (n=3). B, strain CSD100 (opi3/cho2) grown on minimal media (M.M.) alone or supplemented with 1 mM Cho or 1 mM MMEtn-P. CSD100 was transformed with the pRS313 vector control (1), GmPLMT (2), and AtPLMT (3).
Classical biochemical studies have suggested that plants are fundamentally different from animals or yeast by not being able to produce PtdCho directly via methylation of PtdEtn (Marshall and Kates, 1973; Datko and Mudd, 1988a; Kinney, 1993). This concept is further supported by the fact that previous efforts using Arabidopsis and spinach cDNA libraries to complement yeast strains defective in the PtdCho methylation pathway only recovered genes encoding enzymes capable of methylating phospho-base substrates (Bolognese and McGraw, 2000; Nuccio et al., 2000). If the hypothesis is true that plant cells cannot synthesize PtdCho directly from PtdEtn, then plant PLMT enzymes, unlike their animal and yeast counterparts, should be incapable of utilizing PtdEtn as a substrate. We tested this by determining whether the Arabidopsis or soybean PLMT genes could complement a yeast strain with knock-out mutations in both the cho2 and opi3 loci. In yeast, choline auxotrophy is not mediated by the cho2 mutation alone because the Opi3p enzyme is capable of catalyzing a low level of PtdEtn methylation (Kanipes and Henry, 1997). Yeast possessing cho2/ opi3 double mutations, however, have an absolute requirement for exogenous choline. Yeast strain CDS100 (cho2/ opi3) was generated through mating of CTY410 (cho2) with CTY411 (opi3). AtPLMT and GmPLMT cDNAs were cloned downstream of the strong constitutive alcohol dehydrogenase promoter of yeast and transformed into CDS100.

As shown in Fig. 3B, the expression of plant PLMTs failed to support the growth of the cho2/ opi3 mutant on minimal media lacking choline. The inability to complement choline auxotrophy in CDS100 suggests that neither AtPLMT nor GmPLMT can serve as PtdEtn N-methyltransferases. To verify that the plant enzymes are functionally expressed in CDS100,
each strain was grown on minimal media supplemented with MMEm-P, a metabolite that can be incorporated into PtdMMEm via enzymes of the nucleotide pathway. Although CDS100 transformed with the empty vector control remained inviable on this media, expression of both plant PLMT genes restored growth (Fig. 3B). Consistent with the results obtained from the in vitro enzyme assays, expression of AtPLMT gave a stronger growth restoration phenotype than GmPLMT. Microsomal membrane preparations of strain CDS100 expressing the plant PLMTs (grown in minimal media supplemented with choline) were also tested in the in vitro methylation assay described above. No traces of radiolabeled PtdMMEm were detected using this assay (data not shown). Cumulatively, the expression studies in yeast support the claim that plant PLMT enzymes are able to carry out the final two steps of the traditional PtdEmt to PtdCho methylation pathway, but they cannot catalyze the initial methylation of PtdEmt.

Expression of AtPLMT

Examination of the Affymetrix-based expression profiling database AtGenExpress suggests that AtPLMT (At1g80860) is expressed at relatively similar levels in most plant tissues, except fully mature or senescent tissue where a modest decrease in transcript accumulation is observed (data not shown). Furthermore, AtPLMT expression levels appear to be largely unaffected by exposure to either biotic or abiotic stresses. In Saccharomyces cerevisiae, PLMT activities are regulated at the level of transcription in response to soluble phospholipid precursors (Gaynor et al., 1991; Kanipes and Henry, 1997). Specifically, CHO2
and OPI3 mRNA accumulation is repressed in media containing inositol and/or choline. To address whether AtPLMT expression is regulated in a similar manner, Arabidopsis seedlings were grown on MS media supplemented with varying levels of choline or inositol. RNA blot analysis revealed no obvious effect on AtPLMT transcript levels in response to either compound (supplemental Fig. 2). Consistent with the observation of two classes of At1g80860-derived ESTs (the 3’-untranslated region either spliced or unspliced as shown in Fig. 2B), AtPLMT transcripts appear on RNA blots as a near equimolar doublet.

**Characterization of AtPLMT Function in Planta**

In animals and yeast, PLMTs serve critical functions, representing the sole route for de novo choline synthesis in these systems. The existence of the well-characterized phospho-base pathway for de novo choline synthesis in plants, however, raises interesting questions regarding the necessity of the PLMT enzymes in plants. To explore this issue, a reverse genetic approach was taken to ascertain the consequences of the loss of AtPLMT gene function in Arabidopsis. A Ds-transposon insertion mutant line, GT9768, was identified in the Cold Spring Harbor Genetrapper Collection (Martienssen, 1998). DNA sequence analysis revealed that the Ds element was inserted at the first nucleotide of the exon1-intron1 junction (supplemental Fig. 3A). PCR-based genotype analysis of numerous GT9768 progeny showed that the line was homozygous for the insertion allele (data not shown). To assess the effect of the insertion event on AtPLMT expression, total RNA was isolated from wild-type Arabidopsis and the GT9768 line and analyzed by reverse transcriptase-PCR. Using
forward and reverse primers corresponding to exon 1 and exon 2, respectively, *AtPLMT*-specific amplification products were readily detected in wild-type RNA preparations but could not be observed using GT9768 RNA (supplemental Fig. 3B). The failure to detect *AtPLMT* transcripts in GT9768 suggests that the Ds element created a null mutation. The insertion mutation in GT9768 at the *AtPLMT* locus was also confirmed by Southern blot analysis (supplemental Fig. 3C).

When grown under standard growth conditions, either in soil or on MS nutrient plates lacking choline, *atplmt* conferred no obvious morphological or developmental phenotype (data not shown). To gain insights into the effect of the mutant *atplmt* allele at the cellular level, experiments were conducted to compare the synthesis and accumulation of phospholipids with methylated amino alcohol headgroups (PtdMMEtn, PtdDMEtn, and PtdCho) in wild-type versus GT9768 plants. Initially, young Arabidopsis seedlings were incubated in the presence of [14C]formate for up to 24 h. Formate is rapidly incorporated into the methyl donor group of AdoMet via the 1-carbon pathway (Hitz et al., 1981). Labeling was carried out in the dark to minimize the incorporation of labeled carbon that had been oxidized to CO₂, which could then be integrated into other carbon moieties of phospholipids through photosynthetic respiration (McNeil et al., 2000). As shown in Fig. 4A, 14C counts incorporated into PtdMMEtn were significantly greater in the mutant line at all time points assayed. The differential was particularly dramatic at the 2-h time point, where 14C counts in PtdMMEtn exceeded that found in PtdCho in the mutant Arabidopsis plants. [14C]PtdDMEtn levels in this study were too low to accurately measure above background.
Arabidopsis atplmt knock-out mutants accumulate elevated levels of PtdMMEtn and PtdDMEtn. A, time course labeling of intact 8–10-day-old plantlets with $[^{14}C]$sodium formate. Data presented show the mean ± S.E. of six biological replications using wild-type Arabidopsis (dashed line) and atplmt mutant line GT9768 (solid line). B–D, steady-state quantification of PtdMMEtn and PtdDMEtn (B), major phospholipids (C), and galactolipids (D) using ESI-MS/MS. Wild-type Arabidopsis is represented by white bars and GT9768 by gray bars. Error bars represent the S.E. of five biological replications. For PtdMMEtn and PtdDMEtn, differences were found to be significant as determined by Student's t test ($p < 0.05$).

To establish whether the lack of AtPLMT activity results in an increase in the steady-state levels of PtdMMEtn and PtdDMEtn in GT9768 plants, whole plant lipid extracts were analyzed using ESI/MS/MS at the Kansas State Lipidomics Research Center. PtdMMEtn and PtdDMEtn levels were increased by ~9- and 3.5-fold, respectively, in atplmt mutant plants compared with wild-type Arabidopsis (Fig. 4B). No significant change, however, was observed in the steady-state pool of PtdCho (Fig. 4C). These observations suggest that...
production of PtdCho strictly through the phospho-base pathway and/or modulation of optimal PtdCho levels via phospholipase activities can compensate for deficiencies in PtdCho synthesis mediated by the PLMT enzyme. Similar to PtdCho, no differences were observed in any of the other major phospholipids or galactolipids in GT9768 plants (Fig. 4, C and D).

**Fatty Acid Composition of Phosphatidylamino Alcohols**

In animal cells it has been shown that PtdCho synthesized through the nucleotide pathway is not necessarily functionally equivalent with PtdCho produced via the methylation pathway. Differences in acyl chain composition between the two sources of PtdCho is believed to be one of the main causes for this lack of redundancy (DeLong et al., 1999; Watkins et al., 2003). In yeast as well, substrate selectivity of the Cho1p and Opi3p enzymes results in the formation of PtdCho pools whose acyl composition differs from that derived through the enzymes of the nucleotide pathway (Boumann et al., 2004). To determine whether the flux of metabolites through the PLMT enzyme has the potential of yielding PtdCho species that differ from that produced strictly through the phospho-base/nucleotide route (Fig. 1), the fatty acid composition of the individual phosphatidylamino alcohols was quantified in both GT9768 and wild-type Arabidopsis by ESI/MS/MS. No differences in PtdCho fatty acid profiles were observed between mutant and wild-type plants (Fig. 5). Although it cannot be concluded from this result that AtPLMT lacks substrate specificity based on acyl chain composition, it is clear that AtPLMT is not solely responsible for producing any specific PtdCho species, as has been shown for PEMT in the animal model. Similarly, there were no
differences observed in the fatty acid compositions of PtdEtn, PtdMMETn, and PtdDMEtn (Fig. 5) or any of the other major phospholipid and galactolipid species (data not shown) between wild-type and atplmt Arabidopsis plants. Even though no differences were observed in the fatty acid compositions of the phosphatidylamino alcohols in mutant versus wild-type plants, one notable observation from this analysis was the lack of 34:3 fatty acid species in PtdMMETn and PtdDMEtn. Although 34:3 fatty acids (presumably composed of 16:0 + 18:3) account for ~15% of the total PtdEtn and PtdCho species in both genotypes, this acyl combination is nearly undetectable in PtdMMETn and represents less than 3% of PtdDMEtn (Fig. 5).

