Chapter 8

Phosphoinositide Metabolism: Towards an Understanding of Subcellular Signaling

Wendy F. Boss, Amanda J. Davis, Yang Ju Im, Rafaelo M. Galvão, and Imara Y. Perera
Department of Plant Biology, North Carolina State University, Raleigh, NC 27695-7612, USA

1. INTRODUCTION

The polyphosphorylated inositol phospholipids have multiple effects on cellular metabolism including regulating cytoskeletal structure, membrane associated enzymes, ion channels and pumps, vesicle trafficking, and producing second messengers (Cockcroft and De Matteis, 2001; Laude and Prior, 2004; Roth, 1999; Roth, 2004; Simonsen *et al.*, 2001; Takenawa and Itoh, 2001; Yin and Janmey, 2002). These diverse functions emphasize the importance of understanding both the spatial and the temporal regulation of phosphoinositide (PI) metabolism and the need to characterize the subcellular pools (Laude and Prior, 2004; Roth, 2004; Sprong *et al.*, 2001; Yin and Janmey, 2002).

Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) pools can be regulated by PI kinases, PtdIns(4,5)P_2-phosphatases, phospholipase C (PLC), and lipid transfer and binding proteins. The contribution of each to PtdIns(4,5)P_2 pools will depend on the metabolic status of the cell. Enzymes involved in PtdIns(4,5)P_2 biosynthesis are shown in Figure 1. Our goal is to convince the reader of the importance of characterizing the metabolic fluxes within the discrete subcellular phospholipid microdomains that make up the lipid signaling pools.

The chemistry of the lipid head group helps to explain the ubiquitous nature of these polar lipids. Sugar phosphates are the most fundamental structures in biology. They provide the backbone of DNA and RNA and are the building

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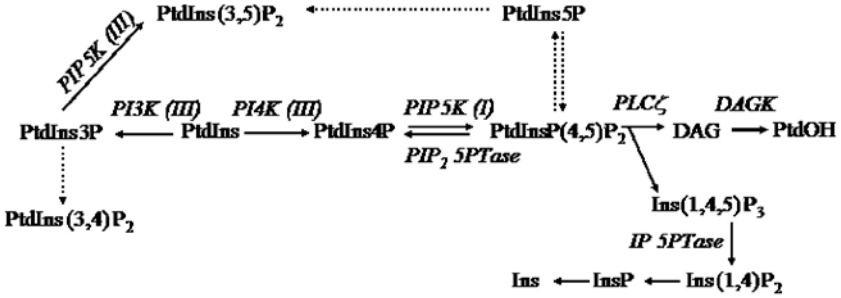

Figure 1. A summary of the PI pathway in plants. The enzymes catalyzing each step are shown in italics. Dashed arrows indicate reactions that have not been reported in plants. *Abbreviations*: DAG, diacylglycerol; DAGK, diacylglycerol kinase; Ins, inositol; InsP, inositol monophosphate; $Ins(1,4)P_2$, inositol (1,4,) bisphosphate; $Ins(1,4,5)P_3$, inositol (1,4,5) trisphosphate; IP 5PTase, inositol phosphate 5-phosphatase; PtdIns, phosphatidylinositol; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns5P, phosphatidylinositol-5-phosphate; $PtdIns(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; $PtdIns(3,4)P_2$, phosphatidylinositol-3, 4-bisphosphate; $PtdIns(3,5)P_2$, phosphatidylinositol-3,5-bisphosphate; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol phosphate 5-kinase; PIP_2 5PTase, phosphatidylinositol(4,5) bisphosphate 5-phosphatase; PLC, phospholipase C.

blocks for carbon metabolism and energy production by the cell. It is likely that glycolipids were part of the original matrix coating RNA as the first cells were formed, but to our knowledge, this remains to be tested (Chen and Szostak, 2004; Hanakahi *et al.*, 2000; Hanczyc *et al.*, 2003). Notably, as organisms evolved, the core proteins that synthesized sugar phosphates became fundamental for life, and inositol phospholipids became a part of the lipid bilayer. In addition, the inositol phosphate head group provides a means for rapidly changing the membrane surface charge. The impact of changing the phosphorylation status of proteins on the biology of the cell is well known. Edmond Fischer and Edwin Krebs were acknowledged in 1992 for their seminal work in this area. However, the impacts of altering lipid phosphorylation on fundamental cellular processes are only beginning to be appreciated (Loewen *et al.*, 2004).

The potential to convey explicit information through the multiple phosphorylation sites of the inositol ring validates the conservation of the PI signaling pathway by biological systems. Furthermore, the stereospecificity of the five hydroxyl groups in the inositol phospholipids that extend from the surface of the bilayer conveys structural identity, which will impact multiple interacting molecules such as metabolites, proteins, and perhaps even nucleic acids. Discrete lipid microdomains are defined by the lipid, protein, and/or nucleic acid scaffold surrounding them. These regions may be part of the bilayer, they may bridge the cytoskeleton/bilayer interface, or they may be scaffolds of molecules that are mobilized within the cell.

It is becoming increasingly clear that the PI lipids are organized within discrete subcellular microdomains. At this stage of our knowledge, however, it is

easier to describe microdomains in terms of function since the structures defining them are poorly characterized. We will use the term plasma membrane signaling pool to describe the microdomains of PtdIns(4,5)P_2 associated with the plasma membrane which are rapidly hydrolyzed in response to a stimulus. Our assumption is that some, but not all, plasma membrane PtdIns(4,5)P_2 microdomains are a part of the same signaling pool. A corollary to this is that different stimuli will activate different signaling pools of PtdIns(4,5)P_2 and thereby mediate different stimulus–response pathways.

The ability to sense and respond to the environment is essential for the survival of all living organisms. The PI pathway is involved in sensing environmental stimuli and is one of the most conserved signaling pathways. Components of the PI pathway are present in both prokaryotes and eukaryotes. For example, membranes from the hyperthermophilic archaeon *Pyrococcus furiosus* will phosphorylate phosphatidylinositol (PtdIns) to form PtdIns3P (Figure 2).

Comparative analysis of the enzymes involved in the PI pathway suggests that as organisms evolved, the complexity of the pathway also increased and multiple families of enzymes were produced as shown in Table 1 (Cockcroft and De Matteis, 2001; Drøbak *et al.*, 1999; Mueller-Roeber and Pical, 2002; Roth, 2004; Stevenson *et al.*, 2000). This increase in diversity would increase the potential for generating discrete subcellular lipid domains.

