The Type B Phosphatidylinositol-4-Phosphate 5-Kinase 3 Is Essential for Root Hair Formation in *Arabidopsis thaliana*  

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Root hairs are extensions of root epidermal cells and a model system for directional tip growth of plant cells. A previously uncharacterized *Arabidopsis thaliana* phosphatidylinositol-4-phosphate 5-kinase gene (*PIP5K3*) was identified and found to be expressed in the root cortex, epidermal cells, and root hairs. Recombinant *PIP5K3* protein was catalytically active and converted phosphatidylinositol-4-phosphate to phosphatidylinositol-4,5-bisphosphate [*PtdIns(4,5)P2*]. *Arabidopsis* mutant plants homozygous for T-DNA–disrupted *PIP5K3* alleles were compromised in root hair formation, a phenotype complemented by expression of wild-type *PIP5K3* cDNA under the control of a 1500-bp *PIP5K3* promoter fragment. Root hair–specific *PIP5K3* overexpression resulted in root hair deformation and loss of cell polarity with increasing accumulation of *PIP5K3* transcript. Using reestablishment of root hair formation in T-DNA mutants as a bioassay for physiological functionality of engineered *PIP5K3* variants, catalytic activity was found to be essential for physiological function, indicating that *PtdIns(4,5)P2* formation is required for root hair development. An N-terminal domain containing membrane occupation and recognition nexus repeats, which is not required for catalytic activity, was found to be essential for the establishment of root hair growth. Fluorescence-tagged *PIP5K3* localized to the periphery of the apical region of root hair cells, possibly associating with the plasma membrane and/or exocytotic vesicles. Transient heterologous expression of full-length *PIP5K3* in tobacco (*Nicotiana tabacum*) pollen tubes increased plasma membrane association of a *PtdIns(4,5)P2*-specific reporter in these tip-growing cells. The data demonstrate that root hair development requires *PIP5K3*-dependent *PtdIns(4,5)P2* production in the apical region of root hair cells.

INTRODUCTION

The uptake of nutrients from the soil into plant roots is facilitated by the formation of root hairs maximizing the root surface area. Root hair formation is enhanced by nutrient-limiting conditions, for instance, in response to limiting iron or inorganic phosphate (Raghothama, 1999; Gilroy and Jones, 2000; Muller and Schmidt, 2004), and may enable plants to colonize otherwise nutrient-restrictive environments. Root hairs form as cytoplasmic protrusions extending from root epidermal cells (Galway et al., 1994) and, besides pollen tubes, are a model system for the study of polar tip growth in plant cells.

The definition of sites of root hair initiation and genetic and cell biological events driving hair cell elongation has been the focus of previous studies (Schiefelbein, 2000; Pesch and Huiskamp, 2004; Samaj et al., 2004; Sieberer et al., 2005; Fischer et al., 2006). The cellular machinery for polar tip growth of root hairs involves the actin cytoskeleton (Voigt et al., 2005), regulatory mitogen-activated protein kinases (Samaj et al., 2002), a variety of regulatory GTP binding proteins (Preuss et al., 2004), and factors required for vesicle trafficking (Yuen et al., 2005; Song et al., 2006). Cytoskeletal structures, including microtubules, help stabilize the elongated hair behind the actively growing tip region and define the area where the plasma membrane is expanded and cell wall material is deposited (Sieberer et al., 2005).

To establish controlled polar tip growth, processes involved in root hair elongation require tight spatial and temporal regulation. Although it is not clear how the various elements of the complex growth machinery are orchestrated, it has been proposed that the polyphosphoinositide, phosphatidylinositol-4,5-bisphosphate [*PtdIns(4,5)P2*], may play a role in the control of root hair growth (Braun et al., 1999; Vincent et al., 2005). *PtdIns(4,5)P2* can affect multiple physiological processes in all eukaryotic cell types studied so far by interacting with various protein partners that are regulated in their biochemical activity or localization (Stevenson et al., 2000; Mueller-Roeber and Pical, 2002; Meijer and Munnik, 2004; Suh and Hille, 2005), plant phospholipase D (Qin et al., 2002), the actin-modifying enzymes profilin, cofilin, and gelsolin (Drobak et al., 1994; Lemmon et al., 2002; Doughman et al., 2003; Wasteneys and Galway, 2003; Wenk and De Camilli, 2004), and mammalian SNARE complex proteins (Vicogne et al., 2006), implying a role for *PtdIns(4,5)P2* in vesicle fusion (Cremona and...
PI4P 5-Kinase 3 and Root Hair Development

PI4P 5-kinases (isoforms 1 to 9) are unique to plants in containing a large enzyme family with regulatory function in pollen tubes (Kost et al., 1999), and PtdIns(4,5)P_2 has been visualized in a plasma membrane microdomain of the pollen tube tip (Kost et al., 1999). As in pollen tubes, the presence of PtdIns(4,5)P_2 in the plasma membrane has previously been demonstrated for the root hair cell tip (Braun et al., 1999), and phosphoinositides have been postulated to contribute to the control of root hair development (Braun et al., 1999; Vincent et al., 2005; Preuss et al., 2006). So far, however, the enzymes and corresponding genes responsible for generating PtdIns(4,5)P_2 with regulatory function in pollen tubes or root hairs have not been identified.

This study addresses the role of PtdIns(4,5)P_2 formation as a requirement for root hair growth. PtdIns(4,5)P_2 is formed by phosphorylation of its more abundant precursor lipid, phosphatidylinositol-4-phosphate (PtdIns4P), in the D-5 position of the inositol ring, a reaction catalyzed by PtdIns4P 5-kinases (PI4P 5-kinases) (Drobak et al., 1999; Mueller-Roeber and Pical, 2002). The Arabidopsis thaliana genome contains 11 genes with similarity to animal PI4P 5-kinases (Mueller-Roeber and Pical, 2002), only two of which have been experimentally characterized in detail and been shown to encode enzymes with PI4P 5-kinase activity in vitro (Elge et al., 2001; Perera et al., 2005). Plant PI4P 5-kinases, including amino acid sequences deduced from putative and uncharacterized genes, can be classified into types A and B (Mueller-Roeber and Pical, 2002). The smaller type A enzymes (isoforms 10 and 11) exhibit a domain structure similar to that of animal and human PI4P 5-kinases, whereas type B kinases (isoforms 1 to 9) are unique to plants in containing a large additional N-terminal extension that includes several membrane occupation and recognition nexus (MORN) repeats (Mueller-Roeber and Pical, 2002). MORN repeats are found in various proteins of both animal and plant origin that mediate protein membrane contacts, such as the Arabidopsis ARC3 protein involved in plastidial fission (Shimada et al., 2004; Maple et al., 2007) or junctophilins, which mediate endomembrane-to-plasma membrane attachment in mammalian cells (Takeushima et al., 2000). A recent report indicates that the N terminus of Arabidopsis PI4P 5-kinase isoform 1 (PIP5K1) has multiple regulatory effects on enzyme activity and may guide subcellular localization of the enzyme (Im et al., 2007). PI4P 5-kinases from different organisms have been reported to associate with different subcellular locations, including the plasma membrane of plants, yeast, and mammals (Perera et al., 1999; Heilmann et al., 2001; Kobayashi et al., 2005; Santarius et al., 2006), the nucleus of yeast and mammals (Ciruela et al., 2000; Audhya and Emr, 2003; Santarius et al., 2006), the actin cytoskeleton of plants, yeast, and mammals (Desvirieres et al., 1998; Doughman et al., 2003; Davis et al., 2007), and endomembranes of plants and mammals (Whatmore et al., 1996; Heilmann et al., 1999; Im et al., 2007).

