Phosphatidylinositol 4,5-Bisphosphate Influences PIN Polarization by Controlling Clathrin-Mediated Membrane Trafficking in Arabidopsis

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INTRODUCTION

An important example of polarity in vegetative plant tissues is the formation and maintenance of a stable auxin gradient in the root tip, which is required for root development and gravitropism (Dhonukshe et al., 2008; Bennett and Scheres, 2010). The directional transport of auxin in the root is mediated by auxin efflux carrier proteins of the PIN-FORMED (PIN) family, membrane integral transporters that exhibit asymmetric plasma membrane localization in root cells (Billing et al., 2005; Tanaka et al., 2006). Directional auxin transport and the perpetuation of a stable auxin gradient in the root require concerted action of different PIN isoforms (Petersson et al., 2009). On the cellular level, the polar distribution of plasma membrane associated PIN proteins is sustained by constant recycling and predominant delivery of the proteins to certain areas of the plasma membrane (Boutté et al., 2006; Kleine-Vehn et al., 2008). Thus, the polarization of PIN proteins to certain areas of the plasma membrane is maintained by a mechanism that involves internalization of the proteins from the membrane, which depends on recruitment to clathrin-coated vesicles (CCVs; Dhonukshe et al., 2007; Kitakura et al., 2011).

The functions of the minor phospholipid phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] during vegetative plant growth remain obscure. Here, we targeted two related phosphatidylinositol 4-phosphate 5-kinases (PI4P 5-kinases) PIP5K1 and PIP5K2, which are expressed ubiquitously in Arabidopsis thaliana. A pip5k1 pip5k2 double mutant with reduced PtdIns(4,5)P2 levels showed dwarf stature and phenotypes suggesting defects in auxin distribution. The roots of the pip5k1 pip5k2 double mutant had normal auxin levels but reduced auxin transport and altered distribution. Fluorescence-tagged auxin efflux carriers PIN-FORMED (PIN1)-green fluorescent protein (GFP) and PIN2-GFP displayed abnormal, partially apolar distribution. Furthermore, fewer brefeldin A-induced endosomal bodies decorated by PIN1-GFP or PIN2-GFP formed in pip5k1 pip5k2 mutants. Inducible overexpression lines for PIP5K1 or PIP5K2 also exhibited phenotypes indicating misregulation of auxin-dependent processes, and immunolocalization showed reduced membrane association of PIN1 and PIN2. PIN cycling and polarization require clathrin-mediated endocytosis and labeled clathrin light chain also displayed altered localization patterns in the pip5k1 pip5k2 double mutant, consistent with a role for PtdIns(4,5)P2 in the regulation of clathrin-mediated endocytosis. Further biochemical tests on subcellular fractions enriched for clathrin-coated vesicles (CCVs) indicated that pip5k1 and pip5k2 mutants have reduced CCV-associated PI4P 5-kinase activity. Together, the data indicate an important role for PtdIns(4,5)P2 in the control of clathrin dynamics and in auxin distribution in Arabidopsis.
Thole and Nielsen, 2008; Heilmann, 2009) contributes to the subcellular polarization of PIN proteins and, thus, to directional auxin transport. For instance, auxin-dependent gravitropic curvature correlated with the production of PtdIns(4,5)P$_2$ in maize (Zea mays; Perera et al., 1999). PtdIns(4,5)P$_2$ controls directional vesicle trafficking in polar growing cells (Ischebeck et al., 2008, 2010a; Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008). Furthermore, PtdIns(4,5)P$_2$ has been proposed to influence clathrin-mediated endocytosis in plants (König et al., 2008b; Zhao et al., 2010). Finally, PIN cycling is perturbed in root tips of an Arabidopsis thaliana pip5k2 mutant (Mei et al., 2012). However, despite these multiple lines of evidence, the mechanism behind these observations has remained obscure.

In Arabidopsis, PtdIns(4,5)P$_2$ is formed by an enzyme family of 11 phosphatidylinositol 4-phosphate 5-kinases (PI4P 5-kinases) (Mueller-Roeber and Pical, 2002). Arabidopsis PI4P 5-kinases often occur as pairs of closely related sister enzymes (Stenzel et al., 2012), and examination of single and double T-DNA insertion mutants indicates that the sister isoenzymes can display redundant functions (Ischebeck et al., 2008; Sousa et al., 2008; Ischebeck et al., 2011). Here, we eliminated two ubiquitously expressed PI4P 5-kinases, PIP5K1, an active PI4P 5-kinase (Mikami et al., 1998) expressed in all plant organs with predominant expression in procambial cells (Elge et al., 2001), and its closely related isoform PIP5K2 (Stenzel et al., 2008; Camacho et al., 2009; Mei et al., 2012). We show that the pip5k1 pip5k2 double mutant displays altered auxin transport and perturbed PIN1-GFP (for green fluorescent protein) and PIN2-GFP recycling. Our data indicate that polarization of PIN proteins requires PtdIns(4,5)P$_2$, which influences the formation of clathrin foci at the plasma membrane and possibly affects the internalization of clathrin coated vesicles.

RESULTS

The PI4P 5-Kinases PIP5K1 and PIP5K2 Are Ubiquitously Expressed in Arabidopsis

To examine the role of PI4P 5-kinases, we first identified Arabidopsis genes encoding PI4P 5-kinase isoforms with potential roles in vegetative tissues using transcript array information accessible through Genevestigator (Zimmermann et al., 2004). In previous experiments, recombinant PIP5K1 and PIP5K2 displayed the highest specific activities among ubiquitously expressed PI4P 5-kinases from Arabidopsis (Stenzel et al., 2008); therefore, we chose these genes for further characterization. The PIP5K1 and PIP5K2 sequences cluster together on a subclade of a phylogenetic tree of all deduced Arabidopsis PI4P 5-kinase amino acid sequences (Stenzel et al., 2012), suggesting related functionality. The third gene in this subclade, PIP5K3, is restricted in expression to roots and affects root hair formation (Kusano et al., 2008; Stenzel et al., 2008). All other PI4P 5-kinase isoforms are more distantly related and notably different from PIP5K1 and PIP5K2. We verified expression of PIP5K1 and PIP5K2 in vegetative tissues using 1500-bp promoter fragments of the genes to drive the expression of a β-glucuronidase (GUS) reporter construct in transgenic plants, followed by histochemical staining, and independently by quantitative real-time RT-PCR (see Supplemental Figure 1 online). The promoter-GUS experiments indicated that PIP5K1 and PIP5K2 are expressed already early in development (see Supplemental Figures 1A and 1B online) and have similar expression patterns in vegetative tissues (see Supplemental Figures 1C to 1J online) as well as in flowers and pollen (see Supplemental Figures 1K to 1N online).

Promoter activity was especially high in procambial tissues (see Supplemental Figures 1G and 1H online) and has similar expression patterns in vegetative tissues (see Supplemental Figures 1A and 1J online) as well as in flowers and pollen (see Supplemental Figures 1K to 1N online). Quantitative real-time RT-PCR analysis supports expression of both PIP5K1 and PIP5K2 in leaves, flowers, roots, whole seedlings, and flower buds (see Supplemental Figure 1O online).

A pip5k1 pip5k2 Double Mutant Exhibits Reduced PtdIns(4,5)P$_2$ and Severely Impaired Growth

We next isolated homozygous Arabidopsis T-DNA mutant lines with exon insertions in the genes PIP5K1 (SALK_146728) or PIP5K2 (SALK_012487) (Figure 1A). We inferred homozygosity from the ability to amplify wild-type alleles by PCR, concomitant with positive amplification of T-DNA–tagged alleles. T-DNA insertions were found in the first exon of pip5k1, 567 bp downstream from the ATG, and between the first exon and the first intron of pip5k2, 1285 bp downstream from the ATG. A pip5k1 pip5k2 double mutant was obtained by crossing homozygous pip5k1 and pip5k2 mutant lines, and the combined genotype was verified by PCR. Quantitative real-time RT-PCR showed that transcripts of PIP5K1 or PIP5K2 were reduced below the limits of detection in the respective single mutants (Figure 1B). Interestingly, reduction of PIP5K1 or PIP5K2 transcripts was accompanied by elevated levels of the transcripts of the other PI4P 5-kinase gene over the level of wild-type controls (Figure 1B). This indicates a compensatory effect at the level of transcription and complicates the interpretation of single mutant phenotypes. By contrast, the pip5k1 pip5k2 double mutant showed reduced levels of both transcripts (Figure 1B).

