**Arabidopsis** Phosphatidylinositol-4-Monophosphate 5-Kinase 4 Regulates Pollen Tube Growth and Polarity by Modulating Membrane Recycling

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Phosphatidylinositol-4-monophosphate 5-kinasers produce phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂] and have been implicated in vesicle trafficking and cytokoskeletal rearrangements. Here, we adopted a reverse genetics approach to investigate the function of the *Arabidopsis thaliana* pollen-expressed gene encoding phosphatidylinositol-4-monophosphate 5-kinase 4 (PIP5K4). Pollen germination, tube growth, and polarity were significantly impaired in homozygous mutant plants lacking *pip5k4* transcript. In vitro, supplementation with PtdIns(4,5)P₂ rescued these phenotypes. In vivo, mutant pollen fertilized ovules, leading to normal seed set and silique length. However, fertilization took longer than in wild-type plants, and the *pip5k4* null mutant allele was transmitted through the pollen at a reduced frequency. Analysis of endocytic events using FM1-43 (or FM4-64) suggested a reduction in endocytosis and membrane recycling in *pip5k4* null mutant pollen tubes. Imaging of elongating tobacco (*Nicotiana tabacum*) pollen tubes transiently transformed with a PIPS5K4-green fluorescent protein fusion construct revealed that the protein localized to the plasma membrane, particularly in the subapical region. Overexpression of PIPS5K4-GFP delocalized the protein to the apical region of the plasma membrane, perturbed pollen tube growth, and caused apical cell wall thickening. Thus, PIPS5K4 plays a crucial role in regulating the polarity of pollen tubes. This study supports a model for membrane secretion and recycling where the apical and subapical regions appear to contain the components required to promote and sustain growth.

**INTRODUCTION**

The pollen grain, upon germination on a receptive stigma, develops a pollen tube that grows through the pistil toward the ovule while carrying sperm cells to the embryo sac. The two main functions of the pollen tube are to transport the sperm cells through the style and to interpret the guidance cues from the female tissue. The elongation of the emergent pollen tube is accomplished via a form of cell extension common in cells of all eukaryotes, from fungal hyphae to nerve cells, known as tip growth. This growth form serves as a paradigm for cell polarity studies because cell extension is restricted to a narrow zone at the apex. The establishment and maintenance of such a growth mode involves multiple signaling pathways, such as Ca²⁺, protein kinases, CAMP, Ras-like small GTPases, targeted vesicle fusion, and specific cytoskeleton arrangements (Cole and Fowler, 2006; Malhó, 2006).

Phosphoinositides have also been reported to play an important role in tip growth. Based on the known central role of the tip-focused Ca²⁺ gradient in pollen tube growth, as well as knowledge of Ca²⁺-related phosphoinositide signaling from animal systems, early work in membrane-related pollen signaling was centered on the expected role of phospholipase C (PLC) activity in phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂ or PIP₂] cleavage and diacylglycerol and inositol 1,4,5-triphosphate [Ins(1,4,5)P₃] production. PtdIns(4,5)P₂ has been reported to accumulate at the pollen tube apex, possibly as a consequence of Rac/ROP GTPase-mediated recruitment of PKS kinase activity (Kost et al., 1999; Dowd et al., 2006; Helling et al., 2006). Monteiro et al. (2005) further showed that photorelease of caged PtdIns(4,5)P₂ led to transient growth arrest, increase of cytosolic free calcium ([Ca²⁺]c), and inhibition of apical secretion. However, photorelease of Ins(1,4,5)P₃ at a concentration causing [Ca²⁺]c transients of similar magnitude stimulated apical secretion and induced severe growth perturbation (Monteiro et al., 2005).

These observations indicate that the role of PtdIns(4,5)P₂ is more complex than its simple conversion to end-products and highlight the multiple functions of this phosphoinositide. Indeed, the intact PtdIns(4,5)P₂ molecule is a central player in actin dynamics, vesicle trafficking, and ion transport (Cremona et al., 1999; Stevenson et al., 2000) due to its ability to bind and regulate many proteins containing PtdIns(4,5)P₂ recognition domains, such as pleckstrin homology domains, basic patches, and epsin N-terminal homology domains (Martin, 1998; Cockcroft and De Matteis, 2001). In pollen, Potocky et al. (2003) observed distinct phospholipase D activities with specific PtdIns(4,5)P₂ and Ca²⁺-requirements and with different dynamics during germination and tube elongation. More recently, Dowd et al. (2006) showed that expressing a catalytically inactive recombinant PLC (PLC1) in petunia (*Petunia hybrida*) pollen tubes caused the apical Ca²⁺-gradient to expand, disrupted the organization of the actin
cytoskeleton, and delocalized growth at the tube tip. These phenotypes were suppressed by depolymerizing actin with low concentrations of latrunculin B, suggesting that PLC1 from petunia regulates actin structure at the growing tip. The authors further reported that a green fluorescent protein (GFP) fusion to Pet PLC1 accumulates in regions of the apical plasma membrane not undergoing rapid expansion, whereas a GFP fusion to the PtDNS(4,5)P2 binding PH domain accumulates in apical regions depleted of PLC. Thus, PLC appears to be involved in the machinery that restricts growth to the very apex of the elongating pollen tube. In a parallel study, Helling et al. (2006) showed that tobacco (Nicotiana tabacum) PLC overexpression inhibited pollen tube growth. The enzyme seems to accumulate laterally at the pollen tube tip plasma membrane in a pattern complementary to the distribution of PtDNS(4,5)P2 and was suggested to be involved in maintaining an apical domain enriched in PtDNS(4,5)P2, which is required for polar cell growth.

These results indicate that regulation of PtDNS(4,5)P2 levels is a crucial step for phosphoinositide and phospholipid signaling in plant cells. However, our knowledge of the enzymes responsible for its synthesis, localization, and function is still limited. In Arabidopsis thaliana, there are 11 type PIP kinases that are predicted to produce PtDNS(4,5)P2 from either PtDNS(4)P or PtDNS(5)P (Mueller-Roeber and Pical, 2002). This activity has now been confirmed for PIP5K1, 3, and 10 (Mikami et al., 1998; Perera et al., 2005; Lee et al., 2007; Kusano et al., 2008; Stenzel et al., 2008). The PIP kinases PIP5K1, 3, and 4 belong to the B subfamily, which contains putative membrane occupation and recognition nexus (MORN) repeats (Elge et al., 2001). The N-terminal MORN domain is critical for regulating PIPK activity, and it affects membrane attachment in vivo (Im et al., 2007). In root hairs, it was recently shown that PIP5K3 synthesizes PtDNS(4,5)P2 and that disruption of the PIP5K3 gene caused reduced growth, whereas overproduction of the enzyme induced aberrant morphologies (Kusano et al., 2008; Stenzel et al., 2008). Similarly, Arabidopsis PIP5K4 was also shown to synthesize PtDNS(4,5)P2 and to be important for stomatal opening (Lee et al., 2007). The authors showed that disruption of the PIP5K4 gene (confirmed by RT-PCR in guard cell protoplasts) caused reduced stomatal opening and that this phenotype was rescued by complementation with full-length PIP5K4. Here, we provide additional genetic, molecular, and cellular evidence that this PIP5K4 gene is highly expressed in pollen and that the PIP5K4 protein is vital for tip growth. The localization and activity of PIP5K4 was found to be essential for proper endocytosis and membrane recycling. Disruption of its activity led to decreased germination and tube growth rate along with perturbations in polarity and deposition of cell wall material. These findings provide further support for the importance of phosphoinositides at the intersection of multiple signaling pathways that regulate cell polarity.