FIGURE 5. ESI-MS/MS derived data showing distribution of acyl chains among PtdEtn, PtdMMETn, PtdDMEtn, and PtdCho in wild-type (white bars) and GT9768 (atplmt) Arabidopsis plants. The 34:3 acyl species found to be disproportionately lower in PtdMMETn and PtdDMEtn are highlighted with an asterisk. Wild-type Arabidopsis is represented by white bars and GT9768 by gray bars.
DISCUSSION

This study represents the first characterization of genes encoding the PLMT branch of the PtdCho biosynthetic pathway in higher plants. Similar to the rat PEMT and yeast Op3p proteins, the predicted soybean and Arabidopsis PLMT enzymes are small, displaying calculated molecular masses of 18.8 and 19.6 kDa, respectively. The observation of multiple predicted membrane-spanning domains is consistent with the localization of PLMT activities within microsomal membrane fractions of disrupted plant cells (Marshall and Katse, 1973; Datko and Mudd, 1988b). Despite catalyzing similar reactions, the Arabidopsis and soybean PLMT enzymes share only ~25% primary amino acid sequence identity with their mammalian and yeast counterparts. Particularly intriguing is the nature of the AdoMet-binding sites in the plant enzymes. Because PLMTs in general do not contain AdoMet-binding motifs that are typical of most non-DNA AdoMet-dependent methyltransferases, Shields et al. (Shields et al., 2003) conducted a study to define the sites involved in AdoMet binding for human PEMT. Two AdoMet-binding sites were identified in human PEMT, a GXG motif (X = any amino acid) located in the middle of the protein, and an EE motif near the C terminus (Fig. 2). Both enzymatic activity and AdoMet binding in the human PEMT were completely abolished when the second glycine residue in the GXG motif or the first glutamic acid of the EE sequence was replaced with conservative amino acid substitutions. The soybean and Arabidopsis enzymes, however, lack these critical residues. A small deletion in the plant PLMTs (relative to the rat and yeast sequences) occurs at the residues corresponding to the XG of the GXG motif, and a non-conservative
lysine residue is found in place of the first, presumably invariant, glutamate in the EE motif (Fig. 2). These observations suggest that plant PLMTs utilize different motifs to bind and orient the AdoMet substrate. Furthermore, the observation that plant PLMTs exclude PtdEtn as a substrate, in contrast to the mammalian enzyme that readily methylates PtdEtn (as well as yeast Opi3p to a lesser extent), also suggests that there are significant differences in the tertiary structures of plant versus animal and yeast PLMTs.

The inability of plant PLMTs to utilize PtdEtn as a substrate coupled with several classical biochemical studies collectively suggests that the direct methylation of PtdEtn does not occur in plants (Marshall and Kates, 1973; Datko and Mudd, 1988a; Kinney, 1993). This means that the de novo synthesis of a choline moiety must originate via the methylation at the phospho-base level by the PEAMT enzyme. MMEtn-P and DMEtn-P can serve as substrates for the nucleotide pathway, which results in the synthesis of PtdMMEtn or PtdDMEtn lipid intermediates. Wang and Moore (Wang and Moore, 1990) showed that the choline-phosphate cytidylyltransferase of castor bean has a similar level of activity using either MMEtn-P or Cho-P as a substrate. Therefore, for most plants species it appears that the flow of metabolites through phospho-base versus phosphatidyl-base intermediates is dictated by the relative efficiencies of the cytidylyltransferase enzyme(s) with the PEAMT enzymes as they compete for common MMEtn-P and DMEtn-P substrates.

The elimination of AtPLMT gene function in Arabidopsis did not lead to any obvious perturbations in normal plant growth and development, but it did result in increased PtdMMEtn and PtdDMEtn accumulation, lipid species that are typically found in only trace
amounts in membrane fractions. The 9- and 3.5-fold increases in PtdMMEtn and PtdDMEtn concentrations, respectively, in atplmt plants elevated the accumulation of these species to levels similar to that observed for phosphatidylserine (Fig. 4). In contrast, the steady-state levels of the end product of the PLMT reaction, PtdCho, remained unchanged. Given that the phospho-base route alone appears to be sufficient for supplying adequate concentrations of PtdCho within atplmt mutant plants (Fig. 4), it is worth speculating on why PLMT function has been maintained during Arabidopsis evolution. Perhaps the most reasonable explanation would be that PLMT functions to optimally channel metabolites of the PtdCho pathway to the desired end product, and thus minimize the accumulation of the PtdMMEtn and PtdDMEtn intermediates. Although the enhanced accumulation of these minor phosphatidylamino alcohols appears to be benign when atplmt plants are grown in an ideal environment, they may be deleterious during growth at suboptimal conditions, or when the plant is exposed to biotic or abiotic stresses. Subjecting atplmt plants to an array of stress conditions would be a high priority for future endeavors to elucidate gene function on whole plant physiology.

Because of the great variability observed among different plant species with regard to the methylation of amino alcohol intermediates in the PtdCho pathway, the conclusions derived from our study of the Arabidopsis atplmt mutant may not necessarily reflect the relative importance of this gene in other higher plants. In soybean, for example, in vivo labeling studies conducted both in cell cultures and leaf disks, and in vitro enzyme assays using cell fractions, provided clear evidence that the final two methylation reactions
occur exclusively at the phosphatidyl-base level in this species (Datko and Mudd, 1988a, 1988b). Therefore, the soybean \textit{GmPLMT} gene would be predicted to be essential for PtdCho synthesis, and its inactivation would likely result in a lethal phenotype. In contrast, although the disruption of PLMT activity in \textit{L. paucicostata} would likely yield a phenotype similar to that observed in Arabidopsis, minimal metabolite flux was shown to occur through phosphatidyl-base intermediates in this system, despite the fact that PLMT activities were readily measured \textit{in vitro} (Datko and Mudd, 1988a, 1988b).

Although no differences were observed in the fatty acid profiles of the phosphatidylamino alcohol lipids from wild-type \textit{versus atplmt} Arabidopsis plants, the lack of 34:3 species within PtdMMEtn and PtdDMEtn of both genotypes was notable, particularly in light of the fact that this combination is very prevalent in PtdEtn and PtdCho (Fig. 5). Because of the virtual absence of unsaturated 16 acyl chain species within all phospholipids of Arabidopsis except phosphatidylglycerol (Browse et al., 1986), it is reasonable to assume that the 34:3 species detected in this analysis were composed of 16:0 plus 18:3. The near absence of this fatty acid combination in PtdMMEtn and its great reduction in PtdDMEtn could be explained by the exclusion of the corresponding diacylglycerol species by the amino alcohol phosphotransferase enzyme when presented with a CDP-MMEtn or CDP-DMEtn substrate. Alternatively, cellular pools of 16:0/18:3 diacylglycerol may be inherently low, and the abundance of this fatty acid combination in PtdCho and PtdEtn could be a reflection of 16:0/18:2 PtdCho and PtdEtn species serving as good substrates for the \textit{FAD3}-encoded \textit{ω}-3 desaturase and the corresponding species of PtdMMEtn and PtdDMEtn.
representing poor substrates for this enzyme. Finally, the paucity of 16:0/18:3 PtdMMEtn and PtdDMEtn could be explained by enhanced susceptibility of these species to phospholipase degradation, or even through an acyl-editing mechanism as recently proposed by Bates et al. (Bates et al., 2007).

In conclusion, through the heterologous expression of candidate cDNAs in yeast, we have demonstrated that the AtPLMT and GmPLMT genes identified in this study encode PLMT enzymes involved in PtdCho biosynthesis. The enhanced accumulation of PtdMMEtn and PtdDMEtn intermediates in an Arabidopsis atplmt mutant line confirmed that the AtPLMT gene product functions within the PtdCho pathway in vivo. The identification and characterization of the genes encoding PLMT activity in plants not only enhance our understanding of the PtdCho pathway per se but also provide an important tool for further defining the great variability observed among different plant species regarding the specific route by which PtdCho is synthesized within the plant cell and how this process is regulated.

ACKNOWLEDGEMENTS

We would like to thank the lab of Dr. Leo Parks for assistance in making the yeast crosses that generated strain CDS100. We also thank Dr. Ruth Welti and the Kansas Lipidomics Research Center for phospholipid and fatty acid analyses of the Arabidopsis plants. Kansas Lipidomics Research Center was supported by National Science Foundation Grants MCB0455318 and D B I 0521587, and National Science Foundation EPSCoR Grant EPS-
0236913 with matching support from the State of Kansas through Kansas Technology Enterprise Corporation and Kansas State University. The Kansas Lipidomics Research Center is also supported by K-INBRE (National Institutes of Health Grant P20 RR16475 from the INBRE program of the National Center for Research Resources). This research was supported by a grant from the National Science Foundation (IBN-9513582) and the Initiative for Future Agriculture and Food Systems (2001-52101-11507) from the USDA Cooperative State Research, Education, and Extension Service.
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of Arabidopsis reveals a critical role for phospholipid metabolism in root system development and epidermal cell integrity. Plant Cell 16: 2020-2034


SUPPLEMENTAL FIGURE S1. Southern blot analysis of genomic soybean DNA (10 μg) digested with BamHI and XbaI, and hybridized with a radiolabeled *GmPLMT* cDNA. The two images are autoradiographs of the same blot in non-adjacent lanes.
SUPPLEMENTAL FIGURE S2. RNA blot analysis of *AtPLMT*. Each lane represents three μg of total RNA from 10 day old Arabidopsis plants grown on a growth medium containing MS salts, 1% sucrose and different concentrations of choline or inositol as indicated. Blots were incubated with radiolabeled *AtPLMT* cDNA as the hybridization probe. Ethidium bromide-stained gel image of the 18S ribosomal RNA is shown as a loading control. Panel on the right highlights the doublet nature of the *AtPLMT* transcripts.
SUPPLEMENTAL FIGURE S3. Analysis of the GT9768 atplmt mutation line. A, Diagram showing the insertion site of the Ds transposon in GT9768 as determined by DNA sequence analysis. Exon sequences are presented in bold type, intron sequence is italicized. B, Reverse transcriptase-PCR of RNA isolated from wild-type (left) or GT9768 (right) Arabidopsis plants. Primers corresponded to sequences specific for exon 1 and exon 2 of AtPLMT. Primers specific for the Actin 8 gene were used as a control. C, Southern blot analysis of wild-type and GT9768 Arabidopsis plants. Blots contained five μg of total genomic DNA digested with EcoRV. Radiolabeled AtPLMT cDNA was used as the hybridization probe.
CHAPTER 3

MUTATIONS IN TWO ARABIDOPSIS PHOSPHATIDYLINOSITOL TRANSFER PROTEIN HOMOLOGS ALTER SEED GERMINATION RESPONSES TO HYPEROSMOTIC STRESS AND ABSCISIC ACID

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INTRODUCTION

Phosphatidylinositol transfer proteins (PITPs) are diffusible carriers of phosphatidylinositol (PtdIns) that were originally characterized because of their abilities to transport PtdIns, and to lesser extent phosphatidylcholine (PtdCho), between two distinct membrane bilayers \textit{in vitro} (Wirtz, 1997). Ubiquitous in eukaryotic species, PITPs can be divided into two major families: (1) those that show structural homology to PITPs originally characterized in mammals; and (2) those that share sequence homology to Sec14p, the prototypical PITP from yeast (Routt and Bankaitis, 2004; Phillips et al., 2006). Mammalian PITPs and yeast Sec14p are very similar in size (~35 kDa) and and display nearly identical \textit{in vitro} PtdIns and PtdCho transfer activities, yet share no primary amino acid sequence homology with each other.

Since its discovery as a protein whose function is required for the transport of secretory proteins from the Golgi to the plasma membrane (Bankaitis et al., 1990), yeast Sec14p has been the subject of intense investigation. Early work focused on a series of mutations that were shown to bypass the requirement of Sec14p for normal Golgi function and cell viability, several of which were shown to encode enzymes of the cytidine diphosphate (CDP)-choline pathway of PtdCho biosynthesis (Cleves et al., 1991). These studies led to a model of Sec14p functioning as a "molecular sensor" that mediates secretory competence through monitoring and maintaining optimal levels of PtdIns and PtdCho within the \textit{trans}-Golgi membranes (Skinner et al., 1995). More recently, considerable evidence has accumulated showing that Sec14p interacts with a specific PtdIns 4-kinase (Pik1p) to generate a pool of PtdIns(4)P that is essential for vesicle trafficking and forespore membrane
biogenesis during sporulation (Phillips et al., 2006). Investigation of five additional yeast proteins that share homology to Sec14p (Sfh1p - Sfh5p) has also lent strong support to the concept that stimulation of PtdIns 4-kinase activity is a common mechanism of function for Sec14p-like PITPs (Routt et al., 2005). This characteristic of Sec14p again resembles that of mammalian PITPs which have been shown to function as activators of the phosphoinositide signaling pathway via interaction with PtdIns 4-kinases. For both Sec14p and mammalian PITPs, stimulation of PtdIns 4-kinase activities is believed to be mediated through an indirect mechanism whereby the PITP enhances delivery of PtdIns monomers to specific PtdIns 4-kinase enzymes (Allen-Baume et al., 2002; Schaaf et al., 2008).

