Development of Transgenic *Physcomitrella patens* lines for visualization of phosphoinositide spatial and temporal localization

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**Abstract**

Tip growth, a type of polarized cellular expansion in plants, algae, and fungi, requires the regulation of many discrete processes. The coordination of multiple events in tip growth depends on a complex signaling system. Phosphoinositides are key signaling lipids that are involved in many cellular processes related to tip growth, such as membrane trafficking and regulation of the actin cytoskeleton. However, there is limited work on PIs in non-vascular plants, such as the moss *Physcomitrella patens*. Due to its susceptibility to reverse genetics and high degree of homologous recombination, *P. patens* has recently emerged as an excellent model system. It is especially suited for tip growth studies because establishment of its structure is dependent on tip growth. To examine PI localization in *P. patens*, we have constructed GFP-tagged lipid binding modules to image PtdIns(4,5)P$_2$ and PtdIns(4)P’s dynamic localization. We have created expression constructs under the control of the constitutive maize ubiquitin promoter, composed of 3xmEGFP fused to the PtdIns(4)P binding PH domains of OSBP and FAPP1, and the PtdIns(4,5)P$_2$ binding domains PH$_{PLC61}$ and the Tubby domain of Tubby. Additionally, constructs using the nonbinding mutants, PH$_{PLC61}$ R40L, PH$_{OSBP}$ R107E, R108E, and PH$_{FAPP1}$ R18L, have been produced. To verify that our lines faithfully report the localization of PtdIns(4)P, we examined the localization of PH$_{OSBP}$-EGFP and PH$_{FAPP1}$-EGFP and verified its specificity with their respective non-binding mutants. We also characterized their growth. Interestingly, lines showing over-expression of OSBP-3xmEGFP displayed a wavy growth phenotype, reminiscent of *Arabidopsis* root hair mutants with disrupted PtdIns(4)P domains. Further characterization and studies with these moss lines will be valuable in understanding how PIs function during polarized tip growth.
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<tr>
<td>ABP</td>
<td>Actin binding protein</td>
</tr>
<tr>
<td>ADF</td>
<td>Actin depolymerizing factor</td>
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<tr>
<td>AGD1</td>
<td>ARF-GAP DOMAIN 1</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>COW1</td>
<td>Can of Worms 1</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FAPP1</td>
<td>Four phosphate adaptor protein-1</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1/YOTB/Vac1/EEA1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-exchange factor</td>
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<tr>
<td>IP₃</td>
<td>Inositol 1,4,5 tri-phosphate</td>
</tr>
<tr>
<td>LBD</td>
<td>Lipid binding domain</td>
</tr>
<tr>
<td>mEGFP</td>
<td>Monomeric enhanced green fluorescent protein</td>
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<tr>
<td>MORN</td>
<td>Membrane occupation and recognition nexus</td>
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<tr>
<td>OSBP</td>
<td>Oxysterol binding protein</td>
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<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PI</td>
<td>Phosphoinositide</td>
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<tr>
<td>PI4K</td>
<td>Phosphatidylinositol-4-OH kinase</td>
</tr>
<tr>
<td>PIP5K</td>
<td>Phosphatidylinositol-4-phosphate 5-OH kinase</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PtdIns(4,5)P₂</td>
<td>Phosphatidylinositol (4,5) bisphosphate</td>
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<tr>
<td>RHD4</td>
<td>Root Hair Defective 4</td>
</tr>
<tr>
<td>ROP</td>
<td>Rho of plants</td>
</tr>
<tr>
<td>Sac1</td>
<td>Suppresor of Actin</td>
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<tr>
<td>SFH1</td>
<td>Sec14 Homologs 1</td>
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<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
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Introduction

Cellular polarization, the establishment of asymmetric organization within a cell, occurs in multiple systems and is an essential component of organismal development and cellular function (Li and Bowerman 2010). One process that involves cellular polarization is polarized cellular growth. In polarized cellular growth, the cell expands in a unidirectional manner through the trafficking of membranous and, in the case of walled cells, flexible cell wall material in exocytic vesicles. In walled cells, this trafficking is coupled to turgor pressure driven cell expansion. Examples of systems that grow purely through polarized growth include neurites, fungal hyphae, moss protonemata, and the root hairs and pollen tubes of higher plants (Li and Bowerman 2010; Inagaki et al. 2011; Rounds and Bezanilla 2013). While polarized cellular growth occurs in a multiplicity of different systems, it is becoming increasingly apparent that many of the molecular players behind establishing and maintaining cellular polarity have been conserved in evolution among the different systems.

In plants, algae, and fungi, this polarized expansion is termed tip growth. The architecture of a tip growing cell is consistent with its mode of growth, where vesicles containing membranous and cell wall material are trafficked to the site of growth to aid in cellular expansion. The apical region of a tip growing cell is highly enriched in vesicles, referred to as the clear zone due to its appearance in electron microscopy (Derksen et al. 1995). Within the clear zone are the endoplasmic reticulum (ER), Golgi dictyosomes, and mitochondria. Behind the clear zone are larger organelles, such as vacuoles and plastids (Rounds and Bezanilla 2013). Thus, vesicles are poised to deliver membrane and secrete flexible cell wall material at the apex of the cell in order for the cell to grow. The
organelles that the vesicles are derived from, the ER and Golgi dictyosomes, are close to the site of growth. The mitochondria, which fuel the trafficking machinery, are also located close to the growth site. This organellar distribution allows for the targeted secretion of new wall and membrane material coupled to turgor driven pressure.

The cytoskeletal arrangement of these tip growing cells is also reflective of their mode of cellular growth. While the mechanisms are not yet well understood, a dynamic cytoskeleton is essential to establishing and maintaining polar growth (Thomas and Staiger 2014). Tip growing plant cells have both microfilaments and microtubules, arranged longitudinally along the shank of the cell within the cortex (Smith and Oppenheimer 2005; Vidali et al. 2009a; Cai and Cresti 2010; Cheung and Wu 2008). While the role of microtubules in polarized growth is not generalizable to all tip growing systems, the importance of an organized actin cytoskeleton in tip growth has been well-established. Actin binding proteins related to turn-over and generation of F-actin play critical roles in tip growth (Vidali et al. 2010; Vidali et al. 2001; Augustine et al. 2011; Augustine et al. 2008; Vidali et al. 2009b; Ketelaar 2013). For example, when actin depolymerizing factor (ADF)-cofilin is knocked down, the F-actin organization is altered and tip growth is inhibited (Augustine et al. 2008). ADF-cofilin is only one example of how proper regulation of the actin cytoskeleton is essential for tip growth. Interestingly, while it is clear that some trafficking occurs along the cytoskeleton, it is still unclear how actin is linked to vesicle delivery, especially because not all actin-related vesicle trafficking events are related to growth (Parton et al. 2003). Although we do not know exactly how the cytoskeleton acts in tip growth, it has been shown that its regulation is essential.
In addition to having an organized architecture and a dynamic cytoskeleton, tip growth involves the formation of ion gradients (Konrad et al. 2011; Hepler et al. 2012; Michard et al. 2009; Steinhorst and Kudla 2013; Cárdenas 2009). Tip growing cells, such as pollen tubes, require calcium to maintain a tip-focused calcium cytosolic gradient and for facilitating exocytosis (Konrad et al. 2011; Hepler et al. 2012; Steinhorst and Kudla 2013). Additionally, K⁺, Cl⁻, and H⁺ gradients have been found to be involved in tip growth regulation (Smith and Oppenheimer 2005). For example, in lily pollen, it was shown that there is a constitutive alkaline band at the base of the clear zone and an acidic domain at the active growth site at the apex of the cell (Feijo et al. 1999). The formation of different ion gradients, especially calcium, is important for tip growth.

An essential process in plants, tip growth requires the interaction of many processes – membrane trafficking, regulation of the cytoskeleton, the formation and maintenance of polarity, and the formation of ion gradients. The coordination of these events during tip growth depends on a complex signaling system that can respond to both inter- and intra-cellular cues. One major class of players within this signaling system are the phosphoinositides (PIs) (Ischebeck et al. 2010).

**Phosphoinositides: Versatile Signaling Molecules**

Initially discovered in physiological experiments with brain tissue samples, the phosphoinositide (PI) signaling pathway is now implicated in a diverse array of different physiological processes. PIs are especially involved in the regulation of intracellular membranes, such as vesicle trafficking, lipid distribution and metabolism, and regulation of ion channels and other membrane proteins. In their capacity as signaling molecules,
PIs have three main roles in eukaryotic systems. They serve as membrane landmarks, regulators of proteins, and as sources for secondary messengers (Balla 2013).

**Synthesis of PIs**

Phosphoinositides (PIs) are highly versatile signaling molecules due to their relatively simple reversibility of form. PIs are generated via phosphorylation of hydroxyls located on the D-3, D-4, and D-5 carbons of the inositol head group of phosphatidylinositol (PtdIns), a minor structural membrane phospholipid found in eukaryotic cells (Fig 1a). Phosphorylation of the hydroxyls via specific lipid kinases generates mono-, bis-, and tri-phosphorylated derivatives (Fig 1b). This phosphorylation is reversible due to the activity of an entire host of phosphatases that dephosphorylate specific PI species. While seven isoforms of PtdIns have been found in mammals and in yeast, the tri-phosphorylated PtdIns(3,4,5)P$_3$ has not been found in plants and the existence of PtdIns(3, 5)P$_2$ is controversial (Ischebeck et al. 2010; Heilmann and Heilmann 2014).