RESULTS

Identification and Functional Characterization of the Arabidopsis Pollen Expressed PIP5K4

PIP5 kinases use PtDNS4P as a substrate for the synthesis of PtDNS(4,5)P2. Several isoforms have been characterized in animal cells, but only one has been reported for yeast: MSS4 (Homma et al., 1998). In Arabidopsis, nine genes of this B subfamily, expressed in different tissues, were identified by genome analysis (Mueller-Roeber and Pical, 2002). To select and characterize our genes of interest, we determined which Arabidopsis PIP5 kinase genes are expressed in pollen using the Genevestigator portal (Zimmermann et al., 2004). This in silico tool suggested that PIP5K4, PIP5K5, and PIP5K6 are highly expressed in pollen. To confirm the tissue-specific expression, the promoter region of each gene was fused to a reporter gene encoding β-glucuronidase (GUS) and transformed into Arabidopsis plants. Histochemical staining of tissues from transgenic plants demonstrated high GUS activity in the pollen grains within the anthers (Figure 1A) and in germinated pollen tubes (Figure 1B) but not in pistils leaves, roots, or root hairs of young seedlings.

To confirm that the isolated PIP5 kinases encode functional enzymes, a yeast mutant phenotype rescue assay was performed. Saccharomyces cerevisiae MSS4 is the functional homolog of mammalian type I PI4P5 kinase (Homma et al., 1998), and we tested the ability of the isolated Arabidopsis PIP5 kinases genes to complement the mss4-1 temperature-sensitive mutant. MSS4 wild-type cells transformed with the empty pGBT9 vector were used as a positive control, and mutant mss4-1 cells transformed with the empty pGBT9 vector were used as a negative control. When the cells were incubated at the permissive temperature (23°C), all of the transformed yeast cells grew normally. However, in the replica plate incubated at the restrictive temperature (37°C), mss4-1 cells transformed with the empty pGBT9 vector did not grow, whereas mss4-1 cells transformed with the PIP5K4, PIP5K5, and PIP5K6 genes from Arabidopsis were viable. These results indicate that the coding regions of the isolated PIP5 kinases encode functional enzymes (Figure 1C; see Supplemental Figure 1 online).

Disruption of PIP5K4 Causes a Male Gametophytic Mutant Phenotype

A reverse genetics approach was used to evaluate the function of PIP5 kinases in pollen. By screening the Salk Institute Genomic Analysis Laboratory collection for T-DNA insertion mutants, we retrieved one T-DNA insertion in the PIP5K4 gene (pip5k4, SALK_001138) and one T-DNA insertion in the PIP5K5 gene (pip5k5, SALK_147475.30.40.X). No T-DNA insertion lines were identified for the PIP5K6 gene. DNA sequencing of the regions adjacent to the T-DNA left border confirmed that in the PIP5K4 gene, the insertion was found to disrupt the coding sequence in the first exon, downstream of the initiation codon (Figure 2A), as had been previously observed by Lee et al. (2007). Similarly, in the PIP5K5 gene, the insertion was found to disrupt the coding sequence also in the first exon. RT-PCR analysis of homozygous mutant lines showed a null expression of PIP5K4 and PIP5K5 (Figure 2B), confirming that the pip5k4 and pip5k5 homozygous mutant plants are knockout mutants.

The pollen grains from pip5k4 homozygous flowers were found to be indistinguishable from wild-type pollen in shape and size. The 4',6-diamidino-2-phenylindole staining showed correctly differentiated, normal sized vegetative and sperm cell nuclei. Alexander and aniline blue staining revealed that there were no
abnormalities in the cytoplasm or wall of the pollen grain or in callose accumulation (see Supplemental Figure 2 online). However, their germination efficiency under in vitro conditions was significantly lower than that of the wild-type controls (18.7% ± 10.4% and 45.5% ± 16.9%, respectively; P = 7E⁻¹¹) (Figures 2C to 2E). Furthermore, the germinated pip5k4 pollen tubes were shorter and exhibited a significantly higher percentage of abnormal morphologies, namely, partial loss of polarity and deformed and/or branch-like growth (Figure 2F and sections below for further details). In wild-type pollen, the frequency of abnormal morphologies was found to be 11.8% (n = 423), a percentage that increased to 41.4% in pip5k4 cells (n = 461). These observations suggested that PIP5K4 is important for pollen germination and plays a crucial role in the maintenance of tip growth. To confirm this phenotype, we evaluated two pip5k4 T-DNA insertion lines complemented with a construct expressing the full-length cDNA of PIP5K4 driven by its own promoter (kindly provided by Lee et al., 2007). Upon confirmation of the T-DNA insertion and of the complementation, pollen from both lines was grown and phenotype rescue was observed. Germination efficiency was restored to values near those of the wild type (Figure 2E; P = 0.175), and no significant morphological differences (i.e., in length and shape) could be found between wild-type and complemented pollen (Figure 2G).

An analysis of pollen from pip5k5 homozygous flowers revealed no significant differences compared with wild-type pollen with regards to shape, size, and germination efficiency. Furthermore, genetic analysis of the progeny of pip5k5 heterozygous plants showed that the pip5k5 allele segregated at the expected Mendelian ratio. Thus, further studies focused on PIP5K4 mutant plants only.

**Figure 1.** Expression Pattern of the Arabidopsis Pollen-Specific PIP5K4.

(A) and (B) Analysis of PIP5K4 expression pattern by promoter-reporter (GUS) fusions. High GUS activity was detected in pollen grains and pollen tubes confirming the Genevestigator database analysis. Similar observations were made for PIP5K5 and PIP5K6 promoter-reporter GUS fusions. Bars = 1 mm in (A) and 10 μm in (B).