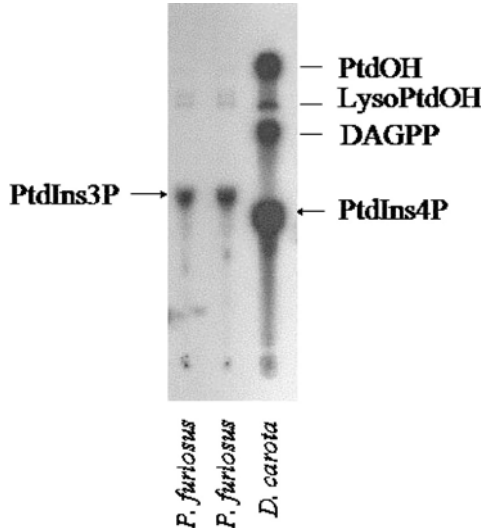

Figure 2. Membranes isolated from *Pyrococcus furiosus* will phosphorylate PtdIns to form PtdIns3P. Microsomal membranes were isolated from *Daucus carota* (wild carrot cells) grown in cell culture and *P. furiosus*. Lipid kinase activity was assayed as previously described using PtdIns as a substrate (Bunney *et al.*, 2000). The lipids were extracted and separated by thin layer chromatography as described by Walsh *et al.* (1991) to separate PtdIns3P and PtdIns4P. The migrations of the lipid standards are indicated (Brglez and Boss, unpublished results).

Table 1. A comparison of the gene families for the major PI pathway enzymes in humans and plants

Gene	Families in humans	Families in plants
PI3K	Type I, II, and III	Type III only
PI4K	Type II and III	Type III and possibly II
PIPK	Type I, II, and III	Type I and III
PLC	β, γ, δ, ϵ, and ζ	ζ

2. LIPID–PROTEIN INTERACTIONS

Insights into PI-mediated signaling domains can be gained from understanding lipid–protein interaction. The inositol phospholipids have been shown to bind to proteins [ATPases, profilin, phospholipase D (PLD), dynamin, and patellin] and to alter their function. For example, PtdIns(4,5)P_2 is required for PLDγ1 and β1 function and stimulates the activity of PLDα1 (Pappan and Wang, 1999; Wang, 1999; Wang, 2001). In a dynamic system such as a living cell this means that if PtdIns(4,5)P_2 is hydrolyzed in response to a stimulus, there would be a decrease in the specific activity of the PLD within the PtdIns(4,5)P_2 microdomain until PtdIns(4,5)P_2 is resynthesized. In this manner, an oscillation of the activity of selected PLD isoforms would be produced in response to sequential changes in PLC activity and PtdInsP kinase activity. This complicates the life of the researcher trying to interpret data on lipid-mediated signaling and emphasizes the need to include time course studies and to simultaneously monitor the suite of lipids and lipid signaling pathways (Li *et al.*, 2004; Meijer and Munnik, 2003; Van Leeuwen *et al.*, 2004; Welti *et al.*, 2002).

Other PI binding proteins include profilins. Profilins are low molecular weight cytoskeletal binding proteins that bind PtdIns(4,5)P_2 and other inositol lipids. Maize profilins have been classified into two groups (Gibbon *et al.*, 1998; Kovar *et al.*, 2000). They are thought to competitively bind actin and regulate actin assembly at different locations within the cell. The class I profilins are more abundant and are proposed to be low affinity G-actin buffers. The class II profilins are in lower abundance but have a higher affinity for G-actin. Both classes of profilins bind PtdIns(4,5)P_2; however, the class I profilins bind with a higher affinity (Kovar *et al.*, 2000). Studies of knockout and T-DNA insertion lines have yet to clearly delineate functions of the profilin isoforms. This is most likely because of the pleotropic effects of the profilins on actin cytoskeleton, vesicle trafficking and signaling, all of which may be mediated through PI pathway intermediates (Drøbak *et al.*, 2004; Huang *et al.*, 2003; Staiger *et al.*, 1997). These studies emphasize the need to understand binding affinities and the *in vivo* dynamics of lipid–protein interactions.

Patellin is a newly identified PI binding protein. Patellin 1 (PATL1) is one of a family of six Arabidopsis proteins containing a Sec14 lipid binding domain

and a Golgi dynamics domain (GOLD). The GOLD domain is found in proteins involved in vesicle trafficking and secretion (Anantharaman and Aravind, 2002). The PATL1 Sec14 domain is homologous to the Sec14p domain in yeast, which binds PtdIns and phosphatidylcholine (Bankaitis *et al.*, 1989). However, biochemical characterization of PATL1 indicates that it binds with highest specificity to PtdIns5P and PtdIns(4,5)P_2. Furthermore, it was shown that PATL1 localized to the cell plate during cytokinesis. The domain structure of PATL1 and its lipid binding properties indicate a role for PATL1 in membrane trafficking to the expanding cell plate and reveal a role for PIs in cell plate formation (Peterman *et al.*, 2004). It will be interesting to determine what role, if any, previously identified lipid transfer proteins play in this process (Kapranov *et al.*, 2001; Monks *et al.*, 2001). Further study is needed to determine more precisely the function of the protein–lipid interactions in these processes.

Another family of lipid binding proteins involved in vesicle trafficking is dynamins. Dynamins are a family of large molecular weight (60–110 kDa) GTP binding proteins involved in membrane trafficking to and from various compartments, cell plate formation, and mitochondrial and chloroplast division. Although dynamins are found in both plants and animals, there are distinct differences between them. All the animal dynamins contain a pleckstrin homology (PH) lipid-binding domain for targeting the protein to the membrane. The PH domain consists of approximately 100 amino acids that bind with varied specificity to inositol phospholipids (Lemmon, 2003; Lemmon *et al.*, 1996). Of the 16 dynamins in Arabidopsis, only two (DRP2A and DRP2B) contain a PH domain (Hong *et al.*, 2003). The most extensively characterized of the two, DRP2A, has been localized to the trans Golgi network and has been shown to be involved in trafficking to the vacuole (Jin *et al.*, 2001). The PH domain in DRP2A (previously ADL6) has a unique structure with three regions of 16–24 amino acids inserted between the characteristic β sheets that make up the PH domain. Lipid binding studies of the full length DRP2A protein shows that it binds strongly to PtdIns3P with minimal binding to PtdIns4P. In contrast, the PH domain alone binds PtdIns3P and PtdIns4P equally. Further studies indicated that the lipid binding specificity of the DRP2A PH domain was determined by interactions of the PH domain and the C-terminus of DRP2A suggesting that this is an important site for lipid–protein regulation *in vivo* (Lee *et al.*, 2002). Further investigation is needed into the exact function of DRP2A in membrane trafficking and how the PH domain and PtdIns3P contribute to its function.