Each PI is produced by a specific kinase. The best characterized member of the PI family, phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P$_2$) is derived via phosphorylation of phosphatidylinositol (4) phosphate (PtdIns(4)P) by phosphatidylinositol-4-phosphate 5-OH kinases (PIP5Ks). PIP5Ks are classified as either Type A or B. Type B PIP5Ks contain N-terminal plant-specific membrane occupation and recognition nexus (MORN) repeats, which are predicted to bind to phospholipids (Mueller-Roeber and Pical 2002). These PIP5Ks are activated by small G proteins, and their regulators GTPase-activating proteins (GAPs) and guanine-exchange factors (GEFs)
Figure 1. Phosphoinositides are versatile signaling molecules. A. Phosphatidylinositol (PtdIns) can be phosphorylated at the 3, 4, and 5 positions (asterisks) to yield different phosphoinositides (PIs). B. PIs are interconverted via phosphorylation by kinases and dephosphorylation via phosphatases.
The kinases that generate PtdIns(4)P from PtdIns are the phosphatidylinositol-4-OH kinases (PI4Ks). PI4Ks are divided first into type II or type III depending on sensitivity to inhibitors, and then into α, β, and γ depending on domain organization (Ischebeck et al. 2010; Balla and Balla 2006). The *Arabidopsis* genome contains 11 genes encoding PIP5Ks and 12 PI4K family members (Mueller-Roeber and Pical, 2002). The presence of different isoforms of these enzymes is not only found in *Arabidopsis*. In rice, multiple isoforms of the different enzymes that generate PI species have also been found (Xue et al. 2009). While each PI species has a specific enzyme that generates them, different systems have multiple isoforms of each kinase, which implies an important role for PI metabolism.

Interestingly, the localization of the kinases involved in PI metabolism is highly compartmentalized, such that each family of kinases is associated primarily with a unique organelle (Balla 2013). The localization of these enzymes have been fairly well-characterized in mammalian cells, yeast, and more recently, in plants. In plants, phosphatidylinositol (PtdIns)-synthases are found in the ER and possibly the Golgi apparatus (Löfke et al. 2008); PI4Ks are found in the *trans*-Golgi network (TGN) (Kang et al. 2011); and PIP5Ks are found in the PM (Kusano et al. 2008; van den Bout and Divecha 2009; Stenzel et al. 2011). These localization patterns coincide largely with the localization of their products, such that their PI products serve as subcellular markers for different organelles. This discrete localization is thought to have functional relevance for the regulatory functions of PIs within the cell.

**PIs as Landmarks for Cellular Membranes**

PIs are landmarks for cellular membranes. PIs constitute only a small proportion of the total amount of phospholipids in a cell. Thus, a protein that recognizes a specific PI
species would localize to where there is a high concentration of these lipids. While PI production is clearly compartmentalized, the PIs themselves are also localized to specific cellular membranes such that each PI species can be considered markers of different organelles. By adapting techniques first developed in mammalian cells to visualize the localization of different PI species in plants, it was determined that, in plants, PtdIns(3)P is primarily found in various vesicular structures and in the growing edges of cell plates (Vermeer et al. 2006); PtdIns(4)P is found in the plasma membrane (PM) and in the trans-Golgi network (TGN) (Vermeer et al. 2009; Simon et al. 2014); and PtdIns(4,5)P$_2$ is found primarily in the cytoplasm of un-stressed cells and in the PM of stressed cells (van Leeuwen et al. 2007). The unique localization of PIs and their relative scarcity within cells allow PIs to serve as cellular markers for the localization and recruitment of different proteins.

**PIs, Regulators of Protein Function**

The concentration of PIs, in addition to their spatial and temporal localization, varies greatly in the cell in response to the activation of cell surface receptors, change in pH, and other stimuli (Balla 2013). Primarily found on the cytosolic side of cellular membranes, different PIs are readily accessible to the PI kinases and phosphatases that can interconvert PIs from one species to the next. The unique pattern of phosphate groups around the inositol head group of the different PI species, maintained by the different enzymes involved in PI metabolism, has been referred to as a “PI code,” which protein domains can recognize and bind to (Kutateladze 2010).

A diverse array of proteins bind to PIs. Examples of these proteins include ion channels, actin regulators, and proteins involved in membrane trafficking (Balla 2013).
These proteins contain lipid binding domains (LBDs) that specifically recognize and bind to PIs, such as the pleckstrin homology (PH) domain, the Phox homology (PX) domain, and the Fab1/YOTB/Vac1/EEA1 (FYVE) domain (Lemmon 2008; Balla et al. 2009; De Matteis and Godi 2004; McLaughlin and Murray 2005). Upon recognition of PIs by these domains, the domain’s associated proteins can then be recruited to sites enriched in a particular PI. By regulating the functions of different proteins via binding, PIs are implicated in a diverse array of cellular processes including controlling ion transport, cytoskeletal structures, GTPase function and/or localization, and membrane trafficking (Balla 2013).

A classic example of the important relationship between PIs and their partner proteins is the relationship between PtdIns(4,5)P$_2$ and actin. In plants, it has been shown that PtdIns(4,5)P$_2$ can affect the actin cytoskeleton directly through binding and regulating distinct actin binding proteins (ABPs) or indirectly through regulating the activity and localization of Rho of plants (ROP) GTPases or through recruiting scaffolding proteins to the plasma membrane (Craddock et al. 2012; Fu et al. 2001; Pleskot et al. 2014; Saarikangas et al. 2010). When phospholipase C (PLC), the enzyme that hydrolyzes PtdIns(4,5)P$_2$ to inositol 1,4,5 tri-phosphate (IP$_3$) and diacylglycerol (DAG), is knocked down in *Petunia* pollen tubes, the actin organization is deregulated in the tip (Dowd et al. 2006). Additionally, when the expression of specific PIP5Ks (kinases involved in generating PtdIns(4,5)P$_2$) is disrupted in *Arabidopsis*, tip localized actin structures are deregulated (Ischebeck et al. 2008; Stenzel et al. 2008; Ischebeck et al. 2011; Kusano et al. 2008; Sousa et al. 2008). Collectively, this shows PtdIns(4,5)P$_2$ is involved in actin regulation and organization. This relationship between PtdIns(4,5)P$_2$
and actin is an example of how PIs can act as protein regulators and are involved in essential cellular functions.

**PIs as Sources of Secondary Messengers**

Phosphoinositides were originally discovered in animal cells because they serve as a source of secondary messengers (Balla 2013). In animal cells, PLC hydrolyzes PtdIns(4,5)P$_2$ into two secondary messengers, IP$_3$ and DAG. IP$_3$ then diffuses into the cytosol and triggers the release of calcium, while DAG recruits and activates members of the protein kinase C (PKC) family. However, secondary messenger signaling differs between plants and animals. Although plants contain 9 PLC isoforms (Tasma et al. 2008), there is no evidence for PKC or IP$_3$ receptors in plants. Additionally, while IP$_3$ has been shown to be involved in Ca$^{2+}$ regulation (Tang et al. 2007), its actual role has been controversial due to the possibility of other, more complicated inositol phosphates being involved (Munnik and Vermeer 2010). Additionally, the role of DAG in plants has not been well-established due to the lack of obvious targets (Dong et al. 2012). Instead, phosphatidic acid (PA), derived from phosphatidylcholine via hydrolysis by phospholipase D (PLD) is an important PI-derived second messenger in plants (Munnik and Vermeer 2010). In plants, there are 12 PLD isoforms (Xue et al. 2009). There are some key differences in secondary messenger signaling between plant and animal systems that have broader implications for how PI signaling functions in these systems.

**PIs in Tip Growth**

PIs have been strongly implicated in tip growth due to their association with different signaling proteins, membrane microdomain formation, and their participation in functions such as regulation of the actin cytoskeleton, ion channels, and membrane
trafficking (Munnik and Nielsen 2011). This was first suggested when it was demonstrated that PIPK activity is associated with Rho GTPases from plants (ROPs), proteins that are involved in symmetry breaking and control of PM domains (Kost et al. 1999). Since this initial report, studies on the role of PIs in tip growth have mainly focused on examining the roles of two PI species, PtdIns(4)P and PtdIns(4,5)P₂, in the pollen tubes and root hairs of the flowering plant model Arabidopsis thaliana. 

\textbf{PtdIns(4,5)P₂} 

Evidence for the involvement of PtdIns(4,5)P₂ in tip growth is mainly from mutational and knock-out studies of Arabidopsis root hairs and pollen tubes. Regulation of PtdIns(4,5)P₂ is essential for proper tip growth of cells; having too much or too little PtdIns(4,5)P₂ causes irregular tip growth. When the expression of different PIP5Ks is disrupted in Arabidopsis, tip-localized actin structures are deregulated. For example, when PIP5K9 is overexpressed, there are decreased actin microfilaments in root elongation zones (Lou et al. 2007). Since tip growth is dependent on proper actin regulation, tip growth in root hairs is also dependent on PtdIns(4,5)P₂. This was especially seen in loss of function mutants of PIP5K3, a root-specific type B PIP5K. These mutants have significantly shorter root hairs (Kusano et al. 2008; Stenzel et al. 2011), while over-expression caused much longer root hairs (Kusano et al. 2008). The theory that PtdIns(4,5)P₂ is related to membrane trafficking and cellular expansion is supported by the localization of GFP-tagged PIP5K3 to the PM of the tips of growing root hairs. When the root hair stopped growing, the fluorescence of the fusion protein disappeared, suggesting that cellular growth rate depends on the levels of PIP5K3 and
thus levels of PtdIns(4,5)P$_2$ (Kusano et al. 2008). Thus, the concentration of PtdIns(4,5)P$_2$ is directly related to tip growth.

The dysregulation of PIP5Ks in pollen tubes also causes irregular tip growth. RNAi of pollen tube specific PIP5K6 caused aggregation of apical membrane material in the pollen tubes, suggesting that there exists an appropriate concentration of PtdIns(4,5)P$_2$ that is necessary for proper membrane trafficking (Zhao et al. 2010). Additionally, mutations in the pollen-specific PIP5K10 and PIP5K11 caused pollen tubes to be hypersensitive to Latrunculin B (Lat B), an actin polarization and organization inhibitor (Ischebeck et al. 2011). Hypersensitivity to Lat B suggests that the normal regulation of actin is disrupted in these pollen tubes, thus correlating the proper generation of PtdIns(4,5)P$_2$ to actin regulation. Furthermore, PIP5Ks are involved in membrane trafficking and linear growth in pollen tubes. Lines of Arabidopsis deficient in PIP5K4 and PIP5K5 showed reduced germination rates, while lines that over-express these kinases showed thickening of the cell wall and irregular tip morphologies in the pollen tubes (Ischebeck et al. 2008; Sousa et al. 2008). In a system as highly regulated as pollen tubes, which are essential for reproduction, such dysregulation in tip growth exemplifies how important a proper concentration of PtdIns(4,5)P$_2$ is to tip growth. Thus, generation and concentration of PtdIns(4,5)P$_2$ in pollen tubes plays an important role in actin cytoskeleton and membrane trafficking, which parallels PtdIns(4,5)P$_2$’s importance in root hairs.