(C) Complementation of temperature-sensitive mss4 yeast mutants. Arabidopsis pollen-specific PIP5K4 was tested for the ability to complement temperature-sensitive mss4 mutants. mss4 mutant cells and wild-type MSS4 yeast cells were streaked on Cm-Trp plates and incubated at the permissive temperature (23°C) or the restrictive temperature (37°C). mss4 and MSS4 cells transformed with the empty pGBT9 plasmid were used as negative and positive controls, respectively. Unlike mss4 cells, mss4 mutant cells expressing PIP5K4 grew at the restrictive temperature, complementing the phenotype. Similar observations were made for PIP5K5 and PIP5K6 genes (see Supplemental Figure 1 online).
Disruption of PIP5K4 Causes a Strong Male Transmission Defect

Despite the defect in in vitro pollen tube growth, the siliques produced by pip5k4 homozygous mutant plants were similar to those of wild-type plants. A comparison of silique length, seed count, incidence of seed gaps, and incidence of deformed seeds revealed no significant differences between pip5k4 and wild-type plants (Figure 2H). However, the impairment of pollen germination and pollen tube growth observed in vitro suggests that the pip5k4 mutation may result in decreased pollen fitness and competitiveness in vivo. Such alterations should result in a distorted transmission rate of the pip5k4 and wild-type alleles to the progeny of heterozygous pip5k4 (+/−) plants. Hence, we performed a genetic analysis of self-fertilized pip5k4 (+/−) plants to evaluate if the mutant allele is transmitted to progeny in the expected 1:2:1 Mendelian ratio. Genotyping of individual plants in the progeny populations was achieved by PCR, using sets of primers annealing to DNA within the T-DNA sequence and on either side of the insertion site. We observed reduced transmission of the mutant allele to the progeny (9.5% of homozygous plants, n = 200; χ² = 99.63; P < 0.001), suggesting a potential defect in either male or female gametophyte (Table 1). To assess whether the defect was due to a problem in the male and/or female gametophytes, reciprocal outcrosses were performed between plants heterozygous for the pip5k4 allele and wild-type plants. When pollen of a mutant heterozygote was used to pollinate wild-type stigmas, there were significantly fewer heterozygotes in the progeny than predicted by Mendelian genetics (Table 2). This indicates a strong and unequivocal male-specific transmission defect in reciprocal outcrosses and an important role for PIP5K4 in pollen function. Most interestingly, when wild-type pollen was used to pollinate pip5k4 (+/−) heterozygous plants, the progeny demonstrated a small deviation from the expected 1:1 Mendelian ratio of heterozygotes to the wild type.

Figure 2. Characterization of the pip5k4 Mutant Phenotype.
The observation that mutant pollen was able to germinate when applied directly to the stigma and to achieve fertilization suggests that the female tissue attenuates the pip5k4 in vitro phenotype. Using the same T-DNA insertion line, Lee et al. (2007) showed that the stomata phenotype they observed could be reverted by the addition of extracellular PtdIns(4,5)P2. We thus investigated whether the same treatment could rescue the pollen tube mutant phenotype. For this purpose, we germinated pip5k4 pollen grains in medium supplemented with 5 μM PtdIns(4,5)P2 coupled to a shuttle carrier (DeWald et al., 2005). To confirm that PtdIns(4,5)P2 was loaded into the cells, a fluorescent version of this hydrophobic formulation was used (see Methods for further details) (Figure 4A). Observations 8 h after sowing revealed that 5 μM extracellular PtdIns(4,5)P2 had no significant effect on wild-type pollen tubes. However, both the pollen germination percentage and the pollen tube growth rate of pip5k4 cells was restored to values that were not significantly different from those of the wild-type control (Figure 4B). Furthermore, pip5k4 pollen tubes loaded with PtdIns(4,5)P2 did not show abnormal morphologies (e.g., branched-like growth and partial loss of polarity), and a blind test analysis could not distinguish between wild-type and pip5k4 pollen tubes supplemented with PtdIns(4,5)P2.

### Table 2. Segregation Analysis of the pip5k4 Mutant Allele: Outcross of pip5k4 Heterozygous Plants

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<td>120</td>
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increasingly useful in exploring the endo- and exocytosis mechanisms in a variety of biological models, including pollen tubes (Parton et al., 2001; Camacho and Malhó, 2003). FM1-43 is a nontoxic, water-soluble dye, virtually nonfluorescent in aqueous medium, and is believed to insert into the outer leaflet of the surface membrane, where it becomes fluorescent. Since the dye stains membranes in an activity-dependent manner, it has proven useful for studies of vesicle recycling, exocytosis, and endocytosis (Cochilla et al., 1999). Confocal imaging of growing Arabidopsis wild-type pollen tubes labeled with the styryl dye FM1-43 for 10 to 15 min revealed a fluorescent staining similar to what had been reported for pollen of other species (Parton et al., 2001; Camacho and Malhó, 2003; Zonia and Munnik, 2008), with a stronger signal in the plasma membrane and an intracellular apical gradient indicative of higher vesicle density in this region (Figure 5A). By contrast, pip5k4 pollen tubes, imaged under the same acquisition settings, had a weaker signal both in the plasma membrane and intracellularly (Figure 5B). The apical fluorescence gradient was partially dissipated (Figure 5C; see Supplemental Figure 3 online), and in cells exhibiting loss of polarity and/or branched-like growth, it was not detectable. A detailed analysis of the intracellular and plasma membrane fluorescent ratios in the apical and subapical regions (Figure 5D) further revealed that pip5k4 pollen tubes mostly differ from the wild type in the amount of internalized dye. The significance of the data presented was confirmed by the $t$ test ($P = 5.94 \times 10^{-14}$). These observations suggest that disruption of PIP5K4 inhibits proper endocytosis and membrane recycling, which may account for the morphological disturbances exhibited by pip5k4 mutant pollen tubes.

