

Arabidopsis Rab-E GTPases exhibit a novel interaction with a plasma-membrane phosphatidylinositol-4-phosphate 5-kinase

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Summary

Rab GTPases of the *Arabidopsis* Rab-E subclass are related to mammalian Rab8 and are implicated in membrane trafficking from the Golgi to the plasma membrane. Using a yeast two-hybrid assay, *Arabidopsis* phosphatidylinositol-4-phosphate 5-kinase 2 (PtdIns(4)P 5-kinase 2; also known as PIP5K2), was shown to interact with all five members of the Rab-E subclass but not with other Rab subclasses residing at the Golgi or trans-Golgi network. Interactions in yeast and in vitro were strongest with RAB-E1d[Q74L] and weakest with the RAB-E1d[S29N] suggesting that PIP5K2 interacts with the GTP-bound form. PIP5K2 exhibited kinase activity towards phosphatidylinositol phosphates with a free 5-hydroxyl group, consistent with PtdIns(4)P 5-kinase activity and this activity was stimulated by Rab binding. Rab-E proteins interacted with PIP5K2 via its membrane occupancy and recognition nexus (MORN) domain which is missing from animal and fungal PtdIns(4)P 5-kinases.

In plant cells, GFP:PIP5K2 accumulated at the plasma membrane and caused YFP:RAB-E1d to relocate there from its usual position at the Golgi. GFP:PIP5K2 was rapidly turned over by proteasomal activity in planta, and overexpression of YFP:PIP5K2 caused pleiotropic growth abnormalities in transgenic *Arabidopsis*. We propose that plant cells exhibit a novel interaction in which PIP5K2 binds GTP-bound Rab-E proteins, which may stimulate temporally or spatially localized PtdIns(4,5)P₂ production at the plasma membrane.

Supplementary material available online at
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Key words: Ypt2, MG132, Proteasome, Root hair, PtdIns(4,5)P₂, Secretion, MORN domain

Introduction

Membrane trafficking in eukaryotes is dependent on the accurate targeting of transport vesicles between the diverse membrane-bound compartments of the biosynthetic and endocytic pathways. The Rab family of small GTPases is a key regulatory protein family which contributes to the specification of membrane identity, the accuracy of vesicle targeting, and the recruitment of molecular motors to membranes (Grosshans et al., 2006). Another class of molecules crucial for the dynamic regulation of the endomembrane system is the phosphoinositide lipids. These are generated transiently on particular membranes and are involved in the regulation of membrane trafficking, cytoskeleton remodelling, signal transduction and cell growth and migration (Vicinanza et al., 2008). Both Rab GTPases and phosphoinositides each localise to particular membranes and represent labile identifiers of the different membrane domains in the endomembrane system (Behnia and Munro, 2005). Intricate and dynamic cross talk between phosphoinositide metabolism and Rab GTPase function occurs at various locations within the endomembrane system of yeasts, animals and plants (Munro 2004, Preuss et al., 2006; Vicinanza et al., 2008).

Genomic data have shown that yeasts, animals and plants have each elaborated distinct sets of Rab proteins. *Arabidopsis* and mammals each have roughly 60 Rab GTPases whereas there are only six to ten in yeasts. Strikingly, however, 80% of the predicted mammalian Rab GTPase subclasses are missing in *Arabidopsis*

(Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003). Phylogenetic analysis shows that all 57 *Arabidopsis* Rab GTPase sequences fall into just eight clades (Rab-A to Rab-H). Six of these clades are clearly related to one of the six RAB GTPase subclasses that are common to all three kingdoms of life, whereas the remaining two are related to the mammalian Rab2 or Rab18 clade (Rutherford and Moore, 2002; Vernoud et al., 2003). Rab GTPases involved in early stages of secretory trafficking and in sorting to the vacuole have been extensively studied (reviewed in Woollard and Moore, 2008), but less is known about the Rab proteins involved in the later stages of secretory trafficking. One of the clades implicated in this stage is the Rab-E clade.

In *Arabidopsis*, the Rab-E clade is represented by five genes, *RAB-E1a* to *RAB-E1e*, that are predicted to form a single functional subclass (Pereira-Leal and Seabra, 2001). These proteins are most similar to Ypt2 of *Schizosaccharomyces pombe* and more loosely similar to Rab8 in mammals and Sec4p of *Saccharomyces cerevisiae* (Rutherford and Moore, 2002). YFP-tagged RAB-E1d and RAB-E1c localise to the Golgi stacks in tobacco leaf epidermis (Zheng et al., 2005) and in *Arabidopsis* root tips where they are also found on the cell plate in dividing cells (Teh and Moore, 2007; Chow et al., 2008; Speth et al., 2009). Localisation to the Golgi occurs via a saturable and apparently nucleotide- and prenylation-dependent mechanism (Zheng et al., 2005). GFP-tagged RAB-E1d and endogenous Rab-E proteins have also been observed at the plasma membrane in *Arabidopsis* leaves, suggesting a role in trafficking

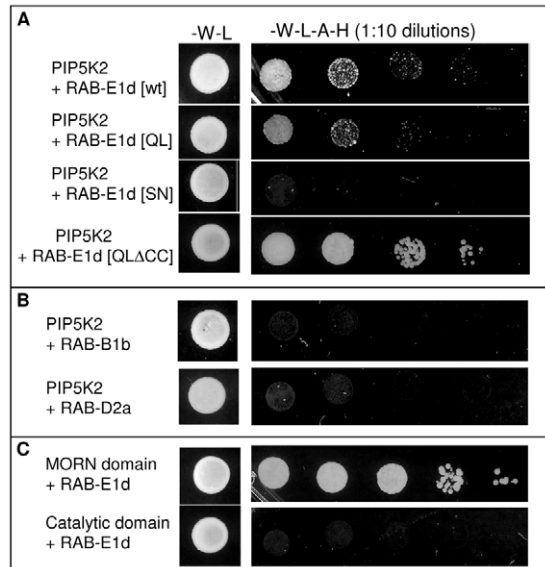


Fig. 1. PIP5K2 interacts specifically with active Rab-E proteins via its MORN domains in yeast two-hybrid assays. (A) Yeast growth on either permissive (-W-L) or stringent selective (-W-L-A-H) media. Strains express a full-length PIP5K2 prey fusion plus RAB-E1d bait fusions carrying either wild-type (wt) RAB-E1d, the Q74L (QL) or S29N (SN) mutants, or the Q74L mutant from which the carboxy-terminal prenylated cysteine residues have been deleted (QLACC). (B) As in A, but with bait constructs carrying Rab GTPases from two other Golgi-associated subclasses, Rab-B (related to Rab2) and Rab-D2 (related to Rab1 and Ypt1), as indicated. (C) As A, but with prey fusions bearing either the MORN domains or catalytic domain of PIP5K2 as indicated.