The specific binding of a PH domain to lipids depends not only on the structure of the parent protein but also on the protein environment surrounding the membrane lipids it will bind. Studies using GFP-PH domains to identify lipid microdomains have shown that some lipids are inaccessible to PH domain binding and emphasize the need to characterize the binding specificity of the PH domain prior to engaging in *in vivo* studies (Balla *et al.*, 2000; Balla and Varnai, 2002).

3. SUBCELLULAR LIPID DOMAINS

The proteins and other molecules that bind to the inositol lipids will affect their turnover rate and the extent of their impact on cellular metabolism. If so many cytoskeletal proteins bind to the inositol lipids, does this mean that the lipids are associated with the cytoskeleton? PtdIns 3-kinase activity is cytoskeletal associated (Dove *et al.*, 1994) and at least one form of PtdIns 4-kinase, (the type III PtdIns 4-kinase) is cytoskeletal associated (Stevenson *et al.*, 1998).

A*t*PI4Kα1 (Figure 3) is probably the major enzyme contributing to the previously reported F-actin associated PtdIns 4-kinase activity (Tan and Boss, 1992; Xu *et al.*, 1992). While it was also reported that PIP5K activity was associated with the actin-enriched fraction from plants, the specific isoform of the enzyme has not been identified (Tan and Boss, 1992). As more specific antibodies and molecular probes become available, it will be important to monitor lipid protein interaction *in vivo* and *in vitro*.

*At*PI4Kα1 is a low abundance protein and is difficult to detect on immunoblots of membranes from Arabidopsis or *Nicotina tobaccum*. When

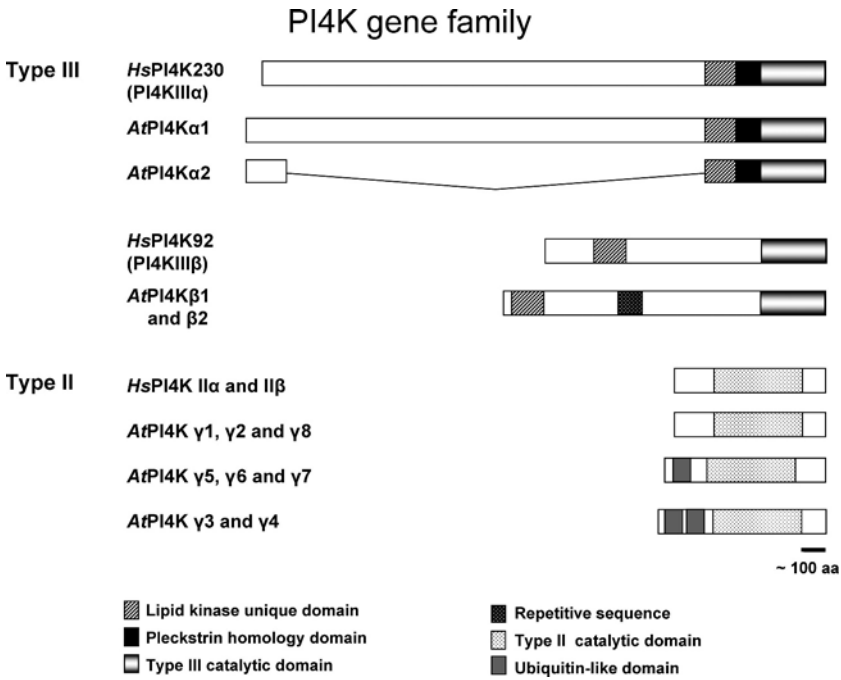

Figure 3. Linear representations the *Arabidopsis thaliana* (*At*) PI4Ks in comparison to human (*Hs*) isoforms. Thus far, only the type III *At*PI4Ks have been shown to be functional enzymes. Conserved domains are indicated and described in the text.

expressed in a heterologous system, however, the protein is readily detectable and has threefold higher specific activity than *At*PI4Kβ1 the other characterized PtdIns 4-kinase isoform (Stevenson-Paulik *et al.*, 2003). Of interest, *At*PI4Kβ1 has a charged repeat domain in the N-terminus that has been proposed to be important for membrane association (Xue *et al.*, 1999). Based on *in vitro* data and heterologous expression in the eukaryotic *Spodoptera frugiperda* (Sf9) insect cells, it is not evident which membrane, if any, is the predominate location. It is possible, that like the yeast and human orthologs, *At*PI4Kβ1 will be involved in membrane secretion from the Golgi apparatus and could potentially supply the plasma membrane PtdIns4P (Stevenson-Paulik *et al.*, 2003; Xue *et al.*, 1999). Like their human orthologs, both *At*PI4Kα1 and *At*PI4Kβ1 can be phosphorylated *in vitro* (Suer *et al.*, 2001; Zhao *et al.*, 2000) (Stevenson-Paulik and Galvão, unpublished results). It will be interesting to see which peptide domains and posttranslational modifications regulate the intracellular distribution of the 4-kinases.

A unique feature of the plant type III PI4Kα is the PH domain that binds to PtdIns4P. Two other PH domains that selectively bind PtdIns4P are the oxysterol binding protein, OSBP, and FAPP1 (phosphatidylinositol-*f*our-phosphate *a*dap*t*or *p*rotein-1) (Dowler *et al.*, 2000; Levine and Munro, 2002). One Arabidopsis PH domain protein, *At*PH1, isolated as a homolog of pleckstrin has been reported (Mikami *et al.*, 1999); however, it binds PtdIns3P and not PtdIns4P (Dowler *et al.*, 2000). The first question asked about the *At*PI4Kα1PH domain was what effect, if any, did it have on enzyme activity? It turns out that *At*PI4Kα1 activity was differentially sensitive to PtdIns4P, the product of the reaction. The specific activity of *At*PI4Kα1 was inhibited 70% by 0.5 mM PtdIns4P in the presence of 1:1 concentration of substrate, PtdIns. The effect of PtdIns4P was not simply due to charge, as AtPI4Kα1 activity was stimulated approximately 50% by equal concentrations of the other negatively charged lipids, PtdIns3P, phosphatidic acid, and phosphatidylserine (Stevenson-Paulik *et al.*, 2003). In contrast, the specific activity of AtPI4Kβ1, which does not have a PH domain, was stimulated twofold by PtdIns4P but not other negatively charged lipids and was inhibited 50% by phosphatidylcholine. Expression of *At*PI4Kα1 without the PH domain compromised PtdIns 4-kinase activity; decreased association with fine actin filaments *in vitro*, and resulted in mis-localization of the kinase *in vivo* in the insect cells. These studies support the idea that the two Arabidopsis type III PI4Kα1 and PI4Kβ1 are responsible for distinct PI pools.