**PIP5K4 Concentrates at the Flanks of the Growing Pollen Tube Tip**

The subcellular localization of the PIP5K4 protein was investigated using a PIP5K4-GFP C-terminal fusion construct containing the complete open reading frame of the PIP5K4 gene. The cDNAs were cloned downstream of the Lat52 promoter (Twell et al., 1990). Wild-type Arabidopsis plants were stably transformed either with pLat52:GFP or with a pLat52:PIP5K4-GFP construct, and pollen tubes expressing fluorescent proteins were imaged using confocal laser microscopy. Control pollen tubes, expressing free GFP, showed a uniform distribution of GFP throughout the cytoplasm (see Supplemental Figure 4A online). By contrast, the fluorescence mainly localized to the plasma membrane and intracellularly (Figure 5B). The apical fluorescence gradient was partially dissipated (Figure 5C; see Supplemental Figure 3 online), and in cells exhibiting loss of polarity and/or branched-like growth, it was not detectable. A detailed analysis of the intracellular and plasma membrane fluorescent ratios in the apical and subapical regions (Figure 5D) further revealed that pip5k4 pollen tubes mostly differ from the wild type in the amount of internalized dye. The significance of the data presented was confirmed by the $t$ test ($P = 5.94 \times 10^{-14}$). These observations suggest that disruption of PIP5K4 inhibits proper endocytosis and membrane recycling, which may account for the morphological disturbances exhibited by pip5k4 mutant pollen tubes.
pollen with the same pLat52:GFP and pLat52:PIP5K4-GFP constructs. This strategy had already been successfully used in previous works (Gu et al., 2005). Tobacco pollen is easily transformed using biolistics, the tubes grow faster than those previously found that, in pollen tubes, modulation of the levels of PIP5K4 is essential for normal growth and maintenance of polarity. In support of this hypothesis, we found that overexpression of PIP5K4 also modifies the apical localization of PtdIns(4,5)P2 levels by PIP5K4 is essential for normal growth and maintenance of polarity. In support of this hypothesis, we found that overexpression of PIP5K4 also modifies the apical localization of PtdIns(4,5)P2 (mapped with the PH pleckstrin homology domain fused to red fluorescent protein [RFP]) (see Supplemental Figure 6 online). However, these data must be critically interpreted due to possible cytotoxic effects in cotransformation experiments (see Supplemental Figure 6 online for further details).

**DISCUSSION**

Phosphoinositides are key players in plant cell signaling, and we previously found that, in pollen tubes, modulation of the levels of PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$ are crucial for apical growth and thus for angiosperm fertilization (reviewed in Malhó, 2006). Here, we extended those observations and describe the genetic and molecular role of the PIP5 kinase PIP5K4 in the growth of pollen tubes in Arabidopsis. Our data are complemented with heterologous expression in tobacco pollen for detailed cellular analysis. The requirement of tight regulation of PIP5K4 activity in the plasma membrane for normal pollen tube development indicates that the localized production and activity of PtdIns(4,5)P$_2$ are essential for the maintenance of polarity.

**A Male Gametophytic Mutant Phenotype Is Associated with Disruption of the PIP5K4 Gene**

PIP5K4 is one of three PIP5 kinases described in databases as being pollen specific (e.g., http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We confirmed that this protein is highly expressed in pollen grains and germinated pollen tubes and that it encodes a functional enzyme, as shown by yeast mutant associated with the plasma membrane, and often the apical region of the plasma membrane exhibited the most intense fluorescent signal (Figures 6D and 6E). This was concomitant with perturbations of polar growth, similar to those observed in Arabidopsis PIP5K4 depleted pollen tubes, including partial loss of polarity, apical cell wall thickening, and inconsistent tube diameter. Equivalent phenotypes were observed in pollen tubes cotransformed with separate pLat52-PIP5K4 and pLat52-GFP constructs (see Supplemental Figure 5 online), thus indicating that fusing PIP5K4 to GFP did not affect its function.

**Overexpression of PIP5K4 Also Disturbs Membrane Recycling**

To further investigate membrane secretion and recycling in tobacco pollen tubes, pollen tubes overexpressing high levels of PIP5K4-GFP fusion protein were loaded with FM4-64 (for 10 to 15 min) and imaged in dual-detection mode. FM4-64 was used instead of FM1-43 to prevent the fluorescence spectrum from overlapping with that of GFP. As previously shown (Castanho Coelho and Malhó, 2006), FM1-43 and FM4-64 generate similar data in confocal imaging. In growing nontransformed pollen tubes, FM4-64 reveals a strong membrane signal and an apical gradient (Figure 7A). Overexpression of PIP5K4 seems to diminish dye uptake and to cause dissipation of the apical gradient (Figures 7B and 7D), suggesting that regulation of PtdIns(4,5)P$_2$ levels by PIP5K4 is essential for normal growth and maintenance of polarity. In support of this hypothesis, we found that overexpression of PIP5K4 also modifies the apical localization of PtdIns(4,5)P$_2$ to suggest that fusing PIP5K4 to GFP did not affect its function.

**Figure 4. Growth Rate of *pip5k4* Mutant Pollen Grains Is Rescued by PtdIns(4,5)P$_2$.**

(A) Confocal imaging of a wild-type and a *pip5k4* pollen tube grown (for 8 h) in the presence of extracellular fluorescently labeled PtdIns(4,5)P$_2$ (5 µM). The images indicate that both types of pollen tubes incorporate the phosphoinositide. Images are projections of optical sections representative of five independent experiments. Bar = 10 µm.

(B) In vitro growth rate of wild-type and homozygous *pip5k4* pollen tubes 8 h after germination in growth medium. Supplementing the medium with 5 µM PtdIns(4,5)P$_2$ increased growth rates of *pip5k4* pollen tubes to values not significantly different from those of the wild type. Error bars (SD) are derived from five independent experiments (n > 200 pollen tubes from each genotype). Germination percentage is also restored to values similar to those of wild-type plants. Similar results were obtained with unlabeled PtdIns(4,5)P$_2$.
phenotype rescue assays (Figure 1). Recently, Lee et al. (2007) tested whether PIP5K4 exhibits PIP5 kinase activity in vitro by assessing the capacity of purified PIP5K4 protein fused to glutathione S-transferase (GST) to use PtdIns4 as a substrate. Their findings indicated that PIP5K4-GST was able to phosphor-ylate PtdIns(4)P to produce PtdIns(4,5)P₂. In the same study, the authors genetically complemented the T-DNA line described here (SALK_001138) by transforming pip5k4 plants with a construct expressing the full-length cDNA of PIP5K4 driven by its own promoter and observed a phenotype rescue.

In vitro tests showed that pip5k4 pollen grains displayed a lower germination percentage and shorter pollen tubes than those of the wild type. We also observed abnormal morphologies in pollen tubes with partial loss of polarity, deformations, and occasional branched-like growth (e.g., exhibiting multiple bifur-cations). The decrease in pip5k4 mutant pollen germination efficiency in vitro might be due to the fact that the media fall short of mimicking the in vivo conditions. Arabidopsis has dry stigma-type flowers, and it is known that the pollen coat is important in adhesion (Zinkl et al., 1999) and facilitates pollen hydration for germination (Johnson and Lord, 2006). Although the mechanisms involved in pollen–stigma interactions are not totally understood, our results indicate that in pistils the pollen mutant phenotype is partly overcome. Silique analysis revealed no differences in silique length and seed set in pip5k4 homozygous siliques, showing that, if given the optimal conditions, mutant pip5k4 pollen grains can still germinate and accomplish their function. However, further insight into the role of PIP5K4 in pollen tube growth demonstrated a partial transmission defect of the pip5k4 allele (see Supplemental Table 1 online for further details). We hypothesize that this transmission defect was due to a competitive disadvantage of the mutant gametophyte compared with the wild type, similar to previously isolated mutants in Arabidopsis (Lalanne et al., 2004; Cole et al., 2005). However, reciprocal outcrosses suggested that disruption of the PIP5K4 gene also mildly affects the female gametophyte. Lee et al. (2007) detected PIP5K4 transcripts in isolated guard cells, and it is possible that transcription of the gene occurs in specific cells of the female tissue (e.g., in cells of the transmitting tract). Further investigation on this topic might thus require RT-PCR analysis of dissected female tissues.