We measured the levels of PtdIns(4,5)P$_2$ and various other phospholipids for wild-type controls, the two single mutants, the pip5k1 pip5k2 double mutant, and for a double mutant line ectopically expressing PIP5K1:EYFP (for enhanced yellow fluorescent protein) under an intrinsic 1500-bp promoter fragment by quantification of unlabeled compounds (Figure 1C; see Supplemental Figure 2 online). Additionally, PtdIns(4,5)P$_2$ was independently quantified also by radiolabeling using [$^{32}$P]P, of wild-type controls, the two single mutants, and the pip5k1 pip5k2 double mutant (Figure 1D). The most prominent changes in lipid levels were observed for PtdIns(4,5)P$_2$, which was reduced in the double mutant by 44 and 32% of wild-type levels according to nonlabeled analysis (Figure 1C) and radiolabeling (Figure 1D), respectively. The single mutants showed a less pronounced reduction of PtdIns(4,5)P$_2$. The reduction of PtdIns(4,5)P$_2$ in the pip5k1 pip5k2 double mutant was accompanied by increases in phosphatidylinositol 4-phosphate and phosphatidylinositol, its biosynthetic precursors (Figure 1C). Among structural phospholipids, the level of phosphatidylcholine was slightly decreased (see Supplemental Figure 2 online), possibly indicating adaptive processes of membranes to a reduction in
Figure 1. Characterization of T-DNA Insertion Mutants Lacking the Closely Related PI4P 5-Kinase Isoforms PIP5K1 and/or PIP5K2.

T-DNA insertion lines for PIP5K1 (SALK_146728) and PIP5K2 (SALK_012487) were isolated and tested for the abundance of relevant transcripts and lipids.

(A) Graphic representation of the position of the insertions. The T-DNA insertion in pip5k1 is located in the first exon and that in pip5k2 at the transition of the first intron to the second exon. Black boxes indicate exons; lines between boxes indicate introns. Arrows indicate the approximate positions of PCR primers used for genotyping and for transcript detection in cDNA preparations.

(B) Changes in transcript levels for PIPS1 or PIPS2 according to quantitative real-time RT-PCR analysis, as indicated. Data represent the means of three independent experiments ± sd. Asterisks represent a significant difference from the wild type according to a Student’s t test (*P < 0.05; **P < 0.01).

(C) Levels of phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdInsP), and PtdIns(4,5)P2 in wild-type controls, the pip5k1 and pip5k2 double mutant, and a double mutant produced phenotypes similar to the single mutants or even the wild-type controls. This observation supports the notion that the fluorescence-tagged variants of PIP5K1 and PIP5K2 retained full functionality in vivo.

Gravitropic Bending and Auxin Distribution Are Impaired in the pip5k1 pip5k2 Double Mutant

The phenotype of the pip5k1 pip5k2 double mutant resembles that of plants deficient in biosynthesis or perception of auxin (Palme and Gälweiler, 1999). To better characterize whether auxin-dependent responses were altered in plants with reduced single mutants, the pip5k1 pip5k2 double mutant, and a double mutant line ectopically expressing PIPS1 under an intrinsic promoter fragment (compl.), according to nonlabeled mass determination. Ten-day-old seedlings were grown on MS agar plates and frozen in liquid nitrogen. Lipids were extracted and separated by thin layer chromatography, and the levels of PtdIns(4,5)P2 were determined by quantifying the associated fatty acids by gas chromatography and mass spectrometry. Data represent the means of three independent experiments ± sd. Asterisks indicate significant differences from the wild type according to a Student’s t test (*P < 0.05; **P < 0.01).

(D) Determination of PtdIns(4,5)P2 levels in wild-type controls, the pip5k1 and pip5k2 single mutants, and the pip5k1 pip5k2 double mutant according to radiolabeling with [32P]Pi. Twelve-day-old Arabidopsis seedlings grown on MS agar plates were incubated for 30 min in liquid MS medium containing [32P]Pi. Lipids were extracted and separated by thin layer chromatography, and the radioactivity on the thin layer chromatography plates determined by liquid scintillation counting. The amount of [32P]-labeled PtdIns(4,5)P2 was calculated as the percentage of the total [32P] incorporated in the phospholipids of each sample. Data represent the means of three independent experiments ± sd. Asterisks indicate significant differences from the wild type according to a Student’s t test (*P < 0.05; **P < 0.01).
Figure 2. pip5k1 pip5k2 Double Mutant Plants Display Severe Growth Defects.

Arabidopsis wild-type controls, single and double mutant plants, and double mutant plants ectopically expressing PIP5K1-EYFP or PIP5K2-EYFP were grown on soil or on MS media, and their phenotype was documented.

(A) Plants grown in soil under 16 h of light/8 h of darkness in the greenhouse for 5 weeks. Inset, identical plants after 4 weeks. Genotypes as indicated. compl. 1, compl. 2, controls ectopically expressing PIP5K1-EYFP or PIP5K2-EYFP under their respective intrinsic promoters in the pip5k1 pip5k2 background.

(B) Seedlings grown for 10 d on MS medium under 24 h of light on a vertical plate. Left, wild-type controls; right, pip5k1 pip5k2 double mutants.

(C) Root length and leaf area were measured from seedlings grown on MS medium under 24 h of light after 7 d (gray bars) or 14 d (black bars). For determination of root length, plants were grown on vertical plates. Data represent mean values ± se of at least 20 seedlings. Double asterisks indicate significant differences from the wild type according to a Student’s t test (***P < 0.01).

(D) Plants were grown under 16 h of light/8 h of darkness in the greenhouse for 10 months. Double mutant plants showed a delay in senescence and a loss of apical dominance. The image is representative for 10 plants observed over the prolonged growth period.

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expression of PIP5K1 and/or PIP5K2, we monitored gravitropic bending of roots and hypocotyls. Seedlings were grown in the dark for 7 d on vertical agar plates, the plates were rotated by 135° for 24 h as previously described (Fukaki et al., 1996), and gravitropic curvature was recorded (Figure 3). Compared with wild-type controls, gravitropic curvature of hypocotyls and roots was slightly reduced in pip5k1 or pip5k2 single mutants and substantially reduced in the pip5k1 pip5k2 double mutant (Figure 3). A complemented double mutant line ectopically expressing pPIP5K1:PIP5K1-EYFP displayed curvature of roots and hypocotyls similar to wild-type controls (Figure 3). The elongation of dark-grown roots (see Supplemental Figure 4A online) and hypocotyls (see Supplemental Figure 4B online) was determined for all lines tested and not found to be significantly different.

Because the phenotype of the pip5k1 pip5k2 double mutant included reduced root growth, apical dominance, and gravitropic bending, we speculated that these aspects might be a consequence of defects in auxin signaling. The wild-type controls and the double mutant showed similar levels of endogenous auxin (Figure 4A). By contrast, the pip5k1 pip5k2 double mutant showed significantly reduced basipetal auxin transport (Figure 4B). Auxin distribution was also tested by monitoring DR5-driven GFP distribution in roots (see Supplemental Figure 5A online). In wild-type controls the DR5-dependent GFP distribution reflected the characteristic auxin maximum in the root tip (see Supplemental Figure 5A online, top left), and the signal was further intensified by the exogenous application of indole-3-acetic acid (IAA; see Supplemental Figure 5A online, top right). By contrast, no GFP fluorescence was observed in pip5k1 pip5k2 double mutants, neither in the absence nor the presence of exogenous IAA (see Supplemental Figure 5A online, bottom panels). The presence of the DR5:GFP construct was confirmed by PCR. To rule out genetic effects of incrossing, an independent DR5:GUS reporter line was created in the pip5k1 pip5k2 background (Figure 4C) and analyzed for responses to endogenous and exogenous auxins. As for the DR5:GFP reporter, the DR5:GUS reporter also did not indicate an auxin maximum in the pip5k1 pip5k2 double mutant. Reporter activity only marginally increased in response to exogenous auxins, such as α-naphthalene acetic acid or 2,4-dichlorophenoxyacetic acid (2,4-D) (Figure 4C). Importantly, the spatial distribution of

**Figure 3.** Altered Gravitropic Response in pip5k1 and pip5k2 Single Mutants and the pip5k1 pip5k2 Double Mutant.

Gravitropic curvature and root growth was tested in wild-type controls, pip5k1 and pip5k2 single mutants, the pip5k1 pip5k2 double mutant, and plants controls ectopically expressing PIPS5K1:EYFP (compl. 1). Bending angles of hypocotyls and roots were recorded 24 h after rotation of 135° and scored in categories of 30°. The length of the bars indicates the percentage of plants in each category. Wild type, data from 242 plants; pip5k1, data from 214 plants; pip5k2, data from 329 plants; pip5k1 pip5k2 double mutant, data from 288 plants; compl. 1, data from 198 plants.

