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# The *Arabidopsis* Stem Cell Factor POLTERGEIST Is Membrane Localized and Phospholipid Stimulated [W][OA]

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**Stem cell maintenance and differentiation are tightly regulated in multicellular organisms. In plants, proper control of the stem cell populations is critical for extensive postembryonic organogenesis. The *Arabidopsis thaliana* protein phosphatase type 2C proteins POLTERGEIST (POL) and PLL1 are essential for maintenance of both the root and shoot stem cells. Specifically, POL and PLL1 are required for proper specification of key asymmetric cell divisions during stem cell initiation and maintenance. POL and PLL1 are known to be integral components of the CLE/WOX signaling pathways, but the location and mechanisms by which POL and PLL1 are regulated within these pathways are unclear. Here, we show that POL and PLL1 are dual-acylated plasma membrane proteins whose membrane localization is required for proper function. Furthermore, this localization places POL and PLL1 in proximity of the upstream plasma membrane receptors that regulate their activity. Additionally, we find that POL and PLL1 directly bind to multiple lipids and that POL is catalytically activated by phosphatidylinositol (4) phosphate [PI(4)P] in vitro. Based on these results, we propose that the upstream receptors in the CLE/WOX signaling pathways may function to either limit PI(4)P availability or antagonize PI(4)P stimulation of POL/PLL1. Significantly, the findings presented here suggest that phospholipids play an important role in promoting stem cell specification.**

## INTRODUCTION

A critical aspect of stem cell maintenance is the asymmetric assignment of cell fate to stem cell daughters so that both self-renewal and differentiation can occur. Proper stem cell regulation is essential for the extensive postembryonic organogenesis that makes up the vast majority of plant organs and tissues. In *Arabidopsis thaliana*, two protein phosphatase type 2C (PP2C) proteins, POLTERGEIST (POL) and PLL1, regulate the asymmetric character of stem cell divisions at both the shoot and root meristems (Yu et al., 2000, 2003; Song and Clark, 2005; Song et al., 2006, 2008; Gagne and Clark, 2007; Gagne et al., 2008). Within the shoot meristem, POL and PLL1 are components of the CLV3/WUS pathway and promote the expression of *WUSCHEL* (*WUS*) in the basal daughter of dividing L3 stem cells (see Supplemental Figure 1A online) (Song et al., 2006, 2008; Gagne et al., 2008). In *pol pll1* mutants, *WUS* expression is not maintained in either L3 daughter, leading to a loss of stem cells and termination of the shoot and floral meristems. During embryogenesis, POL and PLL1 are potential components of the CLV3/WUS-related CLE40/WOX5 pathway where they promote the expression of *WOX5* in the apical daughter of the dividing hypophyseal cell (see Supplemental Figure 1B online) (Song et al., 2008). In *pol pll1* mutants, the hyphophyseal cell daughters lose both *WOX5* expression and morphological asymmetry, resulting in loss of the root meristem. Furthermore, postembryonically POL and PLL1 are continually required at the root meristem for stem cell maintenance, presumably by participating in the CLE40/WOX5 pathway. Importantly, all of the shoot and root stem cell defects found in *pol pll1* mutants can be overcome by ectopic expression of *WUS* and *WOX5*, respectively (Song et al., 2006, 2008; Gagne et al., 2008).

Significant parallels have been identified between the CLV3/WUS and the CLE40/WOX5 pathways (Sarkar et al., 2007). Specifically, CLV3 and CLE40, which are both members of the CLE family, can function in both the root and shoot meristems (Hobe et al., 2003; Fiers et al., 2005; Ito et al., 2006). Also, WUS and WOX5, which are evolutionarily related homeodomain transcription factors, can functionally replace each other (Mayer et al., 1998; Haecker et al., 2004; Sarkar et al., 2007; Nardmann et al., 2009; Stahl et al., 2009). Furthermore, *WUS*, *WOX5*, and other members of the *WOX* family often display asymmetric expression in the daughter cells following cell division, showing a consistent regulatory mechanism for *WOX* genes (Haecker et al., 2004; Breuninger et al., 2008).

Beyond the shoot and root meristems, POL and PLL1 are also required for the asymmetric division of the procambial cells during embryonic development (Song et al., 2008). In *pol pll1* mutants, morphological and developmental asymmetry are lost for the procambial cell daughters, which adopt neither apical nor basal identity. These defects result in a failure to specify the central vasculature. Interestingly, another CLE protein, CLE41, and other *WOX* genes have been shown to function in or are

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expressed in the vasculature, suggesting that other POL/PLL1-dependent CLE/WOX pathways may also exist (Haecker et al., 2004; Deveaux et al., 2008; Whitford et al., 2008). In summary, POL and PLL1 are required for at least two CLE/WOX signaling pathways that specify asymmetric cell divisions during development, including those that give rise to and maintain the shoot and root stem cell niches. Furthermore, because the hypophyseal and procambial cells in *pol pll1* mutants exhibit defects in the normally asymmetric positioning of the division plane, POL/PLL1-dependent asymmetry must be specified in some cases prior to cell division (Gagne and Clark, 2007; Gagne et al., 2008; Song et al., 2008).

Much of what is known about POL/PLL1-dependent pathways has come from studies in the shoot meristem where POL and PLL1 are signaling intermediates for the CLV3/WUS pathway (Yu et al., 2000, 2003; Song and Clark, 2005; Song et al., 2006). Key CLV3/WUS pathway components include the receptor-kinase CLV1, the transmembrane kinase CORYNE (CRN), the receptor-like protein CLV2, and the secreted ligand CLV3 (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999; Muller et al., 2008). Based on genetic analyses, when CLV3 is present, CLV1, CLV2, and possibly CRN negatively regulate POL and PLL1 via an unknown mechanism, while in the absence of CLV3, POL and PLL1 are functional (Song et al., 2006; Muller et al., 2008) (see Supplemental Figure 1A online). This signaling pathway is essential to specify differential expression of *WUS* in the apical and basal daughters of periclinally dividing L3 stem cells (Brand et al., 2000; Schoof et al., 2000; Song et al., 2006, 2008; Gagne et al., 2008). One model for this regulation is that CLV signaling acts upon the L3 stem cell prior to cell division, resulting in the polarization of POL/PLL1 protein and/or activity (see Supplemental Figure 1C online) (Gagne et al., 2008). As the ligand CLV3 is expressed apically to the periclinally dividing L3 stem cells, CLV3 would function as the directional signal for this polarization (Fletcher et al., 1999; Gagne et al., 2008). This polarization of POL/PLL1 protein and/or activity results in the apical daughter receiving little or no active POL/PLL1, while the basal daughter receives active POL/PLL1. The difference in POL/PLL1 activity between the two daughter cells then drives them to differential cellular fates, including differential *WUS* expression. A similar model would apply to POL/PLL1 function in other tissues, such as the root meristem. While this model is consistent with all of the development defects in *pol pll1* mutants, there currently is no shoot meristem–specific data that rules out the original model for the CLV3/WUS pathway, namely, that CLV signaling drives the apical and basal daughters to different cellular fates following cell division.

While genetic and expression studies have revealed that POL and PLL1 are required for the asymmetric cell divisions during stem cell maintenance and embryonic development, information is lacking about how and where POL and PLL1 are controlled at the molecular level in planta. This lack of understanding of POL/PLL1 localization and regulation strongly limits our ability to test and develop models for POL/PLL1-dependent pathways. To further explore the mechanism of POL/PLL1-dependent signaling, we performed a series of experiments to examine POL/PLL1 modification, localization, and enzymatic regulation.

## RESULTS

### POL and PLL1 Are Dual Acylated Plasma Membrane Proteins

While the *POL* gene family has been extensively characterized genetically, little is known about the function of the corresponding proteins at the cellular and biochemical levels (Yu et al., 2000, 2003; Song and Clark, 2005; Song et al., 2006, 2008). Given that POL and PLL1 serve as a link between plasma membrane–localized receptors, like CLV1 and possibly ACR4, and the nuclear regulation of *WUS* and *WOX5* expression, it is critical to address POL/PLL1 localization in planta (Yu et al., 2000, 2003; Song and Clark, 2005; Song et al., 2006, 2008; Stahl et al., 2009). Through sequence analysis, we identified putative N-terminal *N*-myristoylation and palmitoylation signal sequences in all POL family proteins (Figure 1A) (Boisson et al., 2003; Podell and Gribskov, 2004). *N*-myristoylation is the irreversible covalent attachment of myristoyl to an N-terminal Gly after removal of the starting Met (Podell and Gribskov, 2004). This acyl modification promotes protein–protein and protein–membrane interactions. Palmitoylation is the reversible covalent attachment of palmitoyl to a Cys found in the correct context (Resh, 1999; Farazi et al., 2001). This acyl modification can regulate the localization, activity, and/or binding of a protein. A prevailing model is that the addition of palmitoyl can serve to stabilize plasma membrane association of a myristoylated protein (Resh, 1999; Farazi et al., 2001). Perhaps then POL and PLL1 localize to the plasma membrane in a myristoylation- and palmitoylation-dependent manner.