The competitive disadvantage of the pip5k4 pollen could arise from defects in germination, tube growth, and/or tube guidance from the stigma to the ovule. To test this, we performed a same conditions and settings as in (A). Each line represents measurements of the fluorescent signal along the transect depicted in the diagram (starting at the plasma membrane) for 40 different pollen tubes. For the sake of clarity, error bars are not displayed. Supplemental Figure 3 online represents the variability found in pip5k4 pollen tubes.

Figure 5. Endocytosis in Wild-Type and pip5k4 Mutant Arabidopsis Pollen Tubes.

(A) Confocal imaging (~2 μm thick optical section) of a growing wild-type pollen tube (grown for 8 h) and labeled with FM1-43 for 10 to 15 min. Along with a high signal in the plasma membrane, fluorescence accumulates in the cell apex, forming a gradient as reported for other species. Bar = 10 μm.

(B) A pip5k4 pollen tube imaged under the same conditions and settings as in (A). The mutant phenotype is evident and fluorescence is largely confined to the plasma membrane. Bar = 10 μm.

(C) Average fluorescence intensity (FI) in the first 20 μm of growing wild-type and pip5k4 pollen tubes loaded with FM1-43 and imaged under the same conditions and settings as in (A). Each line represents measurements of the fluorescent signal along the transect depicted in the diagram (starting at the plasma membrane) for 40 different pollen tubes. For the sake of clarity, error bars are not displayed. Supplemental Figure 3 online represents the variability found in pip5k4 pollen tubes.

(D) Ratio of the FM1-43 fluorescent signal between distinct regions of wild-type and pip5k4 pollen tubes (n = 40): apical area (Ap) versus subapical area (sub-Ap); apical plasma membrane (Ap memb) versus subapical plasma membrane (sub-Ap memb); apical plasma membrane versus apical area; and subapical plasma membrane versus subapical area (measurements were made in the lines/circles depicted in the diagram).
time-course analysis of pollen tube growth in wild-type pistils pollinated with either wild-type or pip5k4 pollen grains. We found that pip5k4 pollen was able to germinate at similar rates to the wild-type pollen when evaluated 4 h after pollination. However, the growth rate of mutant pollen tubes was much slower than that of wild-type pollen, with pip5k4 pollen tubes requiring about twice the amount time (42 h after pollination) to fully traverse the pistil (Figure 3). These observations are consistent with the observed reduced transmission of the mutant allele to the offspring. Thus, the reduced growth rate of the mutant pollen tube does not prevent a self-crossed pip5k4 homozygote from producing full-length siliques filled with viable seed, but apparently becomes a detrimental factor when the mutant pollen must compete with wild-type pollen. In an outcross with pollen from a heterozygote, the impaired competitive capacity resulted in a nonuniform distribution of seeds bearing the mutant allele in the resultant siliques, with the mutant allele being less prevalent at the end of the siliques farthest from the stigma.

Along with a reduced germination percentage, we observed that in vitro–grown pip5k4 pollen tubes exhibit perturbations of tip growth that could be rescued by addition of extracellular PtdIns(4,5)P2 (Figure 4). Lee et al., (2007) also observed that external addition of 10 μM PtdIns(4,5)P2 but not PtdIns4P or PtdIns(3,4)P2 reverted delayed stomatal opening in this same SALK_001138 line. These results strongly suggest that PIP5K4, and its product PtdIns(4,5)P2, are required for germination and polar growth and thus for pollen competitiveness. The differences highlighted by in vitro and in vivo experiments also alert us to the possible redundant function of other PIP5 kinase genes not silenced in the mutant plants. This could explain why pollen germination and growth was only partially impaired. In support of this hypothesis, preliminary observations of pollen from the double mutant pip5k4 pip5k5 revealed an incapacity to germinate in vitro (see Supplemental Figure 7 online).

**PIP5K4 Deletion Inhibits Membrane Recycling**

Polarized pollen tube growth involves the rapid and localized exocytosis of secretory vesicles and coupled membrane recycling (Holdaway-Clarke and Hepler, 2003; Malhó et al., 2005). Time-lapse imaging of the endocytic uptake of the membrane dye FM4-64 (Parton et al., 2001, 2003) indicated that excess plasma membrane material is retrieved by endocytosis at the flanks of the pollen tube tip and rapidly recycled to the secretory system. Further studies on this process using pulse-chase labeling with FM1-43 and FM4-64 (Moscatelli et al., 2007; Zonia protein seemed to accumulate mostly at the flanks of the plasma membrane near the pollen tip. Insets are bright-field images of the same pollen tube apex displayed on the left. Numbers refer to the time interval (in seconds). Bar = 10 μm.

**Figure 6.** Subcellular Localization of the PIP5K4-GFP Fusion Protein in Tobacco Pollen Tubes.

(A) to (C) Time-course series of a growing tobacco pollen tube transiently transformed with the AtPIP5K4-GFP construct showing the subcellular localization of PIP5K4 (observation made 6 h after transformation). The protein seemed to accumulate mostly at the flanks of the plasma membrane near the pollen tip. Insets are bright-field images of the same pollen tube apex displayed on the left. Numbers refer to the time interval (in seconds). Bar = 10 μm.

(D) and (E) Pollen tubes expressing high levels of AtPIP5K4-GFP exhibited disturbed growth and phenotypes similar to pip5k4 Arabidopsis pollen tubes. Observations made 8 h after transformation show that the protein delocalizes to the tip of the pollen tube, which is accompanied by apical cell wall thickening (arrowheads). Bars = 10 μm.
and Munnik, 2008) suggested the existence of a second internalization mechanism at the very apex.