It has recently been reported that Arabidopsis plants carrying a T-DNA insertion in the gene encoding the putative PI4P 5-kinase isoform, PIP5K9, have elevated transcript levels for this gene, correlated with overall reduced root length (Lou et al., 2007). No enzymatic activity was demonstrated for a PIP5K9 gene product in that study, and the restriction of the described phenotype to roots has not been rationalized in light of the fact that the disrupted gene is ubiquitously overexpressed throughout the plant (Lou et al., 2007). Here, we show that a different member of the Arabidopsis PI4P 5-kinase family, PIP5K3, is expressed in root epidermal cells and root hairs and that recombinant PIP5K3 is catalytically active as a PI4P 5-kinase in vitro. Both under- and overexpression of the PIP5K3 gene result in severe disturbance of root hair directional growth and formation of short or morphologically aberrant root hairs, respectively. The data presented indicate that the formation of PtdIns(4,5)P_2 by PIP5K3 in the apical region of root hair cells is essential for polar tip growth.

RESULTS

PIPK5K3 Is Expressed in Root Epidermis Cells and Root Hairs

In preparation of experiments to define possible physiological roles of Arabidopsis PI4P 5-kinases, transcript array information publicly accessible through the Genevestigator portal (Zimmermann et al., 2004) was reviewed for the tissue-specific expression patterns of all members of the Arabidopsis PI4P 5-kinase family (Mueller-Roeber and Pical, 2002). Based on the Genevestigator data available at the onset of this study, PIP5K3 was identified as the only Arabidopsis PI4P 5-kinase gene with exclusive expression in roots, suggesting a specific function in root development and making PIP5K3 the focus of this investigation. Direct experimental verification of root-specific expression patterns of PIP5K3 was achieved by RT-PCR (Figure 1A) and by expression of fusions of a 1500-bp fragment of the 5' untranslated region upstream of the PIP5K3-coding region with a β-glucuronidase (GUS) reporter, followed by histochemical staining of transgenic plant lines for GUS activity (Figures 1B to 1K). The respective promoter GUS expression patterns were also tested for other PI4P 5-kinase isoforms suggested by Genevestigator analysis to be ubiquitously expressed, including in roots, and are described and discussed in Supplemental Figure 1 and Supplemental Text and References online). The promoter GUS experiments confirmed root-specific expression of the PIP5K3 gene (Figures 1B and 1C) and revealed that the PIP5K3 promoter is active in cells of the root cortex and epidermis (Figures 1D to 1F) and in root hairs (Figure 1G). Only weak or no staining was detected in the vascular tissue (Figure 1D), as evident especially in root cross sections (Figures 1E and 1F). No GUS staining was observed with the PIP5K3 promoter fragment in organs other than roots, as indicated in Figures 1H to 1K.
Arabidopsis PI4P 5-Kinase Isoforms Expressed in Roots Are Active and Catalyze the Conversion of PtdIns4P to PtdIns(4,5)P2

A prerequisite for further genetic and phenotypic studies is the characterization of catalytic activity of the PIP5K3 protein or other putative PI4P 5-kinase isoforms present in roots. Because the PIP5K3 gene has not yet been functionally characterized, PIP5K3 was heterologously expressed in Escherichia coli and the recombinant protein tested for activity in vitro. The catalytic activity of recombinant PIP5K3 and of some engineered PIP5K3 derivatives relevant to experiments described in Figures 3 to 5, 7,
activity, whereas the activity of the other PIP5K3 derivatives tested, only full-length PIP5K3 and described below (pip5k3-4). Of the PIP5K3-derived proteins pip5k3-4 by the T-DNA–disrupted allele were also tested (Figure 2) included a truncated PIP5K3 variant lacking the N-terminal domains (ΔNT-MORN), a PIP5K3 derivative with mutated ATP binding site (K442A), a PIP5K3 derivative mutated in three conserved Ser residues in the catalytic domain for Ala residues, respectively. (B) Catalytic activity in recombinant E. coli extracts, as indicated. Concentrations of recombinant proteins expressed in E. coli were balanced according to protein gel blot analysis. Data represent the means of three to four independent experiments ± SD.

and 9 are given in Figure 2. Proteins derived from PIP5K3 that were also tested (Figure 2) included a truncated PIP5K3 variant lacking the N-terminal domains (ΔNT-MORN), a PIP5K3 derivative with mutated ATP binding site (K442A), a PIP5K3 derivative mutated in three conserved positions of the catalytic site (S673A/S680A/S682A; termed TripleA), and a truncated protein encoded by the T-DNA–disrupted allele pip5k3-4 used for mutant studies described below (pip5k3-4). Of the PIP5K3-derived proteins tested, only full-length PIP5K3 and ΔNT-MORN had catalytic activity, whereas the activity of the other PIP5K3 derivatives tested was reduced to the limits of detection (Figure 2B). A minor activity phosphorylating PtdIns3P to PtdIns(3,4)P2 was observed for all full-length Arabidopsis PI4P 5-kinases tested, whereas no activity was detected against PtdIns5P (Table 1). In addition, the activities of root-expressed PI4P 5-kinase isoforms 1, 2, 7, 8, and 9 with the preferred substrate, PtdIns4P, were tested and are given in Supplemental Figure 2 online. All proteins were recombinantly expressed as fusions to N-terminal maltose binding protein (MBP) tags. Note that catalytic activity was not precluded by the N-terminal addition of an MBP tag (Figure 2).

Figure 2. Catalytic Activity of Arabidopsis PIP5K3 and Derived Proteins.
Proteins were recombinantly expressed in E. coli as fusions to N-terminal MBP tags and were tested for their ability to convert PtdIns4P to PtdIns(4,5)P2 in vitro. (A) Schematic representation of PIP5K3 and derived proteins tested. NT, N terminus (PIP5K31-49); MORN, MORN repeat domain (PIP5K350-227); Lin, linker domain (PIP5K3228-307); Dim, dimerization domain (PIP5K3308-381); Cat, catalytic domain (PIP5K3382-705). Arrowheads in K442A and TripleA indicate mutagenized amino acid positions exchanging a catalytic Lys or Arg for Ala residues, respectively. (B) Catalytic activity in recombinant E. coli extracts, as indicated. Concentrations of recombinant proteins expressed in E. coli were balanced according to protein gel blot analysis. Data represent the means of two independent experiments ± SD.

T-DNA Disruption of the PIP5K3 Gene Locus (At2g26420) Reduces Root Hair Growth
To identify the physiological role of the PIP5K3 gene, a loss-of-function approach was pursued. A search of the Salk Institute Genomic Analysis Laboratory collection for T-DNA mutants of the PIP5K3 gene locus (At2g26420) identified several independent T-DNA insertions (Figure 3A) that were screened for homozygosity. Homozygosity was inferred by the inability to amplify the wild-type allele of PIP5K3 by PCR concomitant with positive amplification of the T-DNA–tagged allele (see Supplemental Figure 3 online). Insertion lines SALK_060590 and SALK_001546, hereafter referred to as pip5k3-1 and pip5k3-2, carry T-DNA insertions in the 5′-untranslated region 248 and 48 bp upstream of the PIP5K3-coding region, respectively. Insertion lines SALK_000024 and SALK_026683 carry insertions in the third intron or the sixth exon, respectively, and will be referred to as pip5k3-3 and pip5k3-4 in the following text. The positions of the T-DNA insertions were confirmed by sequencing of the corresponding genomic loci. To test for altered PIP5K3 transcript abundance in the T-DNA insertion lines, RNA gel blot experiments were performed; however, PIP5K3 transcript levels were too low to detect. The more sensitive RT-PCR analysis (Figure 3B) and quantitative real-time RT-PCR analysis (Figure 3C) indicated that the insertion mutants pip5k3-2 and pip5k3-4 had severely reduced PIP5K3 transcript levels compared with the wild type, whereas only moderate transcript reduction was observed in pip5k3-1 and pip5k3-3 plants. Homozygous pip5k3-4 plants were chosen for a detailed characterization of the T-DNA–disrupted transcript, and it was established that the corresponding truncated mRNA encoded a truncated protein, pip5k3-4, that lacks the extreme C terminus, including the activation loop region (AL) involved in substrate binding (Kunz et al., 2000), and was catalytically inactive after recombinant expression in E. coli (Figure 2B).