To explore whether the localizations and/or functions of POL family members are controlled by acylation, we examined the roles of myristoylation and palmitoylation in POL and PLL1 localization in planta. Constructs were generated encoding POL-green fluorescent protein (GFP) and PLL1-GFP fusion proteins under control of the *35S* promoter (Figure 1B). Additional constructs were also generated that were identical except for substitutions in the first 18 amino acids designed to eliminate myristoylation ($myr^m$) and/or palmitoylation ($pal^m$) (Boisson et al., 2003). Specifically, POL$myr^m$-GFP and PLL1$myr^m$-GFP have the Gly residues presumed to be required for myristoyl attachment changed to Ala residues and the third amino acids altered to residues that would block myristoylation (Boisson et al., 2003). POL$pal^m$-GFP and PLL1$pal^m$-GFP have the Cys residues that are the putative palmitoylation sites changed to Gly residues. POL$myr^mpal^m$-GFP and PLL1$myr^mpal^m$-GFP have both sets of key residues disrupted.

To determine if POL and PLL1 are membrane localized, the constructs were transiently transformed into tobacco (*Nicotiana benthamiana*) (Voinnet et al., 2003). Protein samples were isolated and subjected to ultracentrifugation, and crude, soluble, and membrane fractions were examined by immunoblots (Figure 1C). POL-GFP and PLL1-GFP were detected only in the membrane fraction, identical to the control CLV1-GFP. Substitutions predicted to disrupt a single acyl attachment site (POL$myr^m$-GFP, PLL1$myr^m$-GFP, POL$pal^m$-GFP, and PLL1$pal^m$-GFP) altered the localization patterns, with the proteins found in both the soluble and membrane fractions. When both sets of substitutions

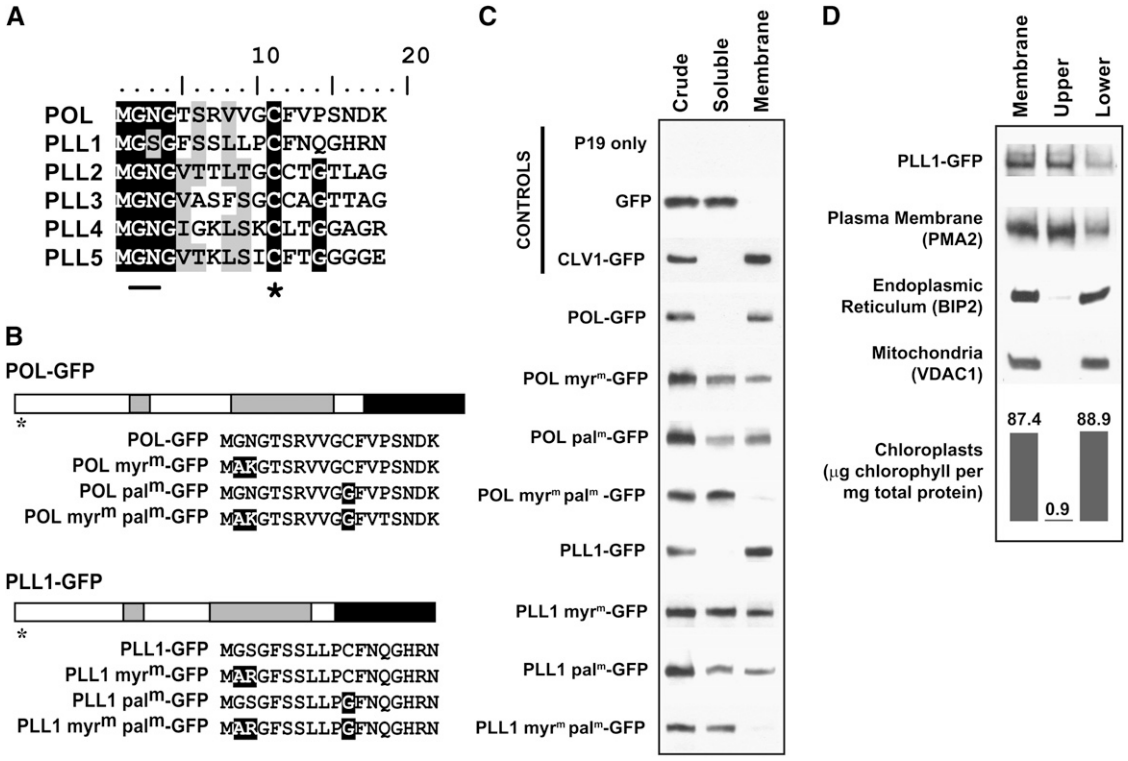


**Figure 1.** *N*-Myristoylation and -Palmitoylation Are Required for Plasma Membrane Localization of POL and PLL1.

**(A)** Alignment of putative *N*-myristoylation and -palmitoylation signal peptides of the *Arabidopsis* POL family members. Positions with >66% amino acid similarity or conservation are indicated. The conserved and similar amino acids are in black and gray boxes, respectively. Residues mutated in this study are underlined. The asterisk marks the conserved Cys residues.

**(B)** Structure of fusion proteins encoded by the *35S:POL-GFP* and *35S:PLL1-GFP* constructs. GFP is in black, while gray designates the regions with homology to the human PP2Ca catalytic domain. The asterisk marks the putative signal peptides. Below the structures are the first 18 amino acids for each of the constructs used with the amino acid substitutions marked by black boxes.

**(C)** Immunoblots of crude, soluble, and membrane protein fractions from infiltrated tobacco plants expressing various GFP fusion proteins.

**(D)** Two-phase partitioning of membranes isolated from infiltrated tobacco plants expressing PLL1-GFP. Various antibodies were used to show the partitioning of PLL1-GFP, the plasma membrane, the ER, and the mitochondrial membrane. Chlorophyll levels were also quantified for each fraction and are expressed in micrograms of chlorophyll per milligram of protein for each sample.

were present (POLmyr$^m$pal$^m$-GFP and PLL1myr$^m$pal$^m$-GFP), and thus presumably both acyl attachment sites were disrupted, there was a complete switch of the proteins to the soluble fraction, similar to GFP alone (Haseloff et al., 1997).

To test if the fusion proteins localize specifically to the plasma membrane, two-phase partitioning was performed on the membrane fractions (Figure 1D). Protein gel blot analysis revealed that PLL1-GFP was enriched in the upper fraction similar to the plasma membrane protein PMA2. The chloroplast, mitochondrial, and the endoplasmic reticulum membranes, on the other hand, remained in the lower fraction, showing that PLL1-GFP does not localize to these membranes.

To verify that POL and PLL1 undergo acylation, POL-FLAG protein, expressed in wheat germ extract, was purified and tested for acylation by liquid chromatography–tandem mass spectrometry (MS/MS) analysis. The N-terminal tryptic fragment MGNGTSR was detected as myristoyl-GNGTSR (801 D). Subsequent MS/MS analysis revealed that this species was indeed the myristoylated form of the N-terminal fragment (see Supplemental Figure 2 online). The adjacent tryptic fragment, VVGCFVPSNDK, which contains the predicted palmitoylation modification site, was not identified in an unmodified form. A mass species of 1400 D was observed though, which corresponds to the palmitoylated form of this tryptic fragment. However, this species was not subjected to MS/MS analysis so the assignment remains tentative.