between these compartments (Speth et al., 2009). Moreover, the expression of RAB-E1d[N128I], a dominant-negative mutant of *Arabidopsis* RAB-E1d, in tobacco leaf epidermal cells caused a secreted GFP marker to accumulate partially in the ER, Golgi and in RAB-F2-positive prevacuolar compartments, owing to increased partitioning of the GFP marker into the vacuolar pathway. It was concluded that RAB-E1d[N128I], and therefore perhaps RAB-E1d or one of its interactors, acts in post-Golgi biosynthetic trafficking to the plasma membrane (Zheng et al., 2005).

Previous studies have identified the *Pseudomonas syringae* pv *tomato* type III virulence effector avrPto as an interactor of the *Arabidopsis* and tomato Rab-E subclass in yeast two-hybrid assays but the interaction could not be confirmed in vivo (Bogdanove and Martin, 2000; Speth et al., 2009). To learn more about the functions of the plant Rab-E subclass in plant cells we used the yeast two-hybrid assay to screen for endogenous interactors of the GTP-bound

protein. We identified an *Arabidopsis* phosphatidylinositol-4-phosphate 5-kinase (PIP5K2; also known as PtdIns(4)P 5-kinase 2), as an interactor of all five *Arabidopsis* RAB-E proteins. We show that in plants the interaction occurs via the MORN (membrane occupancy and recognition nexus) domain of PIP5K2. As this domain is found only in plant PtdIns(4)P 5-kinases, the interaction described here may have evolved in the plant lineage.

Results

Identification of interactors of *Arabidopsis* RAB-E1d by a yeast two-hybrid assay

To identify interactors of the GTP-bound form of RAB-E1d, a yeast two-hybrid screen was performed using the Q74L mutant form of the protein as the bait. This mutant protein is predicted to be GTPase-deficient and thus to be stabilised in the GTP-bound state (Walworth et al., 1992; Olkkonen and Stenmark, 1997). The C-terminal prenylation sites were also removed from the bait to prevent membrane association (Zheng et al., 2005). A screen of 24×10^6 colonies from two pre-mixed *Arabidopsis* cDNA libraries yielded two putative interactors under high-stringency conditions, one of which encoded a truncated protein of the *Arabidopsis* PIP5K2 (At1g77740), a putative sub-family B type I/II PtdIns(4)P 5-kinase (Mueller-Roeber and Pical, 2002).

The interaction between PIP5K2 and RAB-E1d was apparently specific for the GTP-bound state, since PIP5K2 interacted with both the wild-type and Q74L mutant of RAB-E1d, but not with the S29N mutant, which is predicted to preferentially bind GDP (Zheng et al., 2005) (Fig. 1A). Deletion of the prenylation sites from RAB-E1d[Q74L] increased the strength of the interaction with PIP5K2, perhaps by facilitating nuclear rather than membrane localisation of the bait protein during the yeast two-hybrid assay (Fig. 1A).

PIP5K2 interacted specifically with all five members of the Rab-E subclass but not with a member of the most closely related subclass RAB-D2, or with members of several other Golgi- or trans-Golgi-localised subclasses such as RAB-B or RAB-A2 (Fig. 1B, Table 1 and supplementary material Fig. S1).

PIP5K2 interacts with the RAB-E proteins via its MORN domain

The members of the subfamily B of the plant type I phosphatidylinositol-4-phosphate 5-kinases are distinguished by the presence of a conserved N-terminal MORN domain that is absent from paralogues in other eukaryotes (Mueller-Roeber and Pical, 2001; Ma et al., 2004). This domain contains several (seven, in the case of PIP5K2) repeats of a 23-residue long motif that is found in several eukaryotic protein families and is implicated in membrane lipid binding, protein interactions and modulation of enzyme

Table 1. PIP5K2 interacts specifically with active Rab-E proteins via its MORN domain in yeast two-hybrid assays

Bait	Prey				
	Rab-E*	Other Rab subclasses†	RAB-E1d Q74L	RAB-E1d Q74L ΔCC	RAB-E1d S29N
PIP5K2 full length	+	-	+	++	-
PIP5K2 MORN	++	-	++	++	-
PIP5K2 catalytic	-	-	-	-	-
PI4Kβ1	-	n.a.	-	-	-

Growth of cells on stringent selective media: -, no growth; +, ++ and +++, increasing growth. RAB-E1d bait fusions carried either the Q74L (QL) or S29N (SN) mutants, or the Q74L mutant from which the C-terminal prenylated cysteine residues have been deleted (QLACC). PI4Kβ1 is a phosphoinositide 4-kinase that interacts with RAB-A4b (Preuss et al., 2006).

*All five members of the Rab-E subclass were tested and gave the same result.

†Rab GTPases tested include RAB-A2a, RAB-A2d, RAB-B1b and RAB-D2a.

activity (Takeshima et al., 2000; Im et al., 2007). The interacting clone recovered from the yeast two-hybrid library encoded the MORN domain of the *Arabidopsis* PIP5K2 protein. As shown in Fig. 1C, Table 1 and supplementary material Fig. S1, the MORN domain interacted with RAB-E1d more strongly than the full-length PIP5K2 protein whereas deletion of the MORN domain eliminated the interaction between PIP5K2 and RAB-E proteins. Furthermore, the MORN domain exhibited the same specificity as the full-length protein towards the RAB-E subclass (Fig. 1C, Table 1 and supplementary material Fig. S1). Thus, the MORN domain is necessary and sufficient for the specific interaction of PIP5K2 with the GTP-bound form of RAB-E proteins in yeast.

The specificity of the interaction in yeast was also tested using the *Arabidopsis* phosphatidylinositol 4-kinase, PI4K β 1. This was identified as an interactor of *Arabidopsis* RAB-A4b, which localises to *trans*-Golgi membranes (Preuss et al., 2006). We did not detect interactions between PI4K β 1 and the RAB-E subclass (Table 1), consistent with the absence of MORN domains in the PI4K β 1 protein.