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Auxin Distribution Is Impaired in the pip5k1 pip5k2 Double Mutant.

Levels and distribution of auxin were analyzed in wild-type controls and in the pip5k1 pip5k2 double mutant. 

(A) Levels of free IAA in 2-week-old seedlings according to HPLC–mass spectrometry analysis. Data represent the mean ± se of two independent experiments, each with three independent biological replicates. A Student’s t test indicates no significant difference between controls and the double mutant (P < 0.23).

(B) Relative basipetal auxin transport was determined in 10-d-old wild-type controls and in 14-d-old pip5k1 pip5k2 seedlings with similar root lengths grown on vertical plates. Data represent the mean ± se of two independent experiments. The asterisk indicates a significant difference from the control according to a Student’s t test (P < 0.05).

(C) Auxin distribution in the wild type (top) and pip5k1 pip5k2 double mutant (bottom) according to DR5::GUS expression. Patterns after application of exogenous auxins, as indicated. The results are representative for three independent treatments. 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthalene acetic acid.

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Overexpression of PIP5K1 and PIP5K2 Results in Auxin-Related Growth Defects

To examine the biochemical functionality of PIP5K1 and PIP5K2 variants used in this study, we recombinantly expressed the proteins as fusions to N-terminal maltose binding protein tags used to enhance solubility (see Supplemental Figure 6A online). Biochemical characterization showed that EYFP-tagged variants of PIP5K1 and PIP5K2 displayed catalytic activity in vitro (see Supplemental Figure 6B online). In vitro catalytic activity did not differ between enzyme variants carrying only an N-terminal maltose binding protein tag or an additional C-terminal EYFP tag (see Supplemental Figure 6B online), consistent with previous reports (Ischebeck et al., 2008, 2011; Stenzel et al., 2008, 2012). Heterologous expression of untagged or fluorescence-tagged variants of either PIP5K1 or PIP5K2 restored the ability to grow at prohibitive temperature in a Saccharomyces cerevisiae strain carrying an allele of MSS4 encoding a temperature-sensitive variant of the PI4P 5-kinase MSS4p (Homma et al., 1998) (see Supplemental Figure 6C online). The data confirm catalytic activity of PIP5K1 and PIP5K2 and indicate that fluorescence-tagged variants of PIP5K1 and PIP5K2 were functional in vitro, corroborating the mutant complementation tests (compared with Figure 2).

To investigate the subcellular distributions of the proteins and to assess the impact of overproduction of the two kinases, we overexpressed fluorescence-tagged versions of PIP5K1 and PIP5K2 in Arabidopsis. As the fluorescently tagged PIP5K1 and PIP5K2 under their own promoters displayed only very weak fluorescence, we replaced the native promoters with estradiol-inducible promoters of the pMDC7 system (Curtis and Grossniklaus, 2003), which should induce gene expression strongly upon estradiol treatment. We verified induction of the transgenes by monitoring EYFP fluorescence (Figure 5A). In root tip cells of these lines, EYFP-PIP5K1 and PIP5K2-EYFP displayed clear plasma membrane localization and weak diffuse cytosolic fluorescence (Figure 5A), characteristic of soluble enzymes that associate peripherally with the plasma membrane, and in

Figure 4. Auxin Distribution Is Impaired in the pip5k1 pip5k2 Double Mutant.

(A) Level of free IAA in 2-week-old seedlings according to HPLC–mass spectrometry analysis. Data represent the mean ± se of two independent experiments, each with three independent biological replicates. A Student’s t test indicates no significant difference between controls and the double mutant (P < 0.23).

(B) Relative basipetal auxin transport was determined in 10-d-old wild-type controls and in 14-d-old pip5k1 pip5k2 seedlings with similar root lengths grown on vertical plates. Data represent the mean ± se of two independent experiments. The asterisk indicates a significant difference from the control according to a Student’s t test (P < 0.05).

(C) Auxin distribution in the wild type (top) and pip5k1 pip5k2 double mutant (bottom) according to DR5::GUS expression. Patterns after application of exogenous auxins, as indicated. The results are representative for three independent treatments. 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthalene acetic acid.

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particular resembling localization patterns previously reported for other plant PI4P 5-kinases in other cell types (Ischebeck et al., 2008, 2010b, 2011; Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008, 2012). Interestingly, both EYFP-PIP5K1 and PIP5K2-EYFP displayed pronounced polarized distribution and localized predominantly to apical and basal plasma membrane areas (Figure 5A). In some cases, this localization pattern was accompanied by nuclear localization (see Supplemental Figure 7 online). To test whether polarized distribution observed for the overexpressed PI4P 5-kinases was independently reflected by corresponding polarization of PtdIns(4,5)P₂, we analyzed the distribution of the lipid by monitoring YFP-PH₆₅₅₁, a reporter that specifically binds PtdIns(4,5)P₂ (van Leeuwen et al., 2007). In plants not overexpressing a PI4P 5-kinase, YFP-PH₆₅₅₁ displayed polarized distribution to apical and basal membrane domains in a pattern similar to that found for EYFP-PIP5K1 and PIP5K2-EYFP (Figures 5A and 5B). We quantified the polarization of EYFP-PIP5K1, PIP5K2-EYFP, and of YFP-PH₆₅₅₁ as ratios of lateral versus apicobasal plasma membrane fluorescence, which were significantly less than one in cells of both the root cortex and the root epidermis (Figure 5C).

**Figure 5.** Polarized Distribution of PIP5K1, PIP5K2, and PtdIns(4,5)P₂ in Root Cells.

EYFP-PIP5K1 or PIP5K2-EYFP was expressed in seedlings driven by an estradiol-inducible promoter and induction with 5 µM estradiol for 24 h. Images were recorded by confocal microscopy. Images are representative of patterns observed in five independent transformation experiments. Bars = 10 µm.

(A) Subcellular localization of overexpressed EYFP-PIP5K1 (left) and PIP5K2-EYFP (middle) in cells of root cortex (C) and epidermis (E), as indicated. EYFP (right) is shown as a control. Top panels, fluorescence images; bottom panels, false-color representation indicating fluorescence intensities.

(B) Subcellular distribution of YFP-PH₆₅₅₁, a fluorescent reporter for PtdIns(4,5)P₂ in wild-type plants. Polarized distribution indicated by arrowheads. Top panels, fluorescence images; bottom panels, false-color representation indicating fluorescence intensities.

(C) Ratio of lateral versus apicobasal plasma membrane fluorescence for EYFP-PIP5K1, PIP5K2-EYFP, and YFP-PH₆₅₅₁ expressed in root cells, as indicated. Plasma membrane-associated fluorescence of the membrane dye FM4-64 was used as a nonpolar control. C, cortex cells; E, epidermal cells. Values <1 signify polarization toward the apicobasal sides. Data represent the mean ratios of 20 cells ± se. The asterisks indicate a significant difference from the FM4-64 control according to a Student’s t test (* P < 0.05; ** P < 0.01).
Plants used for localization studies were only mildly induced. Consistent with overproduction of the PI4P 5-kinases, strongly induced overexpressing lines accumulated increased amounts of PtdIns(4,5)P₂ (Figure 6A). When the EYFP-PIP5K1 or PIP5K2-EYFP overexpressors were grown on vertical agar plates containing up to 5 µM estradiol, the plants exhibited a loss of gravitropic growth concurrent with random nondirectional root growth (Figures 6C and 6D). By contrast, estradiol treatment did not affect the growth of control plants overexpressing EYFP (Figure 6B). Random root growth was not observed in noninduced plants (data not shown). Auxin-related phenotypes of the overexpressors were not limited to gravitropic bending. Seven-day-old seedlings of all selected overexpressor lines exhibited changes in root hair morphology after 24 h of incubation on agar plates containing 5 µM estradiol. Root hairs of the induced overexpressors were shorter and thicker compared with noninduced controls (Figures 5E, 5F, and 5H). In addition, root hairs in the induced overexpressors often initiated at multiple sites of the trichoblasts (Figures 5G and 5I). The pattern observed was similar to that previously reported for root hair-specific overexpression of the PI4P 5-kinase isoform, PIP5K3 (Kusano et al., 2008; Stenzel et al., 2008) and resembles phenotypes described for plants with perturbed auxin distribution in roots (Fischer et al., 2006). The effect on root hair morphology likely results from the ectopic PI4P 5-kinase expression, as PIP5K1 and PIP5K2 are normally expressed mainly in the vascular

![Figure 6. Overexpression of PIP5K1 and PIP5K2 Results in Increased Levels of PtdIns(4,5)P₂ and Auxin-Related Growth Defects.](image)

(A) Accumulation of PtdIns(4,5)P₂ in induced transgenics displaying strong induction, as indicated by fluorescence intensity detected for EYFP-PIP5K1 (left) and PIP5K2-EYFP (right). Data represent means ± sd from three independent experiments. Asterisks represent significant differences in PtdIns(4,5)P₂ levels upon induced versus the noninduced controls according to a Student’s t test (*P < 0.05). AU, arbitrary units.