The same constructs were also stably transformed into *Arabidopsis* Columbia *erecta-2* (Col *er-2*). While transgenic plants were recovered for all of the constructs, plants expressing POL fusion proteins were not identified (see Supplemental Table 1 online). This result is consistent with our previous observation that *Arabidopsis* POL overexpression lines cannot be readily recovered even when the native promoter is used (Song and Clark, 2005). As POL and PLL1 are also involved in the CLV3/WUS-related CLE40/WOX5 pathway, which regulates the root meristem, we used confocal microscopy to examine the

localization patterns of the fusion proteins in root tissues (Song et al., 2008; Stahl et al., 2009) (Figure 2). Furthermore, root tissues were examined because analyses of the *POL* and *PLL1* *cis*-regulatory elements have shown that these genes are normally expressed in the root meristem, and genetic studies have shown that the corresponding proteins function in these tissues (see Supplemental Figure 3 online) (Song and Clark, 2005; Song et al., 2008). We observed that PLL1-GFP localized to the cell periphery similar to the integral plasma membrane protein BRASSINOSTEROID INSENSITIVE1 (BRI1-GFP; Jin et al., 2007) (Figure 2). The signal intensity of PLL1-GFP was very low compared with the control GFP-tagged proteins, presumably due to cosuppression (see Methods). Furthermore, the localization of PLL1-GFP was distinctly different from that of the endoplasmic reticulum (ER) and Golgi membrane markers, ER-yk and G-yk (Nelson et al., 2007; Thole et al., 2008). These results again indicate that PLL1 localizes to the plasma membrane. PLL1myr$^m$-GFP and PLL1pal$^m$-GFP were also at the cell periphery but accumulated additional signals within the cells. These results are consistent with the tobacco fractionation data where

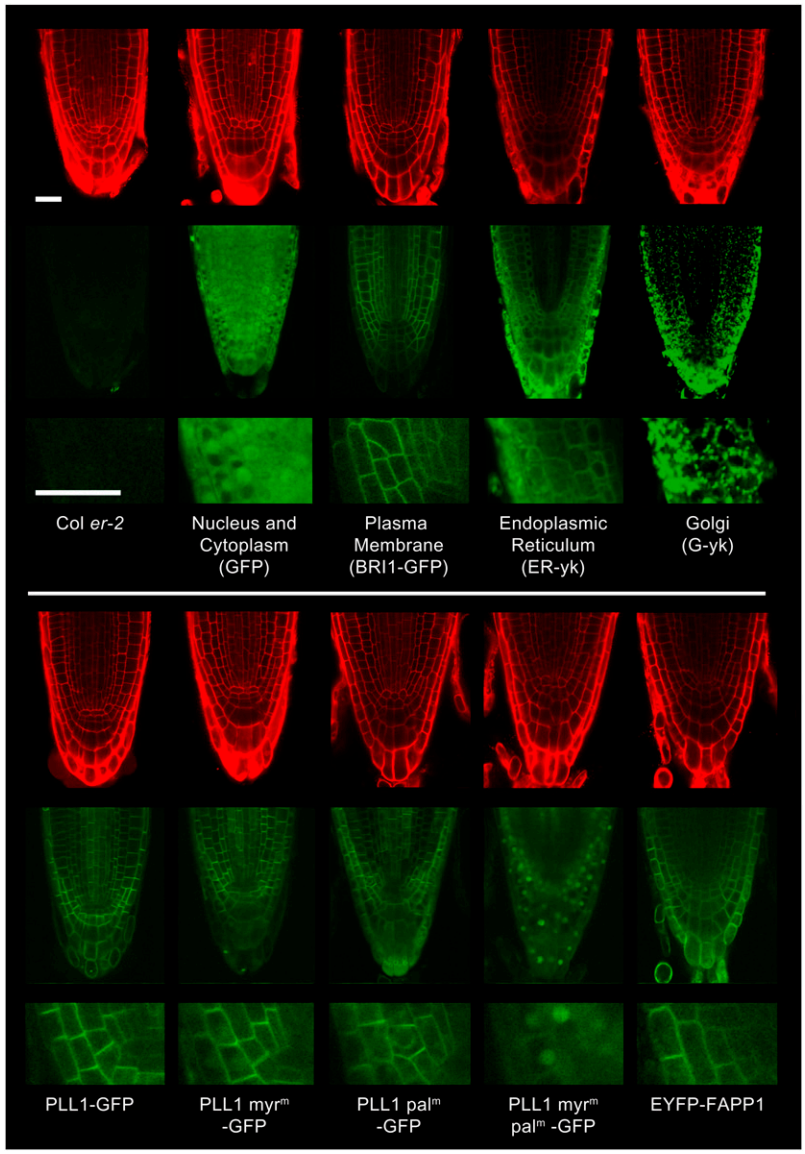


**Figure 2.** Myristoylation and Palmitoylation Are Required for Plasma Membrane Localization of PLL1 in *Arabidopsis.*

Images are of roots of 4-d-old transgenic *Arabidopsis* expressing various fusion proteins and controls. The top images show the propidium iodide–stained cell walls (red), while the middle images show the localization patterns of the GFP (or YFP) fusion protein (green). The bottom images are enlargements of the same regions (bottom left of the middle images) from each of the GFP (or YFP) images to more clearly show the localization patterns. Bars = 25 μm.

these versions of PLL1 were in both the soluble and membrane fractions. Finally, PLL1myr$^{m}$pal$^{m}$-GFP localized predominantly to the nucleus and cytoplasm, similar to GFP alone and again consistent with the tobacco fractionation data (Haseloff et al., 1997). This confocal data in combination with plasmolysis studies of these lines (Figure 3A) and the tobacco fractionation data (Figure 1) support that PLL1 localizes to the plasma membrane in a myristoylation- and palmitoylation-dependent manner in *Arabidopsis*.

It should be noted that some degree of asymmetric distribution of PLL1-GFP was observed within individual cells when the sensitivity of the confocal detector was reduced (Figure 3B). Under the same conditions, the BRI1-GFP control protein did not show irregular distribution within individual cells. Given the resolution of GFP imaging, it is difficult to determine the extent of PLL1-GFP asymmetry; however, it is clear that it does not uniformly accumulate across these cells like BRI1-GFP.

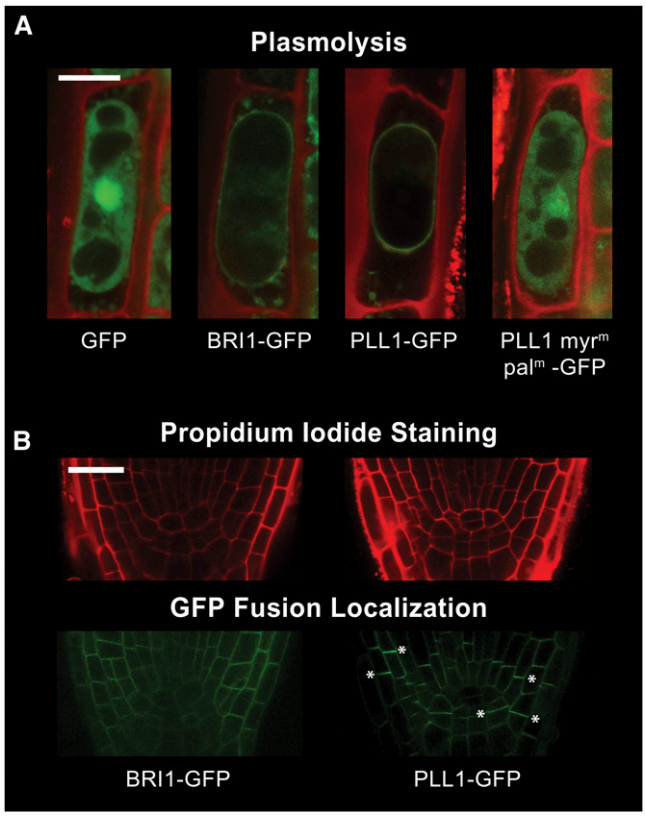


**Figure 3.** PLL1 Is Irregularly Distributed in the Plasma Membrane of *Arabidopsis* Root Cells.

**(A)** Plasmolysis of *Arabidopsis* root epidermal cells expressing various GFP fusion proteins. Images are an overlay of propidium iodide staining showing cell outlines in red with the localization of the GFP fusion proteins in green. Images were taken of 4-d-old seedlings following 2 h treatment with 0.8 M mannitol. Bar = 5 μm.

**(B)** Images of roots of 4-d-old transgenic *Arabidopsis* expressing BRI1-GFP or PLL1-GFP taken using decreased sensitivity of the photomultiplier detector tube. The top images are of propidium iodide staining, while the bottom images show localization of the GFP fusion proteins. A few of the cell faces showing increased accumulation of PLL1-GFP are marked with asterisks. Bar = 25 μm.

### Membrane Localization Is Required for Proper PLL1 Function

To test the importance of plasma membrane localization for proper POL/PLL1 function, we attempted to complement the *pol-6 clv3-2* double mutant with the various PLL1 fusion protein constructs. This double mutant combination was used because it is more sensitive to changes in POL/PLL1 protein levels than the wild type or the phenotypically wild-type *pol* and *pll1* single mutants (Yu et al., 2000, 2003; Song and Clark, 2005). Furthermore, this specific double mutant was used instead of *pll1-1 clv3-2* because POL and PLL1 can cross-complement and *pol-6* is a stronger suppressor of *clv3-2* than *pll1-1* (Song and Clark, 2005). For the PLL1-GFP construct, transgenic plants were recovered with enlarged and fasciated meristems (Figure 4A). These phenotypes are consistent with increased PLL1 activity. For the PLL1myr$^{m}$pal$^{m}$-GFP construct, plants were recovered that showed repeated meristem termination and/or produced their first few flowers without carpels and with decreased stamen number (Figure 4B). Additionally, many of the pedicels on these plants were extremely short. All of these phenotypes are consistent with PLL1myr$^{m}$pal$^{m}$-GFP having a dominant-negative effect.