One key group of PI regulators are the Sec14-like phosphatidylinositol transfer proteins (PITP) (Bankaitis et al. 2010; Mousley et al. 2007). Sec14p is a PITP in yeast that is essential for membrane trafficking and phospholipid metabolism (Bankaitis et al.
The Sec14 superfamily of proteins are found in all eukaryotic systems, yet their physiological functions vary. One such PITP is the CAN OF WORMS/SEC FOURTEEN HOMOLOGS 1 (COW1/SFH1) protein, found in *Arabidopsis* (Bohme et al. 2004; Vincent et al. 2005). The PITP COW1 is essential for normal root hair morphogenesis, as *cow1* mutants exhibit short, wide, and sometimes double root hairs (Grierson et al. 1997). Furthermore, while cytoplasmic, COW1 localizes to and is enriched in discrete membrane domains in the PM (Vincent et al. 2005). COW1 appears to co-localize with PtdIns(4,5)P₂, suggesting that the two may interact in normally growing root hairs. Additionally, it appears that COW1 may regulate PI polarity landmarks (Vincent et al. 2005). In the *cow1* mutant, PtdIns(4,5)P₂ localization, the cytoskeletal actin structures, and Ca²⁺ signaling are disrupted (Vincent et al. 2005). This suggested that the proper regulation of PtdIns(4,5)P₂ in root hairs is essential to the regulation of actin, in the proper formation of Ca²⁺ gradients, and in the morphology of a tip growing cell.

**PtdIns(4)P**

While the role of PtdIns(4,5)P₂ in tip growth has been fairly well-characterized, we are still learning more about the involvement of its precursor, PtdIns(4)P. In plants, PtdIns(4)P is the most abundant PI, accounting for 80% of all PIs (Munnik and Vermeer 2010). Using fluorescent biosensors, it was determined that PtdIns(4)P is found in the lateral flanks adjacent to the growing root hair’s apex, implicating it in tip growth (Yoo et al. 2012). While PtdIns(4)P was originally considered solely as a precursor with redundant functions to PtdIns(4,5)P₂, recent research has revealed that it serves critical roles in membrane trafficking and other processes during polarized cellular growth.
PtdIns(4)P’s role in membrane trafficking was revealed when PI4Kβ1, was found to be recruited to tip localized secretory vesicles containing newly synthesized cell wall material budding from the TGN in root hairs (Preuss et al. 2006) and in pollen tubes (Szumlanski and Nielsen 2009). Additionally, PI4Kβ1 co-localized with RabA4b in root hairs (Preuss et al. 2006) and RabA4d in pollen tubes (Szumlanski and Nielsen 2009). Because the Rab family of GTPases regulate membrane trafficking (Hutagalung and Novick 2011), this suggests that PI4Kβ1 and PtdIns(4)P play a role in regulating membrane trafficking. Consistent with this hypothesis, plants that are defective in both PI4Kβ1 and PI4Kβ2 show mutant root hair morphologies and altered structure of Rab4Ab-labeled TGN compartments (Preuss et al. 2006; Kang et al. 2011). This suggests that PtdIns(4)P is important for TGN organization and for secretion from the Golgi during growth.

While the generation of PtdIns(4)P is important for proper signaling, the turnover of PtdIns(4)P has also proven to be essential. ROOT HAIR DEFECTIVE 4 (RHD4) encodes a Sac1-like PI phosphatase that has a preference for PtdIns(4)P (Thole et al. 2008). Sac1 (Suppressor of Actin) is a PtdIns(4)P phosphatase found in yeast and is known to be involved in the hydrolysis of PtdIns(4)P to PtdIns in the early and medial Golgi (Strahl and Thorner 2007). In rhd4-1 plants, root hairs are short and distorted, showing random bulges during tip growth (Thole et al. 2008). Additionally, during tip growth, RabA4b and PtdIns(4)P localization was disturbed in the mutant, such that both were no longer tip localized but was found in internal membrane components of the root hair (Thole et al. 2008). These results suggest that improper turnover of PtdIns(4)P causes disrupted membrane trafficking, which leads to changes in root hair morphology;
root hairs were able to initiate but not continue to grow. Additionally, RHD4 colocalized with PI4Kβ1 (Thole et al. 2008), suggesting that the two work together to maintain normal PtdIns(4)P levels in root hairs. Clearly, the proper regulation of PtdIns(4)P synthesis is essential to the continuation of normal tip growth.

In addition to being important for the continuation of tip growth, PtdIns(4)P is essential for directional tip growth. AGD1 (ARF-GAP DOMAIN 1) encodes an ADP-ribosylation factor (Arf). Arf GTPase-activating proteins (GAPs) that contains a PH domain (Yoo et al., 2012). The class I Arf-GAPs are regulators of small GTPases that play critical roles in many steps of membrane trafficking in mammalian and plant cells (Randazzo and Hirsch 2004). In agd-1 mutants, root hairs are wavy, showing a loss of unidirectional growth (Yoo et al. 2008). These plants also showed disrupted PtdIns(4)P localization such that, instead of localizing along the flanks of the growing root hair, PtdIns(4)P preferentially accumulated along the concave side of the root hair (Yoo et al. 2012). This disrupted PtdIns(4)P localization was accompanied by altered actin organization and irregular membrane trafficking (Yoo et al. 2012; Yoo et al. 2008). Additionally, the tip-focused Ca^{2+} oscillations that are required for normal tip growth were dampened, suggesting that PtdIns(4)P is also involved in ion signaling (Yoo et al. 2012). Thus, PtdIns(4)P signaling is linked to regulation of the cytoskeleton, Ca^{2+} dynamics and membrane during root hair tip growth. No longer just a precursor to PtdIns(4,5)P_2, PtdIns(4)P serves essential roles in the plant tip growth on its own.

**Examining PI localization**

To determine the spatial and temporal localization of PIs in living cells, LBDs that are specific to the PI species in question are tagged at either the N or C terminus by a
fluorescent reporter protein, such as GFP. Once expressed, this chimeric protein can then be tracked in cells using confocal microscopy (Halet 2005). The localization of the fluorescence indicates the localization of the PI species. By analyzing the localization of the fluorescence and translocation of the fluorescence upon addition of a stimulus, information on the cellular distribution of the PIs and their dynamics at a cellular and an organismal level can be obtained. As mentioned previously, LBDs with varying PI specificities have been identified and characterized. The main LBDs used in imaging are pleckstrin homology (PH) domains, the Phox homology (PX) domain, and the Fab1/YOTB/Vac1/EEA1 (FYVE) domain (Lemmon 2008; Balla et al. 2009; De Matteis and Godi 2004; McLaughlin and Murray 2005). Studies conducted with these fluorescent reporters have revealed the localization of all PI species sans PtdIns(5)P in mammalian cells and in yeast (Várnai and Balla 1998; Halet 2005). In plants, PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P_2 have been imaged (Heilmann 2009).

The most commonly used domain for examining PI localization is the pleckstrin homology (PH) domain, which derives its name from pleckstrin, the major protein kinase C found in platelets (Mayer et al. 1993; Harlan et al. 1994). PH domains exhibit a highly conserved tertiary structure that is based on seven β strands that form two orthogonal β sheets, where the PIs bind to basic residues, and a C-terminal alpha helix (Lemmon and Ferguson 2000). While most proteins containing these domains show low affinity and little specificity towards specific PIs, there are several proteins containing PH domains that are both specific and bind with high affinity. One is the PH domain from human phospholipase C (PLCδ1), which binds preferentially to PtdIns(4,5)P_2 (Várnai and Balla 1998). First utilized in mammalian cells (Várnai and Balla 1998), PH_{PLCδ1} has been also
used in yeast (Szentpetery et al. 2009), Arabidopsis, and tobacco cells (van Leeuwen et al. 2007). In all cases, labeling for PtdIns(4,5)P₂ using PH\textsubscript{PLCδ₁} shows enrichment at the plasma membrane. A method of confirming the localization of PtdIns(4,5)P₂ is to pair the use of wild-type PH\textsubscript{PLCδ₁} with a non-functional mutant PH\textsubscript{PLCδ₁ R40L}, which cannot bind PtdIns(4,5)P₂ (Várnai and Balla 1998).

While PH\textsubscript{PLCδ₁} has been widely used to image PtdIns(4,5)P₂, it also binds inositol 1,4,5-trisphosphate (IP₃). Thus, it may be possible that a change in signal when using PH-PLCδ₁ could be due to changes in IP₃ levels more so than PtdIns(4,5)P₂. The Tubby domain, characterized from the Tubby proteins found in mice, is thought to be a more specific binder to PtdIns(4,5)P₂ because it does not bind to IP₃ (Quinn et al. 2008). Thus, Tubby is a more accurate reporter of PtdIns(4,5)P₂ as it has a higher affinity to PtdIns(4,5)P₂. However, PH\textsubscript{PLCδ₁} is more sensitive to the concentrations of PtdIns(4,5)P₂ and responds well to manipulations of PtdIns(4,5)P₂ level, especially when PLC is active (Szentpetery et al. 2009). Tubby has been utilized in yeast (Szentpetery et al. 2009), mammalian cells (Szentpetery et al. 2009; Quinn et al. 2008; Hammond et al. 2012), and in Arabidopsis (Simon et al. 2014).

Two proteins that have been used to visualized PtdIns(4)P dynamics \textit{in vivo} are the PH domains of oxysterol binding protein (OSBP) and four phosphate adaptor protein-1 (FAPP1) (Dowler et al. 2000; Levine and Munro 2002; Balla et al. 2005). OSBP, found in mice and humans, translocates from the cytoplasm to the Golgi apparatus after binding to oxygenated derivatives of cholesterol (Levine and Munro 1998) and has been shown to bind to the Golgi in yeast and mammalian cells (Levine and Munro 2002). PH\textsubscript{FAPP₁} interacts with PtdIns(4)P and the small GTPase ADP-ribosylation factor (ARF) and
localizes to the TGN on nascent carriers (Godi et al. 2004; Lenoir et al. 2010; He et al. 2011). Interestingly, PHOSBP and PHFAPP1 also reveal slightly different localization in Arabidopsis: PHFAPP1 appears to label more vesicular bodies than PHOSBP, which is more membrane bound (Simon et al. 2014; Vermeer et al. 2009). Point mutants of PHOSBP and PHFAPP1 that do not bind to PtdIns(4)P have been used to confirm the specificity of localization signal with the reporters (Balla et al. 2005).