Correlated to the reduction in PIP5K3 transcript levels (Figure 3C), all homozygous T-DNA insertion mutants exhibited reduced root hair growth (Figure 4). Compared with wild-type plants (Figure 4A), the reduction in root hair growth was most severe in the lines pip5k3-2 (Figure 4C) and pip5k3-4 (Figure 4E) and less

Table 1. Relative Activities of Arabidopsis PI4P 5-Kinase Isoforms Expressed in Roots

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PtdIns3P</th>
<th>PtdIns4P</th>
<th>PtdIns5P</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>2 ± 0.3</td>
<td>4 ± 0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PIP5K1</td>
<td>5 ± 0.8</td>
<td>205 ± 17</td>
<td>–</td>
<td>41.0</td>
</tr>
<tr>
<td>PIP5K2</td>
<td>52 ± 8</td>
<td>1206 ± 156</td>
<td>–</td>
<td>23.0</td>
</tr>
<tr>
<td>PIP5K3</td>
<td>178 ± 27</td>
<td>1972 ± 66</td>
<td>–</td>
<td>11.0</td>
</tr>
<tr>
<td>PIP5K7</td>
<td>2 ± 0.3</td>
<td>37 ± 6</td>
<td>–</td>
<td>18.5</td>
</tr>
<tr>
<td>PIP5K8</td>
<td>3 ± 0.5</td>
<td>27 ± 3</td>
<td>–</td>
<td>9.0</td>
</tr>
<tr>
<td>PIP5K9</td>
<td>4 ± 0.6</td>
<td>142 ± 23</td>
<td>–</td>
<td>35.5</td>
</tr>
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Catalytic activities were tested in vitro against different phosphatidylinositol-monophosphate substrates and indicate the rate of product formation in fmol min⁻¹. Recombinant protein concentrations were balanced according to protein gel blot analysis. Data are the means of two independent experiments ± SD.
Abundance of full-length transcript levels. Arrows indicate the position of primers used for RT-PCR analysis of transcript levels detected in wild-type plants and represent the mean ± SE of two independent experiments. 

pronounced in pip5k3-1 (Figure 4B) and pip5k3-3 (Figure 4D), as summarized in Figure 4F.

Root Hair Development Is Reestablished in PIP5K3 T-DNA Insertion Mutants Expressing Full-Length PIP5K3

The observation that the loss of PIP5K3-transcript was correlated with reduced root hair growth (Figures 3 and 4) was unexpected because the expression of five other active PI4P 5-kinase isoforms in roots (see Supplemental Figures 1 and 2 online) had suggested some functional redundancy. To verify that the observed reduction in root hair growth was indeed due to the disruption of the PIP5K3 gene, complementation of the pip5k3-4 mutant phenotype (Figures 4E and 4F) was attempted using full-length PIP5K3 or various PIP5K3 derivatives, as indicated (Figure 5). The complementation constructs contained the respective cDNA clones behind the 1500-bp PIP5K3 promoter fragment used for the GUS expression experiments shown in Figure 1 and encoded the respective proteins as fusions to N-terminal enhanced yellow fluorescent protein (EYFP) tags. Complementation was evaluated in comparison to nontransformed wild-type plants (Figure 5A) and to pip5k3-4 mutants (Figure 5B) grown in parallel. The genotypes of the plant lines used and the presence of correctly sized transcripts corresponding to the ectopically introduced PIP5K3 constructs were determined by PCR and RT-PCR, respectively (Figure 5G). Expression of PIP5K3 in the pip5k3-4 mutant resulted in reestablishment of normal root hair growth (Figure 5C), and root hairs were at an average even longer than those of wild-type controls (Figure 5H). Using the reestablishment of root hair growth in the pip5k3-4 mutant (cf. Figure 3) as a bioassay, the functionality of the PIP5K3-derived proteins was tested. The truncated ΔNT-MORN or the inactive K442A or TripleA proteins did not reestablish root hair formation in the pip5k3-4 background (Figures 5D to 5F), as summarized in Figure 5H. Root hair stubs forming with expression of K442A (Figure 5E) appeared thinner than those seen in other transgenics. As the levels of the introduced proteins in root hairs were too low to be detected by EYFP fluorescence or by immunoblotting, expression of the transgenes was verified by monitoring transcript levels for the various PIP5K3 derivatives by quantitative real-time RT-PCR (Figure 5I), indicating that the expression levels of the EYFP-tagged PIP5K3 transgenes introduced into the pip5k3-4 mutant did not exceed the level of PIP5K3 expression in wild-type plants by more than an average of ~30%.

Aberrant Root Hair Growth and Morphology with Overproduction of PIP5K3

The underexpression and complementation data for the PIP5K3 gene (Figures 4 and 5, respectively) indicated that PIP5K3 was an essential regulator of root hair development and led us to ask whether root hair formation would also be disturbed by overproduction of EYFP-tagged PIP5K3. The stronger root hair-specific promoter of Arabidopsis expansin 7 (EXP7) (Cho and Cosgrove, 2002) was used to drive ectopic overexpression of PIP5K3 in root hairs of wild-type Arabidopsis plants. Compared with nontransformed wild-type plants (Figure 6A), increasing degrees of overexpression (Figures 6B to 6D) resulted in increasingly deformed root hairs. From the highly polar appearance in wild-type plants (Figure 6E) root hair morphology was altered with increasing PIP5K3 overexpression toward thicker hairs (Figure 6F), curling hairs (Figure 6G), and, with the highest degree of overexpression, globular structures (Figure 6H), indicating a gradual loss of cellular polarity. Increased PIP5K3 transcript levels in the overexpressor lines were documented independently by quantitative
real-time RT-PCR (Figure 6I) and by RT-PCR (Figure 6I, inset). Data shown are from representative transgenic lines. Morphological changes as those reported were found to correlate with transcript accumulation in eight of eight independent transgenic lines.

**Effects of Ectopic Expression of K442A and Truncated ΔNT-MORN on Root Hair Morphology**

Because under- and overexpression of PIP5K3 both affected root hair morphology, the effects of overexpressing the inactive K442A protein or the truncated ΔNT-MORN protein were tested.

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**Figure 4. Reduced Root Hair Growth of T-DNA Insertion Mutants of PIP5K3.**

Plants were grown on agar plates for 8 d, and digital images were taken.

(A) to (E) Representative root hair phenotypes. Bars = 100 μm.

(A) Wild-type plants.

(B) pip5k3-1 mutant.

(C) pip5k3-2 mutant.

(D) pip5k3-3 mutant.

(E) pip5k3-4 mutant.

(F) Quantification of root hair length in the wild type and in plants carrying T-DNA insertions, as indicated. The asterisks indicate significantly reduced root hair length compared with wild-type controls according to a Student’s t test (*, P < 0.05; **, P < 0.01; n > 300).
Figure 5. Root Hair Phenotypes of pip5k3-4 Plants Ectopically Expressing PIP5K3 or Derived Proteins.

Complementation of the pip5k3-4 mutant phenotype was tested by ectopic expression of PIP5K3 or various derived proteins under a 1500-bp fragment of the intrinsic PIP5K3 promoter in the pip5k3-4 background. 

(A) and (B) Root hair phenotypes of wild-type plants (A) and pip5k3-4 mutant plants (B) grown in parallel. 

(C) to (F) Root hair phenotypes of pip5k3-4 plants with expression of PIP5K3 (C), ΔNT-MORN (PIP3228-705; [D]); K442A (E), and TripleA (F). Bars = 200 μm. Images are representative for results obtained with at least five independent transgenic lines.