To quantify the phenotypic differences in the overexpression lines, we analyzed the number of carpels per flower and the extent of the ectopic fifth whorls (Figure 4C). These measurements serve as sensitive indicators of changes in the number of stem cells (Clark et al., 1993, 1995; Dievart et al., 2003). Specifically, an increase in either the carpel count or the size of the fifth whorl is indicative of an increase in the number of stem cells, while a decrease in the carpel count or the size of the fifth whorl denotes a decrease in the number of stem cells. Using these measurements, it is clear that, compared with lines expressing GFP alone, the PLL1-GFP lines have an increase in the number of stem cells, while the PLL1myr$^{m}$pal$^{m}$-GFP lines have a decrease in the number of stem cells. Furthermore, when the fifth whorls are classified according to phenotype (Figures 4D and 4E), it is clear that flowers from PLL1-GFP lines have the largest and most developed fifth whorls, while most of the flowers from the PLL1myr$^{m}$pal$^{m}$-GFP lines have no fifth whorl at all. All of these data demonstrate that PLL1-GFP is functional, while PLL1myr$^{m}$-pal$^{m}$-GFP has a dominant-negative effect.

### POL and PLL1 Are Lipid Binding Proteins

The *Arabidopsis* and tobacco localization studies revealed that even when POL and PLL1 undergo only myristoylation (pal$^{m}$ mutants) or only palmitoylation (myr$^{m}$ mutants), a measurable portion of the proteins are still directed to the plasma membrane (Figures 1C and 2). It is only when both of the modifications are blocked that there is a dramatic change in the distribution of POL and PLL1. These results are inconsistent with the kinetic bilayer trapping hypothesis, which states that more than one acyl modification is required for stable interaction of a protein with the plasma membrane (Resh, 1999; Farazi et al., 2001). This

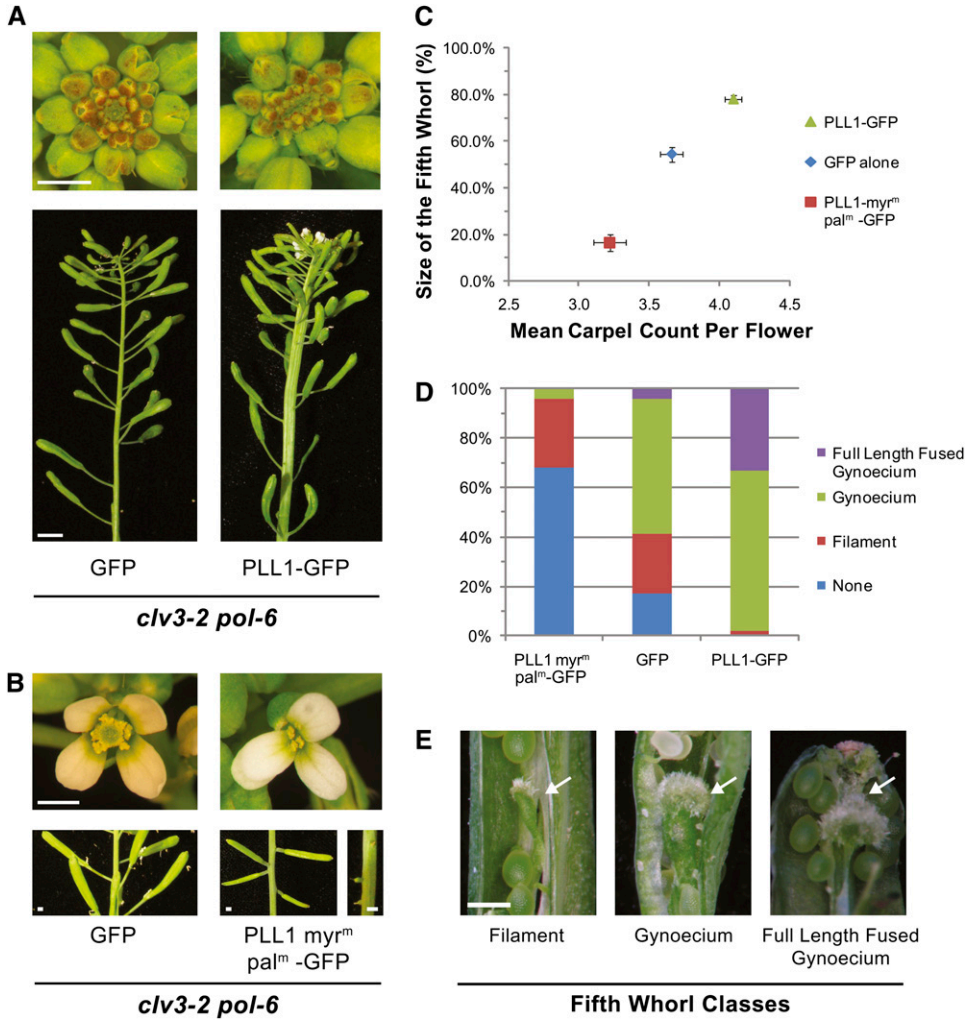


**Figure 4.** Plasma Membrane Localization Is Required for Proper PLL1 Function in Planta.

**(A)** and **(B)** Phenotypic analysis of *pol-6 clv3-2* double mutants transformed with GFP, PLL1-GFP, or PLL1-myr$^m$pal$^m$-GFP. Images are of the shoot meristems and primary inflorescences **(A)** (bars = 5 mm) and representative flowers, siliques, and pedicles **(B)** (bars = 1 mm) of T1 plants.

**(C)** Relative size of the fifth whorl and the mean number of carpels per flower for *clv3-2 pol-6* plants transformed with GFP, PLL1-GFP, or PLL1-myr$^m$pal$^m$-GFP ($n$ = 70, 50, and 50, respectively). Measurements and carpel counts are the average of the first 10 flowers from the primary inflorescences of 32-d-old T2 plants. The measured size of the fifth whorl is represented as a percentage of total silique length. The size of the silique is defined as the length between the attachment site for the sepals, petals, and stamens and the top of the gynoecium. The same definition was used to measure the fifth-whorl length. The vertical and horizontal bars represent SE of the mean for the relative fifth-whorl size and the number of carpels, respectively.

**(D)** Graph showing the phenotypic class distribution of the fifth whorls for the flowers analyzed in **(C)**.

**(E)** Representative images of the three different classes of fifth whorls (bar = 0.5 mm). Arrows are used to denote the fifth whorls.

requirement for two acyl groups for proper localization has been observed for a number of proteins, including the myristoylated and palmitoylated *Arabidopsis* calcineurin B–like protein, which is mislocalized if either acylation site is blocked (Resh, 1999; Farazi et al., 2001; Batistic et al., 2008). The fact that a large portion of the singly acylated POL and PLL1 still localizes to the plasma membrane suggests that there are other mechanisms allowing POL and PLL1 to stably interact with the plasma membrane.

The presence of another mechanism allowing POL and PLL1 to interact with the plasma membrane was also suggested when we found that hSOS-POL and hSOS-PLL1 fusion proteins that are missing both acylation sites autoactivate in the Stratagene CytoTrap yeast two-hybrid system. This system is similar to a traditional yeast two-hybrid system but is based at the plasma membrane. It has previously been shown that lipid binding by the bait protein can result in autoactivation in this system (Santagata et al., 2001). Perhaps POL and PLL1 autoactivate in this system

because they also bind lipids. Lipid binding by POL and PLL1 would also not be surprising as several plasma membrane proteins that only undergo myristoylation are also known to bind lipids to stabilize their membrane interaction (Resh, 1999).

To test if POL and PLL1 themselves contain functional lipid binding domains, POL-FLAG and PLL1-FLAG proteins, expressed in and purified from wheat germ extract, were used to probe lipid strips containing 22 different important lipids (Figure 5). These assays revealed that POL-FLAG and PLL1-FLAG bind phosphatidylinositol (3) phosphate [PI(3)P], phosphatidylinositol (4) phosphate [PI(4)P], phosphatidylinositol (5) phosphate [PI(5)P], phosphatidylserine (PS), and cardiolipin. As we have not identified any similarity between regions of POL or PLL1 and known phospholipid binding domains, these results suggest that POL and PLL1 contain a novel phospholipid binding domain.