All characterized PITPs in higher plants are of the Sec14p-like family. Although genes encoding proteins with homology to Sec14p are prevalent in plant genomes, very few of these genes have been studied. Arabidopsis, for example, possesses 31 open reading frames (ORFs) that encode sequences with homology to Sec14p (Vincent et al., 2005). However, most of these predicted protein sequences are much larger than Sec14p, having structures comprised of a Sec14p-homologous lipid binding domain within a protein containing additional functional motifs. Eleven of the 31 Sec14p-homologous Arabidopsis sequences possess a COOH-terminal nodulin domain that has been associated with targeting proteins to the plasma membrane (Vincent et al., 2005). Two groups have shown that one of these Sec14p-nodulin domain proteins is required for normal root hair growth. Bohme et al. (2004) isolated a Sec14p-nodulin domain gene called Can of Worms 1 (COW1) by the mapped-based cloning of an Arabidopsis mutant displaying a shortened root hair morphology.
In an independent study, a T-DNA insertion mutation within this same gene (designated \textit{AtSfh1} in this report), also yielded plants with shortened, morphologically abnormal root hairs (Vincent et al., 2005). \textit{Atsfh1p} appears to function by generating phosphoinositide landmarks at the tip of the root to direct vesicle deposition and maintain a single focal point of polar growth. Expression of \textit{Atsfh1p} in yeast lacking endogenous \textit{Sec14p} stimulated synthesis of PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P$_2$ (Vincent et al., 2005).

In addition to the 11 \textit{Sec14p}-nodulin gene names, the Arabidopsis genome possesses six genes with \textit{Sec14p} domains immediately upstream of a \textit{Golgi} dynamics (GOLD) domain. One of these, designated \textit{Patellin 1} (\textit{PATL1}) for "little-plate," was determined to be an F-actin interacting protein that is localized to the cell plate during cell division (Peterman et al., 2004). \textit{PATL1} was shown to bind specific phosphoinositides, and it was proposed that the protein functions to promote vesicle formation during cell plate expansion or maturation.

Although most plant proteins that share homology with \textit{Sec14p} contain a \textit{Sec14p} lipid binding domain within the context of a larger protein, homologs also exist that are similar in size to \textit{Sec14p} that do not appear to possess additional motifs. The best characterized of these is \textit{Ssh1p}, a 37 kDa soybean \textit{Sec14p} homolog that was identified through complementation of a yeast strain possessing a temperature-sensitive \textit{sec14} mutation (Kearns et al., 1998). \textit{Ssh1p} is unique among \textit{Sec14p}-like proteins in that it appears to serve as a component in a hyperosmotic stress-inducible signal transduction pathway. When expressed in either yeast or plants, \textit{Ssh1p} is rapidly phosphorylated upon exposure of cells to severe hyperosmotic stress,
a modification that reduces its ability to bind cellular membranes (Kearns et al., 1998; Monks et al., 2001). In soybean, osmotic stress-inducible phosphorylation of Ssh1p is mediated by SPK1 and SPK2, members of the SnRK2b subfamily of the SNF1-related family of protein kinases (Monks et al., 2001). These observations, coupled with the demonstration that recombinant Ssh1p can stimulate the synthesis of the PtdIns(4)P and PtdIns(3)P in vitro, lead to the speculation that Ssh1p functions within an osmotic stress signaling cascade through altering some aspect of phosphoinositide metabolism (Monks et al., 2001).

Although these previous studies have yielded compelling evidence implicating Ssh1p as an intermediate in an osmotic stress-inducible signaling pathway, the role this protein plays at the whole plant level remained unclear. In this study, we characterize Arabidopsis plants with T-DNA mutations in AtSec14-1 and AtSec14-5, two closely related homologs of the soybean SSH1 gene. Plants possessing mutations at both loci display hypersensitivity to high osmotic stress and abscisic acid (ABA) during germination. Double mutant plants also synthesize and accumulate less PtdIns monophosphate (PtdInsP) species than wild type Arabidopsis. Collectively, these results suggest that Ssh1p-like PITPs function in planta to stimulate PtdInsP synthesis, and that the pools of PtdInsP that are regulated by these proteins are important in maintaining germination competence under suboptimal conditions, such as high osmotic stress.
RESULTS

*Arabidopsis Ssh1p Homologs*

Although the Arabidopsis genome possesses 31 genes encoding proteins that share some level of homology to yeast Sec14p, only two of these display greater than 30% amino acid identity with the soybean Ssh1p protein (data not shown). Arabidopsis genes *At1g55840* and *At5g47730* encode Sec14p-like proteins that share 71% and 63% identity with Ssh1p, respectively, and 69% identity with each other. Similar to Ssh1p from soybean, the *At1g55840* gene product was originally identified because of its ability to complement the temperature sensitive *sec14-1ts* mutation of yeast (Jounnic et al., 1998).
Figure 1. Structure, sequence and analysis of T-DNA insertion mutations for Arabidopsis SSH1 homologs. A, Genomic structures of AtSec14-1 (At1g55840), AtSec14-5 (At5g47730) and soybean SSH1. Black boxes, depicting exons, are drawn to scale; lines indicating intron regions are not to scale. Location of T-DNA insertion mutations in AtSec14-1 and AtSec14-5 loci in Salk lines 120972 and 039575, respectively, are indicated. B, Predicted amino acid sequences of AtSec14-1, AtSec14-5 and soybean Sh1p. Positions where amino acids are identical among the three proteins are box-shaded black; residues identical between two of the three proteins are shaded gray. C, RT-PCR expression analysis of leaf tissue from T-DNA insertion mutation lines. Genotypes of plants assayed are indicated at the top, and the gene that is the target of a specific RT-PCR amplification is shown on the left. Gene-specific primer sequences can be found in “Materials and Methods”.
Figure 1 shows the gene structures and predicted amino acid sequences of the \textit{At1g55840} and \textit{At5g47730} putative Sh1p orthologs, hereafter designated \textit{AtSec14-1} and \textit{AtSec14-5}, respectively. These genes display similar gene structures, both to each other and to the soybean \textit{SSH1} locus. The \textit{AtSec14-5} and soybean \textit{SSH1} genes are comprised of 11 exons and 10 introns, with the location of each exon/intron junction precisely conserved. The structure of \textit{AtSec14-1} differs by the absence of intron 9; the sequences corresponding to exons 9 and 10 in \textit{SSH1} and \textit{AtSec14-1} are represented by a single exon in \textit{AtSec14-1} (Fig. 1A). Although the two Arabidopsis predicted proteins and soybean Sh1p are very similar in size (~37 kDa), examination of Arabidopsis expressed sequenced tagged databases suggests the possibility of alternative splicing which may give rise to different sized products (data not shown).
In the study by Jouanne et al. (1998), RNA blot analysis revealed that *AtSec14-1* transcripts were present in each of four tissues tested, with floral tissue showing the highest levels of transcript accumulation. Examination of the gene expression profiling databases AtGenExpress ([http://jsp.weigelworld.org/expviz/expviz.jsp](http://jsp.weigelworld.org/expviz/expviz.jsp)) and Genevestigator ([http://www.genevestigator.com/](http://www.genevestigator.com/)) revealed that: (1) *AtSec14-1* is more highly expressed than *AtSec14-5*; and (2) both genes maintain relatively consistent levels of expression throughout normal plant growth and development. The two major exceptions are mature pollen and mature seed tissue, where transcript accumulation for both genes is substantially reduced in comparison to other plant tissues (data not shown).

**T-DNA Insertion Lines**

A survey of the Salk T-DNA insertion collection curated by the Arabidopsis Biological Resource Center (Columbus, Ohio) revealed one T-DNA insertion mutant for each of the Arabidopsis **SSH1** homologs. For each mutant line, plants were genotyped by PCR to identify individuals that were homozygous for the T-DNA insertion. The specific location of T-DNA integration was confirmed by DNA sequence analysis of PCR products spanning the insertion sites. Line 120972 possesses a T-DNA insert in exon two of *AtSec14-1*; line 039575 contains a T-DNA insertion in exon 10 of *AtSec14-5* (Fig. 1A). Despite being located near the end of *AtSec14-5*, the T-DNA insert in line 039575 would truncate the predicted protein product by approximately one-third, including domains that have been shown to be critical for Sec14p function in yeast. To determine the effects of the T-DNA insertions on wild type
gene expression, reverse transcriptase polymerase chain reaction (RT-PCR) was conducted using RNA extracted from wild type Arabidopsis plants (ecotype Columbia), individuals homozygous for the AtSec14-1 and AtSec14-5 insertion mutations, and double homozygous mutant plants that were generated by crossing the individual mutants, followed by molecular genotyping at the F2 generation. As shown in Figure 1C, no evidence of wild type AtSec14-1 or AtSec14-5 transcripts could be detected in the mutant lines by RT-PCR analysis using primers that flank the T-DNA insertion sites.