PIP5K2 and its MORN domain also interacted with Rab-E proteins *in vitro* (Fig. 2A). As in yeast, the interaction was stronger with the RAB-E1d[Q74L] mutant than with S29N mutant (Fig. 2A). Interactions with RAB-B1b, a member of the Rab2-related *Arabidopsis* Rab-B subclass (Woollard and Moore, 2008), were also weaker than with RAB-E1d[Q74L] and showed no apparent specificity for the nucleotide state as the RAB-B1b[Q65L] and [S20N] mutants each interacted with similar efficiency (Fig. 2A).

PIP5K2 has phosphatidylinositol-4-phosphate 5-kinase activity that is stimulated by Rab binding

PIP5K2 is a member of the *Arabidopsis* sub-family B of type I phosphatidylinositol-4-phosphate 5-kinases (Mueller-Roeber and Pical, 2002). These enzymes are predicted to catalyse the formation of PtdIns(4,5) P_2 from PtdIns(4) P . To investigate the activity of PIP5K2, bacterially expressed protein was used in *in vitro* kinase assays using various phosphoinositide lipids as the substrate. As shown in Fig. 2B, PIP5K2 did not exhibit phosphatidylinositol kinase activity, but was able to phosphorylate PtdIns(3) P and PtdIns(4) P indicating that the protein had phosphatidylinositol phosphate kinase activity. Neither PtdIns(5) P nor PtdIns(3,4) P_2 were substrates for the enzyme, consistent with the hypothesis that PIP5K2 has phosphatidylinositol phosphate 5-kinase activity. Other PtdIns(4) P 5-kinases, including yeast Mss4, the mammalian type I PIP5Ks and the *Arabidopsis* PIP5K1, have been shown to exhibit dual activity *in vitro*, towards both PtdIns(3) P and PtdIns(4) P (Desrivieres et al., 1998; Toliás et al., 1998; Westergren et al., 2001).

To investigate the significance of the interaction between Rab-E and PIP5K2 we examined whether the activity of the enzyme was altered in the presence of the Rab protein. As shown in Fig. 2C the activity of PIP5K2 towards PtdIns(4) P *in vitro* was stimulated approximately fivefold in the presence of GST:RAB-E1d. No such stimulation was observed with GST only and no PtdIns(4) P -kinase activity was detected in preparations of GST:RAB-E1d (data not shown). Thus Rab-E proteins may stimulate the production of PtdIns(4,5) P_2 by PIP5K2 at their site of interaction *in vivo*. We next investigated where PIP5K2 localises and whether Rab-E and PIP5K proteins interact in plant cells.

PIP5K2 fusion proteins localise to the plasma membrane in tobacco leaf epidermal cells

Phosphoinositides and the enzymes responsible for their metabolism are restricted to distinct membranes (De Matteis and Godi, 2004).

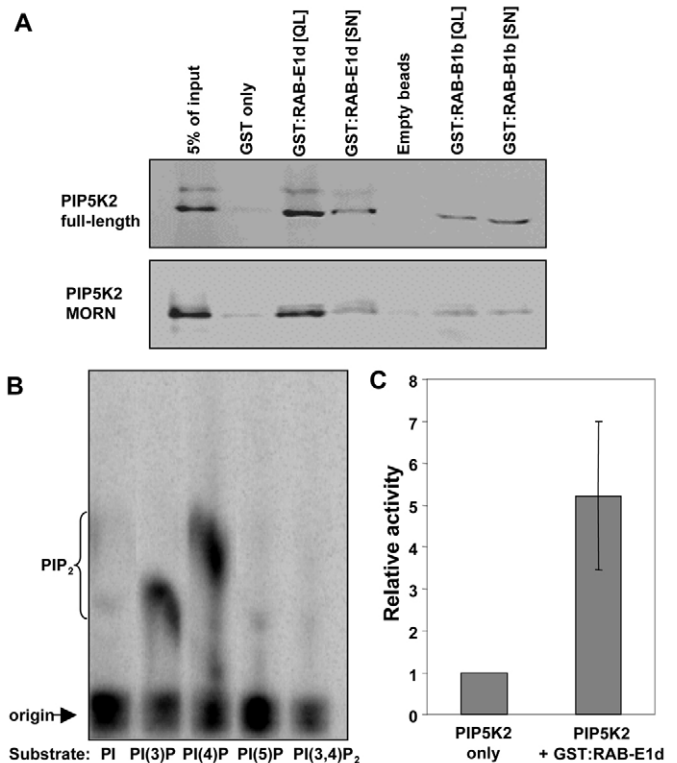


Fig. 2. PIP5K2 has phosphoinositide kinase activity *in vitro*.

(A) Autoradiograph of *in vitro* pull-down assay of radio-labelled PIP5K2 (upper panel) or the MORN domains (lower panel) with glutathione beads alone (empty beads) or beads coated with the indicated fusions. (B) Autoradiograph of a chromatogram indicating the kinase activity of recombinant PIP5K2 towards phosphoinositol (PI) and various phosphoinositol phosphates or diphosphates (PIP₂) as indicated. The positions of phosphates are indicated in brackets. (C) Relative kinase activity (error bars indicate \pm s.d.) towards PtdIns(4) P of recombinant PIP5K2 in the presence or absence of purified GST:RAB-E1d. Activity in the absence of GST:RAB-E1d was defined as 1 in each of three independent experiments.

To investigate the localisation of PIP5K2 in live plant cells, the full-length PIP5K2 protein was fused at its N-terminus to either the Venus variant of YFP (Nagai et al., 2002) or to EGFP and transiently expressed in tobacco leaf epidermal cells (Batoko et al., 2000; Zheng et al., 2005). Confocal laser-scanning microscopy of fluorescently tagged PIP5K2 protein at the early stages of transient expression showed that it accumulated predominantly at the plasma membrane (Fig. 3A,B). By contrast, fusions that lacked the MORN domain or the catalytic domain were observed in the cytosol and in aggregates that increased in size and abundance with expression level (supplementary material Fig. S2).

