Phosphoinositides Regulate Clathrin-Dependent Endocytosis at the Tip of Pollen Tubes in *Arabidopsis* and Tobacco

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Using the tip-growing pollen tube of *Arabidopsis thaliana* and *Nicotiana tabacum* as a model to investigate endocytosis mechanisms, we show that phosphatidylinositol-4-phosphate 5-kinase 6 (PIP5K6) regulates clathrin-dependent endocytosis in pollen tubes. Green fluorescent protein–tagged PIP5K6 was preferentially localized to the subapical plasma membrane (PM) in pollen tubes where it apparently converts phosphatidylinositol 4-phosphate (PI4P) to phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2]. RNA interference–induced suppression of PIP5K6 expression impaired tip growth and inhibited clathrin-dependent endocytosis in pollen tubes. By contrast, PIP5K6 overexpression induced massive aggregation of the PM in pollen tube tips. This PM abnormality was apparently due to excessive clathrin-dependent membrane invagination because this defect was suppressed by the expression of a dominant-negative mutant of clathrin heavy chain. These results support a role for PI(4,5)P2 in promoting early stages of clathrin-dependent endocytosis (i.e., membrane invagination). Interestingly, the PIP5K6 overexpression-induced PM abnormality was partially suppressed not only by the overexpression of PIP2, which breaks down PI(4,5)P2, but also by that of PI4KII1, which increases the pool of PI4P. Based on these observations, we propose that a proper balance between PI4P and PI(4,5)P2 is required for clathrin-dependent endocytosis in the tip of pollen tubes.

INTRODUCTION

Endocytosis internalizes extracellular materials and retrieves excessive components from the plasma membrane (PM), which can then be either degraded in lysosomes/vacuoles or recycled back to the PM. An increasing number of fundamental cellular processes, such as cell polarity establishment, cytokinesis, polarized cell growth, and cellular signaling, have been shown to require endocytosis. In plant cells, endocytosis creates the polar localization of PIN auxin efflux proteins (Dhonuksh et al., 2008; Kleine-Vehn and Friml, 2008; Yang, 2008), enhances brassinosteroid signaling (Geldner et al., 2007), and appears to regulate polarized cell growth (Helling et al., 2006; Moscatelli et al., 2007; Zonia and Munnik, 2008). Multiple pathways of endocytosis are known, but clathrin-dependent endocytosis is most extensively studied in yeast and in animal cells (Conner and Schmid, 2003; Mousavi et al., 2004). Clathrin-dependent endocytosis involves several stages, including assembly of clathrin and its adaptor proteins (such as AP-2 complex and AP-3) onto the inner leaflet of the PM to form coated pits, invagination of coated pits, dynamin-mediated pinching of coated vesicles, and uncoating of clathrin from endocytic vesicles (Conner and Schmid, 2003; Mousavi et al., 2004). Evidence suggests that phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] localized in the inner leaflet of the PM promotes the formation and invagination of coated pits by binding to the AP-2 complex, AP-3, Dab2, and Epsin in yeast and animal cells (Mousavi et al., 2004; DiPaolo and De Camilli, 2006). Acute depletion of PI(4,5)P2 caused loss of coated pits in mammalian COS-7 cells (Zoncu et al., 2007). PI(4,5)P2 may also be important for membrane fission, as it also binds dynamin. Several recent studies show that PIP phosphatase-dependent metabolism of PI(4,5)P2 is required for late stages of endocytosis in mammalian and yeast cells (Sun et al., 2005; Perera et al., 2006; Sun et al., 2007).
In contrast with yeast and mammalian cells, the molecular mechanism for clathrin-dependent endocytosis in plant cells remains poorly characterized, although the existence of clathrin-dependent endocytosis has been well documented and implicated in the polar localization of PI4P proteins to the PM and polarized cell growth in plants. As a single cell system, pollen tubes are particularly well suited for investigating the mechanism for endocytosis and its role in polarized cell growth (Cheung and Wu, 2008; Lee and Yang, 2008; Yalovsky et al., 2008). Pollen tubes rapidly expand via tip growth that is dependent upon massive tip-targeted exocytosis. Thus, it is conceivable that tip-localized endocytosis must be coordinated with exocytosis to regulate rapid tip growth. Indeed, evidence suggests that clathrin is preferentially present at the subapical PM of pollen tubes and that disruption of clathrin-dependent endocytosis in tobacco (Nicotiana tabacum) pollen tubes causes growth inhibition (Derksen et al., 1995; Blackbourn and Jackson, 1996; Moscatelli et al., 2007). Signaling mechanisms regulating polarized pollen tube growth, including tip-localized ROP GTPases (Lin et al., 1996; Kost et al., 1999; Li et al., 1999; Fu et al., 2001; Gu et al., 2005; Hwang et al., 2008; Lee et al., 2008), calcium gradients (Holdaway-Clarke et al., 1997), calcium-dependent protein kinases (Yoon et al., 2006; Myers et al., 2009), F-actin dynamics (Fu et al., 2001; Vidali et al., 2001), Rab GTPases (de Graaf et al., 2005), and PI(4,5)P2 (Dowd et al., 2006; Helling et al., 2006), have been extensively studied. However, the mechanisms regulating clathrin-dependent endocytosis in pollen tubes are yet to be characterized.

PI(4,5)P2 has been reported to localize to the apical PM of pollen tubes and root hairs using a green fluorescent protein (GFP)-tagged pleckstrin-homology (PH) domain from phospholipase C (PLC), which is an in vivo PI(4,5)P2 marker and has been implicated in the regulation of tip growth (Kost et al., 1999; Dowd et al., 2006; Helling et al., 2006). Recent studies suggest that the PM accumulation of PI(4,5)P2 in pollen tubes may require one or more phosphatidylinositol-4-phosphate 5-kinase (PIP5K), which phosphorylates the D-5 position of the inositol ring of phosphatidylinositol-4-phosphate (PI4P). The Arabidopsis thaliana genome encodes 11 PIP5Ks divided into two groups: type B (PIP5K1-9) containing membrane occupation and recognition nexus (MORN) repeats in the N terminus and type A (PIP5K10 and 11) lacking MORN repeats (Mueller-Roeber and Pical, 2002; Im et al., 2007a). In Arabidopsis root hairs, the production of PI(4,5)P2 is through a type B PIP5K, PIP5K3 (Kusano et al., 2008; Stenzel et al., 2008). Overexpression of PIP5K3 causes abnormal root hair morphology, and disruption of PIP5K3 inhibits root hair growth. In pollen tubes, alteration of pollen-expressed PIP5K4 and PIP5K5 similarly affected pollen tube growth (Ischebeck et al., 2008; Sousa et al., 2008). It was proposed that these PIP5Ks affect either pectin exocytosis or recycling of endocytic vesicles (Ischebeck et al., 2008; Sousa et al., 2008). Although several studies have implicated phosphoinositides in the regulation of endocytosis in pollen tubes (Monteiro et al., 2005a, 2005b; Helling et al., 2006; Sousa et al., 2008), the role and the mode of action for phosphoinositides in endocytosis remain unclear.