The fact that *At*PI4Kα1 activity is inhibited by PtdIns4P and inhibition is relieved by adding rPH domain suggests a mechanism whereby other PtdIns4P-binding proteins such as profilin (Drøbak *et al.*, 1994), dynamin-like proteins (Kim *et al.*, 2001b), actin depolymerizing factor (Gungabissoon *et al.*, 1998), phospholipid transfer proteins (Cockcroft, 1996; Cockcroft and De Matteis, 2001; Monks *et al.*, 2001), and potentially PIP5K should affect PI4Kα1 activity or subcellular distribution and illustrates the importance of studying specific

microdomains within the cell. In addition, the PH domain may be involved in the previously characterized activation of PI4K by eEF1A, a translational elongation factor and actin binding and bundling protein (Yang and Boss, 1994a). eEF1A was shown to bind PLCδ PH domain and increase PLC activity (Chang *et al.*, 2002). The potential role of eEF1A in regulating PI4K, PLC, and PIP5K (Davis *et al.*, unpublished results), in additional to its function in actin bundling, provides a mechanism where the status of the inositol lipids, cytoskeletal structure, and protein translation would be coordinately regulated.

In addition to the cytoskeleton, the nucleus is an area where PI metabolism is prevalent. An important and often overlooked observation is that the plant type III PtdIns 3-kinase activity is present in nuclei and immunodetection of the enzyme indicates co-localization with putative transcription initiation sites (Bunney *et al.*, 2000). Also, PtdIns 3-kinase activity and transcript increase significantly during root nodulation (Hernandez *et al.*, 2004; Hong and Verma, 1994) consistent with a role in membrane trafficking and/or transcriptional regulation. As previously reported (Bunney *et al.*, 2000; Drøbak, 1992; Mueller-Roeber and Pical, 2002; Stevenson *et al.*, 2000), the only family of PtdIns 3-kinases found in plants is the type III Vps34-like family that is associated with vacuolar trafficking in yeast (De Camilli *et al.*, 1996; Herman and Emr, 1990; Simonsen *et al.*, 2001) and may have a similar function in plants (Kim *et al.*, 2001a; Matsuoka *et al.*, 1995). Like *S. cerevisiae*, terrestrial plants do not have the plasma membrane type I and II PtdIns 3-kinases associated with PtdIns(3,4,5)P_3 biosynthesis and with much of the human signaling. It should be noted, however, that in a heterologous system, Arabidopsis PIP5K1 will produce PtdIns(3,4,5)P_3 by phosphorylating PtdIns(3,4)P_2 (Elge *et al.*, 2001).

Early studies indicated that nuclei isolated from protoplasts have a high percentage of [^{3}H]inositol PtdIns(4,5)P_2 relative to the total [^{3}H]inositol phospholipids (Hendrix *et al.*, 1989). In addition, histones cause an 80-fold increase in microsomal PtdIns 4-kinase (Yang and Boss, 1994b) activity suggesting that PtdIns4P and PtdIns(4,5)P_2 along with PtdIns3P (Bunney *et al.*, 2000) may be important components of the nuclear matrix or nuclear membrane. Hydrolysis of the inositol phospholipids by PLC and the formation of diacylglycerol would facilitate membrane fusion and could be an integral part of nuclear membrane formation during karyokinesis. The presence of PtdIns(4,5)P_2 in the nucleus is also consistent with a role for Ins(1,4,5)P_3 derived Ins(1,4,5,6)P_4-mediated transport of RNA from the nucleus (Odom *et al.*, 2000). Whether the lipids or inositol phosphates would interact directly or indirectly with RNA is not known. Analysis of inositol lipid or inositol phosphate binding to RNA is virtually uninvestigated in plants. The role of the PI pathway in plant nucleus is far from resolved and provides a rich area for future studies (Irvine, 2003).

One caveat of the studies of nuclei is that the ER is closely associated with the nuclear envelope. Lipid kinase activity has been associated with ER and the role of the inositol lipids in membrane trafficking is well accepted (Cockcroft and De Matteis, 2001; Godi *et al.*, 2004; Roth, 2004; van Meer and Sprong,

2004). The early biochemical studies of the lipid kinases were done prior to having the molecular and biochemical tools that we have today. Future *in vivo* studies of the enzymes and lipids should help to sort out the functional biology. The genomics and biochemistry of the enzymes involved in inositol lipid metabolism have been reviewed in detail quite recently (Drøbak, 1992; Mueller-Roeber and Pical, 2002; Van Leeuwen *et al.*, 2004).

The question of lipid trafficking in plants has been addressed thus far primarily with inhibitor studies and the expression of lipid binding peptides. These studies have led to intriguing results consistent with a role of PtdIns 3-kinase in trafficking to the vacuole (Kim *et al.*, 2001a; Matsuoka *et al.*, 1995). Future studies should focus on identifying the scaffolds of interacting proteins in order to understand more precisely the mechanisms involved. The role of lipid transfer proteins in plants is still a mystery. While orthologs of the yeast phosphatidylinositol lipid transfer proteins have been identified, their precise function in regulating membrane lipid distribution is not clear (Drøbak *et al.*, 1999; Kapranov *et al.*, 2001; Monks *et al.*, 2001). Knockouts of one isoform of these lipid transfer proteins lead to aberrant root hair morphology, but otherwise normal plant growth suggesting that there is functional redundancy within this multigene family that helps to maintain a normal phenotype (Vincent *et al.*, 2005). More complete biochemical and genetic characterization of the family of the putative lipid transfer proteins will reveal specific functions.

4. REGULATION OF PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE POOLS

Regulation of PtdIns (4,5) P_2 biosynthesis begins with the families of PI4Ks. The properties of *At*PI4Kα1 and *At*PI4Kβ1 are described above and more extensively in recent review (Mueller-Roeber and Pical, 2002). These PI4Ks are orthologs of the yeast and mammalian Stt4p/PI4Kα and Pik1p/PI4Kβ, respectively. Although two other type III PI4Ks, *At*PI4Kα2 and β2 can be identified in the genomic database, there are no ESTs reported for these two isoforms and attempts to recover a full length cDNA using PCR have been unsuccessful (Galvão, Stevenson-Paulik *et al.*, unpublished results) suggesting that these may not encode functional enzymes. Curiously, there are several 60–70-kDa polypeptides detected with antibodies raised against the *At*PI4K α1 that were proposed to be encoded by *At*PI4Kα2 (Shank *et al.*, 2001; Stevenson-Paulik *et al.*, 2003), but the lack of ESTs and the failure to obtain PCR products suggest that these bands either represent proteolytic products of *At*PI4Kα1 or β1 or are alternative splice variants encoded by these transcripts. While it is possible that *At*PI4Kα2 and *At*PI4Kβ2 transcripts are expressed in specific cell types under developmental control, they must be in relatively low abundance as, to our knowledge, they have not been detected in any of the publicly available transcript array databases.