**Physiological Analysis**

Because our previous work strongly suggested that soybean Ssh1p was involved in osmotic stress signaling (Kearns et al., 1998; Monks et al., 2001) we hypothesized that plants lacking AtSec14-1 and/or AtSec14-5 function may be compromised in some aspect of hyperosmotic stress tolerance. To test this possibility, mutant and wild type Arabidopsis plants were subjected to a variety of hyperosmotic stress conditions. For the majority of the treatments tested, no significant differences in growth or morphology were observed between wild-type plants and either single AtSec14 mutant, or double mutant individuals (data not shown). The one notable exception involved growth assays of seedlings germinated on high-osmotic media. In these assays, the double mutant lines consistently displayed a reduced germination phenotype in comparison to either wild type plants or plants homozygous for the individual atsec14-1 and atsec14-5 T-DNA insertion mutations. As shown in Figure 2, a 40% reduction in germination was observed when atsec14-1/atsec14-5 seeds were germinated on MS media.
containing 300 mM mannitol, a treatment that did not significantly reduce germination for the other three genotypes tested. Although supplementation of MS agar with 150 mM NaCl resulted in a approximately 30-35% reduction in germination for wild type, *atsec14-1*, and *atsec14-5* genotypes, the *atsec14-1/atsec14-5* double mutant plants averaged a 70% reduction in germination frequency. More modest levels of osmotic stress (50 mM NaCl; 100 mM mannitol) failed to impair germination for any of the four genotypes included in this study (Fig. 2). To ensure that differences in germination phenotype would not be confounded by differences in seed lot age, for all of the above-described studies, age-matched seed from all genotypes was collected from plants that had been grown simultaneously on the same growth chamber shelf. All seeds were ripened for 4 weeks at room temperature and stratified for three days at 4°C prior to germination at 22°C in the light.
Figure 2. Plants homozygous for the atsec14-1/atsec14-5 double mutation are hypersensitive to hyperosmotic stress during seed germination. Stratified seeds of each genotype were plated on 1% phytoagar media with MS salts, 3% sucrose (w/v), and the indicated osmoticum. A, Percentage of seeds that had germinated after nine days post-stratification. B, Time course (in days) of seed germination on media containing 300 mM mannitol. Values shown are the means ± SE of two independent experiments.
Several groups have shown that ABA hypersensitive mutants frequently demonstrate lower germination performance on high-osmotic media (Guo et al., 2009; Magnan et al., 2008; Nishimura et al., 2007). To test whether plants lacking Atsec14-1 and/or Atsec14-5 function display enhanced sensitivity to ABA during germination, seeds of each genotype were plated on media containing ABA and incubated in the light. Plates were scored daily for both protrusion of the radicle through the testa and cotyledon greening. Minimal inhibition or delay in emergence or greening was observed when wild type or single mutant plants were plated on MS media containing 0.25 µM or 0.5 µM ABA (Fig. 3). In contrast, radicle emergence and greening of cotyledons from seed of atsec14-1/atsec14-5 double mutant plants were clearly inhibited by ABA.
Figure 3. *atsec14-1/atsec14-5* double mutant plants are hypersensitive to ABA in seed germination and the development of green cotyledons. Stratified seeds of each genotype were plated on 1% phytoagar medium containing MS salts with the indicated amount of ABA (or DMSO solvent only) and scored for germination (left column) and cotelydon greening (right column) over the course of seven days. Values shown represent the means of three independent experiments ± standard error (n = 39).
As described above, the germination studies were all conducted using after-ripened seed that had been stratified by incubation at 4°C for three days under moist conditions. The stratification treatment is routinely used to help break seed dormancy in Arabidopsis, and ensures maximal germination frequencies for this species (Bentsink 2008). There is a general consensus that ABA is the hormone that is primarily responsible for mediating seed dormancy (Gubler et al., 2005; Finkelstein et al., 2008), and in Arabidopsis, stratification has been associated with a rapid decline in ABA content once the seeds are transferred to warmer temperatures for germination (Ali-Rachedi et al., 2004). Because of this association between stratification and ABA in seed germination, we tested the germination profile of atsec14-1/atsec14-5 seeds that had not been stratified. As shown in Figure 4 A, the germination percentage of double mutant seeds was less than half of that observed for wild type Arabidopsis, or plants homozygous for the individual atsec14-1 and atsec14-5 mutations.
Figure 4. Germination assays of non-stratified \textit{atsec14-1/atsec14-5} seeds. A, sterile, non-stratified seeds were plated on 1% (w/v) phytoagar media and scored for germination after 6 days. B, the addition of \textit{GA}_3 partially overcomes the inhibition of germination in \textit{atsec14-1/atsec14-5} seeds. Values represents average of three independent experiments ± standard error (n = 39).

One of the proposed mechanisms by which ABA may function in maintaining seed dormancy is through the inhibition of the germination stimulating hormone, gibberellic acid (GA). In germination studies of both barley and Arabidopsis, increases in the accumulation of active GA in the embryo were shown to occur immediately after ABA content was reduced (Jacobsen et al., 2002; Ogawa et al., 2003). To test whether the poor germination phenotype of \textit{atsec14-1/atsec14-5} mutant plants can be rescued by GA, non-stratified seeds
from double mutant plants were plated on media containing various concentration of GA$_3$. Supplementation by GA$_3$ at concentrations of 50 µM or greater enhanced the germination frequency of non-stratified atsec14-1/atsec14-5 seed to levels approaching that observed for wild type Arabidopsis (Fig. 4B).

Seed morphology of atsec14-1/atsec14-5 mutants was studied to determine if they display any morphological abnormalities. Scanning electron microscopy was used to compare wild type versus double mutant seeds. No apparent differences were observed in overall seed size or morphology. Similarly, no differences were detected in the size or shape of seed epidermal cells. Examples of typical micrographs are shown in Supplemental Figure 1.

**Biochemical Function of AtSec14-1 and AtSec14-5**

Among the most notable characteristics of the soybean Sh1p protein was its ability to stimulate the synthesis of PtdIns(4)P and PtdIns(3)P in vitro (Monks et al., 2001). The consequences of eliminating AtSec14-1 and AtSec14-5 function on PtdInsP biosynthesis was investigated in vivo. Radiolabeling assays were conducted to measure the incorporation of $^{32}$Pi into polyphosphoinositide species in atsec14-1/atsec14-5 plants. Although germinating seed tissue would represent the optimal material for these assays (based on the results described above), this tissue was not amenable to efficient incorporation of the $^{32}$Pi (data not shown). As an alternative, the radiolabeling experiments were conducted using 10-14 day-old seedlings. This developmental stage displays no phenotypic differences from
wild-type plants, yet actively accumulates \textit{AtSec14-1} and \textit{AtSec14-5} transcripts in wild-type plants. To help normalize for variability in overall uptake across independent experiments, the data were analyzed as the percentage of $^{32}$P-PtdInsP with respect to the entire $^{32}$P-labeled phospholipid pool. As shown in Figure 5A, after a 25 minute incubation with in the presence of $^{32}$Pi, the proportion of radiolabel incorporated into PtdInsP species in \textit{atsec14-1/atsec14-5} plants was about 20% lower than that observed in wild type Arabidopsis ("no salt" lane).
Figure 5. *Arabidopsis atsec14-1/atsec14-5* plants synthesize and accumulate less PtdInsP than wild type plants. A and B, synthesis of PtdInsP and PtdIns(4,5)P2 in wild type and *atsec14-1/atsec14-5* double mutant plants incubated with [32P]-orthophosphate and varying concentrations of NaCl. For each experiment, six Arabidopsis seedlings (10 - 14 days old) were treated with [32P]-orthophosphate (100 µCi/mL final concentration) for 15 min. Salt or mock control medium was added and incubated an additional 10 min with NaCl at the concentrations indicated. Labeled phospholipid products were resolved using thin-layer chromatography and quantified with a Bioscan System 500 imaging scanner. Data are presented as the amount of [32P]-PtdInsP (A) and [32P]-PtdIns(4,5)P2 (B) detected as a percentage of total [32P]-labeled phospholipid extracted. Values shown are the means of eight biological replications ± standard error. Absolute amount of counts per minute ranged from 479 - 1590 cpm or 3650 - 12,002, depending on treatment or genotype for PtdInsP and PtdInsP2 respectively. C and D, quantification of phospholipids using mass spectroscopy. C, PtdInsP content of wild type and *atsec14-1/atsec14-5* plants as percentage of total phospholipids recovered through acid extraction. Each individual experiment represents a pool of 30 - 40 plants (10 - 14 days after germination). Values shown are the means of three to five independent experiments ± standard error. D, Comparison of common phospholipid species between wild type and *atsec14-1/atsec14-5* plants. Each individual experiment represents a pool of 30 - 40 plants (10 - 14 days after germination). Values shown are the means of six independent experiments ± standard error.
### Graphs and Tables

**A**
- **X-axis:** no salt, 250mM NaCl, 500mM NaCl, 1M NaCl
- **Y-axis:** Percent Incorporation
- **Legend:** Wild-type, Double Mutant

**B**
- **X-axis:** no salt, 250mM NaCl, 500mM NaCl, 1M NaCl
- **Y-axis:** Total PtdInsP as % total phospholipids
- **Legend:** Wild-type, Double Mutant

**C**
- **X-axis:** no salt, 0.4 M NaCl
- **Y-axis:** Total PtdInsP as % total phospholipids
- **Legend:** Wild-type, Double Mutant

**D**
- **X-axis:** PtdCho, PtdEtn, PtdIns, PtdSer, PtdOH
- **Y-axis:** nmol mg⁻¹ dry weight
- **Legend:** Wild-type, Double Mutant

**Notes:**
- Wild type: n=6, Double Mutant: n=5 for no salt; n=4 for 0.4 M NaCl.
- Data points indicate mean ± standard error of the mean (SEM).
Because polyphosphoinositide pools in plants are altered in response to hyperosmotic stresses (Dewald et al., 2001; Im et al., 2007; König et al., 2007), the $^{32}$P-labeling experiments were also conducted in the presence of varying levels of NaCl stress. Under conditions of salt stress, $[^{32}P]^{-\text{PtdInsP}}$ levels remained reduced in double mutant plants compared to wild type, but the differential between the two genotypes was much less pronounced (Fig. 5A). Despite the differences observed in $[^{32}P]^{-\text{PtdInsP}}$ levels in wild type versus atsec14-1/atsec14-5 plants, the proportion of $^{32}\text{Pi}$ incorporated into the bis-phosphorylated PtdInsP$_2$ species was not significantly different between the two genotypes, in either the presence or absence of salt stress (Fig. 5B).

To determine whether the differences in newly synthesized PtdInsP that were observed between wild type and atsec14-1/atsec14-5 plants are also reflected in the total endogenous levels of these lipid species, the PtdInsP levels in 10-14 day old seedlings were quantified using mass spectrometry. As shown in Figure 5C, PtdInsP levels were decreased 37% in the double mutant as compared to wild-type plants grown on standard MS agar plates. In contrast to the radio-labeling results, no significant changes in unlabeled PtdInsP levels were observed in wild-type plants exposed to 0.4 mM NaCl. For atsec14-1/atsec14-5 plants, however, NaCl treatment lead to a modest decrease in unlabeled PtdInsP to a degree similar to that observed in plants transiently labeled with $[^{32}P]$-orthophosphate. Quantification of the major plant phospholipids (and PtdOH) by mass spectrometry revealed no significant differences between these lipid species in wild type versus atsec14-1/atsec14-5 plants (Fig. 5D). To test whether there are qualitative, as well as quantitative, differences in total...
endogenous levels PtdInsP content between wild type and atsec14-1/atsec14-5 plants, mass spectrometric analysis was also used to group PtdInsP species according to fatty acid composition (total number of carbons and double bonds per molecule). This analysis showed that all molecular PtdInsP species were reduced in atsec14-1/atsec14-5 plants. These data suggest that the decrease in synthesis and accumulation of PtdInsP in atsec14-1/atsec14-5 plants is not selective with respect to fatty acid composition (Supplemental Fig. 2).

**DISCUSSION**

Our previous investigation of the soybean S sh1p protein presented compelling evidence implicating this Sec14p-like protein in hyperosmotic stress signaling. Furthermore, recombinant S sh1p was shown to stimulate PtdIns kinase activities in vitro (Kearns et al., 1998; Monks et al., 2001). The results from our current study of two closely related SSH1 homologs in Arabidopsis demonstrate the in vivo relevance of these previous observations. In all assays conducted, abnormal phenotypes were observed only in atsec14-1/atsec14-5 double mutant plants, suggesting functional redundancy between the AtSec14-1 and AtSec14-5 loci, at least with respect to seed germination. Radical emergence was inhibited when atsec14-1/atsec14-5 seeds were germinated on media containing a high concentration of NaCl or mannitol. Furthermore, double mutant Arabidopsis seedlings showed reduced levels of PtdInsP synthesis and accumulation. These observations strongly suggest that the Arabidopsis Ssh1p-like proteins are functioning to help confer osmotolerance during seed
germination through a mechanism involving the regulation of specific PtdInsP species, molecules that serve as intermediates in phosphoinositide signaling pathways.