Of the five lipids that bind POL and PLL1, cardiolipin is an unlikely candidate for interacting with POL and PLL1 in planta because it is found predominantly in the inner mitochondrial and thylakoid membranes, which are not where POL and PLL1 localize (Depalo et al., 2004; Nowicki et al., 2005) (Figure 1E). The phosphatidylinositol (PI) monophosphates [PI(3)P and PI(4)P specifically] and PS, on the other hand, are excellent candidates for interacting with POL and PLL1 in planta because these lipids are present, to varying degrees, in the inner leaf of plasma membrane (Uemura et al., 1995; Vance and Steenbergen, 2005; Skwarek and Boulianne, 2009; Vermeer et al., 2009). For a few of these lipids, in-depth studies have been performed to visualize how the lipids behave and are distributed in living plant cells. PI(3)P was shown in the root tip to localize mainly to small vesicles and the vacuolar membrane (Vermeer et al., 2006). PI(4)P was found in the root tip to localize predominantly to the plasma membrane (Vermeer et al., 2009). As this previously observed PI(4)P localization pattern strongly matches the distribution of PLL1-GFP in the root meristem, we performed confocal microscopy on plants expressing the same PI(4)P marker, enhanced yellow fluorescent protein (EYFP)-FAPP1, for direct comparison (Figure 2). This analysis confirms that PI(4)P does indeed localize in a pattern similar to PLL1-GFP and that it also displays some irregular distribution at the plasma membrane of individual cells.

These POL/PLL1-interacting lipids are known to play important roles in regulating cellular functions. PI(3)P is required for vesicle trafficking particularly between the trans-Golgi network and the vacuole (Kim et al., 2001). PI(4)P is involved in a number of processes, including regulating root hair polarity and trafficking between the trans-Golgi network and the plasma membrane (Vermeer et al., 2009). PI(5)P binds to and negatively regulates the *Arabidopsis* histone methyltransferase, *ARABIDOPSIS* TRITHORAX1 (ATX1), which results in an alteration of the expression of ATX1-controlled genes (Alvarez-Venegas et al., 2006). PS is a cofactor for a number of enzymes, such as protein kinase C, and can also activate various proteins, including the $Na^+/K^+$ ATPase (Vance and Steenbergen, 2005).

The existence of three mechanisms that allow POL and PLL1 to interact with the plasma membrane (myristoylation, palmitoylation, and lipid binding) when only two appear to be required for stable interaction suggests an additional role for one of these mechanisms (Figures 1, 2, and 5) (Resh, 1999; Farazi et al., 2001). As the lipids that interact with POL and PLL1 are known to regulate a variety of cellular processes and many of them have been shown to directly modulate the activity of an enzyme, one possibility is that the primary cellular function of lipid binding is to regulate POL and PLL1 catalytic activity.

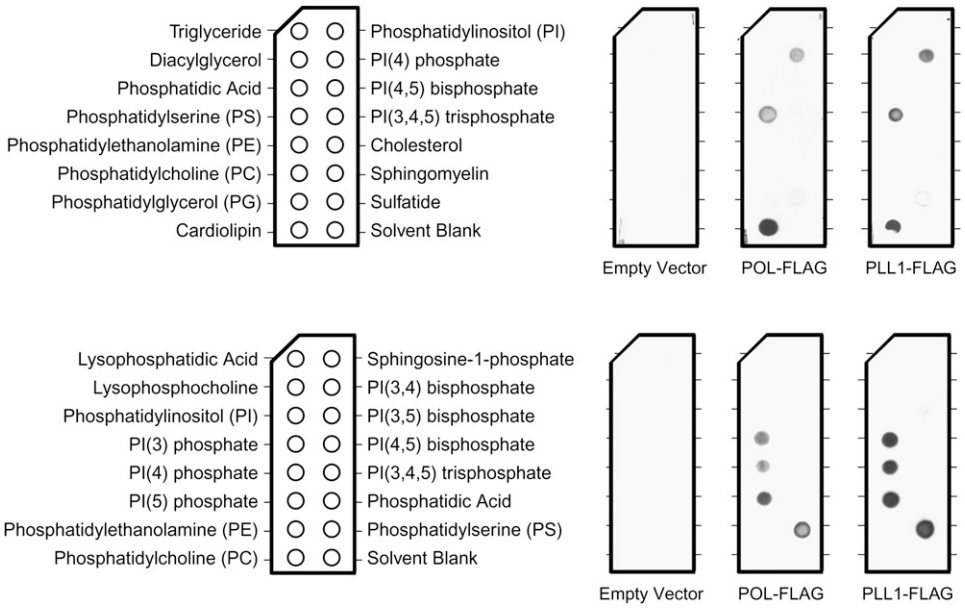


**Figure 5.** POL and PLL1 Bind Phospholipids.

POL-FLAG and PLL1-FLAG proteins were used to probe membranes spotted with the indicated lipids, and the fusion proteins were then detected with anti-FLAG antibody. Purified extract from an empty vector preparation was also tested as a negative control.

### Stimulation of POL Catalysis by PI(4)P

To study the effects of the various membrane lipids on POL and PLL1 activity, we developed a phosphatase assay system using purified MALTOSE BINDING PROTEIN (MBP)-POL fusion protein. Phosphatase assays revealed that inhibitors known to affect PP2C proteins (NaF and EDTA) strongly inhibit MBP-POL activity, while compounds known to inhibit other classes of phosphatases ($NaVO_3$, okadaic acid, and EGTA) have little to no effect on MBP-POL activity, which confirms that POL is a PP2C (Figure 6A) (Cohen, 1989).

To test the effects of the PI monophosphates and PS on POL activity, we added the water-soluble diC8 versions of these lipids directly to the phosphatase reactions using ratios of protein to

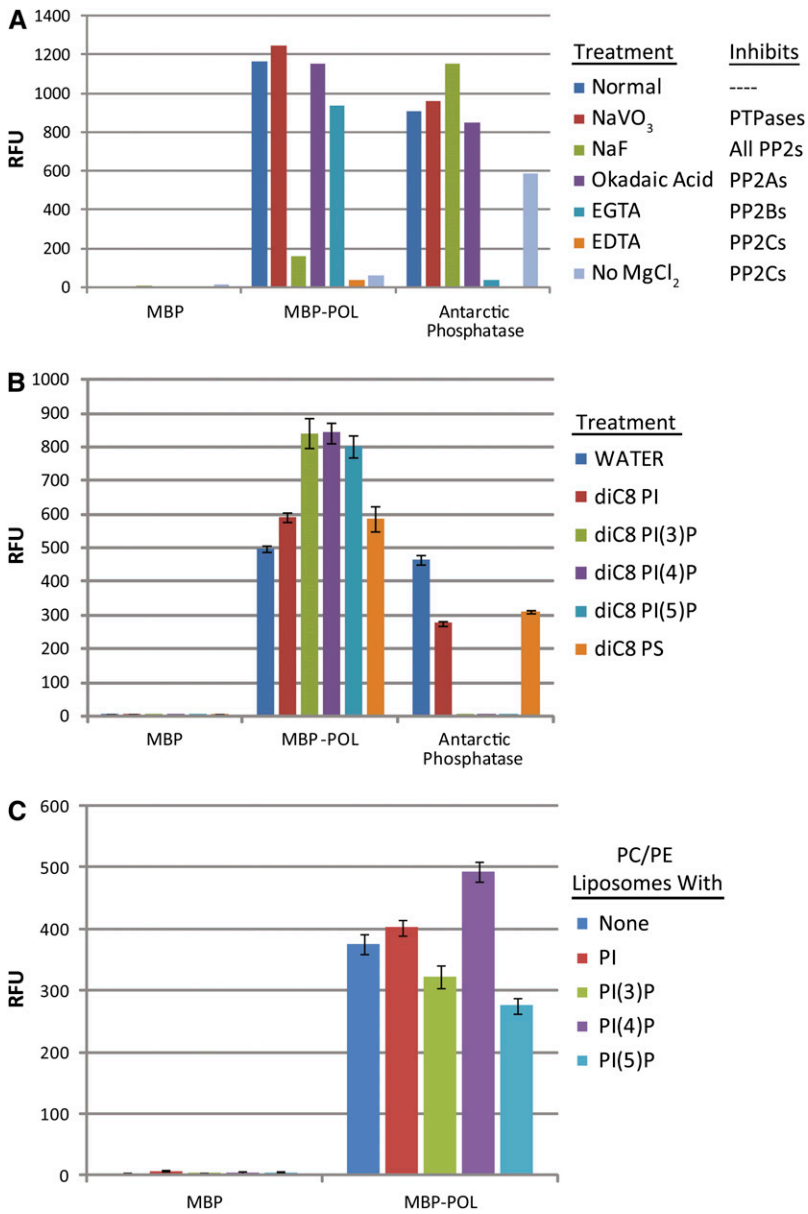


**Figure 6.** POL Activity Is Stimulated by PI(4)P.

**(A)** MBP-POL phosphatase activity is specifically inhibited by PP2C phosphatase inhibitors and not by inhibitors that affect other classes of phosphatases. MBP and antarctic phosphatase are included as controls.