(B) to (D) Effects of overexpression of EYFP-PIP5K1 or PIP5K2-EYFP on root growth of 7-d-old seedlings. Patterns are representative for 24 plants for each treatment. Seedlings were transferred to vertical plates containing 3 or 5 µM estradiol, as indicated. Plants were grown for 24 h and root growth was documented. Arrows indicate the position of the root tip at the point of transfer. Bars = 1 mm.

(B) Control seedlings expressing EYFP.

(C) Seedlings expressing EYFP-PIP5K1.

(D) Seedlings expressing PIP5K2-EYFP.

(E) to (I) Arabidopsis plants expressing EYFP-PIP5K1 or PIP5K2-EYFP under an estradiol-inducible promoter were grown on solid MS medium for 7 d and transferred onto media containing 5 µM estradiol for 24 h. Equally treated plants expressing EYFP were used as a control. Images were recorded by bright-field or confocal microscopy. Images are representative for patterns observed in five independent transformation experiments. Bars = 200 µm in (E), (F), and (H) and 50 µm in (G) and (I).

(E) Control expressing EYFP.

(F) and (G) EYFP-PIP5K1.

(H) and (I) PIP5K2-EYFP.

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tissue of roots and only to a low degree in trichoblasts and root hairs (Stenzel et al., 2008). No difference was observed between the N-terminally tagged EYFP-PIP5K1 and the C-terminally tagged PIP5K2-EYFP with regard to subcellular localization, accumulation of PtdIns(4,5)P_2, or any of the auxin-related phenotypes.

**Polarization of PIN2-GFP Is Disturbed in the pip5k1 pip5k2 Double Mutant and upon Overexpression of PIP5K1 or PIP5K2**

To test whether the phenotypes and changed auxin distribution in the pip5k1 pip5k2 double mutant was linked to changed patterns of PIN distribution, we crossed *Arabidopsis* lines expressing PIN1-GFP or PIN2-GFP into the pip5k1 pip5k2 double mutant background and monitored the fluorescence distribution of PIN1-GFP or PIN2-GFP in root tips (Figure 7). Clear polar plasma membrane localization was observed in wild-type controls expressing PIN1-GFP (Figure 7A) or PIN2-GFP (Figure 7C). The polarized patterns observed corresponded well with previously reported patterns and in cells of the central cylinder PIN1-GFP localized predominantly to the basal side of the cells, whereas PIN2-GFP localized predominantly to the apical sides of cortex cells. In the pip5k1 pip5k2 double mutant background, fluorescence intensities observed for PIN1-GFP and PIN2-GFP were reduced. PIN1-GFP localized slightly more diffusely compared with the sharply defined patterns found in wild-type controls (Figure 7B). Plasma membrane association of PIN2-GFP (Figure 7D) was not limited to the basal or apical sides of the cells. Instead, in the double mutant, PIN2-GFP fluorescence was also sometimes detected in lateral plasma membrane areas (Figure 7D). These observations indicate perturbed polarization of PIN proteins as a consequence of reduced levels of PtdIns(4,5)P_2. To test whether effects of overexpressing EYFP-PIP5K1 or PIP5K2-EYFP would also result in altered distribution of PIN proteins, overexpressor lines were grown on vertical agar plates and induced by the addition of estradiol. Plants displaying gravitropic root growth (compared with Figures 6B to 6D) were selected and the distribution of endogenous PIN1 and PIN2 was analyzed using immunodetection by specific antibodies against *Arabidopsis* PIN1 or PIN2 (Figures 7E to 7J). In estradiol-treated plants expressing an EYFP control, PIN1 was detected at the basal plasma membrane of cells of the central cylinder (Figure 7E), whereas PIN2 was detected at the apical plasma membrane of cortex cells (Figure 7H), representing wild-type distribution. PIN1 localization in the EYFP-PIP5K1 or PIP5K2-EYFP overexpressors resembled that found in the control plants (Figures 7F and 7G). PIN2 localization in the overexpressors differed from that in the EYFP controls by consistently displaying weaker membrane association under identical conditions for immunodetection and fluorescence imaging (Figures 7I and 7J). These observations suggest that overexpression of EYFP-PIP5K1 or PIP5K2-EYFP altered the membrane association of PIN2, which might result from perturbed endocytic trafficking.

**Endocytic Cycling of PIN1-GFP and PIN2-GFP Is Reduced in the pip5k1 pip5k2 Double Mutant**

To assess effects of reduced PtdIns(4,5)P_2 levels on endocytic cycling of PIN1-GFP and PIN2-GFP, we successively applied cycloheximide (CHX) and brefeldin A (BFA), which inhibit de novo protein synthesis and the reinsertion of membranous compartments into the plasma membrane, respectively, resulting in the formation of BFA bodies. Care was taken not to exceed 25 µM BFA. When CHX and BFA were applied to wild-type roots expressing PIN1-GFP (Figure 8A) or PIN2-GFP (Figure 8C), BFA bodies decorated by PIN1-GFP or PIN2-GFP became visible within a few minutes and increased over 60 min, indicating a substantial rate of endocytic recycling of PIN1-GFP and PIN2-GFP between the plasma membrane and endosomal compartments. In sharp contrast, in roots of pip5k1 pip5k2 double mutants expressing PIN1-GFP (Figure 8B) or PIN2-GFP (Figure 8D), we observed fewer and smaller BFA bodies within 60 min of CHX/BFA treatment. The data indicate that in the pip5k1 pip5k2 double mutant, endocytic cycling of PIN1-GFP and of PIN2-GFP from the plasma membrane was impaired upstream of BFA-sensitive endosomes. Overview images of CHX/BFA-treated root tissue are provided in Supplemental Figure 8 online. To test whether defects in endocytosis affected overall membrane internalization, wild-type controls, the pip5k1 and pip5k2 single mutants, and the pip5k1 pip5k2 double mutant were subjected to CHX/BFA treatment, stained with the styryl dye FM4-64, and the subcellular distribution of the dye was monitored over a period of 90 min (Figures 8E and 8F; see Supplemental Figure 9 online). In all cases, the dye was internalized and decorated BFA bodies. In the controls, the single mutants and the double mutant the number of BFA bodies per cell increased with time of dye exposure, indicating that membrane internalization was essentially functional. Quantitative analysis (Figure 8G; see Supplemental Figure 9B online) revealed a decreased number of BFA bodies per cell in the mutant lines; for the pip5k1 pip5k2 double mutant, the reduction compared with wild-type controls was significant after 50 and 90 min of dye application. The data indicate that reduced levels of PtdIns(4,5)P_2 impaired endocytosis of certain components, while other aspects remained largely as in the wild type. This observation is in line with the notion of alternative routes of endocytosis, only some of which might depend on PtdIns(4,5)P_2.

**The Dynamics of Clathrin Light Chain Association with the Plasma Membrane Are Altered in the pip5k1 pip5k2 Double Mutant**

The endocytic cycling of PIN proteins from the plasma membrane requires clathrin-mediated endocytosis (CME) (Dhonukshe et al., 2007; Kitakura et al., 2011). Furthermore, CME has been linked to phosphoinositides in tobacco (*Nicotiana tabacum*) pollen tubes (Zhao et al., 2010). To further substantiate the hypothesis that CME of PIN proteins requires PtdIns(4,5)P_2 in *Arabidopsis*, we monitored the dynamic membrane association of GFP-tagged CLATHRIN LIGHT CHAIN 2 (CLC2-GFP) (Wang et al., 2013) in 7-d-old roots of wild-type controls and pip5k1 pip5k2 double mutants by spinning disc confocal live imaging (Figure 9; see Supplemental Movies 1 and 2 online). In wild-type controls, CLC2-GFP decorated small punctae in the focal plane of the plasma membrane and larger, fast-moving structures outside the focal plane likely representing the trans-Golgi network (Figure 9A; see Supplemental Movies 1 and 2 online). The smaller,
plasma membrane–associated CLC2-GFP foci appeared in wild-type controls at a mean density of 150 ± 13 per 100 µm² plasma membrane (Figure 9B) and were characterized by their transient appearance and disappearance within a time interval of ~26 ± 2 s, as indicated by kymograph analysis (see Supplemental Figure 10 online). In pip5k1 pip5k2 double mutants the plasma membrane–associated foci were present at a substantially reduced density of only 63 ± 12 per 100 µm² plasma membrane (Figure 9C). Individual CLC2-GFP foci did not display alterations in dwell time at the plasma membrane (~28 ± 6 s; see Supplemental Figure 10 online) but appeared overall much larger in the double mutant than in wild-type controls (Figures 9B and 9D). The data indicate that recruitment of CLC2-GFP to the plasma membrane of pip5k1 pip5k2 double mutants was not impaired but that CLC2-GFP displayed altered distribution into fewer but larger plasma membrane–associated foci. This observation is consistent with the notion that clathrin-dependent internalization of PIN1-GFP and PIN2-GFP was impaired in the pip5k1 pip5k2 double mutant (compared with Figure 8).