(G) The presence of the T-DNA insertions was shown by PCR using genomic DNA as a template and a combination of primers specific for the T-DNA.
Both proteins were expressed as fusions to the fluorescence tag RedStar. Root hairs of nontransformed wild-type plants (Figure 7A) and of plants expressing RedStar alone (Figure 7B) did not differ in length. The introduction of RedStar-tagged K442A under the EXP7 promoter resulted in root hair deformation and branching (Figure 7C). Overexpression of RedStar-tagged ΔNT-MORN protein (Figure 7D) resulted in a phenotype similar to that observed with K442A. Note that ΔNT-MORN was catalytically active (Figure 2B) but lacks an N-terminal domain of unknown physiological role. Phenotypes were observed in eight out of eight (K442A) and six out of six (ΔNT-MORN) transgenic lines; however, root hairs were never globally altered, and all plants also had root areas with wild-type-like root hairs. The presence or absence of K442A and ΔNT-MORN transcripts of the correct sizes was verified by RT-PCR (Figure 7E). The PIP5K3 expression levels in the control lines and the overexpressors were determined by quantitative real-time RT-PCR (Figure 7F) and were found to be substantially increased over wild-type PIP5K3 transcript levels. Note that the real-time RT-PCR does not allow discrimination between the wild-type allele and the mutated or truncated variant of the PIP5K3 gene. The data are consistent with dominant-negative effects resulting from overexpression of K442A or ΔNT-MORN.

Expression of PIP5K3 Increases Plasma Membrane Levels of PtdIns(4,5)P2

To determine whether plasma membrane PtdIns(4,5)P2 levels were influenced by the expression of PIP5K3 in tip-growing plant cells, PIP5K3 and K442A were each transiently expressed in tobacco (Nicotiana tabacum) pollen tubes, together with the RedStar-tagged Pleckstrin homology (PH) domain of the human PLCδ1 (HsPLCδ1-PH domain), which specifically recognizes PtdIns(4,5)P2 (Varnai and Balla, 1998) (Figure 9). Pollen tubes were chosen instead of root hairs because the system is similar to root hairs in many respects, it can easily be transiently transfected by particle bombardment, and its autofluorescence is negligible. The active and inactive proteins both localized to the apical plasma membrane of the pollen tube tip in a pattern resembling the distribution of EYFP-PIP5K3 in root hair cells (see Supplemental Figure 4 online; compare Figures 8B and 8D). The HsPLCδ1-PH domain reporter (Figures 9A and 9B, middle panels) also localized to the apical plasma membrane of the pollen tubes, as previously reported (Kost et al., 1999; Dowd et al., 2006). The right panel in Figure 9A illustrates roughly equal intensities of PIP5K3 and HsPLCδ1-PH domain, resulting in a yellow color on the merged image, whereas unequal intensities of K442A and HsPLCδ1-PH domain shown in Figure 9B result in green plasma membrane fluorescence of the merged image. When fluorescence intensities were quantified (Figure 9C), the fluorescence distribution of RedStar-HsPLCδ1-PH with coexpression of active PIP5K3 was clearly plasma membrane associated (Figure 9C, top right panel), whereas equivalent expression of the inactive K442A resulted in a more diffuse distribution of the PtdIns(4,5)P2 reporter (Figure 9C, bottom right panel), indicating that a higher proportion of the reporter remained in the cytoplasm. While synchronous imaging of PIP5K3-derived proteins...
with the HsPLCδ1-PH domain was successful in pollen tubes (Figure 9), no interpretable images were obtained in corresponding experiments on root hairs because of interfering autofluorescence at the low HsPLCδ1-PH expression levels required for meaningful interpretation.

**DISCUSSION**

The data presented indicate an essential role for the PI4P 5-kinase PIP5K3 in the development of root hairs in *Arabidopsis*. The requirement of PIP5K3 for root hair formation must be reviewed in light of its catalytic activity and its capability to generate PtdIns4P$_2$ (Figure 2). The in vitro activity of recombinant PIP5K3 protein indicates a clear preference for the conversion of PtdIns4P to PtdIns(4,5)P$_2$ (Table 1), which is consistent with the presence of a conserved Glu residue (Glu-668) in the AL of PIP5K3 implicated in determining PI4P 5-kinase specificity (Kunz et al., 2000). A low but detectable activity of PIP5K3 and other isoforms against PtdIns3P (Table 1) is consistent with side activities previously reported for PIP5K1 (Elge et al., 2001) and PIP5K10 (Perera et al., 2005), which also exhibit minor activity with this substrate. For the ubiquitous PIP5K1, enhanced activity

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**Figure 6.** Aberrant Root Hair Morphology with Overproduction of PIP5K3.

The wild-type PIP5K3 allele was expressed under the root-hair-specific *EXP7* promoter in wild-type plants.

(A) Root hair morphology of wild-type controls grown in parallel.

(B) to (D) Overexpressor lines shown were chosen to document morphological alterations with increasing levels of PIP5K3 transcript.

(E) to (H) Magnifications of root hairs exhibiting morphologies characteristic for wild-type plants (E) or for plants with increasing levels of PIP5K3 expression (F) to (H). Bars = 200 μm in (A) to (D) and 100 μm (E) to (H).

(I) PIP5K3 transcript accumulation was monitored independently by real-time RT-PCR, using a primer combination amplifying a 64-bp fragment of PIP5K3 as described above and by RT-PCR-analysis (inset) using a primer combination amplifying the full-length PIP5K3 transcript. Real-time RT-PCR data are given relative to transcript levels detected in wild-type plants and represent the mean ± se of two independent experiments. PIP5K3, transcript levels; ACT8, control. A to D refer to panels above. All plants were analyzed 8 d after germination. Altered root hair morphology correlated to PIP5K3 expression levels was observed in eight out of eight transgenic lines analyzed.
was reported with removal of the MORN domain of that protein, indicating an autoinhibitory function for the MORN domain (Im et al., 2007). In our in vitro activity tests with the PIP5K3-derived ΔNT-MORN protein, no such enhancement of PI4P 5-kinase activity was observed (Figure 2B). Possible explanations include that autoinhibitory effects previously described (Im et al., 2007) were specific for PIP5K1 or that in this study the MBP tag N-terminally attached to the MORN domain of PIP5K3 interfered with its regulatory functions. The latter scenario appears unlikely, however, in light of the observation that in vivo functionality of PIP5K3 was given with expression of PIP5K3 fused to an N-terminal EYFP reporter (Figure 5C), which is roughly equal in size and bulk to the MBP tag. The activity tests demonstrate the importance of the extreme C-terminal end of the PIP5K3 protein, including the AL, for catalytic activity because the truncated pip5k3-4 protein was not active (Figure 2B). The loss of catalytic activity with exchange of three conserved Ser residues in the TripleA mutant (Figure 2B) attributes importance to the extreme C-terminal portion of the PIP5K3 protein and suggests PIP5K3 positions Ser-673, Ser-680, and Ser-682 as candidate sites for posttranslational regulation.

The results of in vitro biochemical characterization of PIP5K3 and its engineered variants (Figure 2) were in congruence with evidence from genetic studies and complementation tests. Independent T-DNA insertion mutants for the PIP5K3 gene, foremost those homozygous for alleles pip5k3-2 and pip5k3-4, were compromised in root hair development (Figure 4), indicating that the observed effects on root hair growth were due to altered expression of PIP5K3, rather than to additional T-DNA insertions elsewhere in the genome. This notion is further supported by the fact that PIP5K3 T-DNA insertion mutants ectopically expressing the wild-type allele of PIP5K3 under its intrinsic promoter exhibited wild-type-like root hair growth (Figure 5C) and that overexpression of the inactive K442A exerted subtle but reproducible dominant-negative effects on root hair growth (Figure 7C). The observation that elimination of a single gene encoding one of 11 PI4P 5-kinases (Figures 3 and 4), six of which are expressed in roots (shown for PIP5K2 and PIP5K9 in Supplemental Figure 1 online) and are all catalytically active (Table 1; see Supplemental Figure 2 online), nonetheless resulted in a severe phenotype indicates that other PI4P 5-kinases, such as PIP5K9 also expressed in root hairs (see Supplemental Figure 1 online), did not functionally compensate for the loss of PIP5K3 expression in regard to root hair development. This result is consistent with results presented in Figure 2 and Supplemental

Effects of K442A or ΔNT-MORN Expression on Root Hair Growth.