Here, we found that, in comparison with the wild type, pip5k4 pollen tubes exhibited a lower FM fluorescent signal, most notably at the intracellular level. This observation is consistent with lower internalization rates and perturbed secretion. PtdIns(4,5)P2 helps to mark the plasma membrane as the appropriate target for vesicle fusion (Wenk and De Camilli, 2004), and its levels are known to positively correlate with the pool of secretory vesicles (Di Paolo et al., 2004; Gong et al., 2005). In pollen tubes, PtdIns(4,5)P2 was detected at the growing apex (Kost et al., 1999; Dowd et al., 2006), and it is thus likely that the effects observed in pip5k4 mutant pollen tubes result from lower amounts of PtdIns(4,5)P2 causing a decrease in membrane recycling and, consequently, in vesicle fusion. This hypothesis is supported by the phenotype rescue observed upon addition of extracellular PtdIns(4,5)P2 (Figure 4). It is also in agreement with the observations of impaired root hair growth in PIP5K3 mutant plants (Kusano et al., 2008; Stenzel et al., 2008), suggesting a critical role of these PIP5 kinases in tip growth. Moreover, we have shown (Monteiro et al., 2005) that PtdIns(4,5)P2 mediates calcium responses that, in turn, modulate secretion. Hence, a decrease in PtdIns(4,5)P2 metabolism may contribute to changes in intracellular calcium in pip5k4 pollen tubes. In an analogous way, lower PtdIns(4,5)P2 levels are likely to affect actin and actin binding proteins (Xiang et al., 2007), which are implicated in membrane internalization pathways (Di Paolo et al., 2004).

**Subcellular Localization of PIP5K4 Is Consistent with a Role in Polar Growth**

The perturbation of the normal membrane recycling machinery exhibited by pip5k4 pollen tubes is also consistent with the morphologies observed in such cells. Indeed, all the phenotypes recorded (partial loss of polarity, changes in tip diameter, and...
branched-like growth) are intimately associated with apical membrane recycling and delivery of wall material. Imaging of a GFP-tagged PIP5K4 allowed us to correlate such phenotypes with the subcellular localization of the protein and its role in polar growth. We found that, in growing pollen tubes, GFP-tagged PIP5K4 was mostly associated with the plasma membrane specifically at the flanks of the pollen tube tip. However, when the tubes slowed down (or eventually stopped growing) the protein seemed to be delocalized to the extreme apex of the tube. In a recent work, Stenzel et al. (2008) also reported that root hair–expressed PIP5K3, when bombarded into pollen tubes, localized at the apex of partly depolarized cells. Although the aim was only to assess the PIPKinase effect on PtdIns(4,5)P2 levels, it nevertheless supports our observations. This PIPKinase mobility indicates that these proteins are tightly involved in the regulation of polar growth by fine-tuning the PtdIns(4,5)P2 metabolism at the cell apex. In two parallel reports, Dowd et al. (2006) and Helling et al. (2006) showed that PLC exhibited a more widespread but also mobile distribution, suggesting that pollen tubes maintain a spatial segregation of these proteins to regulate (and confine) phosphoinositide levels. A coupled action between these mobile PIP kinases and PLCs could account for the putative higher concentration of PtdIns(4,5)P2 at the extreme apex; the activity of PLC would prevent its accumulation in older regions of the plasma membrane, thereby reinforcing the PtdIns(4,5)P2 gradient as suggested by Helling et al. (2006). Changes (up or down) in the expression and/or activity levels of PIP5K4 could then occur concomitantly with partial loss of polarity, oscillations in growth rates, and the redirection of pollen tube growth [e.g., as we demonstrated with the photorelease of caged PtdIns(4,5)P2 in Agapanthus umbellatus (Monteiro et al., 2005)]. Interestingly, we observed that tobacco pollen tubes overexpressing the GFP-tagged PIP5K4 also exhibited partial loss of polarity and occasional branched-like growth. In parallel to our observations, Kusano et al. (2008) reported that when a PIP5K3 gene was ectopically expressed in wild-type plants, an abnormality was also found in the morphology of root hair cells. Multiple protruding sites or hair structures on the surface of a single trichoblast were often found, which were absent from wild-type roots and in addition to straight long root hairs; winding root hairs were also observed in some lines. In our tobacco pollen tube observations, the apical regions often exhibited what seemed to be thick deposits of wall material, possibly resulting from a perturbed secretion (Figures 6D and 6E). Preliminary data obtained with ruthenium red staining indicate that this material is mostly of pectin origin (see Supplemental Figure 8 online). This is consistent with continued vesicle fusion that is uncoupled from adequate membrane recycling. This unbalance can also help to explain the branched-like growth. When PIP5K4 levels rise above a threshold, perturbations start occurring; these include reduction in growth rate, altered deposition of wall material, and loss of apical polarity. The larger volume of the expanded apical dome could allow the appearance of subdomains where PIP5K4 levels are brought down to near normal levels (either by spatial segregation or protein turnover). This, in turn, could lead to formation of new polar axes and the emergence of a new normal tip, which would extend until PIP5K4 levels surpass the threshold. An extreme example of an aberrant morphology consistent with this hypothesis is shown in Supplemental Figure 9 online.

Phosphoinositides Are Involved in Defining Functional Zones in Pollen Tubes

Underlying the structural zonation described for pollen tubes is the concept of a continuous flux of components (i.e., proteins and wall precursors) toward the apex. However, recent observations of vesicle dynamics (Parton et al., 2003; Castanho Coelho and Mählö, 2006; Moscatelli et al., 2007; Zonia and Munnik, 2008) suggest that most of the secretory vesicle processing is confined to the subapical and apical region, with fusion taking place mainly at the apical flanks and recycling at the apex and subapex involving two distinct subpopulations of endocytic vesicles. Similar observations have been made in root hairs (OVECKA et al., 2005; VOIGT et al., 2005), where the endocytic markers were shown to rapidly recycle between the endosomes, the trans-Golgi network and the plasma membrane. Budding profiles of the trans-Golgi network were suggested to invade the root hair apex (PREUSS et al., 2006), and similar structures were observed in pollen tubes (MAHLÖ et al., 2005). Interestingly, such a fusion-recycling loop superimposes several other events that can enforce a positive feedback and thus maintain a highly polarized domain. A proton influx-efflux that maintains an electric dipole has been described for these apical/subapical regions (HEPLER et al., 2006; CERTAL et al., 2008). Oscillations in cytosolic free calcium concentration ([Ca2+]c) are also confined to the apical and subapical regions (Holdaway-Clarke and Hepler, 2003), and recent findings on GTPase localization (HWANG et al., 2005), the presence of an exocyst complex (COLE et al., 2005), and actin dynamics (YOKOTA and SHIMMEN, 2006) further suggest that the apical and subapical regions contain all the necessary enzymatic machinery to maintain growth. PtdIns(4,5)P2 is known to intersect with all of these signaling pathways (KOST et al., 1999; MONTEIRO et al., 2005; XIANG et al., 2007), and our data confirm that the spatial distribution of enzymes such as PIP5K4 may help to define the functional zonation of growing pollen tubes. Further elucidation of the regulation of PtdIns(4,5)P2 levels will require the analysis of other pollen-expressed PIP kinases, namely, PIPK10 and PIPK11. These kinases have no MORN domain and will certainly be important in the cytoplasmic regulation of PtdIns(4,5)P2 subdomains.