**PIP5K1 and PIP5K2 Are Required for CCV-Associated Formation of PtdIns(4,5)P₂ and PI4P 5-Kinase Activity**

To verify effects of PtdIns(4,5)P₂ production on CLC2-GFP dynamics (compared with Figure 9), we examined the rates of PtdIns(4,5)P₂ formation in subcellular fractions enriched for CCVs. In Arabidopsis, salt stress can trigger increased formation of CCVs (König et al., 2008b). Furthermore, the levels of PtdIns(4,5)P₂ in Arabidopsis increase upon salt treatment and associate with CCVs (König et al., 2008b). Therefore, we performed salt stress experiments to test whether PIP5K1 and PIP5K2 contributed to the formation of CCV-associated PtdIns(4,5)P₂ in Arabidopsis. Hydroponically grown wild-type controls and pip5k1 and pip5k2 mutants were subjected to salt treatment by the application of 0.4 M NaCl in culture medium, and CCVs were isolated from leaves prior to stimulation and after 60 min, precisely as previously reported (König et al., 2008b). Only the single pip5k1 and pip5k2 mutants could be used for hydroponic growth, as the pip5k1 pip5k2 double mutants are too small to provide the amount of material required for these tests. In wild-type plants, CCV-associated PI4P 5-kinase activity increased upon salt stress (Figure 10), consistent with either translocation of a PI4P 5-kinase from the plasma membrane to CCVs or with activation of CCV-associated PI4P 5-kinase(s). By contrast, in the pip5k1 mutant CCV-associated PI4P 5-kinase activity exhibited only a limited increase and no increase was detected in the pip5k2 mutant upon stress treatment (Figure 10). The data indicate that the CCV-associated PI4P 5-kinase activity is attributable largely to PIP5K2 and, to a lesser degree, PIP5K1. The pattern is consistent with the in vitro catalytic activities of the recombinant enzymes, as PIP5K2 displays five- to sixfold higher activity than its close homolog, PIP5K1 (compared with Supplemental Figure 6B online). The combined characterization of CCVs formed upon salt treatment supports the notion that

**Figure 7. Misexpression of EYFP-PIP5K1 or PIP5K2-EYFP Results in Altered Localization and Polarization of PIN1 and PIN2.**

(A) to (D) The subcellular distribution of PIN1-GFP and PIN2-GFP was monitored by confocal fluorescence imaging in 10-d-old seedlings of wild-type controls and the pip5k1 pip5k2 double mutant. Images are representative for >10 independently grown plants. Bars = 10 µm.

(A) and (B) PIN1-GFP. (A) Wild type control. (B) pip5k1 pip5k2 double mutant.

(C) and (D) PIN2-GFP. (C) Wild type control. (D) pip5k1 pip5k2 double mutant.

(E) to (J) The localization of PIN1 and PIN2 was determined in induced overexpressors of EYFP, EYFP-PIP5K1, or PIP5K2-EYFP by immunodetection using specific antibodies against PIN1 (aPIN1) or PIN2 (aPIN2). Images are representative for >20 independently grown plants. Bars = 10 µm.

(E) and (H) EYFP control. (F) and (I) EYFP-PIP5K1 overexpressor. (G) and (J) PIP5K2-EYFP overexpressor. (E) to (G) PIN1. (H) to (J) PIN2.

Arrowheads, basal (PIN1) or apical (PIN2) localization. Arrows, mislocalization or reduced membrane associated signal. [See online article for color version of this figure.]
Endocytic Cycling of PIN1-GFP, PIN2-GFP, and FM 4-64 Is Reduced in the pip5k1 pip5k2 Double Mutant.

The dynamic distribution of PIN proteins was visualized in 10-d-old seedlings of wild-type controls and the pip5k1 pip5k2 double mutant expressing PIN1-GFP or PIN2-GFP upon pretreatment for different times with 50 µM CHX and 25 µM BFA. The internalization of PIN1-GFP and PIN2-GFP into BFA bodies was monitored in medial confocal sections of 2-µm thickness over a period of 60 min. Additionally, the internalization of the styryl dye FM4-64 was also monitored. Panels left to right, CHX/BFA treatment for 0, 30, and 60 min, as indicated. Bars = 10 µm.

(A) Wild-type controls, PIN1-GFP.
(B) pip5k1 pip5k2 double mutant, PIN1-GFP.
(C) Wild-type controls, PIN2-GFP.
(D) pip5k1 pip5k2 double mutant, PIN2-GFP.
(E) Wild-type controls, FM 4-64.
(F) pip5k1 pip5k2 double mutant, FM 4-64.

The abundance of punctate signals per cell was quantified for all treatments based on digital imaging data for wild-type controls (black bars) and the pip5k1 pip5k2 double mutant (white bars). Data represent mean ± so for 100 cells analyzed for each treatment. Asterisks indicate significant differences from the wild type according to a Student’s t test (*P < 0.05; **P < 0.01).

[See online article for color version of this figure.]
PIP5K1 and PIP5K2 associate with CCVs, consistent with a role for PtdIns(4,5)P2 in CME.

**DISCUSSION**

The strong phenotype of the pip5k1 pip5k2 double mutant and less pronounced phenotypes of the individual pip5k1 or pip5k2 mutants (Figure 2) indicate functional redundancy of PIP5K1 and PIP5K2. This observation is consistent with the high sequence similarity of PIP5K1 and PIP5K2 (Stenzel et al., 2012), which are 87% identical at the amino acid level, and with the similar expression patterns of PIP5K1 and PIP5K2 (see Supplemental Figure 1 online). The mild phenotypes reported previously for a pip5k2 single mutant have been attributed to altered auxin distribution (Mei et al., 2012). However, the interpretation of data reported for the single pip5k2 mutant (Mei et al., 2012) or obtained for both the pip5k1 and the pip5k2 mutants in this study is complicated by the accompanying upregulation of at least one other PI4P 5-kinase (Figure 1B), which might lead to compensatory effects or even overcompensation. Therefore, the characterization of the pip5k1 pip5k2 double mutant combined with comprehensive lipid analysis is more informative and enables interpretation of previously reported data (Mei et al., 2012). The severe phenotype of the pip5k1 pip5k2 double mutant (Figures 2 and 3) indicates that PtdIns(4,5)P2 is a critical factor in plant function and development. It must be noted that the severe phenotype manifested in the pip5k1 pip5k2 double mutant, despite a reduction in PtdIns(4,5)P2 levels by only ~40 to 50% in these plants (Figures 1C and 1D). A possible explanation for this effect arises from the notion of distinct functional pools of PtdIns(4,5)P2 and the recent report that the regulatory consequences of PtdIns(4,5)P2 in plant cells are partially directed by the identity of the PI4P 5-kinase isoform generating the lipid (Stenzel et al., 2012). Clearly, residual PtdIns(4,5)P2 generated in the pip5k1 pip5k2 double mutant by other PI4P 5-kinase isoforms was not

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**Figure 9.** Altered Dynamics of Clathrin Light Chain Association with the Plasma Membrane in the pip5k1 pip5k2 Double Mutant.

The dynamic association of clathrin with the plasma membrane was monitored by spinning disc confocal live imaging in 7-d-old roots of wild-type controls and pip5k1 pip5k2 double mutants expressing CLC2-GFP. Images presented are stills from Supplemental Movies 1 and 2 online.

(A) Plasma membrane foci decorated by CLC2-GFP in wild-type controls (left) and the pip5k1 pip5k2 double mutant (right). Bars = 3 μm.

(B) Individual foci were analyzed for size. Representative foci shown are marked in (A) by arrowheads.

(C) The density of CLC2-EYFP foci per plasma membrane area was calculated for wild-type controls and pip5k1 pip5k2 double mutants. Data represent the mean ± so of six independent experiments. Asterisks indicate a significant difference according to a two-tailed Student’s t test (**P < 0.01).