**(B)** The effects of various water-soluble diC8 lipids on MBP-POL phosphatase activity. Antarctic phosphatase and MBP are included as controls. Data represent the average of three replicates, and the SD is shown.

**(C)** The effects of PC/PE liposomes, containing no additional lipids, 5% PI, 5% PI(3)P, 5% PI(4)P, or 5% PI(5)P on MBP-POL phosphatase activity. Data represent the average of three replicates, and the SD is shown.

phospholipid similar to those used to show activation of the insulin receptor kinase by PI (Figure 6B) (Sweet et al., 1987). We found that PI(3)P, PI(4)P, and PI(5)P all stimulated POL phosphatase activity, while PI and PS had no effect. Furthermore, the presence of the lipids had no effect on the control MBP reactions, and all of the PI monophosphates had a negative effect on the activity of the control antarctic phosphatase. In a separate experiment when the inhibitor EDTA was added to MBP-POL reactions, with or without PI(4)P, the phosphatase activity for all of the reactions was consistently reduced by 70%, indicating that the PI monophosphates had specifically stimulated POL PP2C activity (see Supplemental Figure 4 online).

To more closely mimic the environment present at the plasma membrane, the MBP-POL phosphatase reactions were also performed in the presence of liposomes composed predominantly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Echelon Biosciences). In these experiments, PC/PE liposomes were used that were supplemented with either no additional lipids or 5% of PI, PI(3)P, PI(4)P, or PI(5)P. Here, where the effect of these lipids on MBP-POL was tested in an environment more similar to what is found in planta, only PI(4)P was found to activate MBP-POL activity (Figure 6C). Furthermore, under these conditions, PI(3)P and PI(5)P had a mild inhibitory effect on MBP-POL activity. These results suggest that a membrane environment is critical for proper regulation of the POL/PLL1 by phospholipids.

## DISCUSSION

Previous studies have revealed that the POL/WOX signaling pathways are vital for proper regulation of shoot and root stem cell populations in *Arabidopsis* (Yu et al., 2000, 2003; Song and Clark, 2005; Song et al., 2006, 2008; Gagne and Clark, 2007; Gagne et al., 2008). Unfortunately, only a few components of these pathways are known, and little is understood about how their signaling is transduced in planta. Specifically, POL and PLL1 are the only proteins identified to date that transduce the signal between the pathway receptors at the plasma membrane and the *cis*-regulatory elements of the target *WOX* genes. This study advances our understanding of where and how this signaling is regulated. Specifically, we provided evidence that POL and PLL1 are dual-acylated plasma membrane proteins whose membrane localization is required for proper function and that POL and PLL1 bind to and POL is activated by PI(4)P.

First, we have shown that POL and PLL1 localize to the plasma membrane, bringing them in close proximity to the receptors that regulate their activity. This suggests that the negative regulation of POL/PLL1 occurs at the plasma membrane, although it should be noted that to date there is no evidence supporting direct interaction between POL/PLL1 and the upstream receptors. Furthermore, we have shown that POL/PLL1 localization is dependent on myristoylation and palmitoylation target sequences. Interestingly, a recent study of the *Arabidopsis* *N*-myristoyltransferase (NMT) genes revealed that mutants and

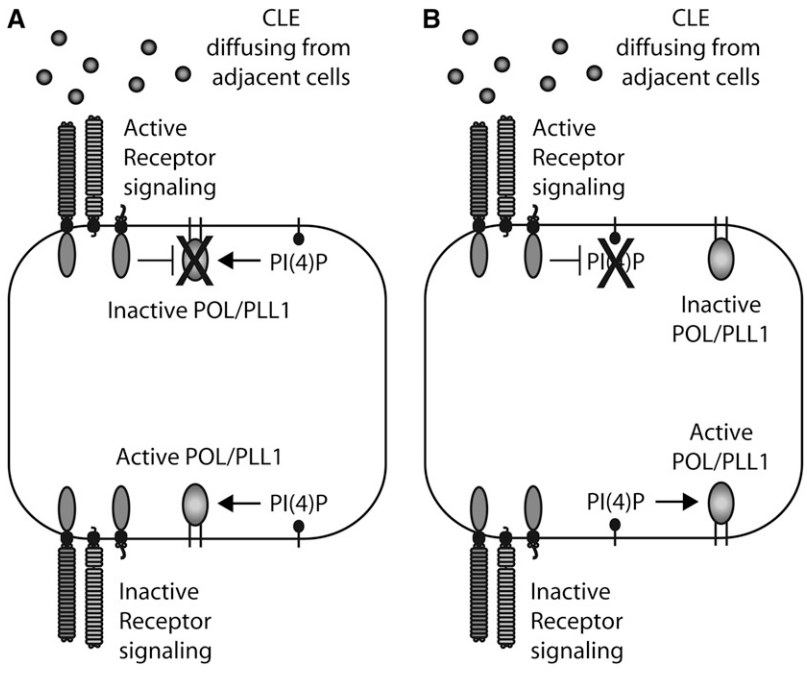


**Figure 7.** Two Potential Models for How CLE Signaling and PI(4)P Binding Regulate POL and PLL1 Activity in Planta.

**(A)** In the first model, the two regulatory signals work antagonistically to set up a polar distribution of POL and PLL1 activity within the cell prior to cell division.

**(B)** In the second model, CLE signaling negatively regulates PI(4)P levels on the apical side of the cell leading to a polar distribution of POL and PLL1 activity.

overexpressors of *NMT1* and *NMT2* have defects in the initiation and/or maintenance of the root, shoot, and floral meristems (Pierre et al., 2007). These *NMT1/NMT2* defects may in part be due to changes in the plasma membrane localization of POL and PLL1. Consistent with this is our result that plants expressing nonacylated PLL1myr$^{m}$pal$^{m}$-GFP have defects in the maintenance of the shoot and flower meristems consistent with the meristem defects seen in the myristoylation-defective *nmt1 nmt2* mutants.

Next, we found that POL and PLL1 bind to the same set of membrane phospholipids. Among the lipids POL and PLL1 bound in vitro, we consider cardiolipin an unlikely candidate for in vivo interaction with POL/PLL1 because it is found and functions within the chloroplasts and mitochondria, while POL/PLL1 localize to the plasma membrane (Nowicki et al., 2005; Wada and Murata, 2007). The other lipids, PS and the PI monophophates, on the other hand, are good candidates for interaction with POL and PLL1 in vivo as most of them are present in the inner leaflet of the plasma membrane (Uemura et al., 1995; Vance and Steenbergen, 2005; Skwarek and Boulianne, 2009; Vermeer et al., 2009).

Interestingly, these POL/PLL1 interacting lipids are all known to play important regulatory functions, including modulating enzyme activity. Thus, we hypothesized that one or more of these lipids might regulate POL/PLL1 activity in planta. To explore this hypothesis, we tested the effects of lipids on POL phosphatase activity. While adding the water-soluble diC8 forms of all three PI monophosphates to the reactions increased POL activity, only PI(4)P activated POL when liposomes were used in the reactions. As a result, we consider PI(4)P to be the most likely candidate to positively regulate POL/PLL1 activity in vivo, but we cannot exclude that under other specific membrane conditions POL would also be activated or inhibited by PI(3)P or PI(5)P. It is interesting to note that the activities of other PP2Cs are also regulated by cellular compounds, such as phosphatidic acid, $H_2O_2$, and polyunsaturated fatty acids (Klumpp et al., 1998; Baudouin et al., 1999; Meinhard et al., 2002; Zhang et al., 2004).

The regulation of PI(4)P levels in planta is poorly understood. Most PI(4)P is formed by phosphorylation of PI by phosphatidylinositol 4-OH kinase. Unfortunately, little is known about the specificity and localization of the majority of the 12 predicted *Arabidopsis* PI(4) kinases (Mueller-Roeber and Pical, 2002; Szumlanski and Nielsen, 2009b). Only two, PI(4)K$\alpha$1 and PI(4)K$\beta$1, have been shown to have lipid kinase activity in vitro, while two others, PI(4)K$\gamma$4 and PI(4)K$\gamma$7, have been shown instead to have in vitro protein kinase activity (Stevenson et al., 1998; Xue et al., 1999; Galvao et al., 2008). Furthermore, extensive phenotypic analysis has only been performed for two of the PI(4) kinases, PI(4)K$\beta$1 and PI(4)K$\beta$2, which function in polarized membrane trafficking in tip growing cells (Preuss et al., 2006; Szumlanski and Nielsen, 2009a).