METHODS

Analysis of Promoter Activity

For PIP5K4 promoter isolation, a 2.0-kb fragment (pPIP5K4, PIP5K4 Promoter) upstream of the translation initiation codon of the PIP5K4 gene was amplified from Arabidopsis thaliana Columbia-0 (Col-0) genomic DNA with forward (5′-CCCCGATCCCCATTTGAGAGCAGTACAATG-3′) and reverse (5′-CCCCCATGGCTTCTTAAACTAATAAAAC-3′) primers carrying BamHI and HindIII restriction sites (underlined, respectively). The PCR fragment was cloned upstream of the GUS reporter gene in pCAMBIA1301 Ti-derived binary vector (Cambia) carrying the hygromycin resistance marker. Plant transformation was performed using the floral dip method (Clough and Bent, 1998). Seeds from transformed
Complementation of Temperature-Sensitive mss4 Yeast Mutants

The coding regions of pollen-expressed PIP5 kinases (4, 5, and 6) were isolated by PCR with the following sets of primers (PIP5K4 forward 5'-CCCCCGGCAGGACATGCGAGGACAAAACG-3' and reverse 5'-CCCCCCCTGCATTACTTATCGAGGAACC-3'; PIP5K5 forward 5'-CCCCCGGCAGGACATGCGAGGACAAAACG-3' and reverse 5'-GGGGGTGCACTCATGTACGTAGAAGACC-3'; PIP5K6 forward 5'-CCCCCGGCAGGACATGCGAGGACAAAACG-3' and reverse 5'-GGGGGTGCACTCATGTACGTAGAAGACC-3'). The PCR fragments were cloned into the yeast expression vector pGBT9 (Clontech) and transformed into the mss4 temperature-sensitive mutant (kindly provided by Yoshikazu Ohya, University of Tokyo, Japan). As a positive control, MSS4 wild-type cells were transformed with the empty pGBT9 vector, and as a negative control, the mutant mss4-1 cells were transformed with the empty pGBT9 vector. For temperature-sensitive mutant complementation, cells were plated on Cm-Trp plates and grown at 23°C (permissive temperature) and 37°C (restrictive temperature).

Plant Growth Conditions

All experiments were performed with Arabidopsis ecotype Col-0. Plants were grown in soil at 22°C with a 16-h-light/8-h-dark photoperiod. For the selection screening for hygromycin-resistant plants, seeds were surface-sterilized for 1 min in 70% ethanol, followed by 10 min incubation in 30% (v/v) bleach containing 0.5% Tween 20 with occasional mixing, and washed five times in sterile distilled water. Seeds were germinated and grown on half-strength Murashige and Skoog plates containing 1% agar. Plants were grown at room temperature with 100% relative humidity. All experiments were performed with Arabidopsis ecotype Col-0. Plants were grown in a greenhouse under standard conditions. Mature pollen was collected and germinated in culture as described by Kost et al. (1999).

Identification of pip5k Mutants

Arabidopsis lines with T-DNA insertions in PIP5K4 and PIP5K5 genes in a Col-0 background were obtained from the Nottingham Arabidopsis Stock Centre (pip5k4, SALK_001138; pip5k5, SALK_147475.30.40). A PCR-based approach was used to identify homozygous lines. PCR genotyping was performed using three primers: a primer to the left border of the T-DNA insertion (Lba1 5'-TGGTTCACTGAGGTGGCC-3'), a pair of PIP5K4-specific primers (PIP5K4-RP 5'-TGAACGGTACTTATTATATGACGC-3' and PIP5K4-FL 5'-AACGACCTCTATTATATATGCAGG-3'), and a pair of PIP5K5-specific primers (PIP5K5-FL 5'-ACAAAGATTTCCTACAGAGAAC-3' and PIP5K5-RP 5'-AATTTCATTTCAGATTGCGAAGAG-3'), and homozygous lines were used for further analysis.

To test whether pip5k4 and pip5k5 homozygous mutant plants generate PIP5K4 or PIP5K5 transcripts, respectively, RT-PCR was performed. Total RNA from inflorescences, flowers, leaves, and roots was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed with the gene primer sets: PIP5K4 forward 5'-CCCTGATGCGAGGACAAAACG-3' and PIP5K4 reverse 5'-CCCCCTGCTTATTCTTACTGAGGAACC-3' and PIP5K5 forward 5'-CCCTGATGCGAGGACAAAACG-3' and PIP5K5 reverse 5'-CCCCCTGCTTATTCTTACTGAGGAACC-3'. As a positive control for RT-PCR, ACTIN8 transcript levels were also analyzed in the same tissues with the primer set: ActIN8Fw 5'-ACCTTTGCTGTGTGCCATTATTGC-3' and ActIN8Rev 5'-GATCCGCCGCTGAGGACAAAACG-3'. The PCR products were separated on 1% agarose gel and visualized with ethidium bromide.

Characterization of pipk4 Mutant Phenotype

To assay in vitro pollen germination, wild-type and pip5k4 pollen grains were collected and cultured as described by Li et al. (1999). The pollen grains were grown at room temperature with 100% relative humidity. From each culture, at least 200 pollen grains were examined to calculate an average germination rate, and 50 pollen tubes were measured to calculate average pollen tube length. As a control for the pip5k4 mutation, we used complemented T-DNA insertion lines with a construct expressing the full-length cDNA of PIP5K4 driven by its own promoter (kindly offered by Y. Lee; see Lee et al. [2007] for further details). Pollen collected from these plants was grown and analyzed as described above.

To perform the pip5k4 mutant phenotype rescue by PtdIns(4,5)P2, pollen germination medium was supplemented with 5 μM PtdIns(4,5)P2, 5 μM ShuttlesPIP Carrier-1, histone H1 (Molecular Probes) was mixed with PtdIns(4,5)P2, and allowed to equilibrate for 5 min. Then, the PtdIns(4,5)P2 Shuttle complex was added to liquid pollen germination medium, and PtdIns(4,5)P2 delivery was confirmed using BODIPY tetramethyl-romamine-XC6-PtdIns(4,5)P2 (Molecular Probes).

Outcroses were performed by applying pollen from newly dehisced flowers onto the stigmas of flowers that had been surgically emasculated just prior to dehiscence. Col-0 was used as a wild-type tester in outcroses. Silique evaluation was performed by harvesting 105 green expanded siliques from selfed pip5k4 homozygous mutant and wild-type adult plants. The siliques were cleared in 70% ethanol for 24 h and then examined under a dissecting microscope.