ABA is the plant hormone that plays the most pivotal role in regulating seed dormancy and mediating adaptive responses to hyperosmotic stresses (Finkelstein et al., 2008; Seki et al., 2007). In several studies, ABA-sensitive mutants have been shown to display increased sensitivity to germination on high osmoticum media (Guo et al., 2009; Magnan et al., 2008; Nishimura et al., 2007). Therefore, it was not surprising to find that the seed germination phenotype of \textit{atsec14-1-1/atsec14-5} plants is also associated with an ABA hypersensitive phenotype at the same stage of development. The reduced frequency of germination for \textit{atsec14-1-1/atsec14-5} seeds that had been supplemented with ABA post-stratification, is also consistent with the observation that non-stratified double mutant seeds display a reduced germination phenotype in the absence of exogenous ABA (Fig. 4A). Because stratification in Arabidopsis leads to a rapid decline in endogenous ABA levels in Arabidopsis (Gubler et al., 2005; Ali-Rachedi et al., 2004), reduced germination phenotypes observed in non-stratified \textit{atsec14-1-1/atsec14-5} seed with no added ABA are likely due to the enhanced endogenous ABA levels being retained in these seeds. Unfortunately, our attempts to address this hypothesis experimentally were unsuccessful, as we were unable to accurately quantify the ABA concentrations in our materials.

The reduced levels of PtdInsP species observed in \textit{atsec14-1-1/atsec14-5} seedlings suggests that perturbations in phosphoinositide-mediated signaling may account for the ABA- and osmotic stress-associated germination phenotype observed in these plants.
Several studies have shown interesting connections between ABA responses and phosphoinositide signaling during seed germination, though no clear model has emerged to explain how these phenomena are mechanistically linked. ABA hypersensitive seed germination phenotypes were observed in Arabidopsis plants with T-DNA insertion mutations in either of two inositol polyphosphate 5-phosphatase (5Ptase) genes, \textit{At5Ptase1} and \textit{At5Ptase2} (Gunsekera et al., 2007). Mutant seedlings showed increased levels of Ins(1,4,5)P$_3$, and reduced levels of PtdIns, PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P$_2$. Conversely, overexpression of \textit{AtPtase2} yielded Arabidopsis seeds that were insensitive to ABA-mediated inhibition of germination (Sanchez and Chua, 2001), a seed germination phenotype similar to that observed in Arabidopsis seeds expressing a human type 1 Ins5Ptase (Perera et al., 2008). Mutations in \textit{CVP2}, another Arabidopsis Ins5Ptase-encoding gene, conferred hypersensitivity during seed germination to levels of ABA in the same range as that observed in \textit{atsec14-1-1/atsec14-5} plants (0.5µM), yet displayed no enhanced sensitivity to hyperosmotic stress (Carland and Nelson, 2004).

Most of the aforementioned studies focused largely on investigating correlations between ABA-associated germination responses and alterations in Ins(1,4,5)P$_3$ levels, which is reasonable given that Ins(1,4,5)P$_3$ is a substrate for the various Ins polyphosphate phosphatase enzymes described in those reports. Nevertheless, it is important to consider that intermediates in the canonical Ins(1,4,5)P$_3$ pathway, including PtdInsP species, have also been shown to serve as signaling molecules (Thole et al., 2008; Leshem et al., 2007; Balla and Balla, 2006). This fact may be particularly relevant for \textit{atsec14-1-1/atsec14-5} plants,
since PtdIns(4,5)P2 levels did not appear to be significantly altered in these plants (Fig. 5).

Although our TLC separations could not distinguish PtdIns(3)P molecular species from PtdIns(4)P, the latter is by far the most prevalent PtdIns species in Arabidopsis seedlings (Welters et al., 1994; Williams et al., 2005), and thus the most likely PtdInsP species impacted in \textit{atsec14-1-1/atsec14-5} mutant plants. Interestingly, in the previously cited study by Gunesekera et al. (2007) and a recent study of a PtdInsP2-specific 5Ptase11 Arabidopsis mutant by Ercetin et al. (2008), reduced levels of PtdIns(4)P and PtdIns(3)P were reported (in addition to increases in Ins(1,4,5)P3) in these germination-deficient lines.

Intriguing similarities can also be found between the germination phenotype of \textit{atsec14-1-1/atsec14-5} plants and the phenotype reported for Arabidopsis seeds germinated in the presence of the compound phenylarsine oxide (PAO). PAO alone did not affect the germination of Arabidopsis seeds, but simultaneous addition of a low level of ABA (0.3 µM) revealed a hypersensitivity to the hormone during germination (Reyes et al., 2006). Although the authors of this study attributed the ABA hypersensitivity-conferring properties of PAO to its reported function as a tyrosine phosphatase inhibitor, other researchers have shown this agent to be a potent inhibitor of plant PtdIns 4-kinase enzyme activities (Krinke et al., 2007). It would be interesting to determine whether PAO treatment of Arabidopsis seedlings leads to reductions in PtdInsP pools similar that observed in \textit{atsec14-1-1/atsec14-5} plants.

The dynamics of the PtdInsP and PtdIns(4,5)P2 pools during exposure to NaCl stress in double mutant versus wild type plants could also be interpreted as providing indirect support for the notion that the PtdInsP species \textit{per se} is the important end product being
regulated by Atsec14-1 and Atsec14-5, as opposed to functioning to help channel PtdIns(4)P through the IP₃ signaling pathway. Despite displaying a ~20% reduction in PtdInsP under normal growth conditions, the levels of PtdIns(4,5)P₂ induction upon exposure to NaCl stress appear to be equivalent between atsec14-1-1/atsec14-5 and wild type plants (Fig. 5A and B). This suggests that under normal growth conditions Atsec14-1 and Atsec14-5 function to maintain a certain pool of PtdInsP within the cell that is not required for osmotic stress-induced PtdIns(4,5)P₂ production. According to this model, the reduction, or absence, of a particular PtdInsP pool would be mediating the hypersensitivity to ABA and osmotic stresses during seed germination, as opposed to the reduction in overall PtdInsP pools limiting the ability of the cell to produce sufficient PtdIns(4,5)P₂, or its cleavage products, as a means of adapting to these stresses. In order to better clarify these possibilities, however, a thorough quantification of all phosphoinositide species involved in cellular signaling needs to be determined in atsec14-1-1/atsec14-5 plants, both in the presence and absence of hyperosmotic stress.

The current model of yeast Sec14p function postulates that the transfer protein stimulates PtdIns(4)P synthesis through the regulated channeling of PtdIns substrates to the Golgi-localized PtdIns 4-kinase, Pik1p (Phillips et al., 2006; Schaaf et al., 2008). Two other yeast Sec14p homologs, Sfh2p and Sfh5p, also stimulate PtdIns(4)P synthesis, but these proteins were shown to stimulate Stt4p, a plasma membrane localized PtdIns 4-kinase, rather than Pik1p (Routt et al., 2005). The observed ~37% reduction in PtdIns(4)P in atsec14-1-1/atsec14-5 plants is consistent with a model whereby plant SSH1p-like proteins similarly...
function to stimulate the synthesis of a particular pool of PtdInsP species under normal growth conditions. Vermeer et al. (2009) recently identified the plasma membrane and Golgi as the sites where the majority of PtdIns(4)P lipids accumulate. Therefore it is tempting to speculate that the AtSec14-1 and AtSec14-5 proteins specifically function at one of these interfaces, similar to the Sec14p (Golgi) or Sfh2p/Sfh5p (plasma membrane) sites of action.

Of particular interest will be defining the specific PtdIns 4-kinase whose activity is stimulated by the Arabidopsis Ssh1p homologs. This task may initially appear to be rather daunting, given that the Arabidopsis genome contains 12 genes that encode enzymes with homology to PtdIns 4-kinases (Mueller-Roeber and Pical, 2002). A recent report by Galvão et al. (2008), however, suggests that some of these PtdIns 4-kinase homologs, AtPI4Kγs, may actually function as protein, rather than lipid, kinases. Of the two Arabidopsis PtdIns 4-kinases whose enzymatic function has been confirmed, one has been found to localize to the peri-nuclear region (AtPI4Kα1) while the other (AtPI4Kβ1) localized to undefined punctate structures (Stevenson-Paulik et al., 2003). Co-localization studies could prove to be useful in helping to elucidate in vivo associations between individual PtdIns 4-kinases and the AtSec14-1 and/or AtSec14-5 proteins, yet are likely to be difficult since only a minority of total Ssh1p protein is membrane associated at any given time, a property shared with yeast Sec14p (Kearns et al., 1998).

In summary, our investigation of Arabidopsis plants mutated at both SSH1 homologous loci, AtSec14-1 and AtSec14-5, has revealed new insights into the function of this novel class of lipid transfer proteins in higher plants. AtSec14-1 and AtSec14-5 appear to
have an important role in seed germination, particularly in hyperosmotic stress environments, through stimulation of PtdInsP synthesis. Loss of AtSec14-1 and AtSec14-5 function also confers hypersensitivity to ABA during germination, providing additional evidence linking ABA germination responses to phosphoinositide signaling pathways.

**MATERIALS AND METHODS**

**Plant Growth**

Sterilized seeds were plated on agar plates containing Murashige and Skoog (MS) salts (Sigma-Adlrich, St. Louis, MO). For plants grown in soil, the 5 to 7 day old plantlets were transferred and grown on wetted Metro Mix 200 (Sun Gro Horticulture, Bellevue, WA). All plants were grown under a 16/8 (day/night) cycle at 22°C ± 2°C with a light intensity of 150 µmol m⁻² s⁻¹.

**Analysis of T-DNA insertion lines**

AtSec14-1 and AtSec14-5 respectively, were obtained from the Arabidopsis Resource Center (Alonso et al., 2003). DNA was extracted from progeny of each line according to the method of Edwards et al. (1991) and used for PCR-based genotyping to select for individuals homozygous for the T-DNA insertion alleles. The diagnostic primers for line 120972 were 5'-ACATCACAAAATGGGCTTTTC3' (forward), 5'-TTGACTTTTCTTCAATGGTGGAG-3' (reverse) for the wild type locus, and the primer 5'-GACCGCTTGCTGCAAC-3', corresponding to the left border of the T-DNA insertion, in conjunction with the 5'-ACATCACAAAATGGGCTTTTC3' wild type primer was used to amplify the mutant locus.
Primers specific for line 039575 were 5' -GCTCCGATTCTATGGTTTTGG-3' (forward), and 5' -TGGTGAGTAAATGGCAGATGG-3' (reverse) for wild type amplification, and the 5' - GCTCCGATTCTATGGTTTTGG-3' primer together with the above T-DNA left border primer were specific for the insertion mutation. The following PCR conditions were used for all primer sets: 94°C incubation for 5 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min and a final 7 min extension at 72°C. Individuals homozygous for each insertion event were cross-pollinated to obtain plants carrying both mutant alleles. Individuals from the F2 generation were genotyped at both loci to identify homozygous double mutant plants.