Another family of PI4Ks that provides further opportunity for study is the type II family. Mammalian and yeast type II PI4Ks were recently cloned and characterized and are thought to function to supply PtdIns4P to the plasma membrane signaling pool (Balla *et al.*, 2002; Minogue *et al.*, 2001; Nishikawa *et al.*, 1998; Wei *et al.*, 2002). There are eight putative type II PI4Ks sequences in the *Arabidopsis* genome (Mueller-Roeber and Pical, 2002); however, to date, none of them have been shown to encode functional lipid kinases. At the amino acid sequence level, the lipid kinase domain from the putative plant type II PI4Ks are 26–30 % identical to the conserved type II lipid kinase domains from either mammalian or yeast enzymes. However, unlike the yeast or mammalian homologs, five of the plant proteins contain N-terminal *ub*iquitin-*l*ike (UBL) domain. Most of UBL domain-containing proteins share the ability to interact with 26S proteasome but their functions vary depending on the other domains present (Eskildsen *et al.*, 2003; Hartmann-Petersen *et al.*, 2003). If these plant proteins are functional lipid kinases, there may be connections between the controlled protein degradation and the PI pathway, that is, they might be regulated by ubiquitin/26S proteasome. Alternatively, the plant type II PI4Ks may not have lipid kinase activity, but rather be like the target of rapamycin (TOR) protein kinases that contain a lipid kinase-like domain but lack lipid kinase activity (Harris and Lawrence, 2003; Helliwell *et al.*, 1994).

Two intriguing conundrums in plant PI signaling are, which PI4K isoform(s) supplies the plasma membrane pool of PtdIns4P and how is the plasma membrane PtdIns(4,5)P_2 signaling pool regulated? Answers to these questions are integral to understanding the dynamics of the plasma membrane PI signaling pathway. Because PI4Kβ1 is product stimulated and because the relative ratio of PtdIns4P to PtdIns(4,5)P_2 is high in plants, PI4Kβ1 might be the major source of PtdIns4P. It remains to be seen whether PI4Kβ1 is trafficked to the plasma membrane and supplies the plasma membrane signaling pool or if that pool is determined by the newly identified family of putative PI4Ks, the type II enzymes.

Although several labs have shown that whole cell PtdIns(4,5)P_2 is rapidly turning over (Munnik *et al.*, 1998), the plasma membrane signaling pool is poorly characterized. A profile of the Arabidopsis PIPKs and PLC is given in Figure 4.

Evidence suggests that under certain physiological conditions, the plasma membrane signaling pool can become limiting. For example, if cells are growing in nutrient depleted medium, the plasma membrane PIP kinase activity increases prior to the production of Ins(1,4,5)P_3 in response to stimulation (Heilmann *et al.*, 2001). An interesting characteristic of plants is that the ratio of PtdIns4P to PtdIns(4,5)P_2 is on the order of 10 or 20 to 1 (Boss, 1989; Gross and Boss, 1993; Meijer and Munnik, 2003; Mueller-Roeber and Pical, 2002; Stevenson *et al.*, 2000), compared to values of 1:1 or 1:2 found in the most reactive animal cells such as brain and iris muscle cells. These observations led many to suggest that PIPKs limit the production of PtdIns(4,5)P_2 and the flux

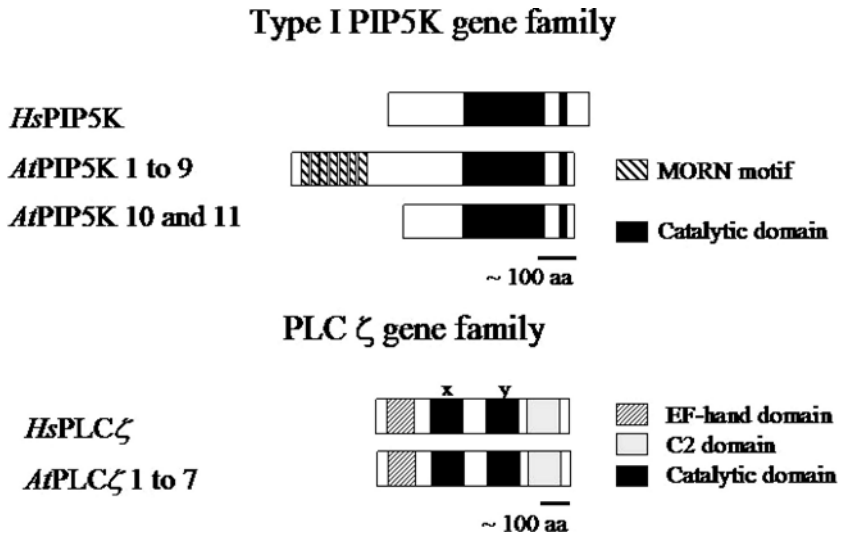

Figure 4. Linear representation of the Arabidopsis PIPKs and PLCs. The Arabidopsis PtdInsP 5-kinases are most similar to the human type I PtdInsP 5-kinases. There are 11 putative type I *At*PtdInsP 5-kinases in Arabidopsis arranged in two subfamilies based on size. Subfamily B contains *At*PIPK1-9, all of which contain membrane occupation and recognition nexus (MORN) repeats. *At*PIPK10-11 are in Subfamily A with molecular weights less than that of the members of subfamily B and contain no MORN repeats. The Arabidopsis phosphoinositide specific phospholipase C family is most similar to the animal PLCζ. There are seven functional PI-PLCs in Arabidopisis (Hunt *et al.*, 2004). All isoforms contain EF-hand motifs, the X and Y catalytic domains characteristic of PI-PLCs and a C2 lipid-binding domain.

through the plant PI pathway. Evidence supporting this hypothesis was reported recently by Perera *et al.* (2002) who showed that when the human inositol polyphosphate 5-phosphatase (InsP 5-ptase) was expressed in plants, there was increased Ins(1,4,5)P_3 turnover and a decrease in PtdIns(4,5)P_2. The human InsP 5-ptase is localized to the plasma membrane and specifically hydrolyzes Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 but not PtdIns(4,5)P_2. As a result of expressing the human InsP 5-ptase the rate of flux through the plasma membrane PI pool increased dramatically. Although the plasma membrane PtdInsP 5-kinase activity increased threefold, there was a decrease in net PtdIns(4,5)P_2 suggesting that synthesis was not keeping up with the hydrolysis. These data indicate that PtdIns(4,5)P_2 biosynthesis was limiting in this transgenic system; however, they do not explain the mechanism nor do they explain the differences in the inositol phospholipid ratios generally reported for plants and animals.