In this research, we investigated the role of PI4P and PI(4,5)P2 in the regulation of clathrin-mediated endocytosis in the tip of pollen tubes. We found that a pollen-enriched Arabidopsis PIP5K, PIP5K6, was preferentially localized to the subapical PM in pollen tubes, as was clathrin. Importantly, downregulation of PIP5K6 inhibited the assembly of clathrin onto the apical PM and endocytosis. By contrast, overaccumulation of PIP5K6 overactivated the early stage of clathrin-dependent endocytosis and was accompanied by a defect in the late stage of endocytosis, resulting in excessive aggregates of invaginated PM. The alteration of the apical PI(4,5)P2 by inhibiting or overexpressing PLC did not cause this membrane deformation phenotype. Furthermore, PIP5K6-induced membrane deformation was suppressed by overexpression of either PLC2 or PI4Kβ1. Taken together, these findings support the hypothesis that PI(4,5)P2 promotes the early stages of clathrin-dependent endocytosis (formation and invagination of clathrin-coated pits), whereas PI4P is required for the completion of clathrin-dependent endocytosis.

**RESULTS**

**PIP5K6 Encodes a PIP5K Localized to the Apical PM in Pollen Tubes**

To investigate the role of the tip-localized PI(4,5)P2 in the regulation of pollen tube tip growth, we sought to identify a PIP5K responsible for the synthesis of PI(4,5)P2 for this particular PI(4,5)P2 pool. Three Arabidopsis PIP5K genes, PIP5K4, 5, and 6, are specifically expressed in pollen (Zimmermann et al., 2004). Recent reports suggest that PIP5K4 and PIP5K5 are preferentially localized to the subapical PM of pollen tubes (Ischebeck et al., 2008; Sousa et al., 2008). We found that PIP5K6-GFP was also enriched at the subapical PM, while GFP alone was only found in the cytosol (Figure 1A; see Supplemental Figure 1A online). In growing pollen tubes, PIP5K6-GFP was preferentially associated with the subapical PM (Figure 1A). By contrast, in tubes that had ceased elongation, PIP5K6-GFP was preferentially localized to the tip of the apical PM (Figure 1B).

To assess whether PIP5K6 is a functional PIP5K kinase in vivo, we visualized pollen PIP(4,5)P2 and PI4P using GFP-based markers, GFP-PLC Domain 1 PH and GFP-FAPP1 PH (GFP-tagged PH domains of the human PLCδ1 and phosphatidylinositol-4-phosphate adaptor protein-1 [FAPP1]), respectively (Furutani et al., 2006). PI(4,5)P2, marked by the GFP-PLCδ1 PH domain, accumulated at the apical PM of pollen tubes (see Supplemental Figure 1B online), consistent with previous reports in pollen tubes (Kost et al., 1999; Dowd et al., 2006). PI4P, marked by the GFP-FAPP1 PH domain, accumulated at the apical PM and in a vesicle-like structure (presumably derived from the trans-Golgi network) in control pollen tubes (see Supplemental Figure 2A online), consistent with the PI4P localization pattern in root hair cells (Thole et al., 2008). Although considered as specific binding proteins for PI4P and PI(4,5)P2 in vitro, the phosphoinositide markers used in this study may compete with native proteins (including downstream effectors and native enzymes) for binding with PI4P and PI(4,5)P2. As a result, overexpression of these two markers might introduce some nonspecific effects on pollen tube growth (Sousa et al., 2008). To minimize the unspecific effects caused by marker
Overexpression of PIP5K6 Causes Overinitiated but Aborted Clathrin-Dependent Endocytosis

We hypothesized that one or both of the following membrane trafficking defects could contribute to the PM deformation induced by PIP5K6 OX: (1) an increase in exocytosis that was not coupled with an increase in endocytosis or cell wall expansion and (2) a defect in a late stage of endocytosis, in which the apical PM was invaginated but not pinched off, as observed in the dysfunction of dynamin (Hill et al., 2001; Kang et al., 2003).

To investigate the effect of PIP5K6 OX on exocytosis, we visualized exocytosis at the tip of tobacco pollen tubes using the fluorescence recovery after photobleaching (FRAP) method (Lee et al., 2008). In pollen tubes expressing GFP-RLK PM marker, we photobleached the apical PM region and tracked the recovery of the GFP signal. In control pollen tubes expressing GFP-RLK alone, the PM-associated GFP signal quickly recovered deformation of the cytoplasm in the tip region. To confirm whether the aggregates were indeed due to invagination of the PM, we coexpressed untagged PIP5K6 with the PM marker RLK-GFP (Lee et al., 2008) and found that the apical PM containing RLK-GFP displayed a similar invagination, as did PIP5K6-GFP (see Supplemental Figure 3 online).

PIP5K6-GFP OX pollen tubes exhibited different degrees of PM deformation. To better describe the severity of this phenotype, we categorized PIP5K6-GFP tubes into three groups: (1) those with the normal smooth PM, (2) those with moderate apical PM deformation in which the apical PM is still a distinguishable intact membrane compartment but is retracted from the apical cell wall, and (3) those with severe apical PM deformation in which the apical PM becomes fragmented membrane patches. At 4 to 5 h after bombardment, the percentage of PIP5K6-GFP tubes falling into these three groups was 18, 40, and 42%, respectively (n = 100; Figure 2A). However, PM morphology was not altered by the overexpression of the GFP-tagged PIP5K6 K443S mutant (n = 30; shown in Figure 2A). Time-lapse imaging showed that the PM invagination started after PIP5K6-GFP localization invaded the extreme tip and became more severe over time (Figure 2C). These results suggest that excessive amount of PIP5K6 activity at the tip of pollen tubes caused the deformation of the PM.