(A) to (D) The inactive PIP5K3-derived K442A protein or the truncated ΔNT-MORN protein was expressed in wild-type plants as fusions to N-terminal RedStar tags under the control of the EXP7 promoter. Images were taken from 8-d-old plants; the transgenic lines are representative for eight (K442A) and six (ΔNT-MORN) independent lines tested. To varying degrees, all lines also exhibited regions with unaltered root hairs. Bars = 200 μm.

(A) Wild-type control.

(B) RedStar control.

(C) and (D) Root hair morphology observed with expression of K442A (C) or ΔNT-MORN (D).

(E) RT-PCR detection of correctly sized ectopic PIP5K3 transcripts for the constructs introduced, as indicated, using a primer combination specific for the cDNA encoding the RedStar tag (sense) and for that of the extreme C-terminus of the PIPS3K3-derived expressed proteins (antisense). RedStar, RedStar transcript, as amplified with RedStar-specific primers; ACT8, actin control.

(F) Real-time RT-PCR analysis of PIP5K3 transcript levels in plant lines, as indicated, using a primer combination for the 64-bp PIP5K3 fragment described above. Dashed line, wild-type PIP5K3 transcript level. Real-time RT-PCR data are given relative to transcript levels detected in wild-type plants and represent the mean ± SE of two independent experiments. Note that the real-time RT-PCR does not distinguish between wild-type and mutated alleles of the PIP5K3 gene. All plants were analyzed 8 d after germination.
of the wild-type allele of the PIP5K3 gene resulted in full complementation of the reporter fluorescence (Figure 9C), indicating increased enzyme localizes to the extreme periphery of the apical tip region of root hair cells, we tested whether expression of PIP5K3 in pollen tubes would increase plasma membrane levels of PtdIns(4,5)P2 (Figure 9). When PIP5K3 and the inactive K442A were each coexpressed with the PtdIns(4,5)P2 reporter HsPLC1-PH, expression of the active enzyme resulted in greatly increased plasma membrane association of reporter fluorescence (Figure 9C), indicating increased localization of PIP5K3 thus remain a subject for future studies.

As results so far indicated that PIP5K3 was an active PI4P 5-kinase possibly localized to the plasma membrane or to vesicles in the apical region of growing root hair cells, we tested whether expression of PIP5K3 in pollen tubes would increase plasma membrane levels of PtdIns(4,5)P2 (Figure 9). When PIP5K3 and the inactive K442A were each coexpressed with the PtdIns(4,5)P2 reporter HsPLC1-PH, expression of the active enzyme resulted in greatly increased plasma membrane association of reporter fluorescence (Figure 9C), indicating increased levels of PtdIns(4,5)P2, consistent with our expectation based on the localization data (Figure 8) and previous data on the topic.

Figure 8. Subcellular Localization of PIP5K3 in Root Hairs.

EYFP-tagged PIP5K3 was expressed in wild-type plants under the control of the EXP7 promoter, and the fluorescence distribution was monitored in 8-d-old plants by confocal laser scanning microscopy. Bright-field and fluorescence channels were imaged synchronously. Images shown are representative for a growing root hair with vivid cytoplasmic streaming expressing EYFP-PIP5K3 under the EXP7 promoter. Bars = 20 μm. (A) Bright-field image. (B) EYFP-PIP5K3 fluorescence. (C) Merge of images (A) and (B). (D) Magnified view of a 1.0-μm confocal section through the root hair apex. The arrowhead indicates a cone-shaped distribution of EYFP-PIP5K3–decorated cytosolic particles close to the apex of the root hair cell. (E) Confocal section (1.6 μm) through the apical region of a root hair, as indicated by the dashed line in (C). Left panel, cell wall autofluorescence; middle panel, EYFP-PIP5K3 fluorescence; right panel, merged image.
Braun et al., 1999; Kost et al., 1999; Perera et al., 1999; Heilmann et al., 2001; Dowd et al., 2006; van Leeuwen et al., 2007). By contrast, plasma membrane fluorescence of the PtdIns(4,5)P$_2$ reporter was much reduced when coexpressed with the inactive K442A (Figure 9B). A low HsPLC$_d$1-PH background signal in pollen tubes expressing K442A can be attributed to the presence of PtdIns(4,5)P$_2$ formed by pollen tube endogenous PI4P 5-kinases. While corresponding experiments with root hairs were not successful because of high root hair autofluorescence, the results from the heterologous pollen tube system (Figure 9) correspond well with the in vitro characterization of PIP5K3 and K442A (Figure 2) and with the effects of the two proteins in the mutant complementation tests (Figure 5). Overall, the results presented are consistent with the hypothesis that PIP5K3 produces PtdIns(4,5)P$_2$ in the tips of growing root hair cells that is required for growth.

A number of reported functions of PtdIns(4,5)P$_2$ can be envisioned to take part in the control of directional tip growth in the apex of growing root hairs, as has been outlined in the Introduction. The loss of cellular polarity observed in Arabidopsis root hairs overexpressing PIP5K3 (Figure 6) is similar to phenotypes observed with increased PtdIns(4,5)P$_2$ levels in pollen tubes due to inactivation of PLC (Dowd et al., 2006; Helling et al., 2006), and pollen tubes strongly expressing the EYFP-PIP5K3 fusions also exhibited tip swelling and a loss of polar tip growth. The observation that expression of PIP5K3 in the pip5k3-4 background resulted in nondeformed root hairs that were slightly longer than those of wild-type plants (Figure 5G), whereas overexpression resulted in severe deformation (Figure 6), suggests that a fine balance of PIP5K3 expression may be required for normal root hair development.

In analogy to an animal model for the involvement of PtdIns(4,5)P$_2$ in synaptic exocytosis (Cremona and De Camilli, 2001), a possible explanation for reduced root hair growth in the absence of PIP5K3 is suggested by the notion that in mammalian cells the assembly of the protein machinery required for the fusion of PtdIns4P-coated vesicles with the plasma membrane depends on the formation of PtdIns(4,5)P$_2$ (Di Paolo et al., 2004; Gong et al., 2005; Milosevic et al., 2005; Vicogne et al., 2006). Compromised vesicle trafficking from the endoplasmic reticulum to the Golgi due to continuous inactivation of the monomeric GTPase, ARF1, in Arabidopsis plants has been shown to result in reduced growth of root hairs and pollen tubes (Song et al., 2006), similar to that seen in this study with disruption of the PIP5K3 gene. In combination with the reported accumulation of PtdIns4P-coated exocytotic vesicles in the tip of growing root hair cells (Preuss et al., 2006) the results presented suggest that PIP5K3 may control root hair elongation by providing PtdIns(4,5)P$_2$ required for vesicle-to-plasma membrane fusion in the apex of the hair cell, either at the surface of the plasma membrane target area or that of the fusing vesicle.

A possible alternative mechanism by which plasma membrane–associated PIP5K3 may affect root hair elongation relates to the control of F-actin dynamics in analogy to the situation in yeast, where the only PI4P 5-kinase, MSS4p, mediates the attachment of F-actin to the plasma membrane and is required for yeast budding (Desrivieres et al., 1998). In analogy to the yeast model, PIP5K3 may yield PtdIns(4,5)P$_2$ controlling F-actin-modulating

![Figure 9. Expression of PIP5K3 in Pollen Tubes Increases Plasma Membrane Levels of PtdIns(4,5)P$_2$.](image)

(A) and (B) EYFP-PIP5K3 and the catalytically inactive EYFP-K442A protein were each coexpressed in tobacco pollen tubes with the RedStar-tagged HsPLC$_d$1-PH, which specifically binds to PtdIns(4,5)P$_2$. EYFP and RedStar fluorescence (left and middle panels, respectively) were synchronously recorded by confocal laser scanning microscopy after incubation of transformed pollen for 10 h. Right panels, merged images. Yellow color indicates colocalization of EYFP and RedStar signals of equal intensity. Bars = 10 μm.