In comparison with other fluorescent fusions, PIP5K2 fusion proteins accumulated poorly in the transfected cells and were visible for only a few hours after the onset of transient expression, which typically occurs approximately 30 hours after infiltration of the leaves with *Agrobacterium* (Zheng et al., 2005) (Fig. 4). GFP:PIP5K2 was stabilised for 2 days or more by incubation of the transfected tissue in the 26S proteasome inhibitors MG132 (Fig. 4), ALLN or PSI (data not shown) implicating proteasomal degradation in the turnover of the GFP:PIP5K2 fusion protein. By contrast, inhibitors of vacuolar proteolysis, such as E64-d (Zheng et al., 2005), had no effect on the accumulation of the fusion protein (Fig. 4).

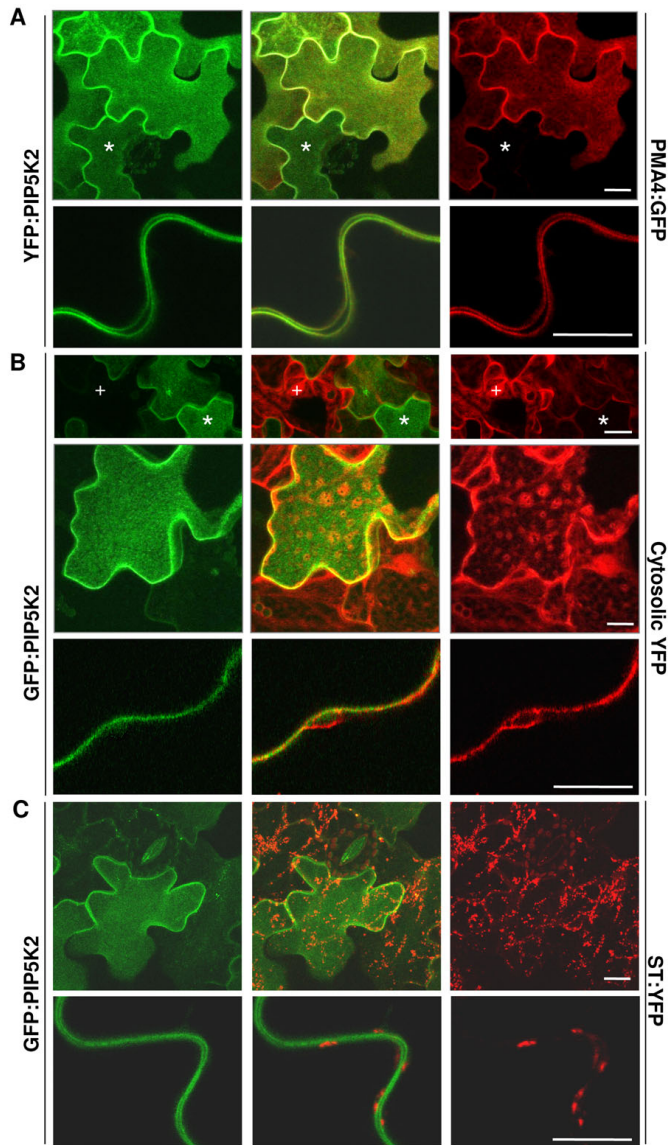


Fig. 3. GFP:PIP5K2 resides at the plasma membrane. Confocal images of tobacco leaf abaxial epidermis following co-transfection with constructs expressing various fluorescent protein fusions; images in the left and right columns are merged in the centre panels. Upper rows are maximum projections in *z* series of optical sections, and the lower row in each section is a single near-medial optical section. (A) YFP:PIP5K2 (green) and the plasma-membrane marker PMA4:GFP (red). (B) GFP:PIP5K2 (green) or cytosolic YFP (red); the asterisks and crosses in the top row indicate cells expressing only GFP:PIP5K2 or YFP, respectively, confirming lack of bleed-through between GFP and YFP detection channels under these conditions. (C) As for B, but with the Golgi-targeted YFP-fusion ST:YFP; in the lower panel the plasma membranes of two adjacent cells are labelled by GFP:PIP5K2. Scale bars: 5 μ m. The *z*-series extend through 24 μ m in A, 36 μ m in B and 33 μ m in C.

GFP:PIP5K2 specifically alters the location of YFP:RAB-E1d in vivo

In tobacco leaf epidermal cells and in *Arabidopsis* root tips, YFP-tagged Rab-E proteins localise to the Golgi apparatus, but not the plasma membrane (Chow et al., 2008; Teh and Moore, 2007; Zheng et al., 2005) (Fig. 5A). By contrast, its putative interactor PIP5K2 appeared to localise at the plasma membrane in both types of cell (Fig. 3A; see also Fig. 6C below), but not at the Golgi apparatus

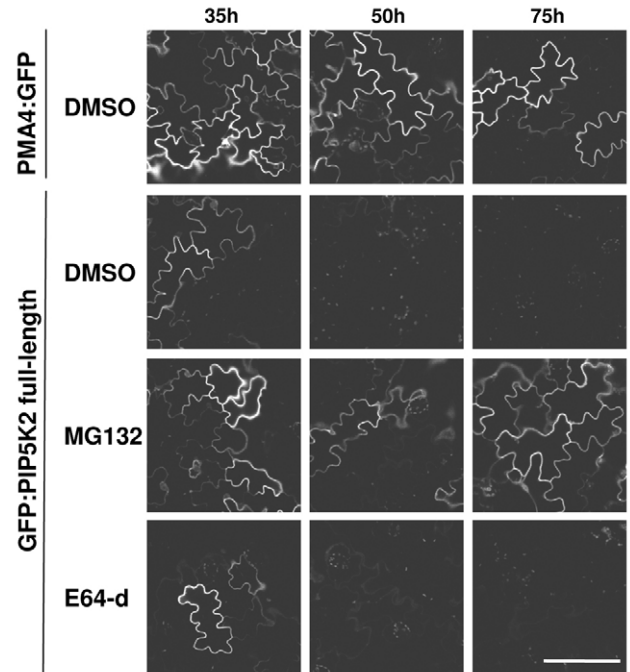


Fig. 4. Stabilisation of GFP:PIP5K2 by proteasome inhibitors in plant cells. Low magnification confocal images of tobacco leaf abaxial epidermal cells transiently expressing GFP fusions to the plasma-membrane ATPase PMA4 or to PIP5K2 at the indicated times (hours) after transfection and incubation in the proteasome inhibitor MG132, the vacuolar protease inhibitor E64-d or the solvent DMSO, as indicated. Scale bar: 50 μ m.