(D) Foci size was quantified for wild-type controls and pip5k1 pip5k2 double mutants. Data represent the mean ± so of six independent experiments. Asterisks indicate a significant difference according to a two-tailed Student’s t test (**P < 0.01).

PIP5K1 and PIP5K2 associate with CCVs, consistent with a role for PtdIns(4,5)P2 in CME.

**Figure 10.** PIP5K1 and PIP5K2 Are Required for Salt-Induced CCV-Associated Increases in PI4P 5-Kinase Activity.

Arabidopsis wild-type controls and the pip5k1 and pip5k2 mutants were grown in hydroponic culture and subjected to salt treatment by 0.4 M NaCl in liquid culture medium. CCVs were isolated before and after 60 min of treatment, and the associated PI4P 5-kinase activity was assayed in the CCV fractions using exogenous phosphatidylinositol 4-phosphate as a substrate. Because the pip5k1 pip5k2 double mutant only yields limited amounts of material and due to the large amount of material required for CCV preparation, this experiment was performed only with single mutants. Data represent means ± so from three independent experiments. Asterisks represent significant differences in the activity after stimulation versus the nonstimulated controls according to a Student’s t test (*P < 0.05; **P < 0.01).
able to compensate for the lack of PIP5K1 and PIP5K2, possibly because it represents one or more PtdIns(4,5)P2 pools that may not be involved in controlling CME.

In line with previous observations on the pip5k2 single mutant (Mei et al., 2012), several phenotypic aspects of the pip5k1 pip5k2 double mutant are consistent with an effect of PtdIns(4,5)P2 on auxin signaling, including impaired root growth (Figures 2B and 2C), reduced apical dominance (Figure 2D), and the attenuated response to gravistimulation (Figure 3). In our experiments, auxin levels in whole double mutant seedlings were not different from those in wild-type controls (Figure 4A). Auxin measurements, particularly on the pip5k1 pip5k2 double mutant, carried a large experimental error. Considering the experimental difficulty in measuring auxin levels, we find our observation consistent with data by Mei et al. (2012), who presented levels of \(-8 \text{ ng g}^{-1}\) fresh weight in a pip5k2 single mutant as differing from \(-10 \text{ ng g}^{-1}\) fresh weight in wild-type controls. Based on the auxin transport assays and two different DR5-based reporters (Figure 4), our data suggest that auxin transport and distribution, rather than auxin amount, were perturbed in the pip5k1 pip5k2 double mutant.

PIN1 is largely responsible for the basipetal auxin transport in the central cylinder, whereas PIN2 mediates the upward transport of auxin in the cortex and epidermis of the root tip and the asymmetric distribution of auxin during gravitropic responses (Sukumar et al., 2009; Rahman et al., 2010). Together, PIN1 and PIN2 contribute to the establishment of the basipetal auxin gradient in the root tip (Grieneisen et al., 2007). Therefore, we focused on the effects of PtdIns(4,5)P2 on the distribution of PIN1-GFP and PIN2-GFP. The influence of PtdIns(4,5)P2 on the distribution of PIN2-GFP is of particular interest in light of the previously reported involvement of phosphoinositides in gravitropic signaling in different plant species (Perera et al., 1999, 2001, 2006). The perturbation of PIN1-GFP distribution in the pip5k1 pip5k2 double mutant (Figure 7B) was not limited to a polarization defect, but rather the protein was not efficiently inserted into the plasma membrane, suggesting a defect in secretory membrane trafficking. This observation is consistent with the report that Arabidopsis PIP5K2 interacts with Rab-E isoforms implicated in the control of Golgi–to–plasma membrane trafficking (Camacho et al., 2009) and suggests a function for PIP5K1 and PIP5K2 in Rab-E–dependent secretion of PIN1-GFP.

Considering how PtdIns(4,5)P2 might contribute to the control of auxin transport, it must also be noted that PtdIns(4,5)P2 has been demonstrated to mediate clathrin recruitment in plant cells (König et al., 2008b; Zhao et al., 2010) and that polarization and PIN cycling involves clathrin-mediated membrane trafficking (Dhonukshe et al., 2007; Kitakura et al., 2011). The link to CME suggests that reduced PtdIns(4,5)P2 levels would influence both clathrin dynamics and PIN recycling, and this notion is supported by this study (Figures 8 to 10). While expression of fluorescence-tagged variants of PIP5K1 and PIP5K2 under their respective intrinsic promoters resulted in complementation of the mutant phenotype (compared with Figure 2), no fluorescence was detected in the complemented plants, likely due to low promoter activity or to limited stability of the PI4P 5-kinase proteins in planta. The polarized subcellular localization of overexpressed EYFP-PIP5K1 and PIP5K2-EYFP in cells of the root tip (Figures 5A and 2C) is consistent with an effect on PIN cycling. While it cannot be ruled out that the localization patterns observed were biased by the overexpression, it must be noted that the YFP-PH\(_{PH_{2,Ca}}\) reporter for PtdIns(4,5)P2 displayed similar polarized distribution in plants not overexpressing a PI4P 5-kinase (Figures 5B and 5C). Together, the data indicate that PIP5K1 and PIP5K2 generate PtdIns(4,5)P2 in polarized apical and basal plasma membrane microdomains important for the asymmetric distribution of PIN proteins. The failure to form BFA bodies decorated by PIN1 or PIN2 and the observation of fewer but larger plasma membrane CLC2-GFP foci in the pip5k1 pip5k2 double mutant (Figure 9) suggest that it is not clathrin recruitment to the membrane but rather cleavage of CCVs from the membrane that might be perturbed in these plants. In the double mutants with reduced PtdIns(4,5)P2 levels clathrin patches might appear larger because fewer CCVs are released and membrane-associated CLC2-GFP foci might coalesce. This concept is consistent with our understanding how CCV formation is mediated by dynamin in a PtdIns(4,5)P2-dependent fashion in mammalian cells (Leemon and Ferguson, 2000).

The involvement of PtdIns(4,5)P2 in the control of plasma membrane cycling of PIN proteins ties in with reports on other membrane constituents. For instance, effects on PIN cycling have been independently attributed also to sterols (Men et al., 2008) and sphingolipids containing very-long-chain fatty acids (Markham et al., 2011), leading us to speculate that PtdIns(4,5)P2, sterols, and sphingolipids might synergistically define membrane domains required for PIN recycling. Association of PtdIns(4,5)P2 with sphingolipids in detergent-insoluble membrane preparations has previously been described (Furt et al., 2010), but no biological function had been attributed to the association. As dynamins must exert a physical force to cleave off a CCV from the plasma membrane, this force requires a counterforce in the membrane bilayer. It appears possible that sterols, sphingolipids, and PtdIns(4,5)P2 form a joint membrane microdomain with particular stabilizing properties that might be required for dynamin function, as has been proposed for membrane microdomains involved in perceiving mechanical force (Anishkin and Kung, 2013).

Besides controlling CME, PtdIns(4,5)P2-containing membrane domains might also influence PIN recycling through indirect effects on PIN phosphorylation. For instance, PtdIns(4,5)P2 might act as a membrane-lipid ligand to activate a protein phosphorylation cascade consisting of 3-phosphoinositide-dependent protein kinase 1, which contains a Pleckstrin homology (PH) domain and phosphorylates the protein kinase PINOID (PID), thereby activating it (Zegzouti et al., 2006). Activated PID in turn phosphorylates PIN proteins, and this phosphorylation is required for their dynamic polar distribution (Robert and Offringa, 2008). In this context, enlarged CLC2-GFP foci in the plasma membrane might also arise from incorrect recruitment of CCVs into apically or basally bound sorting pathways. The data presented here are thus also consistent with a mechanism where PtdIns(4,5)P2 controls PIN-localization through effects on the PIN phosphorylation machinery, as was previously proposed (Zegzouti et al., 2006), in analogy to the polar localization of Glc transporters mediated by protein kinases B and C in mammalian cells (Watson et al., 2004).
The dwarf phenotype of the pip5k1 pip5k2 double mutant suggests that one consequence of reduced PtdIns(4,5)P2 formation is a defect in the balance of cell proliferation and cell differentiation. Because auxin promotes stem cell proliferation (Dello ioio et al., 2008) and a dwarf phenotype implies a shift toward differentiation, the dwarf phenotype observed in the pip5k1 pip5k2 double mutant is consistent with impaired distribution of auxin. Thus, altered growth in consequence of the reduced PtdIns(4,5)P2 production is consistent with a role of PtdIns(4,5)P2 in the control of directional auxin transport in root tips (Bennett and Scheres, 2010). While effects of PtdIns(4,5)P2 on the plasma membrane association of clathrin and, thus, the polar distribution of PIN proteins were evident, other facets of the pip5k1 pip5k2 double mutant phenotype may have additional causes not related to auxin. The pip5k1 pip5k2 double mutant reported may thus represent a tool to further dissect regulatory roles of PtdIns(4,5)P2 in Arabidopsis.