The PM deformation occurred in PIP5K6-GFP OX tubes that had already stopped elongating (Figure 2C). At 4 to 5 h after bombardment, PIP5K6-GFP OX tubes were shorter in length and wider in tips compared with control tubes expressing GFP alone (Figures 2D and 2E). The mean length decreased from 180 μm in control tubes to 113 μm in PIP5K6-GFP OX tubes (n = 30, Student’s t test value P < 0.05). The mean width increased from 8.1 μm in control tubes to 12.4 μm in pollen tubes overexpressing PIP5K6-GFP (n = 30, Student’s t test value P < 0.05). However, pollen tube growth arrest induced by other treatments, such as overexpression of a dominant-negative form of ROP1, did not cause PM deformation (data not shown), suggesting that the PM deformation is a specific effect of PIP5K6 overexpression, which is further supported by the suppression of the PM deformation by the inhibition of the early stage of clathrin-dependent endocytosis (see below).

Overexpression of PIP5K6 Causes Overinitiated but Aborted Clathrin-Dependent Endocytosis

In the course of examining PIP5K6-GFP localization, we found that many PIP5K6-GFP–overexpressing pollen tubes exhibited PIP5K6-GFP aggregates at the tip (Figure 2A). Similar phenotypes were observed in tobacco pollen tubes overexpressing PIP5K4-GFP and PIP5K5-GFP (Ischebeck et al., 2008; Sousa et al., 2008). To assess whether these aggregates were due to PM deformation, multiple scanning at different focal planes was conducted in the tip of PIP5K6-GFP OX tubes. As shown in Figures 2A and 2B, in these abnormal tips PIP5K6-GFP appeared to be associated with the PM, which apparently retracted from the cell wall as a result of invagination, causing the overall
after photobleaching ($n=10$; average curve shown in Figures 3A and 3B). The PM signal recovered to $\approx 70\%$ of the original signal intensity within 3 min after photobleaching. In pollen tubes coexpressing GFP-RLK and PIP5K6, even at the early times when PIP5K6 OX had not caused PM deformation, the recovery of the apical PM-associated GFP signal was slower than in control tubes (Figures 3A and 3C; Student’s $t$ test value $P < 0.05$). At 3 min after photobleaching, the PM signal recovered to $\approx 50\%$ of the original signal intensity. This observation suggested that PIP5K6 OX did not enhance but might suppress exocytosis in pollen tubes. In further support of this conclusion, we found that PIP5K6 OX did not change the localization of a marker for exocytic vesicles yellow fluorescent protein–RabA4d (Lee et al., 2008), as shown in Supplemental Figure 4 online.

We then investigated whether PIP5K6 OX altered endocytosis. FM 4-64 dye is commonly used as a marker for endocytosis.
because it is incorporated in the PM and enters the cytoplasm only through endocytosis when applied to culture media. In normal elongating tobacco pollen tubes, FM 4-64 dye labeled the PM, particular structures (likely endosomes), and the apical cytoplasm as an inverted cone pattern that is thought to contain both recycling and secretory vesicles (Figure 4A). Ten to fifteen minutes after 2.5 \( \mu \text{M} \) FM 4-64 was applied to \( \text{PIP5K6-GFP} \) tubes, FM 4-64 strongly stained the PM region and weakly stained the cytoplasm (Figures 4C to 4E). \( \text{PIP5K6-GFP} \) overexpression inhibited pollen tube elongation (Figure 2). However, inhibition of FM 4-64 uptake in \( \text{PIP5K6-GFP} \)–overexpressing tubes was the direct specific effect of \( \text{PIP5K6-GFP} \) overaccumulation because treatments with brefeldin A (BFA), which also induced the arrest of pollen tube elongation, did not inhibit FM 4-64 entry into the pollen tubes (Figure 4B). These observations are consistent with a previous report that \( \text{PIP5K4} \)OX inhibited FM dye uptake (Sousa et al., 2008) and support the hypothesis that \( \text{PIP5K6} \)OX overactivates the early stages (i.e., membrane invagination) but inhibits the later stage (i.e., fission of invaginated membrane) of endocytosis, resulting in excessive accumulation of deformed membranes at the tip of pollen tubes.

PI(4,5)P\(_2\) is involved in recruiting clathrin and associated proteins that are required for the formation and invagination of coated pits in yeast and animal cells (Mousavi et al., 2004; Di Paolo and De Camilli, 2006). Thus, a simple explanation for the \( \text{PIP5K6} \)OX-induced membrane deformation phenotype is that overaccumulation of PI(4,5)P\(_2\) at the tip overactivated membrane invagination but not closure and pinching of endocytic vesicles during clathrin-dependent endocytosis. We tested this hypothesis using a dominant-negative form of \( \text{Arabidopsis} \) clathrin heavy chain (called Clathrin Hub) (Liu et al., 1995; Dhonukshe et al., 2007), which contains the C-terminal part of the clathrin heavy chain that can bind to and trap the clathrin light chain. Overexpression of \( \text{HUB} \) alone in tobacco pollen tubes slightly inhibited growth (see Supplemental Figure 5 online). When \( \text{PIP5K6-GFP} \) was co-overexpressed with \( \text{HUB} \), the PM invagination phenotype was greatly suppressed (Figures 4F and 4G).

Five to six hours after bombardment, the percentage of \( \text{PIP5K6-GFP} \)–overexpressing tobacco pollen tubes that displayed little, moderate, and severe PM invagination was 15, 40, and 45%, respectively (Figure 4F; \( n = 60 \)). Under the same condition, 65% of pollen tubes overexpressing both \( \text{PIP5K6-GFP} \) and \( \text{HUB} \) showed normal smooth PM, while 35% exhibited moderate PM invagination and none had severe PM invagination (Figure 4G; \( n = 60 \)).

To further test this hypothesis, we used a marker for early stage of clathrin-dependent endocytosis, \( \text{Arabidopsis} \) AP180.
protein. AP180 is an adaptor protein responsible for clathrin cage assembly and thus participates in the initiation of clathrin-mediated endocytosis (Barth and Holstein, 2004). In control tobacco pollen tubes, GFP-AP180 was localized to the subapical PM, similar to clathrin localization pattern (see Supplemental Figures 6A and 6B and Supplemental Movie 3 online). In pollen tubes co-overexpressing GFP-AP180 and PIP5K6, the GFP-AP180 accumulated in both of the apex and the subapical PM, especially the invaginated sites of PM (see Supplemental Figures 6C to 6E online). Taken together, these results strongly support the hypothesis that overinitiated but aborted clathrin-dependent endocytosis accounted for the PM deformation in PIP5K6-GFP OX pollen tubes.