(Fig. 3C). As the Rab-E subclass is implicated in membrane trafficking from the Golgi to the plasma membrane (Zheng et al., 2005; Speth et al., 2009), we considered whether the overexpression of either partner was able to influence the steady-state location of the other in tobacco leaf epidermis. Strikingly, when YFP:RAB-E1d was expressed in cells that also accumulated GFP:PIP5K2, it was no longer observed on the Golgi but instead accumulated at the plasma membrane (Fig. 5B). The extent of this relocation correlated with the expression level of GFP:PIP5K2 in individual cells. By contrast, YFP:RAB-E1d[S29N] was not recruited to the plasma membrane but remained cytosolic as previously reported (Zheng et al., 2005) suggesting that GTP-binding by RAB-E1d is necessary for its recruitment to the plasma membrane by GFP:PIP5K2 (Fig. 5C). The ability of GFP:PIP5K2 to relocate Rab GTPases from the Golgi was apparently specific for the Rab-E subclass, as another Golgi-localised Rab GTPase fusion (YFP:RAB-B1b) did not relocate to the plasma membrane when coexpressed with GFP:PIP5K2 (Fig. 5D). Furthermore, expression of an alternative plasma-membrane GFP marker, PMA4:GFP, failed to alter the localisation of YFP:RAB-E1d (Fig. 5A). When YFP:RAB-E1d was coexpressed with a GFP fusion to the catalytic domain of PIP5K2, it again remained localised at the Golgi (supplementary material Fig. 2A). By contrast, coexpression with GFP-tagged PIP5K2 MORN domains caused YFP:RAB-E1d, but not YFP:RAB-B1b, to co-accumulate in the cytosolic aggregates (supplementary material Fig. 2B,C).

Thus, fluorescently tagged RAB-E1d and PIP5K2 can apparently interact in plant cells because the accumulation of PIP5K2 at the plasma membrane can cause redistribution of RAB-E1d from the Golgi apparatus. As in the yeast two-hybrid assay and in vitro, this