Insights into the reasons for the high ratio of PtdIns4P to PtdIns(4,5)P_2 in plants have come from comparative biochemical studies of recombinant proteins. The *Km* of the human PIPKβ1 for PtdIns4P is close to 70-fold lower than

that found for *At*PIPK1 [μM compared to 69 μM, respectively (Davis *et al.*, 2004; Kunz *et al.*, 2002)]. Moreover, the V_{max} of the Arabidopsis enzyme is sixfold less. This means that at low concentrations, PtdIns4P will rapidly be converted to PtdIns(4,5)P_2 in animal cells, but a much higher threshold concentration of PtdIns4P would be required in plants. These data provide a logical explanation for the relative high ratio of PtdIns4P:PtdIns(4,5)P_2 in plants.

In animal cells, selectively increasing intracellular PtdIns(4,5)P_2 can decrease apoptosis and stabilize intracellular membranes. For example, increasing PIP5K1α expression 2.6-fold inhibited caspase activity and decreased apoptosis in HeLa cells (Mejillano *et al.*, 2001). Furthermore, increasing PtdIns(4,5)P_2 either by overexpressing PIP5K or a constitutively active PIP5K-regulatory protein, Arf6-GTP, resulted in internalization and trapping of membranes in PtdIns(4,5)P_2-positive, actin-coated endosomal vesicles that fused to form vacuolar-like structures (Brown *et al.*, 2001). These data make a compelling argument that altering PIP5K can affect cellular metabolism.

Could similar studies be done with plant enzymes? Only 1 of the 14 putative plant PIPKs, *At*PIPK1, has been well characterized (Davis *et al.*, 2004; Elge *et al.*, 2001; Mueller-Roeber and Pical, 2002; Westergren *et al.*, 2001). *AtPIPK1* encodes a 752 amino acid protein with a predicted molecular weight of 86 kDa. Studies using promoter-Gus fusions indicate high levels of *AtPIPK1* expression in procambial tissue of leaves flowers and roots (Elge *et al.*, 2001). Using recombinant protein, Westergren *et al.* (2001) have shown that the enzyme activity is downregulated by protein phosphorylation and that the substrate preference was for PtdIns4P although PtdIns3P and PtdIns5P were also phosphorylated, but little else is known about the regulation of the plant PIPKs. The substrate specificity and enzyme activity of the human type I and II PIPKs are regulated by an activation loop (Anderson *et al.*, 1999; Doughman *et al.*, 2003; Kunz *et al.*, 2002). Interchanging the activation loops of PIPKs predictively alters the site of inositol phosphorylation converting a type I PtdIns4P 5-kinase into a type II PtdIns5P 4-kinase and vice versa (Kunz *et al.*, 2000). Furthermore, using site-directed mutagenesis, demonstrated that a conserved Glu (362) in the activation loop of the human type I PIPKβ is essential for efficient PtdIns4P 5-kinase function. Similarly, the activation loop of the human type IIβ enzymes has a conserved Ala (381) that is essential for PtdIns5P 4-kinase function (Kunz *et al.*, 2002). Because the activation loop plays such a critical role in the human PIPKs, it is possible that evolutionary changes in the activation loop of the plant PIPKs are responsible, in part, for the differences in the specific activities.

The Arabdopsis PtdInsP kinase isoforms, *At*PIPK1-11, all have the conserved Glu of the human type I activation loop suggesting that the plant proteins are type I PIPKs. Biochemical data are more equivocal, but also indicate that PtdIns4P is the preferred substrate for PIPK1 (Davis *et al.*, 2004; Elge *et al.*, 2001; Westergren *et al.*, 2001). In addition, biochemical data using recombinant *At*PIP5K10

indicates that it too is a type I PtdIns4P 5-kinase (Perera *et al.*, 2005). *At*PIPK10 and *At*PIPK11 are both members of subfamily A of PIPKs in contrast to *At*PIPK1-9, subfamily B (Mueller-Roeber and Pical, 2002). *At*PIPK10 (At4g01190) and *At*PIPK11 (At1g01460) both lack putative membrane occupation and recognition nexus (MORN) repeats in the N-terminus suggesting that they will reside in distinct subcellular locations. Transcript profiles have indicated that *At*PIPK11 is prevalent in pollen (Becker *et al.*, 2003) so they may also represent a tissue-specific isoform of the enzyme. Whether this is the isoform that is involved in Rac regulation of pollen tube growth remains to be seen (Kost *et al.*, 1999). Regulation of plant PIPKs by Rac is an important finding that identifies a signaling complex for generating PtdIns(4,5)P_2 microdomains and a mechanism for altering PIPK activity *in vivo*.

Based on predicted amino acid sequences and biochemical data there is no evidence of type II PIPKs in Arabidopsis. The type II PIPKs are PtdInsP 4-kinases that preferentially phosphorylate PtdIns5P. Putative type III kinases (*At*Fab1a-d) have been identified in the Arabidopsis genome. The predicted amino acid sequences of the *At*Fabs are similar to the human type III kinases and the yeast FAB enzymes that have a PtdIns3P binding domain (FYVE domain) and that phosphorylate PtdIns3P to form PtdIns(3,5)P_2. At least one isoform, *At*Fab1a, has been shown to be a functional PtdIns3P 5-kinase (Drøbak, unpublished results).

Although *in vitro* kinase assays have led to important insights into the regulation of the pathway, it is important to appreciate the potential limitations of these assays that are usually conducted under optimal conditions. Until we can characterize the enzymes within their subcellular microenvironments, we will not truly understand their impact. The effects of altering Rac expression on PIPK activity, actin cytoskeleton and pollen tube growth is one example (Kost *et al.*, 1999). Other examples of how microdomains can affect the product produced by endogenous enzymes comes from recent studies of yeast lacking PTEN, a protein that dephosphorylates PtdIns(4,5)P_2. The PTEN mutant produced PtdIns(3,4,5)P_3, a lipid not normally present in the cells (Mitra *et al.*, 2004). Presumably as a result of the cell not being able to dephosphorylate PtdIns(4,5)P_2 there was a sufficient increase in inositol lipids to favor additional phosphorylation. Elge *et al.* (2001) showed that when expressed in insect cells and given excess substrate *At*PIPK1 produced PtdIns(3,4,5)P_3. Whether this would actually happen under normal or stressed conditions might vary with different species (DeWald *et al.*, 2001; Dove *et al.*, 1997; Pical *et al.*, 1999). It is not difficult to imagine that alterations of PI flux during a sustained stress or as a result of genetic manipulation could result in PtdIns(3,4,5)P_3 production in plants.