**Figure 4.** PIP5K6 OX Induces Clathrin-Dependent Abortive Endocytic Compartments in Pollen Tubes.

(A) FM 4-64 dye uptake in a control tobacco pollen tube. Tobacco pollen tubes were treated with 2.5 μM FM 4-64 for 15 min before imaging under a Leica SP2 confocal microscope. Bar = 10 μm.

(B) Effect of BFA treatment on FM 4-64 dye uptake in a tobacco pollen tube. Tobacco pollen tubes were treated with 10 μM BFA and 2.5 μM FM 4-64 for 15 min before imaging under a Leica SP2 confocal microscope. Bar = 10 μm.

(C) FM 4-64 dye uptake in tobacco pollen tubes with a low level of PIPS6K-GFP overexpression. Tobacco pollen tubes were treated with 2.5 μM FM 4-64 for 15 min before imaging under a Leica SP2 confocal microscope. Bar = 10 μm.

(D) FM 4-64 dye uptake in tobacco pollen tubes with a high level of PIPS6K-GFP overexpression. Tobacco pollen tubes were treated with 2.5 μM FM 4-64 for 15 min before imaging under a Leica SP2 confocal microscope. Bar = 10 μm.

(E) Quantitative analysis of PIPS6K-GFP OX’s effect on FM 4-64 uptake in tobacco pollen tubes. FM dye uptake is significantly suppressed by PIPS6K-GFP OX, as revealed by the fact that FM 4-64 relative localization on the PM is significantly increased by PIPS6K-GFP OX (n = 20, Student’s t test value P < 0.05, error bars indicate SD).

(F) PM invagination phenotype of PIPS6K-GFP OX in tobacco pollen tubes. Five to six hours after bombardment, the percentage of PIPS6K-GFP-overexpressing tobacco pollen tubes that displayed little, moderate, and severe PM invagination was 15, 40, and 45%, respectively (n = 60). Bar = 10 μm.

(G) HUB OX suppresses the PM invagination phenotype induced by PIPS6K-GFP OX in tobacco pollen tubes. Five to six hours after bombardment, 65% of pollen tubes overexpressing both PIPS6K-GFP and HUB showed normal smooth PM, while 35% exhibited moderate PM invagination, and none had severe PM invagination (n = 60, Fisher’s exact probability test value P < 0.05). Bar = 10 μm.

**PIPS6K RNA Interference Inhibits Endocytosis by Blocking the PM Recruitment of Clathrin Heavy Chain**

If the PM-localized PI(4,5)P₂ indeed regulates the formation of coated pits by recruiting clathrin in pollen tubes as in yeast and animal cells, the suppression of PM-localized PIP5Ks is expected to inhibit clathrin-dependent endocytosis. We generated transgenic Arabidopsis plants expressing a PIPS6K RNA interference (RNAi) construct under the control of LAT52 promoter. PIPS6K RNAi lines exhibit reduced PIPS6K mRNA levels in pollen compared with wild-type Arabidopsis plants (Figure 5A). Two representative lines examined showed similar phenotypes that are correlated with the reduction of mRNA levels in these lines. The PIPS6K RNAi pollen tubes were greatly shorter (~60% reduction).
Figure 5. PIP5K6 RNAi Inhibits Clathrin-Dependent Endocytosis in Arabidopsis Pollen Tubes.
than wild-type Columbia-0 (Col-0) pollen tubes (Figures 5B and 5C), similar to the previous findings with the pip5k4 mutant (Sousa et al., 2008). We also found that FM dye uptake was greatly inhibited in PIP5K6 RNAi pollen tubes (Figures 5D and 5E).

We then analyzed the localization of clathrin heavy chain (CHC) in PIP5K6 RNAi pollen tubes using an immunostaining method (Blackbourn and Jackson, 1996; Kim et al., 2001). In control pollen tubes, anti-CHC antibody staining was mainly localized to the subapical PM (Figure 5F). In PIP5K6 RNAi pollen tubes, CHC antibody stained weakly at the PM (Figure 5F). In pip5k4 knockout pollen tubes (Sousa et al., 2008), CHC localization to the apical PM was also greatly reduced (Figure 5F). The relative PM-associated CHC signal was significantly decreased in both PIP5K6 RNAi and pip5k4 mutant tubes (Figure 5G; n = 20, error bar indicates SD, Student’s t test value P < 0.05). Thus, the apparent deficiency in PIP5K-dependent conversion of PI4P to PI(4,5)P2 led to a defect in clathrin-dependent endocytosis. These results, together with PIP5K6–induced excessive clathrin-dependent PM invagination, suggest that PI(4,5)P2 production via type B PIP5Ks is important for the activation of the early stages (i.e., formation and invagination of coated pits) of clathrin-mediated endocytosis in the apical PM of pollen tubes.

Both Overaccumulation of PI(4,5)P2 and Depletion of PI4P in the Apical PM Induced PM Deformation in Pollen Tubes

Since PIP5K6 RNAi not only caused apparent overaccumulation of its product PI(4,5)P2 but also depletion of its substrate PI4P in the apical PM (see Supplemental Figures 1 and 2 online), PI4P depletion and/or PI(4,5)P2 overaccumulation could contribute to the observed PM invagination induced by PIP5K6 overexpression. To assess which of the PIP5K6 RNAi effects was responsible for the PIP5K6 RNAi phenotype, we first co-overexpressed PIP5K6-GFP with Arabidopsis PLC2 in tobacco pollen tubes. PLC hydrolyzes PI(4,5)P2 into the second messengers inositol-3-phosphate and diacylglycerol. Arabidopsis PLC2 mRNA accumulates in pollen, and GFP-tagged PLC2 was localized to both apical and subapical PM of tobacco pollen tubes (see Supplemental Figure 7 online). Overexpression of PLC2 suppressed the overaccumulation of PI(4,5)P2 marker caused by PIP5K6 overexpression (see Supplemental Figure 8 online). When PIP5K6-GFP was coexpressed with PLC2 in tobacco pollen tubes, the PM invagination phenotype was partially suppressed. At 4 to 5 h after bombardment, 18, 40, and 42% of tobacco pollen tubes overexpressing PIP5K6-GFP displayed smooth normal PM, moderate PM invagination, and severe PM invagination, respectively (Figure 6A; n = 100). By contrast, 46, 39, and 15% of pollen tubes overexpressing both PIP5K6-GFP and PLC2 exhibited smooth PM, moderate PM invagination, and severe PM invagination (Figure 6B; n = 100, Fisher’s exact probability test value P < 0.05), respectively. Thus, the apparent removal of PI(4,5)P2 by PLC2 overexpression greatly but did not completely suppress PIP5K6–induced PM invagination. This result supports the notion that overaccumulation of PI(4,5)P2 contributed to, but was not solely responsible for, the PM invagination phenotype. Consistent with this finding, we found that PLC2 RNAi partially suppressed the growth phenotype of PIP5K6-OX (Figures 6D and 6E; n = 30, Student’s t test value P < 0.05).