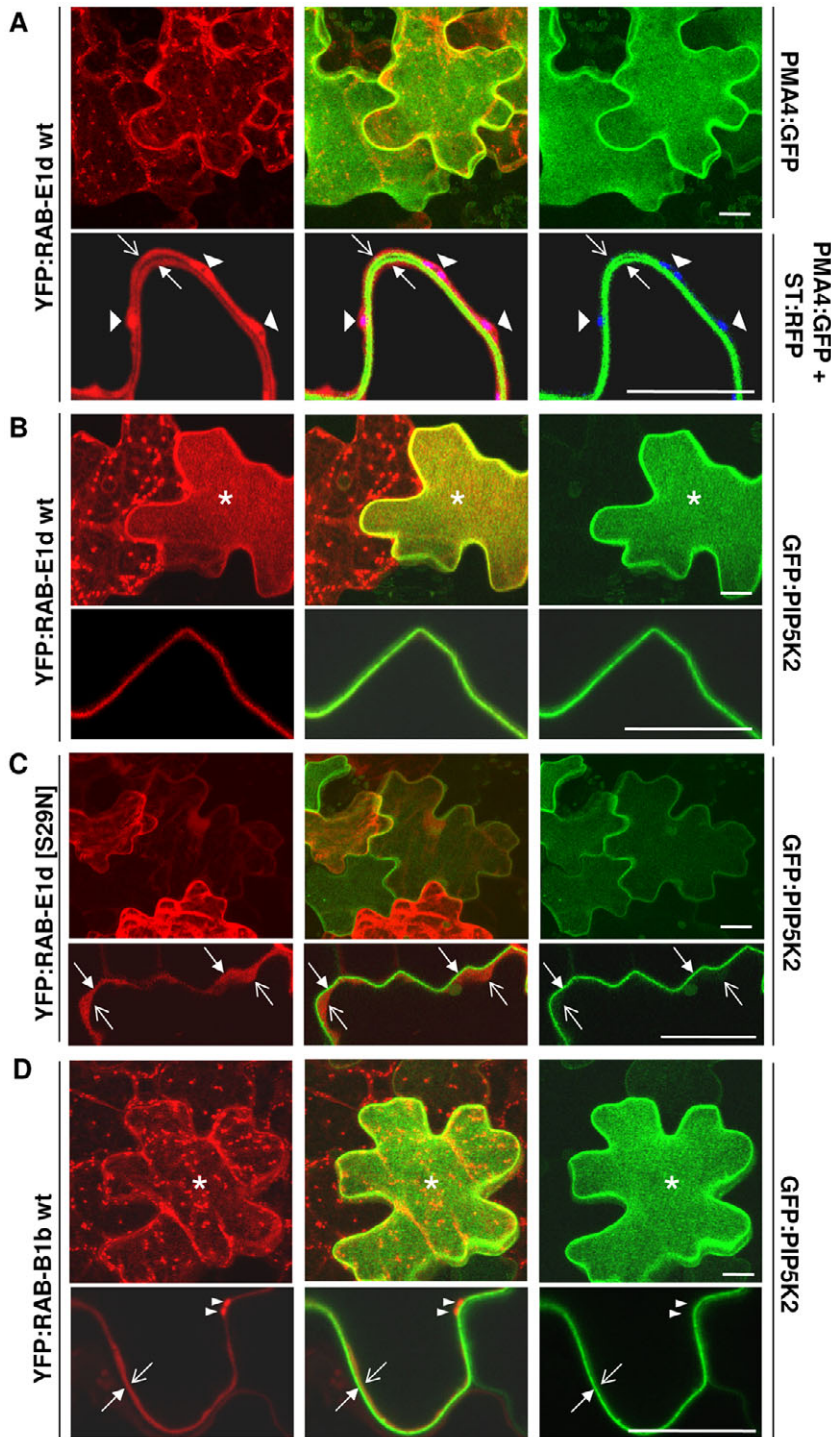


Fig. 5. Transient expression of GFP:PIP5K2 shifts the steady-state membrane localisation of YFP:RAB-E1d from the Golgi to the plasma membrane. Confocal images of tobacco leaf abaxial epidermis following co-transfection with constructs expressing various fluorescent protein fusions; upper rows are maximum projections in z-series and lower rows are single optical sections; images in the right and left columns are merged in the centre column. (A) Upper row, control transfections with YFP:RAB-E1d (red) and the plasma-membrane marker PMA4:GFP (green); lower row, YFP:RAB-E1d (red), PMA4:GFP (green), and the Golgi marker ST:RFP (blue) confirming that YFP:RAB-E1d accumulates in the cytosol (arrows) and is concentrated on Golgi membranes (arrowheads) but not at the plasma membrane. (B) Co-transfection with YFP:RAB-E1d (red) and GFP:PIP5K2 (green) showing that in cells coexpressing both proteins (asterisks) YFP:RAB-E1d adopts a plasma-membrane localisation in contrast to the Golgi localisation in neighbouring cells that lack GFP:PIP5K2. (C) As for B, but with the S29N mutant of YFP:RAB-E1d. In the lower panel, note that YFP:RAB-E1d[S29N] remains cytosolic (open arrows) with no evidence for accumulation at the plasma membrane with GFP:PIP5K2 (solid arrows). (D) As for B, but with YFP:RAB-B1b; in the lower panel the cytoplasmic pool of YFP:RAB-B1b can be resolved from the adjacent plasma membrane with no evidence of accumulation at the latter location; arrowheads, Golgi stacks. Scale bars: 5 μ m. The z-series extend through 40 μ m in A, 27 μ m in B, 26 μ m in C and 36 μ m in D.

interaction in plant cells is mediated via the plant-specific MORN domain of PIP5K2 and is dependent on GTP binding by RAB-E1d.

Overexpression of YFP:PIP5K2 alters *Arabidopsis* seedling growth and reproductive development

The instability of YFP and GFP-tagged PIP5K2 in transient plant expression suggested that the PIP5K2 protein levels in the plasma membrane in plant cells may need to be tightly controlled *in vivo*. We therefore investigated the consequences of overexpressing YFP:PIP5K2 or YFP alone in *Arabidopsis* plants. Many of the

transformants expressing YFP:PIP5K2 were noticeably smaller than those generated with YFP alone, and when the YFP:PIP5K2-expressing plants were transferred to the greenhouse the most severely dwarfed plants died. Nevertheless, 68 transformants survived and exhibited a range of phenotypes (Fig. 6A), including stunted rosette and inflorescence growth (29%), stunted rosette development with sterile flowers (43%), normal rosette development but with sterile flowers (9%), or normal development and fertility (19%). All control plants generated (expressing YFP alone) exhibited normal development and fertility. A small quantity of seeds

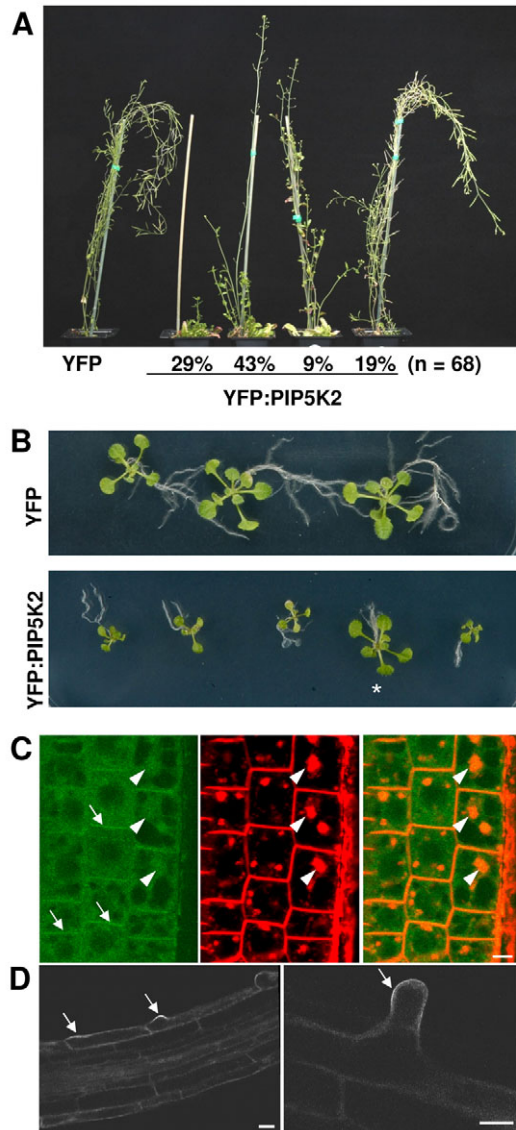


Fig. 6. Overexpression of YFP:PIP5K2 perturbs plant development. (A) Phenotypes of primary *Arabidopsis* transformants expressing YFP only or YFP:PIP5K2. The percentages are the proportion of 68 primary transformants that exhibited phenotypes similar to the individuals shown. (B) T2 generation seedlings from plants expressing YFP only (upper panel) or YFP:PIP5K2 (lower panel); asterisk indicates a segregant that lacks YFP:PIP5K2. (C) Localisation of YFP:PIP5K2 (green) in *Arabidopsis* root tips following incubation in the endosomal dye FM4-64 (red) and treatment with brefeldin A (BFA); arrows, plasma membrane; arrowheads, BFA-bodies labelled by FM4-64. (D) In the differentiation zone of the root YFP:PIP5K2 concentrates at the tips of young and incipient root hairs (arrows). Scale bars: 10 μ m.

was recovered from the YFP:PIP5K2 transformants with abnormal development and, when these families were plated on non-selective medium, they segregated plants that were stunted (Fig. 6B). The stunted phenotype correlated with YFP expression (data not shown). YFP:PIP5K2 expression was barely detectable in these seedlings, in contrast to the free YFP in control plants. The YFP:PIP5K2 protein was enriched in the plasma membrane of the root meristem of the most brightly fluorescing seedlings (Fig. 6C), and in mature cells it was apparently enriched at the tips of emergent and growing root hairs (Fig. 6D). YFP:PIP5K2 did not accumulate in the 'BFA

bodies' induced in *Arabidopsis* root meristematic cells by the drug brefeldin A (BFA), which traps recycling plasma-membrane proteins in aggregated endosomal membranes (Fig. 6C). Thus, YFP:PIP5K2 localises to the plasma membrane in *Arabidopsis* roots, as it does in tobacco leaf epidermis, but apparently it does not recycle with other plasma-membrane proteins via the BFA-sensitive trafficking pathways.

Discussion

Arabidopsis PtdIns(4)*P* 5-kinases have recently been implicated in several cellular processes including apical growth (Ischebeck et al., 2008; Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008), water stress and stomatal opening (Mikami et al., 1998; Lee et al., 2007), and sugar signalling (Lou et al., 2007) but the regulation of plant PtdIns(4)*P* 5-kinase membrane-binding and activity is not understood (Ischebeck et al., 2008).