RNA was isolated from wild type, single and double mutant plants using TRIzol® according to manufacturer’s instructions (Invitrogen Corporation, Carlsbad, California). First, cDNA was synthesized using the Superscript™ First Strand cDNA synthesis kit (Invitrogen). RT-PCR was conducted to assay for the presence of AtSec14-1 and AtSec14-5 transcripts in wild type and mutant lines using the following primers: 5' -GAAGAAGCAGTGAAGCAATTGCG-3' and 5' -GGTCGTCCCTGTTTCTTTGAAG-3' (AtSec14-1 specific); and 5' -GCTATCACACAACGTTCAGCTTCAGG-3' and 5' -AGAAGAATGGGAGGCCAATCAC-3' (AtSec14-5 specific). Primers corresponding to the Actin2 gene used as a control were 5' -CTTTCCGGTTACAGCGTTTG-3' and 5' -GAAACGCGGATTTAGCTGCCT-3'. The following PCR conditions were used: 94°C incubation for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final 7 min extension at 72°C.
**Seed Germination Assays**

All germination assays were conducted using age-matched seed sets from plants that were grown and harvested together in the same location, and ripened for 4 weeks. Prior to germination, seeds were surface sterilized with 50% (v/v) bleach for 12 minutes, washed 3 times with sterile dH₂O and resuspended in sterile 0.1% (w/v) agarose in a 1.5 mL microcentrifuge tube. Sterilized seeds were stratified by incubation at 4°C for 3 d in the dark, then germinated on MS agar plates in the light at 22°C. Seeds were evaluated every 24 hrs for radical emergence and/or development of an open green cotyledon. Plantlets were scored as having transitioned into an open green cotyledon if the cotyledons had become green and were bending toward the hypocotyl.

**[32P]-orthophosphate labeling of phospholipids**

Six 10-14 day old seedlings grown on MS phytagar media with 1% (w/v) sucrose (pH 5.6) were transferred to 24-well plates containing 0.5 mL of liquid MS, 1% (w/v) sucrose, 10 mM MES buffer, pH 5.6, and allowed to equilibrate overnight in a shaker (120 rpm) at room temperature. Labeling was initiated by the addition of [³²P]-orthophosphoric acid at 100 µCi/mL final concentration. Samples were subsequently shaken for a discrete amount of time 25 minutes at room temperature and terminated by the addition of ice-cold 10% (v/v) perchloric acid (final concentration) followed by 20 min incubation on ice. Plants were removed and gently blotted dry prior to transfer into a borosilicate test tube. Phospholipid
extraction and thin-layer chromatography (TLC) separation was carried out as described by Perera et al. (2002).

**Lipidomics analysis**

Total phospholipids were extracted from 14 day old whole Arabidopsis seedlings as previously described (Welti et al., 2002). Quantification of major phospholipids (PtdIns, PtdCho, PtdEtn, PtdOH, PtdSer) from total lipid extracts was conducted by the Kansas Lipidomics Research Center (Manhattan, KS) using electrospray ionization tandem mass spectrometry (ESI/MS-MS). Extracts enriched for acidic phospholipids were prepared as follows. Plants were transferred to 40 mL tubes with Teflon tops containing 10 mL 10% (v/v) perchloric acid. Perchloric acid was removed and 10 mL of 5% (w/v) TCA, 1 mM EDTA was used to wash the plants, followed by two washes with 1 mM EDTA, pH 8.0. Neutral lipids were extracted by adding 8 mL 2:1 MeOH:CHCl₃ with 0.01% (w/v) BHT and shaking at 100 rpm for 10 min at room temperature. After removal of the neutral lipid-containing solvent, the remaining plant tissue was incubated with 5 mL CHCl₃:MeOH:12 N HCl (80:40:1) + 0.01% (w/v) BHT and shaken (100 rpm) for 15 min at room temperature. To each tube, 1.5 mL CHCl₃ + 0.01% (w/v) BHT and 2.7 mL 0.1 N HCl was added and thoroughly mixed using a vortex, and centrifuged at 1,000 x g for 5 min. The lower phase was transferred to a clean test tube, and the upper phase re-extracted by adding 2 mL CHCl₃ + 0.01% (w/v) BHT, vortexed, and centrifuged as before. The lower phase was removed and pooled with the previous extract. Six mL 1:1 1 N HCl:MeOH was added to the pooled
extracts, vortexed, and centrifuged on medium speed 1,000 x g for 5 minutes. After discarding the upper phase, 6 mL of 1:1 1N HCl : MeOH was added, the tubes vortexed and centrifuged at 1,000 x g for 5 minutes. The lower phase was transferred to a clean test tube and upper phase re-extracted with 1mL CHCl₃ + 0.01% (w/v) BHT. The pooled lower phase extracts were evaporated to dryness and resuspended in 1mL 2:1:0.01 CHCl₃: MeOH:dH₂O. Samples were shipped on dry ice to the Kansas Lipidomics Research Center where PtdInsPs were quantified using ESI/MS-MS.

ACKNOWLEDGEMENTS
Kansas Lipidomics Research Center was supported by National Science Foundation Grants MCB0455318 and DBI 0521587, and National Science Foundation EPScor Grant EPS-0236913 with matching support from the State of Kansas through Kansas Technology Enterprise Corporation and Kansas State University. The Kansas Lipidomics Research Center is also supported by K-INBRE (National Institutes of Health Grant P20 RR16475 from the INBRE program of the National Center for Research Resources). This research was supported by a grant from the National Science Foundation (IBN-9513582) and the Initiative for Future Agriculture and Food Systems (2001-52101-11507) from the USDA Cooperative State Research, Education, and Extension Service.
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Supplemental Figure 1. Wild-type and atsec14-1/atsec14-5 double mutant seeds are similar in total size and epidermal cell size on the seed coat. A, Representative scanning electron micrographs of wild-type and double mutant seeds (300x magnification). Bar graph shows average seed size of 10 randomly selected seeds (n=10) ± standard error. Scale bars below are 300 µm. B, Representative scanning electron micrographs of wild-type and double mutant seed coat cells (1000x magnification). Bar graph indicates the average diameter of 10 epidermal cells on each of 10 randomly selected seeds ± standard error. Scale bars below are 100 µm.
Supplemental Figure 2. Mass spectrometric data showing distribution of acyl chains of PtdInsP species in wild-type and atsec14-1/atsec14-5 plants grown in the presence or absence of 0.4M NaCl. Species are grouped according to total number of carbons and double bonds in a given PtdInsP molecule.
CHAPTER 4

METHOD FOR QUANTIFICATION OF PHOSPHATIDYLINOSITOL
MONOPHOSPHATES IN PLANTS

Matthew R. Keogh, Pamela Tamura, Ruth Welti, and Ralph E. Dewey
INTRODUCTION

Phosphatidylinositol monophosphates (PtdInsPs) are PtdIns-based lipids that possess a single phosphate molecule at either position 4 [PtdIns(4)P] or position 3 [PtdIns(3)P] of the inositol ring. Both of PtdIns(4)P and PtdIns(3)P have been shown to be bioactive lipids that have roles in the regulation of vesicle trafficking (Falasca and Maffucci, 2006; D'Angelo et al., 2008; Thole and Nielsen, 2008). Although both of these lipids are found in plants, PtdIns(4)P constitutes nearly all of the PtdInsP species observed in most plant tissues, with PtdIns(3)P typically being either undetectable or present in trace amounts (Welters et al., 1994; Williams et al., 2005). Several different approaches have been used to study PtdInsPs, such as assaying the properties of enzymes that synthesize or degrade them (ref). Alternatively, in vivo radiolabeling assays have been used to determine the rate of synthesis of PtdInsPs within the cell. Although informative, neither of these approaches can be used to definitively establish the total endogenous levels of these lipids. Directly quantifying the amount of unlabeled PtdInsPs molecules within a given tissue is the only way to determine their true unlabeled levels.

Although methods to quantify PtdInsPs from whole tissue have been developed, they tend to be technically difficult, costly, and often unreliable. One commercially available method uses lipid binding polypeptides specific for a PtdInsP of interest which is incubated with a membrane upon which a sample is spotted (typically a complex extract) along side a dilution series of known amounts of pure lipid of interest. The membrane is subsequently incubated with an antibody specific to the lipid binding polypeptide, then developed in a
manner similar to immunoblotting (Echelon Biosciences, Salt Lake City, Utah). Though useful for some applications, difficulties in data interpretation can arise when: (1) the amount of lipid in the mixture of interest is not within the linear range of the pure standards on the membrane; (2) the lipid binding protein is not 100% specific toward the specific PtdInsP of interest; or (3) the binding properties of the lipid binding protein are different when the lipid of interest is dispersed within a complex mixture versus purified preparation. In addition to these issues, this system provides no information regarding the acyl composition of the lipid of interest. Another method that has been employed to yield quantitative data on PtdInsPs levels uses an initial thin layer chromatography (TLC) step to purify the PtdInsPs from other lipid species. TLC purified lipid is then chemically converted to fatty-acid methyl esters which can be easily quantified by gas chromatography (Konig et al., 2008). The primary limitation to this system lies in the inability of TLC to completely purify the PtdInsPs away from other polyphosphoinositides. In another report, HPLC instrumentation equipped with a suppressed conductivity detector was used to measure unlabeled PtdInsPs within complex mixtures (Nasuhoglu et al., 2002), but the use of this system has not been widely reported.

Mass spectrometry is one of the favored methods for analysis of biological compounds from complex mixtures. There have been reports of successful mass spectrometric analysis of phosphoinositides, but these were conducted using samples of mammalian or yeast origin (Wenk et al., 2003; Milne et al., 2005). Although phosphoinositides are low abundance lipids in all organisms, their relative proportion is even
smaller in plants due to the large amount galactolipids (MGDG, DGDG) found in plastid membranes. Furthermore, plant extracts contain compounds that can suppress the ionization of PtdInsPs (R. Welti, unpublished results). We report the development of a facile method for the quantification of PtdInsP species from plant tissue using mass spectrometry.

RESULTS

Optimization of extraction protocol

To maximize the recovery of intact lipids, inactivation of phospholipases prior to phospholipid extraction is critical. Trichloroacetic acid (TCA) has been used to precipitate macromolecules and has been favored over perchloric acid (PCA), likely because of perceived safety issues involved in handling PCA when in reality at the concentration used in the type of procedure it is no more dangerous than other strong acids. In yeast, however, it was shown that PCA is much more effective than TCA in maximizing the extraction of intact PtdInsPs (Bonangelino et al., 2002). 10-14 day-old Arabidopsis seedlings were grown in liquid MS medium and incubated with [32P]-orthophosphate for 60 minutes. Prior to extraction of lipids, plants were pretreated with 10% (v/v) PCA or 10% (w/v) TCA and incubated on ice for 20 minutes. As a control, plants were also directly extracted without any acid pretreatment. Phospholipids were extracted using chloroform:methanol:HCl (Cho and Boss, 1995) and separated using TLC. The [32P]-PtdInsPs were quantified using an imaging scanner. As shown in Table 1, PCA pretreatment was most effective for allowing maximal recovery of polyphosphoinositides. The amount of labeled PtdInsPs as a proportion of total
radiolabeled lipid was much greater using PCA as the pretreatment rather than TCA or direct extraction. Further confirmation that PCA is superior for extracting polyphosphoinositides comes from the observation that bis-phosphorylated lipid species (PtdInsP₂) were only detectable above base-line using the PCA pretreatment (Table 1).