Since suppression of PI(4,5)P2 overaccumulation did not completely restore normal pollen tube tips, we hypothesized that PIP5K6–induced depletion of PI4P in the apical PM of pollen tubes also contributed to the PIP5K6-OX phenotype. To test this hypothesis, we examined the effect of overexpressing Arabidopsis PI4Kβ1, which rescued the reduction of PI4P marker localization caused by PIP5K6 overexpression (see Supplemental Figure 9 online). Co-overexpression of PI4Kβ1 with PIP5K6-GFP not only partially suppressed PI4Kβ1 RNAi pollen tubes than wild-type Col-0 pollen tubes (Figures 6D and 6E; n = 30, Student’s t test value P < 0.05) but also partially suppressed the PM invagination phenotype induced by PIP5K6-GFP overexpression (Figure 6C). At 4 to 5 h after bombardment, the percentage of PIP5K6-GFP and PI4Kβ1 co-overexpressing tobacco pollen tubes that displayed little, moderate, and severe PM deformation was 30, 45, and 25%, respectively (n = 100, Fisher’s exact probability test value P < 0.05). Furthermore, treatment of wild-type pollen tubes with PLC inhibitor U73122, which increased PI(4,5)P2 level without decreasing PI4P level on the PM (see Supplemental Figure 10 online) (Helling et al., 2006), did not cause PM invagination (see Supplemental Figure 10 online; n = 30). These results further support the notion that overaccumulation of PI(4,5)P2 at the apical PM alone was insufficient to cause the PM invagination phenotype induced by PIP5K6-OX. Taken together, our data suggest that the PM invagination phenotype in PIP5K6-OX pollen tubes was the result of simultaneous overaccumulation of PI(4,5)P2 and reduction of its precursor PI4P.
DISCUSSION

Several recent reports support a role for both PI4P and PI(4,5)P2 in the regulation of tip growth in root hairs and pollen tubes (Monteiro et al., 2005a, 2005b; Dowd et al., 2006; Hellerg et al., 2006; Preuss et al., 2006; Ischebeck et al., 2008; Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008; Thole et al., 2008; Szumlanski and Nielsen, 2009), but the mechanism by which these membrane-localized signaling molecules affect polarized cell growth remained unclear. Our results support the hypothesis that both PI4P and PI(4,5)P2 play a critical role in the regulation of clathrin-dependent endocytosis at the tip of pollen tubes and that a proper balance between PI4P and PI(4,5)P2 accumulation in the apical PM is important for clathrin-dependent endocytosis. We propose that PI(4,5)P2 promotes the formation and invagination of clathrin-coated pits as shown in yeast and animal cells, while PI4P participates in the final stage of clathrin-dependent endocytosis at the tip of pollen tubes.

PI(4,5)P2 could be converted into PI(3,4,5)P3 by PI(4,5)P2-3-kinase. Homologs of this enzyme are absent in plants, and...
Plants lack PI(3,4,5)P3. Thus, PI(3,4,5)P3 is unlikely to participate in clathrin-dependent endocytosis in plant cells. PI(4,5)P2 could also be converted into inositol-3-phosphate and diacylglycerol by PLC. However, overexpression of PLC in tobacco pollen tubes did not induce excessive PM invagination seen in PIP5K-overexpressing tubes (Helling et al., 2006), arguing against a role for PLC products in the promotion of early clathrin-dependent endocytosis. Importantly, we found that the clathrin-dependent PM invagination in PIP5K6-overexpressing tubes was partially suppressed when PM-localized PLC2 was overproduced. PI(4,5)P2 has been found in clathrin-enriched vesicles upon salt stress, supporting a link between clathrin-dependent endocytosis and PI(4,5)P2 (König et al., 2008). Therefore, we propose that PI(4,5)P2, not its products, promotes early steps of clathrin-dependent endocytosis.

A role for PI(4,5)P2 in the regulation of clathrin-dependent endocytosis in pollen tubes is consistent with the findings in animal and yeast cells (Sun et al., 2005, 2007; Zoncu et al., 2007). Through the study of PI(4,5)P2 dynamics and clathrin-dependent endocytic compartment, it was shown in yeast cells that PI(4,5)P2-enriched region is important for the initiation of clathrin-dependent endocytosis (Sun et al., 2005, 2007). The most convincing study that supports a direct involvement of PI(4,5)P2 in clathrin-dependent endocytosis was the demonstration that acute depletion of PI(4,5)P2 by inducible activation of PI(4,5)P2 phosphatase causes loss of coated pits in mammalian COS-7 cells (Zoncu et al., 2007). Moreover, several PI(4,5)P2 binding proteins, including AP2 and AP180, are involved in the assembly of clathrin coat and/or invagination of clathrin-coated pits. Importantly, homologs for AP180 are present in plants and are expressed in pollen (Holstein and Oliviusson, 2005). Therefore, PI(4,5)P2 regulation of the early stage of clathrin-dependent endocytosis appears to be conserved in eukaryotic kingdoms.

PI(4,5)P2 also regulates the organization and dynamics of the actin cytoskeleton, which impacts clathrin-mediated endocytosis in yeast (Yin and Janmey, 2003; Kaksonen et al., 2006; Sun et al., 2007). Could PI(4,5)P2 regulation of actin be part of the mechanism by which PI(4,5)P2 promotes the early stages of clathrin-dependent endocytosis? When overexpressed, a dominant-negative mutant of PLC was found to increase F-actin in pollen tubes, implicating the involvement of PI(4,5)P2 in the regulation of the actin cytoskeleton (Dowd et al., 2006). A previous report disagreed with the idea that the membrane invagination induced by PIP5K4 and PIP5K5 overexpression could be attributed to changes in the actin cytoskeleton (Ischebeck et al., 2008). Nonetheless, actin as a potential bridge between PIP5Ks and endocytosis is worthy of further investigation, given that PI(4,5)P2 regulation of endocytosis is conserved in different eukaryotic systems.