We show that Rab GTPases of the plant RAB-E subclass exhibit a novel interaction with PIP5K2. The fact that all five members of the *Arabidopsis* RAB-E subclass interact with PIP5K2 is consistent with the prediction that they represent a single functional subclass within the plant Rab GTPase phylogeny (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). Rab GTPases and phosphoinositide lipids are among the most important labile markers of membrane identity in eukaryotic cells. In several cases, these signals are known to be interdependent, as Rab proteins can either recruit enzymes of phosphoinositide metabolism (Christophoris et al., 1999; de Graaf et al., 2004; Preuss et al., 2006; Shin et al., 2005; Stein et al., 2003) or interact with proteins that bind specific phosphoinositides (Chung et al., 1998; Nielsen et al., 2000; Peterson et al., 1999; Simonsen et al., 1998). However, interactions between Rab GTPases and PtdIns(4)*P* 5-kinases have not previously been described.

Arabidopsis PIP5K2 targets GFP to the plasma membrane where its product, PtdIns(4,5)*P*₂ is known to accumulate in diverse organisms (Várnai and Balla, 1998; van Leeuwen et al., 2007). In plants, however, the concentration of PtdIns(4,5)*P*₂ in the membrane is lower than in other organisms and 10- to 100-fold lower than its precursor PtdIns(4)*P* (Meijer and Munnik, 2003). The concentration of PtdIns(4,5)*P*₂ can be increased in plant cells by inhibiting phospholipase C activity, which suggests that PtdIns(4,5)*P*₂ is rapidly turned over (van Leeuwen et al., 2007). Im et al. failed to image GFP-PIP1 in transgenic tobacco cells or *Arabidopsis* plants (Im et al., 2007). We found that the PIP5K2 protein was also extremely labile unless inhibitors of the 26S proteasome were applied, reinforcing the view that PtdIns(4,5)*P*₂ accumulation in plant cells is tightly regulated. Consistent with this, we found that plants expressing YFP-tagged PIP5K2 exhibited severe pleiotropic developmental abnormalities. Similarly, overexpression of the *Arabidopsis* pollen-specific PtdIns(4)*P* 5-kinases PIP5K4 or PIP5K5, resulted in reduced pollen tube growth rates and aberrant pollen tube morphology (Ischebeck et al., 2008).

One situation in which PtdIns(4,5)*P*₂ is concentrated on membranes in plant cells is at the final stages of cell plate expansion, where it accumulates at the cell plate margins just prior to its fusion with the plasma membrane (van Leeuwen et al., 2007). Interestingly, the Rab-E subclass was recently shown to be one of three Rab GTPase subclasses that can target YFP to the growing margins of the cell plate in *Arabidopsis* root tips (Chow et al., 2008). Labelling of the cell plate by YFP:RAB-E1d was, however, observed less consistently than with RAB-A2 or RAB-A3 proteins (Chow et al., 2008), and this may reflect the observation that PtdIns(4,5)*P*₂ accumulates on the cell plate predominantly at the later stages of

cytokinesis (van Leeuwen et al., 2007). PtdIns(4,5) P_2 was also observed to accumulate at the tips of growing root hairs (van Leeuwen et al., 2007) and, consistent with this, we found that GFP:PIP5K2 also concentrated at such sites in *Arabidopsis* seedlings.

Rab-E proteins interact with PIP5K2 via its MORN-repeat domain. Among eukaryotic PtdIns(4) P 5-kinases, only those from the type I subfamily B in plants possess the MORN domain, accounting for the novel interaction between PIP5K2 and plant Rab-E GTPases. The MORN domain, which inhibits the activity of the PtdIns(4) P 5-kinase catalytic domain and can bind phosphatidic acid (Im et al., 2007), is shown here to be essential for plasma-membrane localisation. Nevertheless, the PIP5K2 MORN domain is apparently insufficient for membrane localisation, as a GFP-tagged form of the amino-terminal domain containing the PIP5K2 MORN repeats failed to localise to the plasma membrane, even though it retained the capacity to interact specifically with the Rab-E GTPases. MORN domains have been found in the mammalian Rab5 GEF ALS2, where they are required for its GEF activity (Otomo et al., 2003), but are also present in membrane-associated protein families that have no known Rab-GTPase interactions, such as junctophilins (Takeshima et al., 2002; retinophilin (Mecklenburg et al., 2000), Arc3 (Shimada et al., 2004), MORN1 and 2 (Gubbels et al., 2006), RSD44 (Shetty et al., 2007) and meichroacidin (Tokuhira et al., 2008).

Overexpression of GFP:PIP5K2 in plant cells was sufficient to shift the steady-state distribution of YFP:RAB-E1d from the Golgi to the plasma membrane. As neither the S29N nor the N128I mutant were recruited to the plasma membrane of cells accumulating PIP5K2, it is likely that PIP5K2 interacts preferentially with the GTP-bound form of RAB-E1d in planta as in yeast and in vitro. The ability of PIP5K2 to relocate YFP:RAB-E1d suggests that it may act as a docking site for the activated form of the protein rather than act purely as an effector that is recruited by GTP-bound Rab-E proteins. A precedent for this is provided by the phosphoinositide 4-kinase PI4K β in mammalian cells, which appears to act as a docking site for Rab11A and B at the *trans*-Golgi (de Graaf et al., 2004). Although we have not demonstrated a direct interaction in planta, PIP5K2 can interact with GTP-bound RAB-E1d in vitro and in yeast two-hybrid assays, so it is possible that PIP5K2 interacts directly with GTP-bound RAB-E1d to stabilise it at the plasma membrane in plant cells. Furthermore, the dominant-negative S29N or N128I mutant of YFP:RAB-E1d failed to detectably alter the distribution of GFP:PIP5K2 or to trap it at the Golgi (Fig. 5C and data not shown), which is consistent with an interaction at the plasma membrane and suggests also that trafficking mediated by Rab-E proteins may not be necessary for localisation of PIP5K2.