PI(4)P levels can also be controlled by PI(4)P phosphatases. Currently, nine putative PI phosphatases have been identified in *Arabidopsis* by sequence homology (Zhong and Ye, 2003; Szumlanski and Nielsen, 2009b). Three of these, SAC6, SAC7, and SAC8, show the closest similarity to yeast PI(4)P phosphatase, and all three are able to complement the yeast mutant, suggesting they are the PI phosphatases that use PI(4)P as a substrate (Despres et al., 2003; Zhong and Ye, 2003; Szumlanski and Nielsen, 2009b). Of these three, only SAC7 (RHD4) has been studied in detail, and it has been shown to regulate root hair tip growth (Thole et al., 2008). Another way PI(4)P levels can be regulated is by conversion between PI(4)P and PI(3,4)$P_2$ or PI(4,5)$P_2$; however, little is known about these methods of regulation. Various studies in *Arabidopsis* have identified at least three putative PI(3)P phosphatases and no PI(3) kinases that may use or generate PI(3,4)$P_2$ (Gillaspy, 2009; Lee et al., 2009). Sequence analyses have also led to the identification of 16 putative PI(5)P phosphatases and four putative PI(5) kinases that may use or generate PI(4,5)$P_2$ (Mueller-Roeber and Pical, 2002; Zhong et al., 2004; Gillaspy, 2009). Additionally, there are other potential ways that PI(4)P levels could be modulated, including lipid hydrolysis and lipid transfer between membranes or membrane bilayers, but again little is known about these processes (Thole and Nielsen, 2008). Finally, understanding how PI(4)P is regulated is further complicated by the presence of different pools of PI(4)P within individual cells, such as the pools in the Golgi or plasma membrane. Given the limited knowledge of the enzymes that regulate PI(4)P levels, which cell types those enzymes function in and which intracellular pools of PI(4)P they act upon, extensive further study is needed to determine how PI(4)P levels might be manipulated to regulate POL/PLL1 activity.

It is interesting to note that recent literature suggests PI(4)P plays a broader and more active role in signaling in plants than it does in animals (Vermeer et al., 2009). In eukaryotes, PI(4)P is traditionally known for its roles in Golgi trafficking and as precursor for the key signaling lipid PI(4,5)$P_2$ (Skwarek and Boulianne, 2009; Vermeer et al., 2009). Recent studies have shown that PI(4)P performs additional roles in plants, including functioning to activate enzymes and regulate polarized root hair growth (Preuss et al., 2006; Meneghelli et al., 2008; Thole and Nielsen, 2008; Thole et al., 2008; Vermeer et al., 2009). Also, plants accumulate a much higher ratio of PI(4)P to PI(4,5)$P_2$ than animals, probably due in part to the product-limited activity observed for plant PI phosphate 5-kinases (Meijer et al., 2001; Meijer and Munnik, 2003; Perera et al., 2005; Im et al., 2007). Thus, as PI(4)P is the major phosphatidylinositol derivative observed in plants and as it appears to have additional functions in planta, it may serve a predominant role in plant intracellular signaling in the place of PI(4,5)$P_2$.

Based on the results presented here, we developed two new models for POL/PLL1 regulation in vivo (Figure 7). In the first model, receptor-mediated CLE signaling and PI(4)P act antagonistically on POL/PLL1 activity (Figure 7A). Specifically, POL and PLL1 are active in cellular domains where PI(4)P is present, and there is an absence of receptor-mediated CLE signaling. In domains where receptor-mediated CLE signaling is present, this signal overrides any PI(4)P stimulation of POL/PLL1 and the phosphatases are inactive. As the CLE ligand is produced in a polar manner relative to the cell of interest, there is a CLE distribution gradient across the cell surface. This gradient then results in an internal gradient of POL/PLL1 activity prior to cell division. Thus, upon cell division, one daughter cell has inactive POL/PLL1, while the other daughter cell has active POL/PLL, and these differences drive the cells to different fates (e.g., stem cell versus differentiated cell). In the second model,

receptor-mediated CLE signaling negatively regulates PI(4)P levels and/or availability (Figure 7B). Here, the polar-generated CLE ligand results in PI(4)P only being available to activate POL/PLL1 on one side of the cell. Thus, upon cell division, one daughter cell has inactive POL/PLL1, while the other daughter cell has active POL/PLL1, which again drives the cells to different cellular fates. It is important to note that in both of these models, directional CLE signaling results in intracellular polarization of POL/PLL1 activity. Once set up, this intracellular polarization is maintained during cell division because the intracellular factors involved, PI(4)P and/or POL/PLL1, are in or are bound to the plasma membrane and not diffusing through the cytosol. It should be noted that these models could also be readily adapted to account for the possibility that POL and PLL1 act after cell division by simply hypothesizing that the activities of POL and PLL1 are differentially regulated in the two cell daughters through a gradient of CLE signal.

On a final note, as PI(4)P is involved in trafficking between the trans-Golgi network and the plasma membrane, another possible model would be that PI(4)P and POL/PLL1 are involved in negatively regulating the trafficking of CLV/WOX pathway components to the plasma membrane (Vermeer et al., 2009). This possibility clearly does not apply to the trafficking of CLV1 or CLV2, based on the observation that *pol pll1* mutants are fully epistatic to *clv* mutants, the opposite of what the trafficking model would suggest (Song et al., 2008). Furthermore, PI(4)P itself has been shown to have nontrafficking functions, including activating the *Arabidopsis* plasma membrane $Ca^{2+}$-ATPase (Meneghelli et al., 2008).

The results presented have expanded our knowledge on how POL and PLL1 are regulated in planta by showing that these two phosphatases are plasma membrane localized through acylation. This work has also provided evidence of a role for phospholipids, tentatively PI(4)P, in the POL/PLL1-dependent CLE/WOX signal transduction pathways. Further studies are needed to better understand the precise roles of PI(4)P in regulating these key developmental pathways. These results also contribute to recent literature that has revealed phosphatidylinositol phosphates as key developmental regulators in multicellular organisms (Skwarek and Boulianne, 2009).

## METHODS

### Plant Materials and Growth Conditions

Unless otherwise specified, *Arabidopsis thaliana* and tobacco (*Nicotiana benthamiana*) were grown at 21°C under 24 h light after 4 d of stratification in water at 4°C. Plants in pots were grown on a mixture of two parts Metro-Mix 360 (Sun Gro), one part vermiculite, and one part perlite supplemented with Osmocote (Scotts). Plants in Petri dishes were grown on 0.5× Murashige and Skoog basal salt mixture (Sigma-Aldrich) containing 2% sucrose and 0.7% agar under a long-day photoperiod. Col *er-2* and *pol-6 clv3-2* are as described (Prigge et al., 2005; Song and Clark, 2005). BRI1-GFP–expressing plants are as described (Jin et al., 2007). Plants transformed with EYFP-FAPP1, ER-yk, and G-yk were obtained from Erik Nielsen (Nelson et al., 2007; Thole et al., 2008). Tobacco seeds were obtained from David Somers. The $P_{CPC}$*:GUS* (for β-glucuronidase), $P_{POL}$*:GUS*, and $P_{PLL1}$*:GUS* lines are as described (Wada et al., 2002; Song and Clark, 2005).

### Transgenic *Arabidopsis*, Infiltrated Tobacco, and Microscopy

The full-length and mutant versions of *POL* and *PLL1* used in the GFP fusion constructs were amplified by PCR. *POL* inserts were generated using the following 5′ primers: 5′ POL, 5′ POLmyr$^m$, 5′ POLpal$^m$, and 5′ POLmyr$^m$pal$^m$ in combination with the 3′ POL primer (see Supplemental Table 2 online). *PLL1* inserts were generated using the following 5′ primers: 5′ PLL1, 5′ PLL1myr$^m$, 5′ PLL1pal$^m$, and 5′ PLL1myr$^m$pal$^m$ in combination with the 3′ PLL1 primer (see Supplemental Table 2 online). Using BP Clonase (Invitrogen), the PCR products were recombined into pDONR207 (Invitrogen). The resulting pDONR207 constructs were then recombined with pMDC83 (Curtis and Grossniklaus, 2003) using LR Clonase (Invitrogen). To generate the non-Gateway *GFP* alone version of pMDC83, the plasmid was digested with *Kpn*I and then ligated shut to remove the Gateway cassette.

For transient expression in tobacco, these constructs and the *P19* construct (obtained from David Somers) were transformed into *Agrobacterium tumefaciens* strain GV3101. Each experimental construct was then coinfiltrated with the *P19* construct into tobacco as described (Voinnet et al., 2003) with minor modifications, including transforming each construct at a final $OD_{600}$ of 0.5. The control CLV1-GFP construct was obtained from Y.F. Guo. To generate this construct, *CLV1-FLAG* was digested out of $P_{ER}$*:CLV1-FLAG* using *Bam*HI and *Sal*I (New England Biolabs) and then cloned into pCHF1 (DeYoung et al., 2006). Then, *mGFP5* was PCR amplified with engineered *Spe*I and *Sal*I sites and cloned into the $P_{35S}$*:CLV1-FLAG* cassette replacing the FLAG sequences.