Plasma membrane PtdIns(4,5)P_2 levels will be regulated both by synthesis and catabolism. Hydrolysis by C-type phospholipases is the best studied PtdIns(4,5)P_2 catabolic pathway (Cote and Crain, 1993; Hunt *et al.*, 2004). PLC activity in plants was first described by Sommarin's lab (Melin *et al.*, 1992; Melin *et al.,* 1987). Analysis of tissue-specific expression profiles and

the calcium sensitivity of the seven active *Arabidopsis* isoforms are reported by Hunt *et al.* (2004). All the data thus far indicate that the plant PLCs are calcium stimulated and belong to the ζ family of enzymes (Hernandez-Sotomayor *et al.*, 1999; Hunt *et al.*, 2004). That is, they are calcium regulated but lack the PH domain of the δ family of PLCs (Mueller-Roeber and Pical, 2002). The PH domain of the PLC δ binds to PtdIns(4,5)P_2 and Ins(1,4,5)P_3 and in doing so facilitates the binding of the enzyme to the membrane lipids until sufficient Ins(1,4,5)P_3 is produced to displace it (Cifuentes *et al.*, 1994). In this manner PLC δ comes on and off the membrane in response to stimuli. It is not known what regulates the subcellular distribution of the plant PtdIns(4,5)P_2 PLCs. Most of the activity is membrane associated and localized increases in cytosolic Ca^{2+} could selectively activate the specific isoforms (Hunt *et al.*, 2004). The specific activities reported in early studies of isolated membranes were similar for plants and animals (Sandelius and Sommarin, 1990). If this proves to be true when purified recombinant proteins are compared, it would support the hypothesis that under normal conditions, plant PLCs do not limit the flux through the PI pathway.

It is important to remember that there are no G-protein regulated (β family) or tyrosine kinase-regulated (γ family) PLCs in Arabidopsis. Of all the enzymes in the plant PI pathway, the PLCs are the least complex and provide the best support for the notion that the plant PI pathway is not as highly evolved as the human brain PI signaling pathway. A corollary to this hypothesis is that the ζ family of PLCs should be the seminal isoforms from which the others evolved. Attempts have been made to alter the expression at least two of these *in planta* (Hunt *et al.*, 2003; Mills *et al.*, 2004; Sanchez and Chua, 2001). While the antisense plants had no reported guard cell phenotype (Sanchez and Chua, 2001), plants expressing the sense construct of PLC 1 and 2 under the control of a guard cell promoter had a wilted phenotype in response to water stress and had reduced, not increased levels, of PLC transcript and reduced levels of guard cell PLC activity. Hunt *et al.* (2003) suggested that the decreased response of the guard cells to water stress and abscisic acid (ABA) was consistent with the lack Ins(1,4,5)P_3-mediated signaling. Comparative analyses of pharmacological and genetic manipulations of PLC activity of guard cells have revealed the complexity of the response (Mills *et al.*, 2004). Until we know more about the subcellular localization of the proteins and how their activity is regulated, it is difficult to know what pleiotropic effects result from changes in PLC activity.

Another potential fate of the PtdIns(4,5)P_2 plasma membrane signaling pool is that it can be dephosphorylated by phosphatases. The InsP 5-ptase and the effects of altering gene expression have been described in Chapter 3. The family of 5PTases most likely to affect the plasma membrane signaling pool are the type II, which preferentially hydrolyze inositol phospholipid substrates. Mutations in these genes have been associated with several diseases in humans (Pendaries *et al.*, 2003; Tronchere *et al.*, 2003). There are at least nine putative inositol phospholipid PTases in Arabidopsis all of which contain a predicted

suppressor of actin (SAC, Novick *et al.*, 1989) domain and are predicted to hydrolyze PtdInsPs (Zhong and Ye, 2003). Some mutations in these SAC domain-containing proteins affect cell wall biosynthesis and have been characterized as fragile fiber mutants (Zhong and Ye, 2003). The lack of some SAC-like proteins during development (Despres *et al.*, 2003) suggests an important role for PtdIns(4,5)P_2 during embryo development and is consistent with increased PtdInsP 5-kinase activity and PtdIns(4,5)P_2 in embryogenic cell cultures (Chen *et al.*, 1991; Ek-Ramos *et al.*, 2003; Wheeler and Boss, 1987). Mutations in tissue- and cell-specific isoforms of the lipid phosphatases that result in increased microdomains of PtdIns(4,5)P_2 will no doubt lead to important insights. One exciting example of cellular and subcellular specificity has been reported for another member of this family, SAC9 (Williams *et al.*, 2005). The SAC9 mutant has flaccid guard cells and is a dwarf plant suggesting that SAC9 is regulating the guard cell plasma membrane signaling pool of PtdIns(4,5)P_2. PtdIns(4,5)P_2 levels in SAC9 mutant plants increase 40-fold. Ins(1,4,5)P_3 levels also increase indicating a shunt of PtdIns(4,5)P_2 into the PLC signaling pool. While one might at first approximation assume that the increased PtdIns(4,5)P_2 and Ins(1,4,5)P_3 might distribute equally throughout the cell, apparently it does not as reflected in the differences in phenotype of the SAC9 mutant and the fragile fiber mutants. The flaccid stomata of the SAC9 mutant is the most intriguing evidence that altering the flux through the plasma membrane PtdIns(4,5)P_2 signaling pathways will affect growth and development. More complete analysis of the impact of altering PtdIns(4,5)P_2 5PTases and PLC on other compensatory pathways and measurements of metabolic flux will surely produce the insights needed to interpret these very intriguing results.

As illustrated by the examples above, the key to understanding the PI pathway is to comprehend the nature of the regulation of discrete subcellular microdomains of the lipids. Any changes in a specific plasma membrane signaling pool, whether a transient oscillation in PtdIns(4,5)P_2 or a more sustained change in rate of flux through the pathway in response to a stimulus, will result in changes in cytoskeletal structure, membrane enzyme activity, and pump or channel activity within the microdomains where the lipid resides.