PI4P May Participate in a Late Stage of Clathrin-Dependent Endocytosis

Our results also support a requirement for the PI(4,5)P2 precursor, PI4P, in the regulation of clathrin-dependent endocytosis. Using a PI4P marker, GFP-FAPPI PH, we found that PI4P is enriched in the apical PM and is localized in putative Golgi-derived vesicles. Similar PI4P distribution was also found in root hairs (Vermeer et al., 2009). In plant cells, PI4P is the most abundant form of phosphoinositide (Im et al., 2007b). Genetic studies of enzymes involved in PI4P synthesis and degradation in root hairs have implicated PI4P in the regulation of polar exocytosis (Preuss et al., 2006; Thole et al., 2008). Because PI4P is the immediate precursor of PI(4,5)P2, however, it has been difficult to determine whether PI4P merely provides a precursor for PI(4,5)P2 or has a direct role in signaling.

The excessive PM invagination phenotype in PIP5K6-overexpressing pollen tubes provided us with a unique opportunity to investigate the role of PI4P. As discussed above, our findings suggest that overaccumulation of PI(4,5)P2 contributes to this phenotype. However, this phenotype cannot be explained solely by overproduction of PI(4,5)P2 because inhibition of PLC, which also causes overaccumulation of PI(4,5)P2, did not induce the PM invagination phenotype. Furthermore, co-overexpression with PLC2 was able to only partially suppress the PIP5K6 overexpression phenotype. Importantly, co-overexpression with PI4K, which synthesizes PI4P, also partially suppressed the PIP5K6 overexpression phenotype. These observations suggest that PI4P not only provides a precursor for PI(4,5)P2 but also plays a more direct role in the regulation of clathrin-dependent endocytosis.

In yeast, knockout mutants for SjL genes [which encode PI(4,5)P2 5-phosphatases] show abnormal PM invagination (Sun et al., 2005, 2007), which highly resembles the PIP5K6 overexpression PM phenotype. Using markers for different stages of endocytosis, it was shown that the abnormal PM invagination in the sjl mutants was caused by a defect in the late stage of clathrin-dependent endocytosis. The PM invagination phenotype in sjl knockout yeast cells was due to overaccumulation of abortive endocytic compartment. Based on these observations, it was proposed that the process of hydrolyzing PI(4,5)P2 into PI4P is required for the later steps of clathrin-dependent endocytosis. Our finding that increasing the pool of PI4P in PIP5K6-overexpressing tubes by overexpressing PI4K can rescue the PM invagination defect clearly supports a direct role for PI4P in the regulation of the late stage of endocytosis. Thus, we propose that a PI4P-dependent process may provide a conserved mechanism underlying the completion of clathrin-dependent endocytosis and that the right balance of (or interconversion between) PI4P and PI(4,5)P2 is critical for the initiation, progression, and maturation of clathrin-dependent endocytosis. Identification of PI4P binding proteins involved in this process will be important for testing this hypothesis.

METHODS

Plant Growth and Pollen Germination Conditions

Arabidopsis thaliana plants (Col-0) were grown at 22°C in growth rooms under a light regime of 16 h of light and 8 h of dark. Arabidopsis pollen grains were germinated on a solid germination medium (Li et al., 1999) at room temperature. Nicotiana tabacum plants were grown in a growth chamber at 25°C under a light regime of 12 h of light and 12 h of dark. Tobacco pollen grains were germinated in a liquid germination medium (Fu et al., 2001) at room temperature.
Cloning of Arabidopsis PIP5K6, PLC2, PI4K1β, HUB, and AP180 Coding Sequences

The PIP5K6 coding cDNA without the stop codon was amplified from cDNA obtained from Col-0 flowers using forward (5’-GGTTGTACAAAAAAGCAGGCTCCATGCCGAGCAAGCTTAAAAAAG-3’) and reverse (5’-TCTAGAAGCGTCTTCAACGAAGAC-3’) primers carrying the XbaI site (underlined). The PLC2 coding cDNA was amplified from cDNA obtained from Col-0 flowers using forward (5’-GCTCTAGAATGTCGGTAGCACACGC-3’) and reverse (5’-GGGATCCCGCCATGCCATTTGAAAGACG-3’) primers carrying XbaI and BamHI sites (underlined). The PI4K1β coding cDNA was amplified from cDNA obtained from Col-0 flowers using forward (5’-GGAGCTCTTAGTAGGTCGCTTACAT-3’) and reverse (5’-GGGACCATATGAGTTCATAATTCTTATGACC-3’) primers carrying NcoI and KpnI sites (underlined). Coding cDNAs for PI4K6, PLC2, and PI4K1β were cloned into the vector pGEM-Teasy vector (Promega) and sequenced. Coding cDNA for HUB was cloned into vector pCR2.1-HUB construct into the pUC pLAT52 vector using the XbaI site (downstream of 3’-end of GFP) to produce the pLAT52:HUB construct. HUB coding cDNA was sequenced. Coding cDNA was amplified from pCR2.1-HUB construct into the pUC pLAT52 vector using Ncol and KpnI sites to produce the pUC pLAT52:HUB construct. PLC2 coding cDNA was subcloned from pGEM-T-easy PLC2 construct into the pUC pLAT52 vector using XbaI and BamHI sites to generate the pUC pLAT52:PLC2 construct. PI4K1β coding cDNA was subcloned from pGEM-T easy PI4K1β construct into the pUC pLAT52 vector using XbaI and BamHI sites to obtain the pUC pLAT52:PI4K1β construct. AP180 coding cDNA was subcloned from Gateway DONR vector to destination vector to obtain the pGWLAT52:GFP-AP180 construct.

PI5PK6 RNAi Construct and Transgenic Lines

To generate the PIP5K6 RNAi construct, a 340-bp antisense cDNA fragment of the specific sequence of the PIP5K6 was amplified using primer combination (5’-AAATCGATTACCTCGACTTCTCC-3’/5’-AACATGCGCTTTAGAGATTTGTGCTT-3’), cloned into the Cia1-Saci sites in the pGEM-TZI vector, yielding the plasmid pGEM-TZI-PIP5K6A. The antisense fragment was cloned as an NcoI-SmaI fragment into pUC pLAT52:GFP vector; the LAT52-PI5PK6A fragment (HindIII/SalI) was subcloned into the pBl121 vector by replacing the cauliflower mosaic virus 35S promoter and GUS cassette, generating the plasmid pBI121 LAT52-PIP5K6A. The sense fragment of PIP5K6 amplified using the primer set (5’-AAGGATCTACTTACTTGACCTTCCTCC-3’/5’-AACCTTCTTAGATTTGTGCTT-3’) was inserted into BamHI-KpnI sites into the pBl121 vector, and then the fragment of intron-PIP5K6S (KpnI/XbaI) was ligated into the pBl121 LAT52-PIP5K6A, generating the plasmid pBl1-LAT52-PIP5K6 RNAi construct.