**Table 1: Comparing perchloric acid to trichloroacetic acid as means to inactive phospholipases in order to maximize phosphoinositide extraction.**

Data are expressed as a percentage of total radioactivity counted and represents the average of 3 experiments ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%PtdInsP₂s</th>
<th>% PtdInsPs</th>
<th>%PtdOH</th>
<th>% other lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct Extraction</td>
<td>Not detected</td>
<td>24.9 ± 2.1</td>
<td>43.6 ± 1.5</td>
<td>26.8 ± 1.4</td>
</tr>
<tr>
<td>10% TCA</td>
<td>Not detected</td>
<td>13.5 ± 1.0</td>
<td>47.0 ± 1.8</td>
<td>21.1 ± 1.3</td>
</tr>
<tr>
<td>10% PCA</td>
<td>2.80 ± 0.3</td>
<td>35.4 ± 1.2</td>
<td>41.4 ± 1.7</td>
<td>16.7 ± 0.3</td>
</tr>
</tbody>
</table>

**Extraction for mass spectrometry analysis**

The determination of a method to produce plant extracts suitable for mass spectrometry analysis was approached by preparing extracts using two different protocols. Both methods utilized are based on chloroform:methanol:HCl extraction, but differ in that one includes the addition of an initial extraction with non-acidified chloroform:methanol in order to remove many of the neutral lipids. After the neutral lipid extraction, the plant material is then re-extracted with chloroform:methanol:HCl. The addition of the neutral extraction step yielded a final extract that was enriched in acidic lipids, presumably containing a higher proportion of PtdInsPs. Dried extracts and extracts re-suspended in a known volume of 2:1:0.01 chloroform:methanol:dH₂O were made from fractions corresponding to total lipid, neutral lipid and acidic lipid extracts. Each sample was shipped on dry ice to the Kansas Lipidomics
Center and analyzed by mass spectroscopy. Diagnostic PtdInsP signals were only detectable in acidic extracts that had been: 1) subjected to the neutral lipid extraction; and 2) resuspended and shipped in the solvent mixture.

**Determination of a diagnostic signal ion**

Determination of diagnostic signal ions was achieved by analyzing a control di palmitoyl PtdIns(4)P standard (shown in Fig. 1A). A typical spectrum of the fragmentation products of the standard is displayed in Figure 1B. A close up of the region of the spectrum containing diagnostic signal ions is shown in Figure 1C. The sodiated inositol bisphosphate minus H$_2$O product (Fig. 1D) was selected as the peak by which to quantify PtdInsPs as it was the strongest signal ion. Other relevant ionization products are indicated on Figure 1C.
Figure 1. Determination of diagnostic signal ion for phosphatidylinositol monophosphate.
**PtdInsP standard:** Dipalmitoyl phosphatidylinositol 4-phosphate (di16:0 PtdInsP) sodiated anion ([M-2H+Na]−); **Formula:** C_{41}H_{78}O_{16}P_{2}Na; m/z 911.4668 (product ion scan for standard performed at this mass)

Mass spectrum of dipalmitoyl phosphatidylinositol-4 phosphate standard
Mass spectrum of dipalmitoyl phosphatidylinositol-4 phosphate standard: a magnification of the region of interest. Daughter ions are labeled including the diagnostic signal ion Sodiated inositol bisphosphate – H₂O.

**Fragment analyzed to quantitate**

**PtdInsPs**: Sodiated inositol bisphosphate anion (above) minus a water molecule;  
**Formula**: C₆H₁₂O₁₂P₂Na-H₂O=  
C₆H₁₀O₁₁P₂Na;  
**m/z**: 342.9602

**Individual PtdInsP species**: Sodiated anion ([M-2H+Na⁺]) of each species (various R’ and R”);  
**Formula**: various;  
**m/z**: various (product ion scan for each species performed at its specific mass)
**Quantification of PtdInsPs from whole plant extracts**

Arabidopsis seedlings were grown on an agar plates, transferred to liquid medium and allowed to equilibrate overnight. Salt stressed individuals were treated for 60 minutes with 0.4M NaCl. Acidic extracts were prepared following PCA pretreatment and neutral lipid extraction as described above. The quantification of PtdInsPs from these materials is shown in Figure 2. The level of PtdInsP observed in these samples is approximately 1/10th of that observed for PtdIns in Arabidopsis seedlings of comparable age analyzed in a similar manner (see Chapter 3, Fig. 5D). This ratio of PtdIns to PtdInsP is similar to the range of ratios that have been reported by other investigators in this field (Hetherington and Drobak, 1992).

There was no significant difference in total PtdInsPs between NaCl treated versus non-treated plants (Fig. 2). Using this same assay, we were able to quantify the PtdInsP content in Arabidopsis plants possessing knockout mutations in the *AtSec14-1* and *AtSec14-2* genes, and demonstrate that the mutant plants accumulate significantly less PtdInsPs than wild type plants. The results of these assays are detailed in Chapter 3 (Fig. 5).
Figure 2: Quantification of total phosphatidylinositol phosphates from wild-type Arabidopsis seedlings stressed with 0.4M NaCl stress or no stress.

**Acyl composition of PtdInsPs**

The various combinations of acyl chains, as represented by total number of carbons and double bonds, that are associated with PtdInsPs extracted from young Arabidopsis seedlings are shown in Figure 3. The results obtained are very similar to the pattern that has been reported previously for PtdIns (the direct precursor of PtdInsPs) in Arabidopsis (Welti et al. 2002). Acyl chains of 34:2 and 34:3 constitute ~90% of the total mol percentage PtdInsP. The remaining ~10% is comprised primarily of 34:1, 36:6, 36:5, 36:4, 36:3, 36:2, and 36:1 acyl chain combinations. The exposure of Arabidopsis plants to 0.4M NaCl did not lead to any significant changes in the acyl composition of the PtdInsP species extracted from these materials. These results are similar to the results shown in Figure 2,
Figure 3: Acyl species associated with phosphatidylinositol phosphate. Different acyl pairs are labeled on the X-axes. The first number represents total number of carbon atoms in both fatty acids and the second number indicates number of double bonds. Data is expressed in terms of percentage of total phosphatidylinositol phosphate.
PROTOCOL FOR THE QUANTIFICATION OF PHOSPHOINOSITIDE MONOPHOSPHATES FROM PLANT TISSUE

Lipid Extraction

Total phospholipids were extracted from 14 day old whole Arabidopsis seedlings as previously described (Welti et al., 2002). Quantification of major phospholipids (PtdIns, PtdCho, PtdEtn, PtdOH, PtdSer) from total lipid extracts was conducted by the Kansas Lipidomics Research Center (Manhattan, KS) using electrospray ionization tandem mass spectrometry (ESI/MS-MS). Extracts enriched for acidic phospholipids were prepared as follows. Plants were transferred to 40 mL tubes with Teflon tops containing 10 mL 10% (v/v) perchloric acid. Perchloric acid was removed and 10 mL of 5% (w/v) TCA, 1 mM EDTA was used to wash the plants, followed by two washes with 1 mM EDTA, pH 8.0. Neutral lipids were extracted by adding 8 mL 2:1 MeOH:CHCl₃ with 0.01% (w/v) BHT and shaking at 100 rpm for 10 min at room temperature. After removal of the neutral lipid-containing solvent, the remaining plant tissue was incubated with 5 mL CHCl₃ : MeOH : 12 N HCl (80 : 40 : 1) + 0.01% (w/v) BHT and shaken (100 rpm) for 15 min at room temperature. To each tube, 1.5 mL CHCl₃ + 0.01% (w/v) BHT and 2.7 mL 0.1 N HCl were added and thoroughly mixed using a vortex, and centrifuged at 1,000 x g for 5 min. The lower phase was transferred to a clean test tube, and the upper phase re-extracted by adding 2 mL CHCl₃ + 0.01% (w/v) BHT, vortexed, and centrifuged as before. The lower phase was removed and pooled with the previous extract. Six mL 1:1 1 N HCl : MeOH was added to the pooled extracts, vortexed, and centrifuged at 1,000 x g for 5 min. After discarding the upper
phase, 6 mL of 1:1 1N HCl : MeOH was added, the tubes vortexed and centrifuged at 1,000 x g for 5 minutes. The lower phase was transferred to a clean test tube and the upper phase re-extracted with 1mL C HCl_3 + 0.01% (w/v) BHT. The pool ed lower p hase ex tracts w ere evaporated to dryness and resuspended in 1mL 2:1:0.01 CHCl_3: MeOH:dH2O.

**Preparation for Mass Spectrometric Analysis of PtdInsPs**

Dipalmitoyl phosphatidylinositol 4-phosphate (di16:0 PtdIns(4)P, Matreya, Pleasant Gap, PA) was used as an internal standard to quantitate the PtdInsPs species in the sample extracts. Standard (0.28 nmole) was added to an aliquot of sample equivalent to approximately 2 mg dry weight of the plant material extracted. The sample/standard mixture was diluted to 1 ml with chloroform:methanol:water (2:1:0.01) and then washed with 500 ml of citrate buffer (150 mM, pH 4). The top, aqueous layer was discarded. This wash step was incorporated to reduce the interfering salts/substances in the sample causing ion suppression in the mass spectrometer, and it is assumed that any partitioning of the sample into the aqueous layer (and its subsequent removal) would occur similarly to the standard, allowing quantitation to remain accurate. The bottom, organic layer was evaporated under a stream of nitrogen to near dryness and then redissolved in 670 μl of a mixture of chloroform:methanol:water:piperidine (2:1:0:1:0.2).
**Mass Spectrometric Analysis of PtdInsPs**

Mass spectra were acquired on an MDS SCIEX/Applied Biosystems Q Star Elite hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Samples were introduced by continuous infusion into the ESI source at a rate of 10 µl/min using the integrated Harvard syringe pump. MS/MS product ion scans were carried out in negative ionization mode with fragmentation of the sodiated PtdInsP species ([M-2H+Na]-).

Sequential scans were performed for 34:4 PtdInsP, 34:3 PtdInsP, 34:2 PtdInsP, 34:1 PtdInsP, 34:0 PtdInsP, 36:6 PtdInsP, 36:5 PtdInsP, 36:4 PtdInsP, 36:3 PtdInsP, 36:2 PtdInsP, 36:1 PtdInsP, 36:0 PtdInsP, and for the internal standard 32:0 PtdInsP during continuous infusion of each sample. The ion spray voltage was set at -4.5 kV, the source temperature at 150ºC, the curtain gas at 25 (arbitrary units), and the ion source gases at 20 and 30 (arbitrary units). The de-clustering potential was set at -80 V, the de-clustering potential 2 at -15 V, and the focusing potential at -300 V. The collision gas, nitrogen, was set at 4 (arbitrary units), and the collision energy was -55 V. Data at each fragmentation mass were collected over the range of \( m/z \) 5-1100, 180 cumulative scans over 3 minutes. Data were collected and processed using the Analyst QS 2.0 software.