Recently, a novel probe for PtdIns(4)P using the SidM protein has been developed (Hammond et al. 2014). Secreted by the Legionella pneumophila, SidM is localized in vacuoles containing L. pneumophila where Rab1 signaling is stimulated by GEFs and AMPylation activities (Müller et al. 2010; Machner and Isberg 2007). In mammalian cells, labeling with P4M showed that PtdIns(4)P localizes to the Golgi, the PM, and to Rab7-positive late endosomes/lysosomes. Previously, when labeling with PHOSBP and PHFAPP1, this was not seen (Hammond et al. 2014). This suggests that P4M binds more specifically to PtdIns(4)P than the previously described domains and can report a wider cellular distribution of PtdIns(4)P. Additionally, P4M is a more sensitive reporter than other PtdIns(4)P reporters; its affinity in vitro matches the cellular concentration of PtdIns(4)P and P4M is able to react to changes in levels of PtdIns(4)P (Hammond et al. 2014; Brombacher et al. 2009). Thus, P4M appears to be both a more accurate and sensitive reporter of PtdIns(4)P localization than the reporters that have been historically used.

While the use of these LBD-fluorescent reporter proteins is well established and well characterized in mammalian cells and in yeast, this technique has not been widely utilized in plant systems. Thus far, the PI reporter proteins have primarily been used in
Arabidopsis, and we have limited knowledge of the localization and spatial function of PIs in other plant systems.

Moss: An Evolutionarily Relevant Model for Tip Growth

While we have gained a greater understanding of PIs in vascular plants such as A. thaliana, our understanding of how PIs function in the most ancient extant land plant group, the byrophytes, is lacking. Around 470 MYA, the colonization of land by plants was made possible due to acquired abilities such as the ability to anchor the plant body to soil, to tolerate desiccation, and to transport water. In byrophytes, which include the moss Physcomitrella patens, tip growth is essential to the establishment of the plant structure and in its propagation (Vidali and Bezanilla 2012). Specifically, the germination of a haploid spore produces the protonemata, which is exclusively composed of two types of tip growing cells, caulonema and chloronema (Menand et al. 2007). There are several key differences between the two cell types. The chloronema, the first cells to emerge from the spore, are comparatively rich in chloroplasts, with cell plates that are perpendicular to the long axis of the filament (Vidali and Bezanilla 2012). In contrast, the caulonemal cells have fewer and less developed chloroplasts, with cell plates that are oblique to the long axis of the filament. Unlike chloronemal cells, caulonemal cells are fairly cytosol rich in their apical region. Furthermore, caulonemal cells grow faster and divide more quickly than chloronemal cells. In addition to the protonemata, the rhizoid cells of the leafy gametophore stage, serving to anchor the plant to the substrate, also grow via tip growth (Vidali and Bezanilla 2012).

The bryophyte P. patens is an excellent model for tip growth. For one, P. patens is easily propagated – tissue damage causes dedifferentiation in the damaged area and
also causes emergence of new protonemal cells, which then become new tissue (Prigge and Bezanilla 2010). Moreover, via enzyme-digestion of the cell wall, individual cells can be isolated. These isolated protoplasts can be transformed via PEG/Ca\(^{+2}\) mediated DNA uptake, and whole plants can then be generated from a single cell. Stable transformants can then be detected in 4-6 weeks (Liu and Vidali 2011). Thus, it is possible to isolate single “colonies” of plants using this technique and create monoclonal populations of the same plant. Additionally, \textit{P. patens} has a dominant haploid state, making them ideal for genetic manipulation via techniques such as RNAi – any recessive mutation of essential genes will show a clear phenotype due to the absence of a second gene copy. In a similar vein, \textit{P. patens} exhibits a high degree of homologous recombination, so gene targeting has a higher chance of success. In addition, most signaling pathways that exist in \textit{A. thaliana} also occur in a simplified form in \textit{P. patens} (Vidali and Bezanilla 2012). For example, in \textit{P. patens}, there are four ROP genes that encode nearly identical proteins while \textit{Arabidopsis} genome encodes 11 ROP protein (Eklund et al. 2010; Craddock et al. 2012). Thus, \textit{P. patens} is an excellent model system for tip growth because it is simple to maintain and propagate, it contains simplified versions of signaling pathways found in more complicated systems, and is amenable to sophisticated genetic manipulations.

Similar to other tip growing systems, the actin cytoskeleton plays a critical role in \textit{P. patens} tip growth (Prigge and Bezanilla 2010). Studies show that a few actin-associated proteins compose the core of the machinery necessary for tip growth and viability in moss. When Actin Depolymerizing Factor (ADF)/cofilin is silenced via RNAi, moss exhibit growth defects where their small plant bodies are composed of small
depolarized cells (Augustine et al. 2008). This is reminiscent of how a deregulated actin cytoskeleton in pollen tubes and root hairs causes irregular morphologies and, in the case of root hairs, extremely short hairs (Smith and Oppenheimer 2005). Profilins, small actin binding proteins, and formins, profilin-interacting proteins that act as actin polymerizers, are also both essential to tip growth and plant viability (Vidali et al. 2007; Vidali et al. 2009b; van Gisbergen et al. 2012). Interestingly, the formins in \textit{P. patens} contain a PTEN (phosphatase and tensin) domain that binds to phosphoinositide-3,5-bisphosphate (PtdIns(3,5)P$_2$), homologous to PTENs in other systems (Cvrcková et al. 2004; van Gisbergen et al. 2012). Additionally, Class XI myosin is involved in tip growth (Vidali et al. 2010). In \textit{P. patens}, we see an essential role for actin in tip growth that parallels the role of actin in vascular plants.

In addition to actin, PIs are essential for plant development and tip growth in \textit{P. patens} (Saavedra et al. 2011). The PI kinase family in \textit{P. patens} is small compared to that of \textit{A. thaliana} PI kinases (Ischebeck et al. 2010). Thus far, only two Type I/II PIPK genes, \textit{PpPIPK1} and \textit{PpPIPK2}, have been characterized (Ischebeck et al. 2010; Saavedra et al. 2009). \textit{In vitro}, \textit{PpPIPK1} phosphorylates PtdIns(4)P and PtdIns(3)P, while \textit{PpPIPK2} prefers PtdIns as a substrate over PtdIns(4)P and PtdIns(3)P (Saavedra et al. 2009). Both are type B kinases, meaning that they contain MORN repeats. Both kinases are essential for plant growth (Saavedra et al. 2009; Saavedra et al. 2011). The \textit{pipk1} knock-out mutant cannot properly elongate its rhizoid and caulonemal cells due to the absence of the essential cortical F-actin gradient (Saavedra et al. 2011). The \textit{pipk1-2} double knock-out lines exhibited dramatic developmental defects in protonemal and gametophore morphology and lowered PtdIns(4,5)P$_2$ levels (Saavedra et al. 2011). This indicates the
importance of PI metabolism in *P. patens*, which reflects the importance of PIs in other plant systems.

Because *Physcomitrella* has similar signaling pathways in tip growth as higher, vascular plants, it is suited to be a model for tip growth. Parallels in tip growth mechanisms between *P. patens* and *A. thaliana* can provide insight on how tip growth has evolved over time and what processes have been conserved. However, our knowledge of tip growth mechanisms in moss is still limited and much more needs to be done to understand them.

**Examining PI localization in Moss**

While we know that the two characterized PIPKs in *P. patens* localize to the plasma membrane (Mikami et al. 2010; Saavedra et al. 2009), we do not know the spatial nor temporal localization of PIs in moss. To determine the localization of two essential PIs in tip growth, PtdIns(4)P and PtdIns(4,5)P\(_2\), we created transgenic lines of moss that express chimeric proteins composed of a PI specific lipid binding domain tagged with 3x-mEGFP. Specifically, to examine PtdIns(4)P localization, we generated constructs composed of the PH domains of four phosphate adaptor protein (FAPP1) and the PH domain of oxysterol binding protein (OSBP) (Levine and Munro 1998; Balla et al. 2005; Godi et al. 2004; He et al. 2011; Liu et al. 2014). To examine the spatial localization of PtdIns(4,5)P\(_2\), we utilized the PH domain of PLC\(\delta\_1\) and the Tubby domain of Tubby (van Leeuwen et al. 2007; Szentpetery et al. 2009; Vármai and Balla 1998; Dowler et al. 2000). In addition to generating constructs containing the wild-type domain, we generated constructs containing the non-functional mutants PH\(_{PLC\delta1}\)R40L, PH\(_{OSBP}\)R107E,R108E, and PH\(_{FAPP1}\)R18L (Vármai and Balla 1998; Balla et al. 2005). After creation of the constructs,
*P. patens* were stably transformed and selected lines were verified for normal growth. We developed a rapid scoring system to determine relative levels of expression and characterized the reporter localization in PtdIns(4)P reporter lines and in the non-functional mutants. Interestingly, we saw that lines that overexpress PH\textsubscript{OSBP}-EGFP have an altered phenotype that is reminiscent of *agd1* mutants, which supports that PH\textsubscript{OSBP}-EGFP is able to bind to PtdIns(4)P (Yoo et al. 2012; Yoo et al. 2008). With further characterization, these lines will be useful for future studies on the roles of PIs in various contexts.