The PIP5K6 RNAi construct was introduced into Agrobacterium tumefaciens GV3101 and transformed into Arabidopsis (Col-0) using the floral dipping method (Clough and Bent, 1998). Independent T3 homozygous plants were isolated using kanamycin selection for further analysis. Pollen mRNA was extracted from wild-type and PIP5K6 RNAi plants and then used for synthesis of cDNA using the Qiagen RNAeasy kit and the Invitrogen Superscript III reverse transcriptase kit. RT-PCR experiments (30 cycles) with primer sets (PIPK6S-F, 5’-ATGTCGCTGAGACAAGCACACCG-3’/5’-GGTTGTACAAAAAAGCAGGCTCCATGCCGAGCAAGCTTAAAAAAG-3’) were performed to determine the mRNA level of PIP5K6 in both wild-type Col-0 and PIP5K6 RNAi pollen.

Constructs for Transient Expression in Tobacco Pollen Tubes

To generate the pLAT52:PI5PK6-GFP construct, PI5PK6 coding cDNA was subcloned from pGEM-T easy PI5PK6 into pUC pLAT52:GFP vector using XbaI site (in frame with the 5’ end of GFP sequence) (Fu et al., 2001). To generate the pLAT52:PI5PK6 K443S-GFP construct, PI5PK6 coding cDNA was subcloned from pGEM-T easy PI5PK6 into the pUC pLAT52:GFP vector using XbaI and BamHI sites (in frame with 5’ end of GFP sequence). To generate the pLAT52:PI5PK6 construct, PI5PK6 coding cDNA was subcloned from pGEM-T easy PI5PK6 into the pUC pLAT52:GFP vector using XbaI site. To generate the pLAT52:PI5PK6 K443S construct, PI5PK6 K443S coding cDNA was subcloned from pGEM-T easy PI5PK6 into the pLAT52:GFP vector using the XbaI site. To generate the pLAT52:PI5PK6 K443S construct, PI5PK6 K443S coding cDNA was subcloned from pGEM-T easy PI5PK6 into the pLAT52:GFP vector using XbaI site. The pLAT52:GFP:RLK PM marker construct has been described previously (Lee et al., 2008). DNA encoding the human PLC1 PH domain was cut using BgII and BamHI from YFP-PLC1c construct (from M. Furutani; Furutani et al., 2006) and then inserted into the pUC pLAT52:GFP vector using the BgII site (downstream of the 3’ end of GFP) to produce the pLAT52:GFP:PLC1 PH construct. The pLAT52:GFP:PLC1 PH domain was cut using BgII and BamHI from pDriveFAPP1 construct (Furutani et al., 2006) and then inserted into pUC pLAT52:GFP using the BgII site (downstream of the 3’ end of GFP) to produce the pLAT52:GFP:FAPP1 construct. 

Transient Gene Expression in Tobacco Pollen Tubes

Mature pollen grains collected from tobacco flowers were used for transient expression using a particle bombardment procedure as described previously (Fu et al., 2001). For all plasmid constructs used in our experiments, 0.2 to 1.0 μg plasmid DNA was used for each bombardment. Bombarded pollen grains were germinated in a liquid germination medium for various times before observation under a microscope (Fu et al., 2001).

Analysis of Arabidopsis Pollen Tube Growth

Flowers from Arabidopsis plants 2 weeks after bolting were used as the source of pollen. Pollen grains were germinated on a solid germination medium (Li et al., 1999). Unless indicated otherwise, ~3 to 5 h after germination, images of pollen tubes were recorded through a cooled CCD camera (model C4742-95; Hamamatsu) attached to an Eclipse inverted microscope (model TE300; Nikon). The images were analyzed using the MetaMorph v4.5 (Molecular Devices) measurement function.

PLC Inhibitor Treatment

U-73122 (Sigma-Aldrich) PLC inhibitor (0.1% DMSO) was added into tobacco pollen to a final concentration of 10 μM immediately following
FM 4-64 dye was applied onto thin layers of solid Arabidopsis pollen germination medium. For Arabidopsis (Invitrogen) was added to final concentrations of 2.5 and 10 μM into liquid pollen germination medium. For Arabidopsis pollen tubes, a droplet (10 μL) of liquid Arabidopsis pollen germination medium containing 10 μM FM 4-64 dye was applied onto thin layers of solid Arabidopsis pollen germination medium. Pollen tubes were incubated with FM 4-64 dye for 10 to 15 min before examination under a confocal microscope (Leica SP2 or Zeiss LSM510).

**FM 4-64 Dye Staining in Pollen Tubes**

FM 4-64 dye staining was performed at 4 to 5 h after pollen germination. For tobacco pollen tubes, FM 4-64 dye (Sigma-Aldrich) was added to a final concentration of 2.5 μM into liquid pollen germination medium. For FM 4-64 dye staining with BFA treatment, FM dye (Invitrogen) and BFA (Invitrogen) were added to final concentrations of 2.5 and 10 μM into liquid pollen germination medium. For Arabidopsis pollen tubes, a droplet (10 μL) of liquid Arabidopsis pollen germination medium containing 10 μM FM 4-64 dye was applied onto thin layers of solid Arabidopsis pollen germination medium. Pollen tubes were incubated with FM 4-64 dye for 10 to 15 min before examination under a confocal microscope (Leica SP2 or Zeiss LSM510).