The data were smoothed, the background of each spectrum was subtracted, and the peaks were centroided and integrated. The fragment at \( m/z \) 342.9602, corresponding to the sodiated inositol bisphosphate anion (minus a water molecule), was used to quantify the PtdInsP species within each sample through comparison of the fragment in individual species scans to the same fragment in the internal standard scan. A 32:0 PtdInsP standard-only...
Mass Spectrometric Lipid Profiling

Lipid profiling was performed on each sample in order to correlate the amount of each PtdInsP species with the amount of total phospholipids and galactolipids present. Although the original two-step sample extraction method did eliminate a large portion of the phospholipids and galactolipids from the samples, lipid profiling allows normalization and comparison of mole percentage data of the various PtdInsP species within this experimental setup. An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition was carried out as described previously (Devaiah et al., 2006) with modifications. The samples were dissolved in 1 ml of a mixture of chloroform:methanol:water (2:1:0.01, v:v:v). An aliquot of 30 to 100 µl of extract, equivalent to 0.3-1 mg dry weight of the plant material extracted, was used for the analysis. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added in the following quantities (with some small variation in amounts in
different batches of internal standards): 0.6 nmol di 12:0-PtdCho, 0.6 nmol di 24:1-PtdCho, 0.6 nmol 13:0-lysoPtdCho, 0.6 nmol 19:0-lysoPtdCho, 0.3 nmol di 12:0-PtdEtn, 0.3 nmol di 23:0-PtdEtn, 0.3 nmol di 14:0-PtdGly, 0.3 nmol di 20:0(phytanoyl)-PtdGly, 0.3 nmol di 14:0-PtdOH, 0.3 nmol di 20:0(phytanoyl)-PtdOH, 0.2 nmol di 14:0-PtdSer, 0.2 nmol di 20:0(phytanoyl)-PtdSer, 0.23 nmol 16:0-18:0-PtdIns, 0.16 nmol di 18:0-PtdIns, 2.01 nmol 16:0-18:0-MGDG, 0.39 nmol di 18:0-MGDG, 0.49 nmol 16:0-18:0-DGDG, and 0.71 nmol di 18:0-DGDG. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform:methanol:300 mM ammonium acetate in water was approximately 300:665:35, and the final volume was 1.3 ml.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µl/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PtdCho and lysoPtdCho, [M + H]\(^{+}\) ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PtdEtn and lysoPtdEtn, [M + H]\(^{+}\) ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); PtdGly, [M + NH4]\(^{+}\) in positive ion mode with NL 189.0 for PtdGly; lysoPtdGly, [M – H]\(^{-}\) in negative
mode with Pre 152.9; PtdIns, [M + NH4]+ in positive ion mode with NL 277.0; PtdSer, [M + H]+ in positive ion mode with NL 185.0; PtdOH, [M + NH4]+ in positive ion mode with NL 115.0; MGDG, [M + NH4]+ in positive ion mode with NL 179.1; and DGDG, [M + NH4]+ in positive ion mode with NL 341.1. The scan speed was 50 or 100 per sec. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PtdEtn, +40 V for PtdCho, +25 V for PtdIns, PtdSer, and PtdOH, +20 V and PtdGly, +21 V for MGDG, and +24 V for DGDG. Declustering potentials were +100 V for PtdEtn, PtdCho, PtdOH, PtdGly, PtdIns, and PtdSer, and +90 V for MGDG and DGDG. Entrance potentials were +15 V for PtdEtn, +14 V for PtdCho, PtdIns, PtdOH, PtdGly, and PtdSer, and +10 V for MGDG and DGDG. Exit potentials were +11 V for PtdEtn, +14 V for PtdCho, PtdIns, PtdOH, PtdGly, PtdSer, and +23 V for MGDG and DGDG. The mass analyzers were adjusted to a resolution of 0.7 full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas were integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class. The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and
molar amounts calculated in comparison to the internal standards of the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the “internal standards only” spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each “internal standards only” set of spectra were used to correct the data from the following 10 samples. Finally, the data were corrected for isotopic overlap, adjusted to account for the fraction of the sample analyzed, and normalized to the sample “dry weights” to produce data in the units nmol/mg. Mole percentages of each species were also calculated.

CONCLUSIONS

There have been different suggestions over the years concerning what method would be preferred for extracting phosphoinositides from plants. The most commonly used procedure involves a chloroform:methanol:HCl-based extraction of the acidic phospholipids (Cho and Boss, 1995). We developed a modified version of the chloroform:methanol:HCl extraction procedure to generate lipid extracts that would be suitable for mass spectrometric analysis. There are two fundamental differences between our extraction method and those reported previously. First, we obtained a much higher yield of PtdInsPs using PCA as a pretreatment to inactivate phospholipases, as opposed to TCA. Second, an initial neutral lipid extraction prior to the extraction of acidic phospholipids served to enrich the PtdInsPs as a proportion of total lipid recovered, but more importantly eliminated contaminating compounds that were inhibitory to the electrospray ionization of the mass spectrometry analysis. These conditions
are similar to the extraction method used by Milne et al. (2005) to extract polyphosphoinositides for mass spectrometric analysis from mammalian cell cultures. Using a dipalmitoyl PtdIns(4)P control standard, researchers at the Kansas Lipidomics Center were able to establish the spectral pattern of PtdInsPs from the organic acid extracts. Furthermore, they determined sodiated inositol bisphosphate (minus dH₂O) to be the ionization product best suited for quantification of PtdInsPs. Together, we have developed a protocol for quantifying PtdInsPs from plant tissue, a tool that could greatly enhance future investigations of these important signaling compounds (and metabolic intermediates) in higher plants.
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FUTURE DIRECTIONS

Matthew R. Keogh
PHOSPHOLIPID METABOLISM

There have been some impressive metabolic studies in the area of plant phospholipid research, most notably the work of Anne Datko and Harvey Mudd. Andrew Hanson took things to the next level by modeling the flux through the phospholipid head-group metabolic pathway. In recent years, most studies involving phospholipids have focused largely on investigating properties of the associated fatty-acids. While soy lecithin (PtdCho) is an important food additive for its use as an emulsifier, it is more or less considered a by-product from the extraction of vegetable oil. The lack of an obvious end product of commercial value is the likely basis for the paucity of research being conducted on the major plant phospholipids (with the exception of the aforementioned studies of fatty acid composition).

A couple of interesting observations came out of my characterization of plant phospholipid \( N \)-methyltransferases (PLMTs) that are worthy of further investigation. Of particular interest would be to establish the basis of differences in fatty-acids associated with the ethanolamine based lipids that I observed. One possibility is that an aminoalcohol phosphotransferase enzyme is discriminating among diacylglycerols (DAGs) with differing acyl chain combinations in concert with its binding to a cytidine diphosphate (CDP)-head-group. The differences could alternatively be achieved if there are differing pools of DAG present in the membrane at the site of phospholipid synthesis. The bigger question that is raised in this field of study, and more difficult to answer, is more of a question of evolution. Why is the flux of metabolites through the PtdCho biosynthetic pathway so
different among plant species? Another related question is why plants maintain PLMTs as essential components of PtdCho biosynthesis in some species, like soybean, but appear to be dispensable in others, such as Arabidopsis? Similarly, the soybean genome possesses 3 open reading frames (ORFs) encoding putative phosphoethanolamine N-methyltransferase activities that one would predict should be able to methylate ethanolamine-phosphate all the way to choline-phosphate, but apparently catalyzes the single methylation of ethanolamine-phosphate. Addressing these questions would make further in vivo studies on the soybean PLMT particularly interesting as it appears to be an essential gene in this species.

**PHOSPHOLIPID SIGNALING AND ATSEC14**

The field of phospholipid signaling in plants is a much more active field of study compared to phospholipid head-group metabolism. One area of particular interest would be to gain further insights into the function of Sec14p-like proteins in plants by investigating some of the other homologs found in Arabidopsis. Although there are 31 different Sec14p-like isoforms in Arabidopsis, studies on only 3 of these 31 have been published. However, there are several hurdles to be considered when studying these proteins. The lack of a direct biochemical assay to assess function of these proteins makes data interpretation challenging. The ties to polyphosphoinositide metabolism that are unfolding are intriguing, but for the work in this area to be meaningful, more studies need to be conducted in vivo to determine whether plant Sec14-like proteins function is truly similar to the yeast Sec14p and Sfh5p proteins (Schaaf et al., 2008). A nother very big hurdle is in the identification of which
Sec14p-like proteins may or may not be functioning redundantly. The large number of Sec14 homologs and the possibility of redundancy make the use of reverse genetics tools tricky, but not impossible.

The findings in Chapter 3 present several interesting future areas of study. Determining which phosphatidylinositol (PtdIns) kinase AtSec14-1 and AtSec14-5 are stimulating would be the first obvious thing to pursue. I would determine this using a couple of different approaches. Co-localization experiments could potentially be suggestive, but ultimately not definitive. Protein-protein interaction experiments would likely be uninformative as the mechanism by which Sec14p and Sfh5p stimulates PtdIns kinases does not involve a direct interaction (Schaaf et al., 2008). Imaging of green fluorescent protein pleckstrine homology (GFP-PH) domain fusion proteins specific to either PtdIns(3)P or PtdIns(4)P within the double mutant background would be the best approach to study as it may show a different distribution of PtdInsPs in the cell compared to wild-type. Such methods have been successfully used in Arabidopsis (Vermeer et al., 2006; Vermeer et al., 2009). Additionally, experiments in which a hyperosmotic stress is applied to plants expressing GFP-PH domain could yield information as the PtdInsP metabolism changes under stress in the double mutant background. Some care must be taken in the interpretation of data produced via this method as expression of PH domains can alter the level of its ligand in vivo.

The connection between AtSec14-1 and AtSec14-5 and seed germination and early seedling growth was unexpected. Developing a method to follow PtdInsPs during seed
germination would greatly assist our understanding of the connection between PtdInsP function in seed germination. The use of GFP-PH domain proteins expressed in Arabidopsis seeds could be helpful for this as well. In depth histological examination of the seeds of the double mutant plant could be helpful in explaining the means by which \textit{atsec14-1/atsec14-5} seeds are compromised in their ability to germinate, yet could also yield nothing for what would be a large investment of effort.

**MASS SPECTROMETRY ANALYSIS OF PHOSPHOINOSITIDES**

Chapter 4 is a starting point for the analysis phosphoinositides from plant extracts. We made an important achievement in successfully developing a method for this analysis specific for PtdInsPs, but there is a lot of room for improvement. Analyzing other phosphoinositides including PtdInsP$_2$ and discriminating between PtdIns(3)P and PtdIns(4)P isomers both would be the next big steps forward. True determination of both fatty acid species on the acyl chains of PtdInsPs would also be more informative, and it has been accomplished for the analysis of phospholipids from yeast but not phosphoinositides (Ejsing et al., 2009). In some cases, based on what is known about the fatty-acids associated with different phospholipids we can somewhat confidently assume what the individual fatty-acid species are. For instance a PtdCho molecule with a fatty-acid signature 34:3 most certainly represents 18:3 and 16:0, but a 36:4 species could be 18:2/18:2 or 18:3/18:1.

Improving the mass spec methodology to be able to analyze PtdInsPs from extracts of total lipids rather than extract enriched for acidic phospholipids would represent a
significant improvement for two reasons. It would allow for simultaneous quantitative analysis of other lipids within the same sample and would simplify the extraction procedure, perhaps enabling more amenable high-throughput studies. Such an analysis could be a snapshot of sorts. The ultimate goal in this area would be to obtain phospholipid metabolomic information in tissue sections. This has been achieved for lipids in mouse brain sections (Murphy et al., 2009). An analysis of this sort was also performed on the waxes on Arabidopsis leaves (Chae et al., 2009), but has yet to be successfully applied to plant phospholipids.
REFERENCES