**Materials and Methods**

**Overview**

In order to monitor phosphoinositide (PI) dynamics in *Physcomitrella patens*, we developed transgenic *P. patens* lines expressing a lipid binding domain (LBD) tagged by 3xm-EGFP. Multisite Gateway® 2-fragment recombination cloning technology was utilized to create constructs encoding wild type and mutant LBD from PLCδ, OSBP, and FAPP1 and the wild type LBD of Tubby fused in frame with 3x-mEGFP (Fig. 2). The creation of these constructs consisted of three main steps: 1) PCR amplification of the lipid binding domain flanked by attB sites; 2) BP recombination of the LBD with pDONR-P1P5r for C terminal tagging or pDONR-P5P2 for N terminal tagging; and 3) recombination of the resulting entry clone into the destination vector, pTHUB1gate, via a LR recombination reaction (Fig. 2, Table 1). The resulting expression clone was then digested by SwaI, and *P. patens* protoplasts were transformed with the resulting plasmid DNA. Lines of moss stably expressing these constructs were established and
Figure 2. Schematic of two-way Gateway® cloning strategy. A. Entry clones production through BP recombination of LBDs with a pDONR destination plasmid. To generate the expression clone, the newly created entry clone went through LR recombination with 3x-mEGFP-L1R5 for N terminal tagging (B) or 3x-mEGFP-L5L2 for C terminal tagging (C) and with the destination vector pTHUBIgate.
characterized for growth and fluorescent signal. (*E. coli* cells harboring entry clones containing wild type LBDs of PLCδ, OSBP, FAPP1, and Tubby and the mutant LBDs of PLCδ, OSBP, and FAPP1 were provided by T. Kaye Peterman (Table 1)).

**LR recombination reaction**

Entry clones containing the LBDs flanked by attB sites were subjected to 2 fragment recombination with 3x-mEGFP into the pTHUBIgate destination vector containing a hygromycin resistance marker and SwaI restriction sites (Fig 2). Wild type and mutant pPLCδ and pFAPP1 were tagged at the C terminus; wild type and mutant pOSBP and wild type pTubby were tagged at the N terminus, reflective of their positions in the wild type protein. Recombination of entry clones with the destination vector was accomplished via a LR recombination reaction (Invitrogen). Entry clone DNA (4 ng/µL), p3x-mEGFP (4 ng/µL), pTHUBIgate (15 ng/µL), and TE buffer (pH 8.0) were mixed with LR Clonase® II Plus™ enzyme mix (2 units) and incubated at room temperature overnight. To terminate the reaction, Proteinase K solution (0.09 µg/µL in 0.9 mM Tris-HCl, pH 7.5, 1.8 mM CaCl2, 4.5% glycerol) was added and the reaction incubated for 10 minutes at 37°C. Chemically competent *E. coli* were transformed with LR recombination reaction product.

**Transformation of Mach1 E. Coli**

Chemically competent *E. coli* (Mach1™, F- φ80(lacZ)ΔM15 ΔlacX74 hsdR(rK-mK+) ΔrecA1398 endA1 tonA) cells were transformed according to the manufacturer’s directions (Invitrogen). A mixture of LR reaction product (5 µL) and chemically competent *E. coli* cells (50 µL) were incubated on ice for 30 mins, then heat shocked for
Table 1. An overview of plasmids used in this study.

<table>
<thead>
<tr>
<th>Type of Plasmid</th>
<th>Name</th>
<th>Residues from Original Protein</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry Clone</td>
<td>pPLCδ</td>
<td>1-170</td>
<td>attL5-PH&lt;sub&gt;PLCδ&lt;sub&gt;1&lt;sub&gt;-attL2</td>
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<tr>
<td>Entry Clone</td>
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<td>Entry Clone</td>
<td>pFAPP1M</td>
<td>1-101</td>
<td>attL5-PH&lt;sub&gt;FAPP1&lt;sub&gt;R18L&lt;sub&gt;-attL2</td>
</tr>
<tr>
<td>Entry Clone</td>
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<td>87-189</td>
<td>attL1-PH&lt;sub&gt;OSBP&lt;sub&gt;-attR5</td>
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<tr>
<td>Entry Clone</td>
<td>pOSBPM</td>
<td>87-189</td>
<td>attL1-PH&lt;sub&gt;OSBP&lt;sub&gt;R107E, R108E&lt;sub&gt;-attR5</td>
</tr>
<tr>
<td>Entry Clone</td>
<td>pTUBBY</td>
<td>243-505</td>
<td>attL1-Tubby-attR5</td>
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<tr>
<td>Entry Clone</td>
<td>p3x-mEGFP-L5L2</td>
<td>677-1384 (3x tandem)</td>
<td>attL5-3x-mEGFP-attL2</td>
</tr>
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<td>677-1384 (3x tandem)</td>
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<td>attB1-3x-mEGFP-attB5-Tubby-attB2</td>
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30 seconds at 42°C. The mixture was then immediately returned to ice. 250 µL of 37°C SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added to the mixture before shaking at 225 rpm in a 37°C horizontal shaker for an hour. Aliquots of each transformation reaction were spread on solid LB media (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agarose) with either carbenicillin (100 µg/mL carbenicillin) or kanamycin (50 µg/mL kanamycin) and incubated overnight at 37°C to yield isolated colonies.

**Mini-Prep Isolation of Plasmid DNA**

A QIAPrep Spin Miniprep Kit (Qiagen) was used to purify plasmid DNA from *E. coli* cultures. Small cultures (5 mL) of selective LB broth with kanamycin (50 µg/mL kanamycin) were inoculated with an isolated colony and grown at 37°C overnight. Approximately 3 mL of each overnight culture was centrifuged for 1.5 mins at 13,000 x g to collect cells. After discarding the supernatant, cells were thoroughly resuspended in 250 µL Buffer P1 (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100ug/mL RNase A), lysed with 250 µL of Buffer P2 (200mM NaOH, 1% SDS), and neutralized with 350 µL Buffer N3 (3.0M KAc, pH 5.5). The mixture was immediately inverted six times to mix. The resulting cell debris was pelleted via centrifugation at 13,000 x g. The clear lysate was decanted into a QIAprep Spin Column in a collection tube and spun at 13,000 x g for 1 minute, to allow binding of plasmid DNA to the column’s silica membrane. The membrane was washed with 500 µL of Buffer PB (5M Guanidinium-HCl, 30% isopropanol) and 750 µL of Buffer PE (10 mM Tris-HCl pH 7.5, 80% ethanol), discarding the flow-through between steps. Residual ethanol from Buffer PE was removed by centrifuging the washed column. The spin column was placed into a sterile
micro-centrifuge tube and plasmid DNA was eluted with 50 µL of Buffer EB (10 mM Tris Cl, pH 8.5). Plasmid DNA concentration was determined using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific).

**Characterization of Expression Clones**

**SwaI Digestion of LR recombination Reaction Products**

To test that the entry clones recombined into the destination vector properly, LR recombination reaction products were digested using SwaI and successful digests were selected for sequence analysis. Plasmid DNA was digested by SwaI enzyme (1 unit/µL DNA, New England BioLabs) in NEBuffer 3.1 (100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂, 100µg/ml BSA, pH 7.9, New England BioLabs), for one hour at room temperature. The products were analyzed via gel electrophoresis (1% agarose, 40 mM Tris, 20 mM acetic acid, 1 mM Na 2 EDTA, pH 8.0). DNA was visualized using SYBR Safe DNA gel Stain (Invitrogen). Gel images were taken under UV trans-illumination using Kodak Gel Logic 200 Imaging System and Kodak ID software.

**Sequence Analysis of Expression Clone Plasmid DNA**

Plasmid DNA was sequenced by the Massachusetts General Hospital DNA Core Facility (https://dnacore.mgh.harvard.edu) using gene specific primers for each domain and primers specific to the maize ubiquitin promoter (Table 2). Primers were designed to cover the recombination junctions between the LBD, 3x-mEGFP, and the ubiquitin promoter in the destination vector.
Table 2. Sequencing primers for PH$_{\text{PLC} \delta 1}$, PH$_{\text{OSBP}}$, PH$_{\text{FAPP1}}$, and TUBBY entry clones.

<table>
<thead>
<tr>
<th>Name</th>
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<tbody>
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<tr>
<td>PLCD R Set 4</td>
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<td>OSBP Set 2</td>
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<td>FAPP Forward Set 5</td>
<td>GTG GAC CAA CTA TCT CAC AGG</td>
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<td>FAPP Set 1</td>
<td>GTC CTT GTA TCA GTC AAA CAT GC</td>
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<td>CCC TTG AAG CCT AGG ACA TTT</td>
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<tr>
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<tr>
<td>UBIP SEQ</td>
<td>CCC TGC CTT CAT ACG CTA TT</td>
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Maxi-Prep Isolation of Plasmid DNA

A NucleoBond Xtra Maxi Plus kit (Macherey-Nagel) was used to purify plasmid DNA from *E. coli* cultures. For maxi-preps, large cultures (250 mL) of selective LB broth with carbenicillin were inoculated with 250 µL of small culture and shaken at 250 rpm for 12-16 hours in a 37°C horizontal shaker. Approximately 250 mL of each culture was centrifuged for 10 mins at 6000 x g to collect cells. After discarding the supernatant, cells were thoroughly resuspended in 10 mL of Resuspension Buffer with RNase A (RES) via vortexing, lysed with 10 mL of Lysis Buffer, mixed gently by inverting 5 times, then incubated at room temperature for 5 mins. The resulting lysate was neutralized with 10 mL of neutralization buffer and immediately mixed by inversion 10-15 times. Sample was loaded onto NucleoBond Xtra Column previously equilibrated with Equilibrium Buffer and allowed to empty by gravity flow. The column was washed with 15 mL Equilibration Buffer, then washed with 10 mL of Wash Buffer. The DNA was eluted with 15 mL Elution Buffer and then precipitated with 10.5 mL room temperature isopropanol with a 2 min incubation at room temperature. The resulting precipitate was loaded onto NucleoBond Finalizer using a 15 mL syringe, and the DNA bound to the membrane. The DNA was washed with 70% ethanol and, the membrane was dried. The DNA was eluted from the membrane with 500 µL of Redissolving Buffer (5mM Tris/HCl, pH 8.5) two times. The eluting DNA was pooled and reinjected through the membrane. DNA was collected in a sterile micro-centrifuge tube. Plasmid DNA concentration was determined using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific).
Production of Transgenic Lines

Linearization of Expression Plasmid for Transformation

To linearize the LR reaction product, DNA was incubated with SwaI enzyme (1.2 µg DNA/unit) in 1X NEBuffer 3.1 at room temperature overnight. Digested DNA was incubated with NaAc (1M, pH 5.2), and ethanol (70%) for >30 minutes at room temperature to precipitate the DNA, then spun at 16,000 x g. The precipitate was washed with ethanol (70%) and then re-spun for 2 minutes. To remove residual ethanol, DNA was re-spun after initial decanting and excess ethanol was removed. The DNA was then allowed to air-dry in a laminar flow hood and resuspended in 50 µL of sterile TE.