**Immunostaining of CHC in Pollen Tubes**

Pollen grains of Arabidopsis flowers were germinated in solid germination medium for 4 h at room temperature and stained as described previously (Hwang et al., 2008). Pollen tubes were treated with fixative (4% paraformaldehyde, 3 mM MgSO₄, 2 mM CaCl₂, 18% sucrose, and 50 mM PIPES buffer, pH 6.9) for 1 h. After washing gently with PBST (0.05% Triton X-100) buffer (Hwang et al., 2008), three times for 5 min each, pollen tubes were treated with digestion buffer (2% cellulase R-10, 400 mM mannitol, 5 mM CaCl₂, and 15 mM MES buffer, pH 5.5) at room temperature for 3 to 5 min. Digested pollen tubes were washed gently with PBST buffer three times for 5 min each and then were blocked with 1% nonfat milk (Nestle) in PBS buffer (Hwang et al., 2008) at room temperature for 1 h. Pollen tubes were incubated with the purified primary anti-CHC (Kim et al., 2001) polyclonal antibody (1:200 dilution with 1% nonfat milk in PBS buffer) at room temperature for 1 h. After washing three times for 10 min each in PBST buffer, pollen tubes were incubated with secondary antibody and fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG (Sigma-Aldrich) (1:300 dilution with 1% nonfat milk in PBS buffer) at room temperature for 2 h. After three washes with PBST buffer, slides were mounted with mount solution (0.1% p-phenylenediamine, 50% glycerol, and PBS buffer). Mounted pollen tubes were observed under a confocal microscope (Leica SP2).

**Confocal Microscopy and Imaging Analysis for GFP Localization**

Localization patterns for GFP-tagged proteins in pollen tubes were observed under a confocal microscope (Leica SP2 or Zeiss LSM510). The signal intensities of GFP on the PM and in the cytoplasm were measured using the MetaMorph v4.5 (Molecular Devices) measurement function. The PM region for GFP intensity measurement was measured by defining the peripheral region of the pollen tube as the PM. Apical PM region for intensity measurement in tobacco and Arabidopsis pollen tubes were defined as the first 6 μm along PM from the tip point, respectively. A circular region (4 μm in diameter) 4 μm away from tip was chosen for measurement of cytosolic intensity. Relative localization of GFP on the PM was calculated to show the degree of internalization of GFP dye.

**FM 4-64 Dye Localization**

FM 4-64 dye–labeled pollen tubes were observed under a Leica SP2 confocal microscope or a Zeiss LSM510 confocal microscope. The signal intensities of FM dye on the PM and in the cytoplasm were measured using the MetaMorph v4.5 measurement function. Apical PM region for intensity measurement in tobacco and Arabidopsis pollen tubes were defined as the first 6 μm along PM from the tip point, respectively. A circular region (4 μm in diameter) 4 μm away from tip was chosen for measurement of cytosolic intensity. Relative localization of FM dye on the PM was calculated to show the degree of internalization of FM dye.

**CHC Localization**

Arabidopsis pollen tubes stained with anti-CHC primary antibody and FITC-conjugated secondary antibody were observed under a Leica SP2 confocal microscope. Median planes of pollen tubes were scanned to show the subcellular localization pattern of CHC.

The signal intensities of FITC antibody on the PM and in the cytoplasm were measured using the MetaMorph v4.5 measurement function. Apical PM region for intensity measurement in Arabidopsis pollen tubes was defined as the first 4 μm along PM from the tip point. A circular region (4 μm in diameter) 4 μm away from tip was chosen for measurement of cytosol intensity. Relative localization of FITC antibody on the PM was calculated as the ratio of PM intensity versus cytosolic intensity.

**FRAP Analysis of Exocytosis at the Pollen Tube Tip**

Tobacco pollen tubes expressing RLK-GFP PM marker alone and coexpressing RLK-GFP PM marker and PIP5K6 were used for FRAP analysis (Lee et al., 2008). The apical region of pollen tubes was photobleached using 100% power with a 488-nm laser (Leica SP2), and the recovery of fluorescence in pollen tubes was tracked in the following 3 min using a confocal microscope (Leica SP2). Time interval between adjacent frames was 10 s. Relative intensity of membrane-localized RLK-GFP compared with fluorescence before photobleaching was used to quantify the speed of fluorescence recovery.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: PIP5K6 (At5g07960), PLC2 (At3g08510), PI4K1 (At1g05020), RLK PM marker (At1g35390), CHC HUB (At3g11130), and AP180 (At1g05020). Sequence data for human genes used in this article can be found in GenBank under accession numbers AF286162 (FAPP1) and NM1130964 (PLC51).

**Supplemental Data**

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

**Supplemental Figure 1.** PIP5K6 OX Increases PI(4,5)P2 on Apical PM of Tobacco Pollen Tubes.

**Supplemental Figure 2.** PIP5K6 OX Suppresses PI4P on Apical PM of Tobacco Pollen Tubes.

**Supplemental Figure 3.** PIP5K6 OX Induces PM Deformation in Tobacco Pollen Tubes.

**Supplemental Figure 4.** PIP5K6 OX Does Not Change RabA4d Localization Pattern in Tobacco Pollen Tube Tips.

**Supplemental Figure 5.** DN-CHC HUB OX Slightly Inhibits Pollen Tube Growth.

**Supplemental Figure 6.** PIP5K6 OX Causes Mislocalization of GFP-AP180 in Tobacco Pollen Tubes.

**Supplemental Figure 7.** GFP-PLC2 Is Preferentially Localized to the Subapical PM of Pollen Tubes.

**Supplemental Figure 8.** PLC2 OX Suppresses Increase of PI(4,5)P2 Induced by PIP5K6 OX.
Supplemental Figure 9. PIP4K1 Ox Suppresses PIP4 Decrease Induced by PIP5K6 Ox.

Supplemental Figure 10. PLC Inhibitor Treatment Increases PIP4 Localization and Does Not Cause PM Imagination in Tobacco Pollen Tube.


ACKNOWLEDGMENTS

This work was supported by the 973 Basic Science Project (2007cb108700), the Department of Energy to Z.Y. (DE-FG02-04ER15555), the National Institute of General Medical Sciences to Z.Y. (GM081451), and the Natural Science Foundation of China to Y.Z. J.A.F.’s lab is funded by the Department of Energy to Z.Y. (DE-FG02-04ER15555), the National Institute of General Medical Sciences to Z.Y. (GM081451), and the Fundacao para a Ciencia e Tecnologia grant PTDC/BIA-BCM/108044/2008.

Received May 18, 2010; revised November 23, 2010; accepted December 6, 2010; published December 28, 2010.

REFERENCES


Kleine-Vehn, J., and Friml, J. (2008). Polar targeting and endocytic...


Phosphoinositides Regulate Clathrin-Dependent Endocytosis at the Tip of Pollen Tubes in Arabidopsis and Tobacco
Yan Zhao, An Yan, José A. Feijó, Masahiro Furutani, Tadaomi Takenawa, Inhwan Hwang, Ying Fu and Zhenbiao Yang
Plant Cell 2010;22;4031-4044; originally published online December 28, 2010;
DOI 10.1105/tpc.110.076760

This information is current as of October 28, 2017