Evidence for the rapid and transient production of Ins(1,4,5)P_3 comes from many studies beginning with those of Satter's group (Morse *et al.*, 1987). Once Rich Crain's group demonstrated the validity of using the commercially available brain binding assay for monitoring InsP(1,4,5)P_3 in plants, the field expanded rapidly (Cote and Crain, 1993; Cote *et al.*, 1996). Prior to this time, many were mislead by the large amounts of [^{3}H]inositol labeled metabolites that could be recovered from plants, many of which co-migrated with Ins(1,4,5)P_3 on the simple gravity-mediated ion exchange chromatography columns used. The rapid and selective Ins(1,4,5)P_3 assays enabled more extensive time course studies that revealed sustained elevations of Ins(1,4,5)P_3 in

addition to the rapid transient increases in response to stimuli (DeWald *et al.*, 2001; Drøbak and Watkins, 2000; Perera *et al.*, 1999; Perera *et al.*, 2001). The shorter transients were predicted to be part of a ubiquitous "wake up call" which is probably transmitted by several signaling pathways in sessile organisms such as plants and the long-term response was predicted to reflect a specific Ins(1,4,5)P_3-mediated event (Perera *et al.*, 1999).

We do not know what roles Ins(1,4,5)P_3 plays in regulating plant growth and development. Of interest, plant cells appear to be able to survive quite well with almost no detectable steady state Ins(1,4,5)P_3 (Perera *et al.*, 2002; Perera *et al.*, unpublished results). It is important to remember that this is not a static system and, as mentioned above, the flux through the pathway will increase as a result of the increased rate of hydrolysis of Ins(1,4,5)P_3 and the attenuation of Ins(1,4,5)P_3 signal. Thus, transgenic plants expressing the human InsP 5-ptase gene may prove to be a useful tool for assessing the impact of the plasma membrane generated Ins(1,4,5)P_3 and PtdIns(4,5)P_2 turnover on plant growth and development. Understanding the source of the Ins(1,4,5)P_3, how these signals intersect and what determines which interacting signaling networks or interactomes will be activated is a challenge for future studies.

5. FUTURE CHALLENGES

Living cells are constantly engaged in sensing, interpreting, and responding to environmental cues. To fully appreciate the dynamics of the signaling microdomains it is essential to think in terms of flux. This is difficult for most of us as our vision is limited to a great extent by the measurement methods we use. For example, we often analyze transcript levels, enzyme activities, or cellular structures at fixed moments in time. Only in death does one reach a truly static state. The challenge for scientists is to envision the dynamics of the living cell and appreciate the constant flux within the cellular milieu. While modern microscopy is making great advances, the ability to investigate microdomains and metabolic fluxes of the PI pathway is limited in part by our lack of nanoprobes. We know from *in vivo* labeling studies that synthesis (phosphorylation) of PtdIns and PtdInsP occurs within seconds indicating rapid turnover of the phosphate group. We also know that the lipid kinases are associated with many of the membranes of the cell, the cytoskeleton, and the nucleus (Drøbak *et al.*, 1999; Mueller-Roeber and Pical, 2002); however, we have yet to fully characterize the subcellular distribution of the specific isoforms of the plant PI metabolizing enzymes.

As more genetic manipulations of the PI pathway are encountered, extensive and more complete analyses of the inositol metabolites as well as the phospholipids will be required. Fortunately, the methods have been refined and

standards are more readily available than in 1985; so there is the potential to characterize the metabolic fluxes in signaling pools (Welti *et al.*, 2002; Wenk *et al.*, 2003) (Chapters 1, 3, and 4 in this book).

In addition, through evolving technologies, we are gaining the tools to do these studies *in vitro* and *in vivo* by using molecular genetics and recombinant proteins. With these new techniques, however, the cautions and comments of one of the great biochemists of the 20th century, Efraim Racker are still appropriate: *"As a biochemist I have stressed the need for obtaining pure proteins more than once; I have also stressed the need to distrust data obtained with crude systems. As a physiologist I want to stress the need to pay attention to data obtained with native membrane or even with intact cells and to distrust data obtained with pure proteins. . . . the only way we can find out whether the whole is the sum of the parts is by putting the pieces together again and learning how they work. It is the task of the physiologist to help the biochemist by pointing out what is missing."* (Racker, 1965)

In vitro analyses of protein–lipid interactions are essential to understand the mechanisms involved, and as Racker indicates, these must be analyzed in the context of the whole organism. An example of the importance of Racker's advice comes from studies of a potential PI pathway inhibitor neomycin. In earlier studies, adding PtdIns(4,5)P_2 increased the specific activity of reconstituted the dog kidney ATPase (Lipsky and Lietman, 1980). If neomycin was added to the PtdIns(4,5)P_2 micelles prior to adding them to the ATPase, neomycin inhibited ATPase activation as one might anticipate; however, if PtdIns(4,5)P_2 was added to the ATPase and then neomycin was added, there was no effect of neomycin on the PtdIns(4,5)P_2-mediated activation. That is, in the reconstituted system, PtdIns(4,5)P_2 bound the ATPase with such a high affinity that neomycin could not competitively bind and displace the lipid. Although many have documented the fact that neomycin at 1:1 concentrations will bind to PtdIns(4,5)P_2 micelles and prevent PLC hydrolysis *in vitro* (Gungabissoon *et al.*, 1998; Staxen *et al.*, 1999), it is unlikely that neomycin would preferentially bind PtdIns(4,5)P_2 already associated with cellular proteins including PLC. Why is it that so many use neomycin to inhibit the PI pathway *in vivo* and see an effect? There are several explanations. One may be that neomycin, which is a positively charged aminoglycoside will bind to the cell wall and will prevent the penetration of elicitors (Cho *et al.*, 1995). Another is that neomycin, once it enters the cell, will inhibit protein synthesis and thereby affect cellular metabolism.

The insights from the studies using neomycin are mentioned to emphasize the need for multifaceted approach to studying these pathways and to highlight the need to characterize individual pools of PtdIns(4,5)P_2. Importantly, genetic manipulations also are not sufficient to predictively alter a pathway as multicellular organisms will induce compensatory mechanisms to survive. Conflicting results from inhibitor and molecular genetic studies may seem insurmountable (Mills et al., 2004), and yet, by combining technologies and doing comprehensive analysis at the cellular and subcellular level, these conflicts will

eventually be resolved and scientists will weave together the interactome of the signaling networks. Advances in nanotechnology will surely lead to new levels of understanding as probes are developed to visualize fluxes in lipid microdomains *in vivo*. Our ultimate goal should be to reach a point where as scientists we can predictively alter plant growth responses by manipulating the flux through a selective signaling pathway.

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