Transformation of Physcomitrella patens

Transformation was performed as described by Liu and Vidali (Liu and Vidali 2011). Protoplasts were generated by incubating week old Gransden (Gran) P. patens in 8% mannitol and 2% Driselase (Sigma D9515-25G) for an hour with gentle shaking at room temperature. The protoplast suspension was filtered through a 100 μm mesh (BD Falcon 352350) and spun at 250xg for 5 minutes. The supernatant was decanted and the protoplasts were resuspended in 10 mL of 8% mannitol and centrifuged at 250xg for 5 minutes. This was repeated twice more. After the final spin, protoplasts were counted, pelleted, and then re-suspended in MMg solution (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.7) to obtain a final concentration of 1.6 million protoplasts/mL. The protoplasts in MMg solution were incubated at room temperature for 20 mins. For each transformation, 600 µL of protoplasts were incubated with 60 µg linearized DNA and 700 µL of PEG/Ca⁺² solution (154mM PEG4000, 310 mM mannitol, 150 mM CaCl₂) for 30 minutes at room temperature with occasional gentle agitation. The mixture was then
diluted with 3 mL of W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES (pH 5.7)) and then spun at 250xg for 5 mins. The protoplasts were then resuspended in 2 mL of melted PRM-T (PpNH4 media, 6% mannitol, 0.6% agarose, 0.01M CaCl2) and plated on PRM-B plates (PpNH4 media, 6% mannitol, 0.8% agarose, 0.01M CaCl2) with cellophane (1 mL per plate).

Selection of Plants

Transformed *P. patens* were grown under a 16/8 photoperiod at 25°C. Four days after transformation, plants were moved onto PpNH4 plates with hygromycin (1.84 mM KH2PO4, 3.4 mM Ca(NO3)2, 1 mM MgSO4, 2.72 mM Diammonium tartrate, 54 μM FeSO4.7H2O, 9.93 μM H3BO3, 1.97 μM MnCl2.4H2O, 0.23 μM CoCl2.6H2O, 0.19 μM ZnSO4. 7H2O, 0.22 μM CuSO4. 5H2O, 0.10 μM Na2MoO4.2H2O, 0.168 μM KI, 0.7% agar, 15 μg/ml hygromycin). After a week, plants were moved off of selection, onto PpNH4 plates without hygromycin. Plants were moved on and off selection for 3 weeks. Stably transformed individual plants were gridded on PpNH4 plates with hygromycin and left to grow for approximately a month. Plants were then analyzed for fluorescence using a Leica M165C stereo fluorescence microscope.

Characterization of Stable Lines

Confocal Imaging of Plants

Plants were grown on PpNO3 solid media (1.84 mM KH2PO4, 3.4 mM Ca(NO3)2, 1 mM MgSO4, 54 μM FeSO4.7H2O, 9.93 μM H3BO3, 1.97 μM MnCl2.4H2O, 0.23 μM CoCl2.6H2O, 0.19 μM ZnSO4. 7H2O, 0.22 μM CuSO4. 5H2O, 0.10 μM Na2MoO4.2H2O, 0.168 μM KI, 0.7% agar) for confocal imaging. Week old plants were mounted on PpNO3 media with 1% agar, and mounts were covered with PpNO3 liquid media. Slides
were viewed using a Leica SP5 confocal microscope. EGFP was excited using the 488-nm line of the argon laser and emission was collected between ~490-540 nm. Chlorophyll was excited using the 594-nm line of the HeNe laser and emission was collected between ~650-700 nm.

**Determining Relative Expression**

Week-old stable transgenic *P. patens* lines grown on PpNH₄ media were imaged using a Leica M165C stereo fluorescence microscope with a Leica 0.63X video objective (10447367) and a DFC310 FX 1.4 megapixel camera. Images were taken under the GFP-3 filter to collect for GFP fluorescence, using auto-exposure settings with brightness at 90%, saturation at 0.75, and gamma at 1. Images were evaluated based on brightness of image and exposure time and lines were separated into ++++, ++, and +. To confirm these scores, lines were further evaluated using confocal microscopy. Gain and offset settings were normalized to lines scored as ++ using the dissecting scope and the relative expressions of lines marked as +++ and + were examined.

**Morphometric Analysis of Lines**

Week-old cultures of stable moss lines were protoplasted as previously described. Protoplasts were suspended in liquid plating media (PpNH₄ media supplemented with 8.5% mannitol and 10 mM CaCl₂) at a concentration of 10,000 cells/mL, and 1 mL of cells was plated on a PRM-B plate with cellophane. Four days after plating, plants were transferred to PpNH₄ plates. Plants were imaged for growth after 2 days via a Leica M165C stereo fluorescence microscope with a Leica 0.63X video objective (10447367) and a DFC310 FX 1.4 megapixel camera. Plants were examined using the GFP-2 filter to collect for both chlorophyll autofluorescence and GFP fluorescence. Exposure settings
were normalized to the wild-type plant. Images were taken at 1392 x 1040 full frame HQ. Collected images were analyzed using ImageJ software using a morphology analysis macro developed by Bibeau and Vidali (2014). Growth was evaluated by measuring the plant area based on the chlorophyll autofluorescence.

Statistics

For comparison of multiple treatments, one-way analysis of variance (ANOVA) was used, and comparison of means were done by the Tukey post hoc test using JMP 11.2.1. An adjusted P value of 0.05 was used as significant. Area values were log transformed to obtain a normal distribution.

Results

To investigate the localization of phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P$_2$) and phosphatidylinositol (4) phosphate (PtdIns(4)P) during polarized cellular growth and plant development in the bryophyte *Physcomitrella patens*, we generated a set of transgenic *P. patens* lines that express a chimeric reporter protein composed of a PI specific lipid binding domain (LBD) tagged with 3x-mEGFP (Fig 2). To visualize the localization of PtdIns(4)P, the pleckstrin homology (PH) domains of oxysterol binding protein (OSBP) and four phosphate adaptor protein (FAPP1) were utilized (Balla et al. 2005). To examine PtdIns(4,5)P$_2$’s localization, the Tubby domain from the Tubby protein and the PH domain of phospholipase C $\delta_1$ (PLC$\delta_1$) were utilized (Quinn et al. 2008; van Leeuwen et al. 2007). The position of the LBD in these constructs reflects the position of the domain in the native protein. Tubby and PH$_{OSBP}$ are tagged on the C terminal, and PH$_{FAPP1}$ and PH$_{PLC\delta1}$ are N terminally tagged (Balla et al. 2005) (Quinn et al. 2008; van Leeuwen et al. 2007) (Várnai and Balla 1998; Balla et al. 2005).
These LBDs have different specificities for their specific PI – PH\textsubscript{OSBP} has a higher affinity for PtdIns(4)P than PH\textsubscript{FAPP1}, \(K_d\) of 3.5 \(\mu\)M vs 18.6 \(\mu\)M (Balla et al. 2005; Simon et al. 2014), and TUBBY is said to have a higher affinity for PtdIns(4,5)P\(_2\) than PH\textsubscript{PLC\delta1} (Carroll et al. 2004; Szentpetery et al. 2009). Additionally, to validate the localization data seen in the reporter lines, lines expressing a reporter protein composed of 3x-mEGFP fused to non-binding mutants of PH\textsubscript{OSBP}, PH\textsubscript{FAPP1}, and PH\textsubscript{FAPP1} were generated (PH\textsubscript{FAPP1} R18L; PH\textsubscript{OSBP} R107E,R108E; and PH\textsubscript{PLC\delta1} R40L (Várnai and Balla 1998; Balla et al. 2005).

**Creation of Stable Lines**

*Creation and Verification of Expression Constructs*

The LBD-3xmEGFP constructs were created by two fragment Gateway® cloning (Fig 2). Entry clones plasmids, in which the LBDs and 3x-mEGFP were flanked by \textit{attL} sites were provided. Chemically competent \textit{E. coli} cells were transformed with the BP reaction product, and kanamycin-resistant clones expressing the product were selected. Plasmid DNA was isolated from selected colonies and sequenced for correct ORF sequence and for \textit{att} sites.

Correct clones were then used for the two fragment recombination reaction using LR Clonase II Plus (Fig2b-c). The entry clones containing the LBD or 3x-mEGFP were recombined into the destination vector pTHUBI\textit{gate}, under the control of a maize ubiquitin promoter. The destination vector pTHUBI\textit{gate} has two arms that target the Pp108 locus flanking the recombination sites of the genes, two antibiotic resistance markers (a carbenicillin resistance gene for use in bacteria, and a hygromycin resistance
Figure 3. Characterization of expression clones by SwaI digestion. Expression clone DNAs were isolated from carbenicillin-resistant *E. coli* cells transformed with the product of the LR recombination reaction. The plasmid DNAs were digested with SwaI for 1 hour at room temperature in NEBuffer 3.1. The expected band sizes for pOBSP-EGFP, pOBSPM-EGFP, pFAPP1-EGFP, and pFAPP1M-EGFP were ~10kB and ~3kB; for pPLCδ-EGFP, pPLCδM-EGFP, and pTUBBY-EGFP, the expected band sizes were ~11kB and ~3kB.
gene for use in plants), and SwaI restriction sites (Schaefer and Zryd 1997). The LR recombination reaction product was then proliferated by transforming and culturing competent *E.coli* cells. The cells expressing the plasmid underwent antibiotic selection, and plasmid DNA was purified from selected colonies. To test for recombination of the entry clones into the destination vector, plasmid DNAs were digested with SwaI and analyzed via gel electrophoresis (Fig. 3). If both genes were successfully recombined into the destination vector, the expected band sizes for pOBSP-EGFP, pOBSPM-EGFP, pFAPP1-EGFP, and pFAPP1M-EGFP were ~10kB and ~3kB; for pPLCδ-EGFP, pPLCδM-EGFP, and pTUBBY-EGFP, the expected band sizes were ~11kB and ~3kB. Clones that exhibited band sizes that corresponded with our predicted masses were obtained for all constructs (Fig 3). The digested clones that exhibited these bands were then sequenced using gene specific primers for junction sequences to ensure that genes had recombined in the proper orientation in the plasmid. We successfully found clones that had both the expected band sizes and the correct sequence to allow for ubiquitin promoter-driven expression of the LBD-3XmEGFP fusion proteins. These clones were then used for the continuation of the project.