(A) PIP5K3.

(B) K442A.

(C) Quantification of relative fluorescence intensities in horizontal sections indicated in (A) and (B). Fluorescence intensities were normalized against the highest value for each scan, set as 1. Transient expression was performed under identical conditions. Images were not individually adjusted for brightness or contrast. Data presented are from a representative experiment. Of 36 transformed pollen tubes observed for PIP5K3 or K442A, 18 and 19 tubes, respectively, exhibited the pattern shown. Expression levels of the remaining tubes were either too low to visualize any RedStar fluorescence with expression of K442A or were too high for meaningful imaging.

(Braun et al., 1999; Kost et al., 1999; Perera et al., 1999; Heilmann et al., 2001; Dowd et al., 2006; van Leeuwen et al., 2007).
proteins, such as profilin or gelsolin, that may be associated with F-actin strands binding to PIP5K3 via its Lin domain (Davis et al., 2007). A lack of PIP5K3 in root hairs of the Arabidopsis mutants described may reduce directional membrane traffic by preventing F-actin polymerization at the plasma membrane.

In summary, the data presented indicate PIP5K3 as a functional component of the machinery controlling directional tip growth of root hairs. The PIP5K3 protein catalyzes the production of PtdIns(4,5)P₂ in the apical region of root hair cells and may control vesicle trafficking and/or cytoskeletal structures required for root hair growth. The identification of the PI4P 5-kinase responsible for the production of PtdIns(4,5)P₂ involved in root hair growth provides a key tool needed to determine the particular physiological processes in the root hair that are controlled by that lipid. Future work will be directed toward elucidating the exact roles of PtdIns(4,5)P₂ in the cell biology of root hair formation.

METHODS

Plant Growth Conditions

All experiments were performed with Arabidopsis thaliana ecotype Columbia-0 (Col-0). Seeds were surface-sterilized for 5 min in 75% ethanol, followed by 20 min incubation in 6% (w/v) NaOCl in 0.1% (w/v) Triton X-100, and washed five times in sterile distilled water. Before plating, seeds were vernalized at 4°C for 2 d, followed by culture on half-strength Murashige and Skoog (MS) medium including basal salt mixture (Duchefa) containing 1% (w/v) sucrose and 0.7% (w/v) agar (Duchefa) at 22°C under continuous light.

Primers

Sequences for all primers named below are provided in Supplemental Table 1 online.

Genotyping of T-DNA Insertion Lines

T-DNA insertion lines (pip5k3-1, SALK_060590; pip5k3-2, SALK_001546; pip5k3-3, SALK_000024; and pip5k3-4, SALK_026683) in the Arabidopsis Col-0 background were obtained from the Nottingham Arabidopsis Stock Centre. A PCR-based approach was used to identify homozygous lines and to confirm the exact insertion position of the T-DNA. For the analysis of the pip5k3-1 mutant and pip5k3-2 mutant, primers used were LBa1, SALK_060590_reverse, and SALK_060590_forw. For the analysis of the pip5k3-3 mutant, primers used were LBa1, SALK_000024_reverse, and SALK_000024_forw. For the analysis of the pip5k3-4 mutant, primers used were LBa1, SALK_026683_reverse, and SALK_026683_forw.

Cloning of cDNA or Genomic Constructs

PI4P 5-Kinase Coding Sequences

The cDNA sequences of Arabidopsis PI4P 5-kinase genes PIP5K1, PIP5K2, PIP5K7, PIP5K8, and PIP5K9 were amplified from cDNA prepared from young Arabidopsis inflorescences using the following primer combinations: PIP5K1, PIP5K1Nco_for/PPIP5K1Nco_rev, PIP5K2, PIP5K2Pci_for/PIP5K2Pcl_rev, PIP5K7, PIP5K7Nco_for/PIP5K7Nco_rev, PIP5K8, PIP5K8Nco_for/PIP5K8Nco_rev, and PIP5K9, PIP5K9Nco_for/PIP5K9Nco_rev. The cDNA of PIP5K3 was isolated by RT-PCR from cDNA of 14-d-old roots grown on agar plates using the primer combination PIP5K3Nco_for/PIP5K3Nco_rev. PCR-fragments were subcloned into the vector pGEM-Teasy (Promega) and sequenced. The resulting plasmids were designated as PIP5K1-pGEM-Teasy, PIP5K2-pGEM-Teasy, PIP5K3-pGEM-Teasy, PIP5K7-pGEM-Teasy, PIP5K8-pGEM-Teasy, and PIP5K9-pGEM-Teasy, respectively.

Mutagenesis of the PIP5K3 Coding Sequence

To obtain the cDNA clone encoding K442A, the PIP5K3 coding sequence was altered using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with the primer combination PIP5K3 K442A QC for/PIP5K3 K442A QC rev. The QuikChange product was cloned into pGEM-Teasy (Promega), yielding PIP5K3-K442A-pGEM-Teasy. The cDNA encoding TripleA was amplified from PIP5K3-pGEM-Teasy using the primer combination PIP5K3_loop_for/PIP5K3_loop4_rev and cloned into pGEM-Teasy, yielding the plasmid PIP5K3-TripleA-pGEM-Teasy. To create the NT-MORN construct, the PIP5K3 cDNA sequence omitting the cDNA encoding the N terminus of PIP5K3 was amplified using the primer combination AtPIP5K3Nco3_for/AtPIP5K3Nco3 rev2, and the PCR product was cloned into the vector pGEM-Teasy, yielding the plasmid NT-MORN-PIP5K3-pGEM-Teasy. The cDNA encoding the truncated pip5k3-4 protein was amplified from PIP5K3-pGEM-Teasy using the primer combination AIP5K3_Nco_for/PIP5K3_as4.

PI4P 5-Kinase Promoter Fragments

For generation of promoter-GUS fusions, the GusPlus gene was amplified from the vector pCAMBIA1305.1 (accession number AF354045) using the primer combination Gus_utr5 for/Gus_utr5 rev, and the PCR product was introduced as a NotI-SacI fragment into the vector pgreen0029 (http://www. pgreen.ac.uk/) (Hellens et al., 2000), yielding the plasmid pgreenGUSPlus. Amplification of 1500-bp genomic sequences upstream of coding sequences for use as promoters was achieved with different Arabidopsis BAC clone templates as indicated, using the following primer combinations: PromPIP5K1, PromPIP5K1_for/for PromPIP5K1_rev from BAC clone F2E2; PromPIP5K2, PromPIP5K2_for/for PromPIP5K2_rev from BAC clone T32E8; PromPIP5K3, PromPIP5K3_for/for PromPIP5K3_rev from BAC clone T3j22; PromPIP5K7, PromPIP5K7_for/for PromPIP5K7_rev from BAC clone T19D16; PromPIP5K8, PromPIP5K8_for/for PromPIP5K8_rev from BAC clone T7P1; and PromPIP5K9, PromPIP5K9_for/for PromPIP5K9_rev from BAC clone F8A24. The PCR products representing PromPIP5K1, PromPIP5K3, PromPIP5K7, PromPIP5K8, and PromPIP5K9 were moved directionally as SalI-NorI fragments into the vector pgreenGUSPlus. The PCR product representing PromPIP5K2 was moved as a NotI-NorI fragment into pgreenGUSPlus. The resulting plasmids were transformed into Agrobacterium tumefaciens strain EHA105 and used for stable Arabidopsis transformation.