METHODS

Constructs for Stable Expression in Arabidopsis

Amplification of 1500-bp genomic sequences upstream of coding sequences for use as promoters was achieved with different Arabidopsis thaliana bacterial artificial chromosome clone templates as indicated, using the primer combinations PromPIP5K1for/PromPIP5K1rev on BAC clone F2E2 and PromPIP5K2for/PromPIP5K2rev on bacterial artificial chromosome clone T32E8. The PCR products representing promoter regions of PIP5K1 or PIP5K2 were moved directionally as SalI-NotI fragments into the vector pUC18Entry-EYFP, creating the plasmids Prom-PIP5K1-EYFP and Prom-PIP5K2-EYFP. The coding regions of PIP5K1 and PIP5K2 were amplified from PIP5K1- pGem-T-easy or PIP5K2- pGem-T-easy (Stenzel et al., 2008), respectively, using the primer combinations PIP5K1for/PIP5K1rev and PIP5K2for/PIP5K2rev. The coding sequences for PIP5K1 and PIP5K2 were moved as NotI-NotI fragments from PIP5K1-pGem-Teasy and PIP5K2-pGem-Teasy into Prom-PIP5K1-EYFP and Prom-PIP5K2-EYFP, respectively. A stop codon between PIP5K1/PIP5K2 and the respective EYFP reading frames was removed by mutagenesis using the primers QCPIP1Kfor/QCPIP1Krev and QCPIP2Kfor/QCPIP2Krev. Finally, the fragments consisting of the respective promoter sequence and the fusion constructs encoding PIP5K1-EYFP and PIP5K2-EYFP, respectively, were transferred to the expression plasmid pCAMBIA3300.0 GC using Gateway technology (Invitrogen) according to the manufacturer’s instructions.

Cloning of P44 5-Kinase Promoter Sequences for Generation of Promoter-GUS Fusions

The same PCR products representing PromPIP5K1 and PromPIP5K2 as used above were moved directionally as SalI-NotI fragments into the vector pgreenGUSPlus (Stenzel et al., 2008). The resulting plasmids were transformed into Agrobacterium tumefaciens strain EHA105 and used for stable Arabidopsis transformation.

Constructs for Stable Expression in Saccharomyces cerevisiae

PIPSK1 and PIP5K2 were moved as NotI-NotI fragments from PIP5K1- or PIP5K2-pGem-T-easy, respectively, into the yeast expression vector, Mss4 was amplified from S. cerevisiae cDNA using the primer combination MSS4for/MSS4rev. After cloning the PCR product into pGEM-T-easy (Promega), it was moved as a NotI-Not fragment into the vector YEp12-A1NEX (provided by Ellen Hornung, Georg-August-University, Goettingen).

Fusion Constructs for Inducible Overexpression in Arabidopsis

The coding region for PIPS K1 was moved from PIPS K1-pGem-T-easy into the plasmid pUC18Entr-ccdb as a NotI-NotI fragment. This introduced a new NcoI site contained in the primer sequence that was used for the introduction of the EYFP coding region, previously amplified using the primer combination EYFPfor/EYFPrev.

The coding region for PIPS K2 was moved from PIPS K2-pGem-T-easy as a SalI-NotI fragment into pUC18Entry-EYFP. Both EYFP-PIPSK1 and PIPS K2-EYFP were then transferred to the expression plasmid pMDC7 (Curts and Grossniklaus, 2003) using Gateway technology (Invitrogen) according to the manufacturer’s instructions.

All primer sequences are specified in Supplemental Table 1 online.

Arabidopsis Culture

Experiments were performed with Arabidopsis ecotype Columbia-0 (Col-0) or in Col-0 plants carrying T-DNA insertions in pip5k1 (SALK_146728) and/or for pip5k2 (SALK_012487). For stress treatments, seeds were surface sterilized by incubation for 15 min at 22°C in 6% (w/v) sodium hypochlorite in 0.1% Triton X-100 and washed five times in sterile distilled water. Seeds were vernalized at 4°C overnight and cultured in sealed jars on 0.5% Murashige and Skoog (MS) medium with modified vitamins (Duchefa) containing 1% Suc and 0.25% Gelrite (Carl Roth). After 14 d, plants were transferred into hydroponic cultures in liquid medium according to Randall and Bouma (1973). Hydroponic cultures were exposed to 140 μmol photons m⁻² s⁻¹ of light under a light/dark regime of 8 h light/16 h dark and continuous aeration. For stress treatments, 8- to 10-week-old plants were stimulated by adding NaCl to a final concentration of 0.4 M to the hydroponic medium. Rosette leaves were harvested before treatment and after various periods of stimulation and immediately frozen in liquid nitrogen.

Arabidopsis Transformation

Recombinant constructs were introduced into Arabidopsis lines using the floral dip method (Clough and Bent, 1998). Independent transformants were selected on MS media containing 50 μg mL⁻¹ kanamycin or 15 μg mL⁻¹ hygromycin, 1% (w/v) Suc, and 0.6% (w/v) agar or grown on soil and sprayed after 7 d with a 0.5% (w/v) Basta solution (Bayer Crop Science).

CCV Isolation

CCVs were enriched according to Harley and Beevers (1989) with minor modifications as previously described (König et al., 2008b). To prevent protein degradation, Pefabloc (Carl Roth) and protease inhibitor cocktail (Carl Roth) were included in all buffers.

Extraction and Analysis of Lipids

Lipids were extracted and analyzed according to König et al. (2008a) with slight modifications, as fatty acid methyl esters were analyzed using a 30 m × 250-μm DB-23 capillary column (Agilent) and a GCMS-QP2010S-EI gas chromatography–mass spectrometry system (Shimadzu).

Radiodetection of PtdIns(4,5)P2

Twelve-day-old Arabidopsis seedlings grown on MS agar plates were transferred to a 24-well plate and equilibrated overnight under continuous light with gentle shaking. Each well contained 500 μL of MS media with 1% Suc and six Arabidopsis seedlings (for wild-type and single mutant lines) and eight seedlings (pip5k1 pip5k2 double mutant). Radiolabel (50 μCi
of $^{32}$P was added to each well, and seedlings were harvested after 30 min, blotted on Kimwipes, and incubated for 20 min on ice in 500 μL of cold 20% perchloric acid. Seedlings were transferred to glass tubes, and lipids were extracted and separated by thin layer chromatography as previously described (Perera et al., 2005). The amount of $^{32}$P-labeled PtdInsP$_2$ was calculated as the percentage of the total $^{32}$P incorporated into the lipid phase of each sample.

**Lipid Kinase Assays**

Lipid kinase activity was determined as previously described (Cho and Boss, 1995). Reaction products were identified according to comigration with authentic standards (Avanti Polar Lipids) and quantified on thin layer chromatography plates by phosphor imaging (Phosphorimager FLA-3000; FujiFilm).

**Heterologous Expression in S. cerevisiae**

PI4P 5-kinases were expressed in S. cerevisiae strain YOC808 and complementation tests performed as previously described (Homma et al., 1998). The parental strain, YOC807, was used as a control.

**Determination of Gravitropic Bending**

Gravitropic bending was determined using plants grown on MS media in vertical Petri dishes as previously described (Fukaki et al., 1996) according to digital images.

**Genotyping**

The absence or presence of T-DNA inserts was detected by PCR on genomic DNA templates. The following primer combinations were used to amplify the wild-type alleles: gPIP5K1f/or/gPIP5K1rev and gPIP5K2f/or/gPIP5K2rev. Mutant alleles were amplified using LbAT1 and the respective reverse primer.

**Real-Time RT-PCR Analysis**

Total RNA was extracted using plant RNA purification reagent (Invitrogen). RNA was incubated with RNase-free DNase I for 30 min at 37°C to remove genomic DNA contamination, and RNA was ethanol precipitated. Five micrograms of total RNA was used as a template for reverse transcription with RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) in the presence of oligo(dT) primers. Equal amounts of first-strand cDNAs were used as templates for real-time PCR amplification using the following primer combinations: qPIP5K1f/or/qPIP5K1rev and qPIP5K2f/or/qPIP5K2rev. The Arabidopsis actin gene, ACT2, was amplified using primer combination ACT2f/or/ACT2rev. Quantitative real-time PCRs were performed in a MiniOpticon System (Bio-Rad), and PIP5K1 and PIP5K2 transcript levels were quantified in relation to ACT2 levels.