**Transformation and Selection of Stable Lines**

To generate stable transgenic lines, protoplasts of week-old, wild-type *P. patens* were transformed with linearized plasmid DNA using a PEG/Ca\(^{+2}\) mediated process (Liu and Vidali 2011). Transformed protoplasts were plated for recovery for four days, then alternated selection on hygromycin and recovery every week for four weeks. To isolate specific lines, plants that survived selection were individually gridded onto media containing hygromycin and left to grow for another month. The plants that survived the
continuous selection were considered suitable for further study, as they are the most likely to have the reporter construct stably integrated into their genome. We generated 110 PH\textsubscript{OBSP}-EGFP lines; 94 PH\textsubscript{OBSPM}-EGFP lines; 39 PH\textsubscript{FAPP1}-EGFP lines; 66 PH\textsubscript{FAPP1M}-EGFP lines; 44 PH\textsubscript{PLCδ}-EGFP lines; 30 PH\textsubscript{PLCδM}-EGFP lines; and 96 Tubby-EGFP lines.

**Initial Characterization of Stable Lines**

For our lines to be useful in future studies, we needed to identify lines that produced our protein at a level that did not interfere with normal growth. To determine which lines expressed LBD-3xmEGFP, plants under continuous selection were screened using a Leica M165C stereomicroscope using the GFP-3 filter. Plants were first scored for relative intensity of GFP signal, a measure of the expression level of the fusion protein in the plant (Fig 4). Scores were obtained by comparing the exposure time and image brightness. The brightest plants, plants with the lowest exposure time and the highest image brightness, were scored as ++++. Medium plants were scored as ++, and dim plants were scored as +. Using these scores, we hoped to find lines with the highest level of reporter expression that did not interfere with normal growth or with imaging.

To confirm that our semi-quantitative scoring system was valid, selected lines were also imaged using confocal microscopy. Settings for confocal microscopy were normalized to a ++ plant to determine relative expression levels. Scores obtained with the confocal microscope were consistent with scores obtained using the stereomicroscope, thus confirming the validity of our rapid stereomicroscope scoring method. In the future, scoring will determined using only the stereomicroscope. For each construct, from our stably transformed lines, two lines of each intensity level were selected for further characterization for a total of 24 lines (Appendix B).
Figure 4. Semi-quantitative scoring for LBD-GFP expression of transgenic \textit{P. patens} lines. Week old plants were imaged for GFP fluorescence under an upright stereomicroscope at 12X magnification using auto-exposure settings from LAS V4 software. GFP intensity was scored as $+++$, $+$, or $+$ as determined from exposure time and individual assessment.
To determine the spatial expression patterns of PtdIns(4)P in moss, selected lines expressing PHOSBP-EGFP, PHOSBPM-EGFP, PHFAPP1-EGFP, and PHFAPP1M-EGFP were imaged for GFP and chlorophyll fluorescence using confocal microscopy (Fig 5, 6). Lines expressing pOBSP-EGFP displayed primarily plasma membrane (PM) localized GFP (Fig 5A), while lines expressing its non-functional mutant, pOSBPM-EGFP, showed cytoplasmic and nuclear localization of GFP (Fig 5B). Additionally, the localization of 3x-mEGFP tagged PHFAPP1 in P. patens differed from that of PHOSBP (Fig. 6A). Although 3x-mEGFP tagged PHFAPP1 also localized to the PM, it was also detected in the cytosol and in punctate structures (Fig 6A). The lines expressing PHFAPP1’s non-functional mutant showed cytosolic and nuclear localization (Fig 6B). We saw similar expression pattern in other lines expressing the same construct (Fig 5, 6). Additionally, the localization pattern between the non-functional mutant and the wild-type LBD differ. Preliminary imaging of the PtdIns(4,5)P$_2$ reporter lines showed localization to the PM, cytoplasm, and nucleus (data not shown). However, due to time constraints, these lines could not be investigated further. The localization pattern of the two 3x-mEGFP tagged PtdIns(4)P binders in the moss is similar to the localization pattern of the same LBDs in the root epidermis of Arabidopsis (Simon et al. 2014). The similar localization pattern of the LBDs in moss with the Arabidopsis root epidermal cells, coupled with the loss of targeted membrane localization between the wild-type LBD and its non-functional mutant in our lines, suggests that the lines are faithfully reporting the localization of PtdIns(4)P in P. patens.

To determine if the production of the reporter protein interfered with normal growth, we assayed selected lines for changes in growth rate by measuring plant size in
Figure 5. Cellular distribution of PHOSBP-EGFP (A) and its non-functional mutant, PHOSBP R107E,R108E-EGFP (PHOSBPM-EGFP) (B) in P. patens. Week-old plants were examined using confocal microscopy. Lines were imaged for EGFP (green) and chlorophyll (red) fluorescence. Scale bars = 25 µm.
Figure 6. Localization of PH<sub>FAPP1</sub>-EGFP (A) and its non-functional mutant, PH<sub>FAPP1 R18L</sub>-EGFP (B) in week old stable <i>P. patens</i> lines. Confocal microscopy was used to analyze EGFP (green) and chlorophyll (red) fluorescence. White arrowheads indicate punctate structures. Scale bars = 25 µm.
week-old plants regenerated from protoplasts. Plant area was estimated using chlorophyll autofluorescence in individual plant images from each line. Area is represented as a percentage of the control, the wild-type moss, Gransden (Gran). Selected lines expressing PHOSBP-EGFP and PHOSBPM-EGFP were assayed for area (Fig 7). Interestingly, this preliminary data showed that PHOSBP-EGFP lines scored as +++ and + had very different morphology from wild-type (Fig 7A). Lines scored as ++ were the most similar to the control plant in terms of area (Fig 7A). In the lines that express PHOSBPM-EGFP, all but OMA3.10 exhibited normal growth across the three variables (Fig 7B).

Interestingly, PHOSBP-EGFP scored as +++ have a different phenotype than ++ and + plants (Fig 8). Plants with relatively high expression levels of PHOSBP-EGFP were significantly smaller than the wild-type plant (Fig. 7A) and had wavy tips (Fig 8). Furthermore, plants that over-express PHOSBP-EGFP appeared to have more branching (Fig 8). This altered phenotype was not seen in the ++ and + PHOSBP-EGFP, or the +++ PHOSBPM-EGFP plants (Fig 6, Fig 8B). This further confirmed that the PHOSBP-EGFP produced was binding specifically to PtdIns(4)P and also suggested that expressing PHOSBP-EGFP at high levels disrupted the natural PtdIns(4)P concentration and caused irregular tip growth.

**Discussion**

Polarized cellular expansion is a complex process that occurs in a multiplicity of systems. In plants, fungi, and algae, this process is called “tip growth.” The bryophyte *P. patens* is an excellent model system for tip growth, yet we do not know how the PIs, an essential component of tip growth, function in these plants. In order to understand the localization of PtdIns(4)P and PtdIns(4,5)P2 in moss during tip growth, we generated
Figure 8. Moss that strongly express PHOSBP-EGFP have altered morphology. Stereomicrographs of week-old wild type (A), PHOSBPm-EGFP (B), and PHOSBP-EGFP (C) plants grown from protoplasts. D. Confocal microscopy of OA2.9. Plants were imaged for EGFP (green) and chlorophyll (red). White arrowheads indicate wavy tips. Scale bar = 100 µm.
stable transgenic *Physcomitrella patens* lines expressing 3x-mEGFP tagged LBDs. To visualize PtdIns(4)P, PH_{OSBP} and PH_{FAPP1} were utilized; for PtdIns(4,5)P$_2$, PH$_{PLC\delta 1}$ and TUBBY were used. Additionally, to verify the specificity of our reporters, we developed lines expressing the non-functional mutants of PH$_{OSBP}$, PH$_{PLC\delta 1}$, and PH$_{FAPP1}$ tagged with 3x-mEGFP. Expression constructs were generated using two-fragment Gateway® recombination cloning, and successful cloning of constructs was verified via SwaI digestion and sequencing of junctions (Fig 3). Wild-type moss were successfully transformed with the expression constructs, and stable lines were isolated via hygromycin selection.

To determine which stable lines were suitable for further characterization and for future use, we first developed a semi-quantitative scoring system to determine relative levels of expression (Fig 4). Using this scoring system, we separated lines into high, medium, and low expressers (+++, ++, +) of their respective constructs. The lines that expressed a PtdIns(4)P binder or the non-functional mutant of a PtdIns(4)P binder were further examined for localization of the chimeric reporter protein and for normal growth (Fig 5-7).

In lines expressing the wild type LBD, the localization of the chimeric reporter protein should report the localization of PtdIns(4)P. We utilized two LBDs with differing affinities for PtdIns(4)P – PH$_{OSBP}$ has a $K_d$ value of 3.5 $\mu$M for PtdIns(4)P, while PH$_{FAPP1}$ has a $K_d$ of 18.6 $\mu$M (Simon et al. 2014). We found that in lines expressing PH$_{OSBP}$-EGFP, the protein primarily localized to the PM (Fig 5A). In PH$_{FAPP1}$-EGFP lines, we saw a slight enrichment in the PM and also in punctate structures in the cytosol (Fig 6A). The specificity of the reporter was supported by the localization of their respective non-
functional mutants. Lines expressing the non-functional mutant form of the reporter proteins exhibited cytosolic and nuclear localization of GFP, which is characteristic of non-targeted GFP localization (Fig 5B, 6B). Because the spatial localization of the wild-type protein and the non-functional mutant clearly differ, we concluded that our stables lines are faithfully reporting the localization of PtdIns(4)P in *P. patens*.