PI5PK3 Overexpression Constructs

To create PIP5K3 overexpression constructs, an 800-bp fragment of the characterized promoter sequence of the EXP7 gene (At1g12560) (Cho and Cosgrove, 2002) was amplified using the primer combination PromAtEXP7_for/PromAtEXP7_rev with genomic Arabidopsis DNA as a template. The PCR product was moved as a SalI-NorI fragment into the vector puc18-Entry (Hornung et al., 2005), yielding the plasmid promEXP7_puc18entry. The complete cDNA sequence of PIP5K3 was amplified with the primer combination PIP5K3Nco_for/for PIP5K3_rev using the plasmid PIP5K3-pGEM-Teasy as a template, and the PCR product was cloned into pGEM-Teasy. The resulting plasmid was named PIP5K3-pGEM-Teasy2. The PIP5K3-cDNA was moved as a NotI-NorI fragment into the vector promEXP7_puc18entry, yielding the plasmid promEXP7_pIP5K3_puc18entry. To distinguish the overexpressed PIP5K3 clone from the endogenous PIP5K3-alleles, the cDNA sequence encoding EF1P was amplified from the plasmid pEYFP (Clontech) using the primer combination
respectively, into the bacterial expression vector

The primer combination

promPIP5K3puc18entry, yielding promEXP7-YFP-PIP5K3puc18entry. The RedStar (Janke et al., 2004) coding sequence was amplified from plasmids carrying the authentic clones obtained from Martin Fulda using the primer combination RedStar_for/RedStar_rev, cloned into pGEM-Teasy, and moved from there as a NotI-NotI fragment first into the vector prom-

EXP7-puc18entry, yielding promEXP7-RedStar-puc18entry. For overex-

pression constructs for K442A and ∆NT-MORN, the respective clones were moved as NotI-NotI fragments from the corresponding pGEM-Teasy plasmids to promEXP7-puc18entry, yielding the plasmids promEXP7- K442A-puc18entry and promEXP7-∆NT-MORN-puc18entry, respectively. Finally, the RedStar sequence without a stop codon was amplified using the primer combination RedStar_for/RedStar_rev2 and moved directly as an Ncol-Ncol fragment into promEXP7-K442A-puc18entry or promEXP7-

∆NT-MORN-puc18entry, respectively. All entry clones were moved into the vector pCambia 3300 using Gateway technology (Invitrogen) according to the manufacturer’s instructions and transformed into the A. tumefaciens strain EHA105.

Mutant Complementation Constructs

To create constructs for mutant complementation tests, the 1500-bp promoter fragment promPIP5K3 was moved as a SalI-NotI fragment into the vector pUC18-Entry (Hornung et al., 2005), yielding the plasmid promPIP5K3puc18entry. The full-length PIP5K3 cDNA sequence was moved from PIP5K3-pGEM-Teasy2 as a NotI-NotI fragment into the vector promPIP5K3puc18entry, yielding the plasmid promPIP5K3- PIP5K3puc18entry. To distinguish the introduced PIP5K3 clone from the endogenous PIP5K3 allele, the cDNA sequence encoding EYFP was moved as an Ncol-Ncol fragment in frame with the PIP5K3 cDNA into the plasmid promPIP5K3-PIP5K3puc18entry, yielding the plasmid promPIP5K3-YFP-PIP5K3puc18entry. The ∆NT-MORN, K442A, or TripleA cDNA clones were moved as NotI-NotI fragments from their respective pGEM-Teasy plasmids into the plasmid promPIP5K3puc18entry, yielding the plasmids promPIP5K3-∆NT-MORN-puc18entry, promPIP5K3-K442A-puc18entry, or promPIP5K3-TripleA-puc18entry, respectively, followed by introduction of the EYFP sequence into the Ncol site of these plasmids to produce promPIP5K3-YFP-∆NT-MORN-puc18entry, promPIP5K3-YFP-K442A-puc18entry, and promPIP5K3-YFP-TripleA-puc18entry. All entry clones were moved into the vector pCambia 3300 using Gateway technology (Invitrogen) according to manufacturer’s instructions and transformed into the A. tumefaciens strain EHA105.

Bacterial Expression Constructs

Constructs for heterologous expression in Escherichia coli were created by moving the cDNA sequences encoding PIP5K1, 3, 7, 8, and 9 as Ncol-Ncol fragments from the plasmids PIP5K1-pGEM-Teasy, PIP5K3-pGEM-Teasy, PIP5K7-pGEM-Teasy, PIP5K8-pGEM-Teasy, and PIP5K9-pGEM-Teasy, respectively, into the bacterial expression vector pETM-41 (EMBL Protein Expression and Purification Facility). The cDNA sequence of PIP5K2 was released from the plasmid PIP5K2-pGEM-Teasy as a PciI-PciI fragment and moved into the Ncol site of pETM-41. The ∆NT-MORN cDNA was moved as an Ncol-NotI fragment from the vector ∆NT-MORN-PIP5K3- pGEM-Teasy into the plasmid pETM-41. The cDNA encoding the N-terminal MORN domain (PIP5K3 639-1122) was amplified from PIP5K3-
pGEM-Teasy using the primer combination 5’-GATCCATGGAGAACGG- TGCTAAAGAAGCCG-3’/5’-GATCCATGCGCTTCTTCCCTCCACCCACCC-3’. The PCR product was cloned into pGEM-Teasy and moved from that plasmid into pETM-41 as an Ncol-Ncol fragment. The coding sequences for K442A, TripleA, and pip5k3-4 were moved as NotI-NotI fragments from the respective pGEM-Teasy plasmids into pETM-41. All clones were introduced into the pETM-41 vector in frame with the cDNA encoding the N-terminal MBP and polyhistidine (His) tags of pETM-41.

Fusion Constructs for Particle Bombardment

The cDNA clones encoding EYFP-PIP5K3, EYFP-K442A, and EYFP-

∆NT-MORN were moved as NotI-NotI fragments into the vector pENTR2b (Invitrogen). The coding sequence for the human PLCγ1-PH domain (Varnai and Balla, 1998) was amplified from plasmid DNA provided by Tamás Balla (National Institute for Child Health and Human Development, Rockville, MD) and modified to encode a seven-amino acid linker (Gly-Gly- Ala-Gly-Ala-Gly) between the PH domain and the RedStar tag, as previously described (Dowd et al., 2006), using the primer combination 5’-GATCCGCGCCGCGGTAGCGTGGACGTGGACGAGAATGAGGATCTACAGGCGC-3’/5’-GATCGATATCTTAGATCTTGCGACGCCAGCA-3’. The amplicon was moved into pENTR2b as a NotI-EcoRV fragment. The pENTR2b constructs were transferred by Gateway technology (Invitrogen) from pENTR2b to the plasmid pLatGW, an expression vector containing the tomato Lat52 promoter (Twell et al., 1990), an attR Gateway cassette, and a pA35S terminator. The pLatGW-vector was a gift from Wolfgang Dröge-Laser (University of Göttingen, Germany).

Detection of Specific Transcripts and Analysis of PIP5K3 Expression Levels by RT-PCR

Expression levels of PIP5K3 in wild-type plants and in pip5k3-1, pip5k3-2, and pip5k3-4 mutants were analyzed by RT-PCR. Total RNA was extracted from roots, stems, leaves, and flowers using Plant RNA purification reagent (Invitrogen). For removal of contaminating genomic DNA, RNA samples were incubated for 30 min at 37°C with RNase-free DNase, and the RNA was subsequently precipitated. Five micrograms of total RNA were reverse transcribed with RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) in the presence of oligo(dT) primers. For determination of relative transcript levels, equal amounts of first-strand cDNA were used as templates for PCR amplification using the primer combination PIP5K3Nco_forward/PIP5K3Nco_rev. The Arabidopsis actin8 (ACT8) gene was amplified using the primer combination AIACT8 Forward/AIACT8 Reverse and served as an internal positive control (Bustin, 2000). Truncated transcripts as shown in Figure 7H were detected using the primer combination YFP_for/PIP5K3Nco_rev. Transcript levels shown in Figure 7B were detected using the following primer combinations: RedStar_for/RedStar_rev, K442A and ∆NT-MORN, RedStar_for/PIP5K3Nco_rev.