Aniline blue staining was performed in pre-emasculated mature wild-type flowers pollinated either with wild-type pollen or pip5k4 pollen. The pollinated pistils were collected 4, 24, and 42 h after pollination and stained as described by Sumie et al. (2001). The stained pistils were observed with an Olympus BX-51 epifluorescence microscope (Labocontrole) and photographed with a 5 Mpx Olympus Camedia digital camera.

Bright-Field Imaging

Bright-field and/or differential interference contrast images were acquired with a PCO Sensicam-QE camera (Labocontrole) attached to an Olympus IX-50 microscope using an Olympus X40 Plan dry (NA = 0.85) objective. The intervals between image acquisitions and the exposure times were controlled by Image Pro Plus 5.0 software (Media Cybernetics).

FM Labeling and Confocal Imaging

Arabidopsis and tobacco pollen tubes were labeled with FM1-43 or FM4-64, as described before (Camacho and Malho, 2003; Castanho-Coelho and Malho, 2006). Briefly, pollen tubes were labeled with 2 μM FM solution (Molecular Probes) prepared in pollen tube growth medium and observed 10 to 15 min after labeling. Thin time-course optical sections (~2 μm thick) were acquired with a Leica SP-E confocal laser scanning microscope using <20% laser intensity and operating in the mode 512 × 512, 400 Hz (~1/4 s per frame). A ×20 Plan Apo dry objective (NA = 0.75) or a ×40 Plan dry (NA = 0.85) (Leica) was used. For quantification purposes, gain and offset settings were kept constant. Spectral settings were such as to prevent bleed-through between channels. Fluorescence was quantified in terms of average pixel intensity (0 to 255 scale for 8-bit images) and statistically analyzed with a Student’s t test (two-tailed distribution).
Transient Transformation and PIPK4-GFP Imaging in Tobacco Pollen Tubes

The PIP5K4 coding cDNA fragment without the stop codon was amplified from Col-0 genomic DNA with forward (5′-CCCAGGGCTCATGAGCAAGGAACAAAGCTG-3′) and reverse (5′-CCCCCGCGGCGCGGAT-TATCCCTAGTGAAGAC-3′) primers carrying Apal and NotI restriction sites (underlined), respectively. The resulting DNA fragment was cloned into the pGreen pLat52-GFP vector (Hellens et al., 2000) in frame with the 5′ end of the GFP coding sequence, resulting in a PIPK4-GFP fusion coding sequence driven by the pLat52 promoter (Twell et al., 1990). As a control, we used the PIP5K4 coding cDNA fragment with the stop codon cloned into the pGreen pLat52 vector (Hellens et al., 2000), resulting in the PIP5K4 gene being driven by the pLat52 promoter (Twell et al., 1990). cDNA fragments encoding the PH domain (amino acids 2 to 175) of human PLC-δ1 (Stauffer et al., 1997) were introduced into the pollen tube expression vector described above at the C terminus of yellow fluorescent protein or RFP to generate plasmids that allow the expression of fusion proteins in pollen tubes.

A helium-driven PDS-1000/He particle delivery system (Bio-Rad) was used for the biolistic transformation of tobacco pollen as described by Kost et al. (1999). Two micrograms of plasmid DNA was bombarded per sample. When two plasmids were cotransformed, particles were coated with 5 μg of DNA (2.5 μg of each plasmid DNA). Pollen grains were then allowed to germinate and transferred onto cover slips for microscopic imaging; since no quantification was intended in this case, the detector gain was adjusted to provide pixel intensity values between 100 and 200 (for an 8-bit image).

Data Analysis

Numerical data extraction was performed using Image-Pro Plus 5.0 software. Fluorescence measurements presented correspond to medium fluorescence intensity in the first 0 to 10 μm and 10 to 20 μm of the pollen tube cytoplasm and/or plasma membrane (apical and subapical regions, respectively). Schematic drawings in Figure 5 indicate regions and areas of measurements. Except where mentioned, numerical data in figures correspond to average measurements performed in that region of the cell. For clarity, error bars were not displayed in Figure 5. For statistical measurements of germination percentage, growth rates, phenotype, and seed count, a χ² test was applied (values from the wild type were considered as “expected” with P < 0.01 and 1 degree of freedom).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PIP5K4 (At3g56960), PIP5K5 (At2g41210), and PIP5K6 (At3g07960).

Supplemental Data

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

Supplemental Figure 1. Complementation of Temperature-Sensitive mss4 Yeast Mutants with Arabidopsis Pollen-Specific PIP5K5 and PIP5K6.

Supplemental Figure 2. Cytological Assays of Wild-Type and pip5k4 Pollen.

Supplemental Figure 3. Variability of FM1-43 Fluorescence Intensity in pip5k4 Pollen Tubes.

Supplemental Figure 4. Subcellular Localization of PIP5K4-GFP Fusion Protein in Arabidopsis Pollen Tubes.

Supplemental Figure 5. Phenotype of Tobacco Pollen Tube Cotransformed with PIP5K4 and GFP Constructs.

Supplemental Figure 6. Tobacco Pollen Tubes Transformed with YFP-PH and PIP5K4-GFP/RFP-PH Constructs.

Supplemental Figure 7. In Vitro Germination of Wild-Type, pip5k4, and pip5k4/pip5k5 Pollen.

Supplemental Figure 8. Tobacco Pollen Tube Expressing the PIP5K4-GFP Fusion Protein and Poststained with Ruthenium Red.

Supplemental Figure 9. Extreme Phenotype of Tobacco Pollen Tube Overexpressing PIP5K4-GFP Fusion Protein.

Supplemental Table 1. Segregation Analysis of Progeny of Selfed Heterozygous Mutant Plants and Reciprocal Crosses between Wild-Type Plants and Heterozygous Mutant Plants.

ACKNOWLEDGMENTS

We thank Youngsook Lee for the generous gift of complemented SALK_001138 T-DNA insertion line and Luı́sa Camacho for critical reading of the manuscript. This work was supported by Fundação Ciência e Tecnologia, Lisboa, Portugal (Grant BCI/37555/2001; FEDER). E.S. also acknowledges Fundação Ciência e Tecnologia for a PhD grant (SFRH/BD/17480/2004).

Received February 15, 2008; revised October 19, 2008; accepted November 9, 2008; published November 25, 2008.

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Arabidopsis Phosphatidylinositol-4-Monophosphate 5-Kinase 4 Regulates Pollen Tube Growth and Polarity by Modulating Membrane Recycling

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Plant Cell 2008;20;3050-3064; originally published online November 25, 2008;
DOI 10.1105/tpc.108.058826

This information is current as of December 14, 2017

Supplemental Data  /content/suppl/2008/11/21/tpc.108.058826.DC1.html
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