In lines expressing PHOSBP-EGFP and PHOSBPM-EGFP, plant area was assayed to determine if expressing the reporter protein interfered with growth. We found that expressing the non-functional mutant, PHOSBPM-EGFP, in *P. patens* did not affect growth in most of the selected lines (Fig 7B). However, growth was affected in PHOSBP-EGFP lines scored as +++ and as +. While the growth assay data is preliminary, we noticed an interesting phenotype associated with +++ PHOSBP-EGFP lines (Fig 8). Aside from being smaller than the wild type control plant, +++ PHOSBP-EGFP lines had non-linear, wavy tips and were generally less spread out than both the non-functional mutant and the control lines. This suggested that higher expressions of PHOSBP-EGFP disrupted normal tip growth in *P. patens*, presumably due to altering PtdIns(4)P concentrations by sequestration. This phenotype further suggests that our reporter is specifically reporting the localization of PtdIns(4)P in *P. patens*. However, it is important to find levels of reporter expression that are tolerated by the plant. The ++ PHOSBP-EGFP plants exhibited a negligible reduction in growth, and it is possible that with more data, these will be the most useful lines for examining PtdIns(4)P dynamics.

Interestingly, the localization of these specific LBDs in *P. patens* agrees with the localization of these proteins when expressed in the root epidermis of the flowering plant *Arabidopsis thaliana* (Simon et al. 2014). In a study of various LBDs conducted by
Simon et. al (2014), they concluded that PHFAPP1 localized at the PM and post-Golgi compartments in the Arabidopsis, while PHOSBP was restricted to the PM. The similar reporter localization between the two systems suggests that the localization of PtdIns(4)P in moss parallels the localization in Arabidopsis, which implies that PtdIns(4)P serves similar functions in both systems. However, unlike Arabidopsis root hairs, we did not see PtdIns(4)P localization in the lateral flanks of the protonemata (Yoo et al. 2012). One possible explanation is that the imaged plants were not actively growing. It will be interesting to image actively growing, younger plants to determine if PtdIns(4)P localization would be focused on the lateral flanks of the growing protonemata tissue.

Additionally, the wavy tips found in our +++ plants is reminiscent of agd-1 root hair mutants (Yoo et al. 2012; Yoo et al. 2008). AGD-1 is an ARF-GAP thought to regulate the localization of PtdIns(4)P (Yoo et al. 2012; Yoo et al. 2008). When AGD-1 expression is knocked down in A. thaliana, directional tip growth is lost in root hairs, which suggested that PtdIns(4)P localization is essential to unidirectional growth. In high expressing PHOSBP-EGFP lines, we saw that the plant area was significantly smaller than that of the wild-type plants and of the +++ PHOSBPM-EGFP lines. Yoo et al (2012) also found that the root hairs of agd-1 mutants are significantly shorter than wild-type. The parallel in phenotypes between the lines over-expressing PHOSBP-EGFP and the agd-1 Arabidopsis suggests that the over-expression of PHOSBP-EGFP causes delocalization or sequestration of PtdIns(4)P that alters tip growth in P. patens. This also suggests that PtdIns(4)P plays similar roles in P. patens as in higher plants, and supports the use of P. patens as a suitable model system for tip growth.
In addition to further examining the PtdIns(4)P reporter lines, we will characterize and validate our PtdIns(4,5)P$_2$ lines: PH$_{PLC\delta1}$-EGFP, Tubby-EGFP, and PH$_{PLC\delta1}$ R40L.

While preliminary imaging suggests that both functional reporters localize to the cytoplasm, PM, and the growing tip (data not shown), we did not collect enough data to substantiate this localization pattern.

Although we are fairly confident that our lines are faithfully reporting the localization of PtdIns(4)P, further validation and characterization is required. Validation of lines would include biochemical assays such as Western blot to determine if our produced protein is of the expected size and protein-lipid overlay assays to determine if our produced protein binds to PtdIns(4)P and with what specificity. Additionally, further growth data on these lines will allow us to determine which lines will be suitable for future studies.

An alternative method of validating the accuracy of our reporters is to determine if they are sensitive and responsive to plant PI levels. Plants can be treated with kinase inhibitors such as the PI4K inhibitor phenylarsine oxide (PAO) to see if decreased levels of PtdIns(4)P would alter the localization of the reporter. Additionally, the hydrolysis of these PIs could be inhibited. For example, the PLC inhibitor neomycin prevents the hydrolysis of PtdIns(4,5)P$_2$, effectively increasing the concentration of PtdIns(4,5)P$_2$. Altered reporter localization or expression of the reporter protein after drug incubation would confirm the reporter’s sensitivity to PI levels.

Another method of altering PI concentration is via genetic modification. Because *P. patens* display a high level of homologous recombination, it is particularly suited for genetic studies. The hygromycin selection cassette can be removed from the genome of
stable lines via Cre-Lox recombination, and new genes can be introduced. With this method, we can knock down the expression of specific regulators of PI metabolism in the moss or over-express them and examine how the localization of the reporters change. To examine how the localization of PtdIns(4)P would change, we would knock-down or over-express specific PI4Ks in the stable lines. In moss, there are orthologs of the Arabidopsis AtPI4Kβ1 and the yeast PI4Ks, Stt4 and Pik1 (Ischebeck et al. 2010; Audhya et al. 2000). We predict that altering the expression level of these moss orthologs of PI4K would change the concentrations of PtdIns(4)P, thus altering the localization patterns of our fluorescent reporter lines. To validate the our PtdIns(4,5)P$_2$ reporter lines, one option would be to knock-down PpPIPK1 in these lines. In pipk1 mutants, PtdIns(4,5)P$_2$ levels decrease significantly, while the pipk2 mutants exhibited a negligible difference in PtdIns(4,5)P$_2$ levels (Saavedra et al. 2011). Alternatively, over-expressing PpPIPK1 should cause an increase in PtdIns(4,5)P$_2$ concentration. By comparing the expression pattern of our reporters between the two conditions (knocking-down expression and over-expressing the PI-specific kinases), we would be able to examine our reporter’s sensitivity to altered PI concentrations.

Concluding remarks

Currently, our understanding of the function and localization of PIs in non-vascular plants is deficient. We have developed stable lines of P. patens expressing a fluorescent reporter proteins to study the localization of PtdIns(4)P and PtdIns(4,5)P$_2$, two essential PIs in growth and development. When suitable reporter lines are available, studies on the spatial and temporal localization of PtdIns(4)P and PtdIns(4,5)P$_2$ in moss will be enhanced. These reporter lines are especially versatile because new plasmids can
be introduced after lines are “cured” of antibiotic resistance and stable lines of these
doubly transformed moss can be maintained. Additionally, lines reporting PtdIns(4)P and
PtdIns(4,5)P$_2$ will be useful for studies on a variety of processes, such as membrane
trafficking, cell signaling, and stress response. As we increase our understanding of the
importance of the PI signaling network in plants, it is essential that we better understand
how PIs function in such essential functions as polarized growth.
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fates of phosphatidylinositol produced by phosphatidylinositol synthase isoforms 

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doi:10.1126/science.1149121

in diverse signaling proteins. Cell 73 (4):629-630

McLaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by 


# Appendix

Appendix A. PCR primers used to amplify PH domains and add attB sites for BP recombination reaction.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ – 3’)</th>
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<tbody>
<tr>
<td>PHCattB1 (Forward)</td>
<td>GGGGACAAGTTGTACAAAAAAGCAGGCTTAATGGACTCGGGCCGGGACCTTC</td>
</tr>
<tr>
<td>PHCattB5r (Reverse)</td>
<td>GGGGACAACCTTTGTATAACAAAAGTTGTCTTCAGGAAGTTCTGAGCTCCTTGAAG</td>
</tr>
<tr>
<td>OSBPattB5 (Forward)</td>
<td>GGGGACAACCTTTGTATAACAAAAGTTGTGGTCTGGCTCGGAGAG</td>
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<tr>
<td>OPBPattB2 (Reverse)</td>
<td>GGGGACCCTTTGTACAAGAAAGCTGGGTATTATCTGCCAGCATCTTCACAGCTTTG</td>
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</tr>
<tr>
<td>FAPP1attB5r (Reverse)</td>
<td>GGGGACAACCTTTGTATAACAAAAGTTGTCTGGCTCTGATCAGTCACACAGCCTTTG</td>
</tr>
<tr>
<td>TUBBYattB5 (Forward)</td>
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</tr>
<tr>
<td>TUBBYattB2 (Reverse)</td>
<td>GGGGACCCTTTGTACAAGAAAGCTGGGTACTACTCGAGCCAGCTTGCTG</td>
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Appendix B. Scores and initial morphological characterization of stable lines.

<table>
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<tr>
<th>Line</th>
<th>Exposure Time</th>
<th>Score</th>
<th>Area %</th>
<th>Circularity</th>
<th>Solidity</th>
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<tbody>
<tr>
<td>OMA7.4</td>
<td>1</td>
<td>+++</td>
<td>106%</td>
<td>0.053724</td>
<td>0.37131</td>
</tr>
<tr>
<td>OMA3.10</td>
<td>0.7768</td>
<td>+++</td>
<td>70%</td>
<td>0.084237</td>
<td>0.42744</td>
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<tr>
<td>OMA7.14</td>
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<td>++</td>
<td>108%</td>
<td>0.110632</td>
<td>0.454598</td>
</tr>
<tr>
<td>OMA5.14</td>
<td>1</td>
<td>++</td>
<td>113%</td>
<td>0.056652</td>
<td>0.376459</td>
</tr>
<tr>
<td>OMA6.8</td>
<td>1</td>
<td>+</td>
<td>133%</td>
<td>0.035177</td>
<td>0.32402</td>
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<tr>
<td>OMA3.14</td>
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<td>+</td>
<td>126%</td>
<td>0.036663</td>
<td>0.340271</td>
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<tr>
<td>OA2.9</td>
<td>0.9</td>
<td>+++</td>
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<td>0.052159</td>
<td>0.51448</td>
</tr>
<tr>
<td>OA3.9</td>
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<td>0.475459</td>
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<tr>
<td>OA8.8</td>
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<td>0.027004</td>
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<td>OA3.2</td>
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<tr>
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<td>0.049864</td>
<td>0.421274</td>
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<tr>
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<td>+++</td>
<td>79%</td>
<td>0.07</td>
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<td>FA3.12</td>
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<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
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<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>FMA2.8</td>
<td>1</td>
<td>+++</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>--</td>
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<td>++</td>
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<td>--</td>
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<tr>
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<td>0.0438</td>
<td>0.3796</td>
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<tr>
<td>FMA1.1</td>
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<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>