**Determination of Auxin Levels**

Plant material was extracted as previously described (Matyash et al., 2008). The analysis of constitutives was performed using an Agilent 1100 HPLC system (Agilent Technologies) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (MDS Sciex). Nanoelectrospray ionization (nanoESI) analysis was achieved using a chip ion source (TriVersaNanoMate; AdvionBioSciences). Reversed-phase HPLC separation was performed on an EC 50/2 Nucleo, C18 gravity 1.8-µm column (50 x 2.1 mm, 1.8-µm particle size; Macherey and Nagel), applying a column temperature of 30°C. For analysis, 10 μL of extract was injected. The binary gradient system consisted of solvent A, water/acetic acid (100:0.1, v/v), and solvent B, acetonitrile/acetic acid (100:0.1, v/v), with the following gradient program: 5% solvent B for 1 min, followed by a linear increase of solvent B up to 95% within 10 min and an isocratic run at 95% solvent B for 4 min. To reestablish starting conditions, a linear decrease to 5% B within 2 min was performed, followed by 10-min isocratic equilibration at 5% B. The flow rate was 0.3 mL min$^{-1}$. For stable nanoESI, 130 μL min$^{-1}$ of 2-propanol/acetone/triwater/acetic acid (70:20:10:0.1, v/v/v/v) delivered by a 2150 HPLC pump (GE Healthcare) was added just after the column via a mixing tee valve. Using another post column splitter, 790 nL min$^{-1}$ of the eluent was directed to the nanoESI chip. Ionization voltage was set to −1.7 kV. IAA was negatively ionized and determined in a scheduled multiple reaction monitoring mode. For the scheduled mode, the multiple reaction monitoring detection window was set to 72 s and a target scan time of 1.2 s was applied. Mass transitions were as follows: for D$_3$-IAA 179/135 (declustering potential −40 V, entrance potential −6.5 V, and collision energy −22 V) and for IAA 174/130 (declustering potential −55 V, entrance potential −4 V, and collision energy −16 V). The mass analyzers were adjusted to a resolution of 0.7 atomic mass units full width at half height. The ion source temperature was 40°C, and the curtain gas was set at 10 (given in arbitrary units). Quantification was performed using a calibration curve of intensity (mass-to-charge) ratios of [unlabeled]/[deuterium-labeled] versus molar amounts of unlabeled (0.3 to 1000 pmol).

**Determination of Auxin Transport**

Seven-day-old Arabidopsis Col-0 and 10-d-old day old pip5k1 pip5k2 seedlings with similar root lengths were grown on vertical plates. A piece of agarose gel containing 10 μM IAA (Sigma-Aldrich) and 100 nm [3H]IAA (Radiochemicals) was placed at the juncture between root tip and hypocotyl. Auxin was allowed to diffuse into the root. After 18 h, the terminal 1-cm tip of the root was removed and radioactivity determined by liquid scintillation counting using an Analyzer Tricarb 1900 TR (Canberra Packard).

**Staining, Fixing, or Application of Pharmacological or Immunological Agents**

**CHX and BFA Treatments**

Plants were treated with 50 μM CHX ~15 min prior to imaging. For the PIN1-GFP and PIN2-GFP recycling kinetic analysis, 25 μM BFA was applied to the plantlets, and confocal images were taken after the times indicated.

**Whole-Mount Immunolabeling of PIN Proteins**

Whole-mount immunolabeling of root tips was done as previously described (Sauer et al., 2008) with modifications: After fixation, root tips were mounted on poly-L-Lys–coated slides, air-dried, and stored at ~20°C. After rehydration and cell wall digestion, blocking was done with 5% BSA in PBS, and antibodies were diluted in 5% BSA/PBS supplemented with 1% acetylated BSA (Aurion). Goat anti-PIN1 (ap-20, diluted 1:250; Santa Cruz Biotechnology) and chicken anti-PIN2 (diluted 1:250; Agrisera) were used as primary antibodies. Donkey anti-goat IgG coupled to AlexaFluor488 and goat anti-chicken IgG coupled to AlexaFluor488 (all from Invitrogen) diluted 1:500 were used as secondary antibodies.

FM4-6 (Molecular Imaging Products) was added to root tips at a final concentration of 10 μM as described (Parson et al., 2001) and visualized after different periods of incubation.

**Histochemical Staining for GUS Activity**

Histochemical staining of plant tissue for GUS activity was performed as previously described (Jefferson et al., 1987). GUS-positive samples were
examined with a bright-field microscope (Olympus BX51) or a stereomicroscope (Olympus SZX12) at low magnification (×4 to ×10), both equipped with a camera, and digital images were recorded.

Microscopy and Imaging

For root length and leaf area measurements, plants were imaged using a flatbed scanner (Canoscan 800S; Canon). Lengths of roots and hypocotyls were determined using ImageJ (v. 1.41; National Institutes of Health), while leaf area was calculated using BlattFlaeche software (Datinf). Bright-field and epifluorescence images were recorded using an Olympus BX51 microscope, a F41-028 HQ filter set for YFP (Olympus), a F41-007 HQ filter set for Redstar, an Olympus ColorView II camera, and analySISDocu3.2 software (Soft-Imaging-Systems). Alternatively, images were recorded using a Zeiss Axioslager M1 microscope with filter sets 43 for DsRed, 46 for EYFP, and 47 for CFP. Confocal 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 and using a Zeiss LSM 510 confocal microscope (Zeiss). EYFP was excited at 514 nm and band-pass filtered. EYFP and Redstar were simultaneously excited at 488 and 561 nm, respectively, and imaged using a 405/488/561-nm major beam splitter and 505- to 550-nm and 575- to 615-nm band-pass filters. Alternatively, confocal microscopy was performed using an LSM 710 confocal microscope (Zeiss). Multi Time macro (Multi Time Series PLUS ZEN 2010-18) was used for automated series of multiple movies. Kymographs were obtained using the multiple kymograph plug-in, while clathrin density measurements were performed using the Fiji plugin TrackMate.

The fluorescently tagged CLC2-GFP marker lines were imaged on a confocal microscope equipped with a Yokogawa spinning disc head fitted to a Nikon Ti-E inverted microscope, a total internal reflection fluorescence (TIRF) oil immersion objective (Nikon CFI Apo TIRF × 100, numerical aperture of 1.49) an evolve charge-coupled device camera (Photometrics Technology), and a ×1.2 lens between the spinning disc and the camera. GFP was excited at 491 nm using a multichannel dichroic and an ET525/50M band-pass emission filter (Chroma Technology), while exposure time was 800 ms for the GFP laser. Image acquisitions were performed using Metamorph online premier (version 7.5). Images were processed with the help of Fiji image analysis software. Background correction was performed using subtract background (rolling ball radius 20 pixels), and walking average plug-ins in Fiji were used to smoothen the movies. Kymographs were obtained using the kymograph plug-in, while clathrin density measurements were performed using the Fiji plugin TrackMate.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACT2, At1g18780; CLC2, At2g40060; PIP5K1, At1g21980; PIP5K2, At1g77740; PIN1, At1g73590; and PIN2, At5g57090.

Supplemental Data

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

Supplemental Figure 1. PIP5K1 and PIP5K2 Are Expressed in Various Organs of Arabidopsis.

Supplemental Figure 2. T-DNA Insertion Mutants Deficient in PIP5K1 and PIP5K2 Have Unaltered Levels of Structural Phospholipids.

Supplemental Figure 3. T-DNA Insertion Mutants with the Genotypes pip5k1−/−/pip5k2−/− or pip5k1−/−/pip5k2−/− Are Phenotypic Intermediates between Single and Double Mutants.

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AUTHOR CONTRIBUTIONS

I.H. designed research, analyzed data, and wrote the article. T.Isch., S.W., P.K., and J.L. designed research, performed experiments, analyzed data, and edited the article. M.M., I.S., C.L., T.W., and T.I., performed experiments and analyzed data. Y.J.I., I.Y.P., and B.H. performed experiments, analyzed data, and edited the article. W.B., T.T., W.F.B., I.F., and S.P. analyzed data and edited the article.

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Phosphatidylinositol 4,5-Bisphosphate Influences PIN Polarization by Controlling Clathrin-Mediated Membrane Trafficking in *Arabidopsis*

Till Ischebeck, Stephanie Werner, Praveen Krishnamoorthy, Jennifer Lerche, Mónica Meijón, Irene Stenzel, Christian Löfke, Theresa Wiessner, Yang Ju Im, Imara Y. Perera, Tim Iven, Ivo Feussner, Wolfgang Busch, Wendy F. Boss, Thomas Teichmann, Bettina Hause, Staffan Persson and Ingo Heilmann

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