Determination of Specific Transcript Levels by Quantitative Real-Time RT-PCR

The levels of specific transcripts in roots were determined by real-time RT-PCR analysis of cDNA reverse transcribed from 1 μg of total RNA using 100 units of Reverse Transcriptase H+ (MBI Fermentas) according to the manufacturer’s instructions. The resulting cDNA was diluted 1:10, and 1 μL was used as a PCR template in a 25-μL reaction containing 2.5 μL 10X PCR buffer (Bioline), 2 mM MgCl2, 100 μM deoxynucleotidetriphosphate, 2.5 μL QuantiTect primer mix (Qiagen), 0.1-fold Sybr green, 10 nM fluorescein, and 0.25 units of Biotaq (Bioline). Samples were denatured for 3 min at 95°C, followed by 40 cycles of 20-s denaturation at 95°C, 20 s of annealing at 55°C, and 40 s of elongation at 72°C. Fluorescence was monitored during each annealing and elongation phase. The program was concluded by 4 min of elongation at 72°C and 1 min of denaturation at 95°C. Renaturing of amplified DNA was followed by the assessment of melting parameters by increasing the temperature in 0.5°C increments while monitoring fluorescence. In addition to the individual QuantiTect primers used, a DNA fragment of the PP2A subunit

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of PDF2 (At1g13320) was used as an internal reference. Transcript levels were calculated according to Livak and Schmittgen (2001).

Heterologous Expression in *E. coli*

Recombinant enzymes were expressed in *E. coli* strain BL21-AI (Invitrogen) at 25°C for 18 h after induction with 1 mM isopropylthio-β-galactoside and 0.2% (w/v) L-arabinose. The PIPS3-MORN domain was expressed in *E. coli* Rosetta II cells (Novagen) at 25°C for 4 h after induction with 1 mM isopropylthio-β-galactoside. Cell lysates were obtained by sonication in a lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol, pH 8.0.

Lipid Kinase Assays

Lipid kinase activity was assayed by monitoring the incorporation of radiolabel from [γ-32P]ATP into defined lipid substrates according to Cho and Boss (1995) using total extracts of BL21-AI expression cultures. Recombinant expression levels were adjusted between individual cultures according to immunodetection of expressed MBP-tagged proteins (data not shown), allowing for the comparison of enzyme activities obtained with different cultures. Radiolabeled lipid reaction products were separated by thin layer chromatography and visualized by autoradiography using Kodak X-OMAT autoradiography film (Eastman Kodak). Reaction products were identified according to comigration with authentic standards (Avanti Polar Lipids). Phosphatidylinositol-bisphosphate bands were scraped according to autoradiography, and incorporated radiolabel was quantified using liquid scintillation counting (Analyzer Tricarb 1900 TR; Canberra Packard). Enzyme activities presented in Figure 2, Table 1, and Supplemental Figure 2 online represent the mean of at least three independent experiments, assayed in duplicates.

Arabidopsis Transformation

Recombinant constructs were introduced into *Arabidopsis* plants through *A. tumefaciens*-mediated transformation using the floral dip method (Clough and Bent, 1998). Independent transformants were subjected to selective conditions on MS medium containing either 50 μg mL^{-1} kanamycin (for promoter-GUS plants) or 10 μg mL^{-1} glucosamine-aminomun (for complementation constructs in T-DNA insertion lines), 1% (w/v) sucrose, and 0.7% (w/v) agar. Resistant seedlings were transferred to soil after 2 to 3 weeks; homozygous T2 plants were used for analysis.

Histochemical Staining for GUS Activity

Histochemical staining of plant tissue for GUS activity was performed as previously described (Jefferson et al., 1987). In brief, tissue samples were vacuum-infiltrated for 5 min in a GUS substrate solution of 100 mM sodium phosphate, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100 and then incubated at 37°C for 3 h. Subsequently, the samples were transferred to 70% ethanol to remove chlorophyll pigmentation. GUS-positive samples were examined with a bright-field microscope (Olympus BX51) or a stereomicroscope (Olympus SZX12) at low magnification (×4 to ×10), and digital images were recorded. For cross sections, stained roots were embedded in polyethylene glycol 1500 according to Hause et al. (1996). Cross sections of 10 μm thickness were cut with a microtome (HM 355; Microm International), transferred to poly-L-lysine-coated slides, and examined by bright-field microscopy with an Axiosmager microscope (Zeiss). Micrographs taken with AxioCam MRCS (Zeiss) were processed through Photoshop 8.0.1 (Adobe Systems). All GUS-stained samples shown represent typical results of at least three independent transgenic lines for each construct.

Quantification of Root Hair Length

Root hair length from 8-d-old plants grown on agar plates was determined on low-magnification (×10) digital images captured using a CCD camera (ColorViewII) and image analysis freeware (ImageJ; http://rsb.info.nih.gov/ij). To ensure comparable results, the area 3 to 5 mm behind the root tip was analyzed. Plants grown on agar plates were carefully removed in ~100 μL of half-strength MS medium (Duchefa) on microscope slides for analysis. Quantification data are the means of 200 to 350 values representing 10 root hairs each of 20 to 35 individual plants measured for each data point.

Pollen Tube Growth and Transient Gene Expression

Mature pollen was collected from four to six tobacco (*Nicotiana tabacum*) flowers of 8-week-old plants. Pollen was resuspended in growth medium (Read et al., 1993), filtered onto cellulose acetate filters, and transferred to Whatman paper moistened with growth medium. Within 5 to 10 min of harvesting, pollen was transformed by bombardment with plasmid-coated 1-μm gold particles with a helium-driven particle accelerator (PDS-1000/He; Bio-Rad) using 1350-psi rupture discs and a vacuum of 28 inches of mercury. Gold particles (1.25 mg) were coated with 3 to 7 μg of plasmid DNA. After bombardment, pollen was resuspended in growth medium and grown for 7 to 10 h in small droplets of media directly on microscope slides.

Microscopy and Imaging

Images were recorded using a Zeiss LSM 510 confocal microscope. EYFP was excited at 514 nm and imaged using an HFT 405/514/633-nm major beam splitter (MBS) and a 530 to 600-nm band-pass filter; RedStar was excited at 561 nm and imaged using an HFT 405/488/561 nm MBS and a 583 to 604-nm band-pass filter; EYFP and FM4-64 were synchronously excited at 488 nm and 561 nm, respectively, and imaged using an HFT 405/488/561-nm MBS and a 518 to 550-nm band-pass filter and a 657 to 754-nm band-pass filter, respectively. Root hair autofluorescence was imaged using an HFT 405/514/633-nm MBS and a 470 to 500-nm band-pass filter to record autofluorescence without fluorescence cross-leakage from the EYFP channel. The autofluorescence signal was then subtracted from the EYFP signal. Fluorescence and transmitted light images were contrast-enhanced by adjusting brightness and γ-settings using image-processing software (Photoshop; Adobe Systems), except where stated differently. Fluorescence intensities were determined using AnalySIS software (Olympus). FM4-64 was added to pollen tubes at a final concentration of 10 μM as described (Parton et al., 2001) and visualized after 5 to 15 min of incubation before dye internalization.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers of genes used in this study are as follows: PIP5K1, At1g12980; PIP5K2, At1g7740; PIP5K3, At2g26420; PIP5K7, At1g10900; PIP5K8, At1g60890; PIP5K9, At3g09920; ACT8, At1g49240; PDF2 (PP2A), At1g13320.

Supplemental Data

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

**Supplemental Figure 1.** Organ-Specific Expression Patterns of PI4P 5-Kinase Isoforms 2 and 9 in *Arabidopsis*.

**Supplemental Figure 2.** Catalytic Activity of *Arabidopsis* PI4P 5-Kinase Isoforms Expressed in Roots.

**Supplemental Figure 3.** Identification of Homozygous T-DNA Insertion Mutants in the PIP5K3 Gene Locus At2g26420.
Supplemental Figure 4. Subcellular Localization of PIP5K3 and K442A Heterologously Expressed in Tobacco Pollen Tubes.

Supplemental Table 1. Primers Used in This Study.

Supplemental Text and References.

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