For the *Arabidopsis* transformations, constructs were transformed into either the *Agrobacterium* strain AGL1 or the strain GV3101, which were then used to transform *Arabidopsis* Col *er-2* and *pol-6 clv3-2* as described (Clough and Bent, 1998). Transgenic plants were isolated and the *Agrobacterium* was killed by selection on plates containing 20 μg/mL hygromycin and 100 μg/mL timentin. Protein gel blot analysis, using rabbit anti-GFP (Torrey Pines Biolabs) and goat anti-rabbit (Biorad) antibodies, was performed to identify the best-expressing lines. Multiple lines were obtained for each construct.

All confocal microscopy was performed with a TCS SP5 DM6000B confocal microscope using an HCX PL APO CS 100.0x1.40 OIL lens and LAS AF software (Leica Microsystems). GFP (or YFP) and propidium iodine signals were observed simultaneously on separate channels. The GFP (or YFP) signal was excited using the argon 488-nm laser line and observed using a bandwidth for PMT2 of 490 to 550 nm. The propidium iodide signal was excited using the DPSS 561-nm laser line and observed using a bandwidth for PMT3 of 598 to 656 nm. Confocal images were obtained from transgenic plants and controls grown at 30°C to reduce silencing (J. Li, personal communication). Prior to imaging, roots were stained for 30 s in 10 μg/mL propidium iodide in water. For the plasmolysis experiments, the roots were treated with 0.8 M mannitol for 2 h with shaking prior to staining with 10 μg/mL propidium iodide in 0.8 M mannitol. GUS staining was performed, as described (Masucci et al., 1996), on 4-d-old seedlings grown at 30°C. Phenotypic quantification was performed using a light microscope and Vernier calipers.

### Fractionation Analysis

Two days after infiltration, protein was extracted from tobacco leaves by grinding samples, using a mortar and pestle on ice, in 2 mL of extraction buffer (50 mM Tris HCl 8.0, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 10 mM NaF, 10 mM $NaVO_3$, 2% protease inhibitor cocktail [P9599; Sigma-Aldrich], 10 μg/mL chymostatin, and 2 μg/mL aprotinin) per gram of fresh weight. Cellular debris were removed by two 10-min, 3300*g* spins at 4°C. Membrane and soluble fractions were isolated by centrifugation at 100,000*g* for 1 h at 4°C. Protein gel blot analysis was performed using rabbit anti-GFP (Torrey Pines Biolabs) and goat anti-rabbit (Bio-Rad)

antibodies. Blots were visualized using SuperSignal West Pico and Femto Chemiluminescent substrates (Pierce).

Two-phase partitioning was performed as described (Marmagne et al., 2006) with the following modifications. Membrane fractions were isolated as described above and then resuspended in 1 mL of microsome resuspension buffer for every 2.5 g of original fresh weight. The microsome resuspension buffer was supplemented with 10 mM EDTA, 10 mM NaF, 10 mM $NaVO_3$, 2% protease inhibitor cocktail (P9599; Sigma-Aldrich), 10 μg/mL chymostatin, and 2 μg/mL aprotinin to prevent degradation. The two-phase partitioning process was scaled down 50-fold. Equal amounts of total protein (9 μg) from the plasma membrane–enriched upper phase and the plasma membrane depleted lower phase were used in gel blot analysis with rabbit anti-GFP (Torrey Pines Biolabs), rabbit anti-PMA2 (Morsomme et al., 1998), mouse anti-BiP (SPA-818; Stressgen), mouse anti-VDAC1 (PM035; Tom Elthon), goat anti-rabbit (Bio-Rad), and goat anti-mouse (Bio-Rad) antibodies. Chlorophyll extraction and quantification were performed as described (Guo and Gan, 2006).

### Recombinant Proteins

To generate the constructs for the C-terminal FLAG-tagged fusion proteins, *POL* and *PLL1* were amplified by two rounds of PCR to add the FLAG tag. The following primers were used for the first round of PCR: for the *POL* construct, 5′ POL FLAG with 3′ POL FLAG, and for the *PLL1* construct, 5′ PLL1 FLAG with 3′ PLL1 FLAG (see Supplemental Table 2 online). The second round of PCR used the first-round product and the 5′ gene-specific primers with the 3′ second-round primer (see Supplemental Table 2 online). PCR products were then cloned into the *Sal*I restriction site in the pSP64 plasmid (Promega). The fusion proteins were generated with the TNT SP6 high-yield protein expression system (Promega) using 5 μg of plasmid per reaction. The proteins were purified using anti-FLAG M2 Affinity Gel (Sigma-Aldrich) and 3X FLAG peptide (Sigma-Aldrich) using the Immunoprecipitation of FLAG Fusion Proteins Protocol with the following modifications: the reaction volume–to–bead volume ratio was 2:1, and the 3X FLAG peptide was used at a final concentration of 150 μg/mL. Protein concentrations were determined using BSA standards. FLAG-tagged proteins were kept at room temperature for all steps of the purification process and were used fresh due to precipitation issues when the proteins were frozen or placed at 4°C. For the liquid chromatography–MS/MS experiment, purified POL-FLAG protein was digested with trypsin and analyzed at the Michigan Proteome Consortium (Ann Arbor, MI).

pMALC2 (New England Biolabs) and MBP-POL constructs were as described (Yu et al., 2003). All constructs were transformed into Rosetta 2(DE3) (Novagen) and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Purification of the fusion proteins was as described (Yu et al., 2003). Protein concentrations were determined using BSA standards.

### Lipid Binding

Hydrophobic membranes prespotted with membrane lipids were obtained from Echelon Biosciences. Interaction was tested using the standard Echelon protocol with the following modifications. All blocking and washing steps contained 3% fatty acid–free BSA (Sigma-Aldrich) instead of nonfat dry milk. The membranes were incubated with 0.38 μg/mL recombinant protein or the equivalent amount of elution from the empty vector reaction purification. The primary antibody used was a mouse anti-FLAG M2 antibody (Stratagene), and the secondary antibody was a goat anti-mouse horseradish peroxidase (Bio-Rad). All steps were performed at 22°C.

### Phosphatase Assays

Protein phosphatase assays were performed using the recombinant proteins at a concentration of 80 nM, unless otherwise noted, and the ProFluor Ser/Thr PPase assay (Promega). The positive control, antarctic phosphatase (New England Biolabs), was used at 0.01 μL per reaction. Phosphatase reactions were run for 2 h before termination and quantification. Measurements were taken using a SpectraMax M2 microplate reader (Molecular Devices) and 96-well opaque plates (Corning). Unless noted as raw data (see Supplemental Figure 5 online), the phosphatase results shown were normalized using the AMC control substrate data. The effects of various inhibitors were tested by adding the inhibitors at the following final concentrations: 50 μM $NaVO_3$, 20 mM NaF, 1 mM EGTA, 10 mM EDTA, or 10 μM okadaic acid (Sigma-Aldrich) (Cohen, 1989). The effects of diC8 lipids (Echelon Biosciences and Avanti Polar Lipids) on POL phosphatase activity were tested by adding the lipids, resuspended in water at 1 mM, to the reactions at a final concentration of 300 μM. The relative ratios of protein to phospholipid used for the diC8 experiment were similar to those used in a study showing activation of the insulin receptor kinase by PI (Sweet et al., 1987). The effects of liposomes were tested by adding various freshly made PolyPIPosomes (Echelon Biosciences) to the reactions at a final concentration, for the experimental lipid, of 10 μM. For the liposome reactions, the recombinant proteins were used at 40 nM.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: POL (At2g46920), PLL1 (At2g35350), CLV1 (At1g75820), CLV3 (At2g27250), PMA2 (At4g30190), BIP2(At5g42020), VDAC1 (At3g01280), and BRI1(At4g39400).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Summary of the Current Knowledge of the CLE Pathways Signaling at the Shoot and Root Meristems.

**Supplemental Figure 2.** LC/MS/MS Analysis of Trypsinized POL-FLAG Protein Shows Evidence of Myristoylation.

**Supplemental Figure 3.** *POL* and *PLL1* Are Expressed in the *Arabidopsis* Root.

**Supplemental Figure 4.** EDTA Can Inhibit POL Stimulation by PI(4)P.

**Supplemental Figure 5.** Raw Data for diC8 and Liposome Activation Experiments.

**Supplemental Table 1.** Summary of Data from Anti-GFP Immunoblots Done to Identify Transgenic Lines in the Col *er*-2 Background Expressing the Various GFP Fusion Proteins.

**Supplemental Table 2.** Oligonucleotide Primers Used in This Study.

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