We have not observed YFP:RAB-E1d or YFP:RAB-E1c at the plasma membrane in either tobacco leaf epidermis, in *Arabidopsis* seedling root tips (Zheng et al., 2005; Teh et al., 2007; Chow et al., 2008) or in *Arabidopsis* leaves, so its association with PIP5K2 may normally be transient or may be below the detection limit at endogenous PIP5K2 abundance in these cells. In leaves of *Arabidopsis* plants however, Rab-E proteins and GFP-tagged RAB-E1d have been detected in substantial but variable quantities at the plasma membrane (Speth et al., 2009) supporting the view that Rab-E proteins visit this membrane. Using a YFP:RAB-E1d fusion from the WAVE collection, weak fluorescent signals were occasionally observed at the plasma membrane in mature *Arabidopsis* leaves (supplementary material Fig. S3) and root tips (Geldner et al., 2009). The significance of the variation in steady-state distribution of Rab-

E proteins and protein fusions between the Golgi and plasma membrane in *Arabidopsis* is currently unclear.

In plant cells, a fluorescent reporter of PtdIns(4) P , the substrate of PIP5K2, was found to be abundant in the plasma membrane and in Golgi membranes labelled by the *trans*-Golgi marker ST-CFP (Vermeer et al., 2008). Consistent with this, PI4K β 1, a phosphoinositide 4-kinase that can generate PtdIns(4) P , has been located at *trans*-Golgi membranes in root hairs (Preuss et al., 2006). Furthermore, disruption of the two redundant PI4K β genes of *Arabidopsis* altered the morphology of *trans*-Golgi cisternae, indicating that PI4K β function is important for maintenance of these compartments (Preuss et al., 2006). PI4K β 1 interacts with the Rab11-related protein RAB-A4b and colocalises on *trans*-Golgi membranes with YFP:RAB-A4b (Preuss et al., 2006). This interaction between phosphoinositide 4-kinases and Rab11-related GTPases at the *trans*-Golgi seems to be common to eukaryotes as paralogues in both yeast and animals exhibit genetic and physical interactions, respectively (de Graaf et al., 2004; Hama et al., 1999; Walch-Solimena and Novick, 1999; Sciorra et al., 2005). As YFP:RAB-E1d and -E1c proteins localise to the Golgi and plasma membrane (Zheng et al., 2005; Speth et al., 2009), Rab-A4 and Rab-E proteins appear to interact at the *trans*-Golgi and plasma membrane with enzymes involved in the sequential conversion of PtdIns to PtdIns(4,5) P_2 . This is consistent with previous data obtained using a dominant-negative mutant of RAB-E1d, which implicated Rab-E proteins, or one of their interactors, in post-Golgi trafficking to the plasma membrane (Zheng et al., 2005).

In *Arabidopsis*, YFP:RAB-A4b appears to remain associated with membranes when the two redundant PI4K β genes are disrupted (Preuss et al., 2006), so it is unlikely that these kinases are essential docking factors for RAB-A4b, as proposed for mammalian RAB11 and PI4K β . Instead we suggest that RAB-A4b recruits PI4K β to the *trans*-Golgi where it generates the substrate for PIP5K2, which in turn stabilises active Rab-E proteins on *trans*-Golgi or post-Golgi membranes such as the plasma membrane. As RAB-E1d can stimulate the kinase activity of PIP5K2 in vitro, the ability of PIP5K2 to stabilise RAB-E1d at the plasma membrane suggests that this interaction may locally stimulate PtdIns(4,5) P_2 production. This would be consistent with the unusually low quantity of PtdIns(4,5) P_2 in the plasma membrane of plant cells and the relative abundance of PtdIns(4) P (van Leeuwen et al., 2007; Vermeer et al., 2008). One possibility is that this interaction alters the identity of Golgi-derived secretory membranes at (or en route to) the plasma membrane. Alternatively, as PtdIns(4,5) P_2 appears to stimulate endocytosis (Huang et al., 2004; Zoncu et al., 2007), it is conceivable that the interaction helps to balance rates of exocytosis and endocytosis to ensure membrane economy.

Materials and Methods

Cloning by Gateway recombinant technology

The coding sequence of the proteins of interest was amplified by polymerase chain reaction (PCR) using primers containing the Gateway attB adaptors and PyroBest DNA polymerase (Takara, Bio Europe, Berkshire, UK). Information on the template and primers used for each PCR is provided in the supplementary material Table S1. When no template plasmid was available, total RNA was extracted from *Arabidopsis thaliana* ecotype Columbia (Col-0) seedlings or leaves, using the RNeasy Mini kit (Qiagen Ltd, Crawley, UK) and reverse transcribed using an oligo d(T) and SuperScript II RNase H-Reverse Transcriptase (Invitrogen, Paisley, UK), following the manufacturer's instructions. The PCR product was cloned by a BP recombinant reaction into the pDONR207 plasmid (Invitrogen, Paisley, UK), following the manufacturer's instructions. *E. coli* strain DH5 α cells (Invitrogen, Paisley, UK) were transformed with 1 μ l of the BP reaction mix by heat-shock, and the plasmid was isolated from the transformants using the QIAprep Spin Miniprep kit (Qiagen). The plasmid DNA was checked by restriction enzymes and by DNA sequencing using

the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems Ltd, Warrington, UK). pENTR207 plasmids containing the appropriate insert were used in a LR recombinant reaction to sub-clone the cDNA of interest into the appropriate destination plasmid.

Yeast two-hybrid screening

Saccharomyces cerevisiae MAT α Y187 and MAT α AH109 strains (Clontech, Palo Alto, CA) were transformed with the GAL4 DNA-binding domain (BD) cloning vector pAS2.1-RABE.1d [QL Δ CC] by the lithium acetate method, following the manufacturer's instructions (Matchmaker, Clontech, Palo Alto, CA) and the transformants were selected in SD agar minimal medium lacking tryptophan for 72 hours at 30°C. AH109 cells expressing the GAL4 BD-RABE.1d [QL Δ CC] fusion protein were tested for autonomous activation in SD agar minimal medium lacking tryptophan (W), alanine (A) and histidine (H) for 72 hours at 30°C. Y187 cells expressing the GAL4 BD-RABE.1d[QL Δ CC] fusion protein were mated with AH109 cells expressing GAL4 activation domain (AD) fused to proteins from two *Arabidopsis* seedling cDNA libraries, synthesized using oligo d(T) and random oligonucleotides and constructed in the pGAD4T7Rec plasmid (Matchmaker). The two cDNA libraries were a generous gift from Brendan Davies (University of Leeds, Leeds, UK). Twenty-four million clones were screened. The putative interactors were selected under high stringency conditions, in SD agar minimal medium lacking W, leucine (L), A and H for up to 10 days at 30°C and the colonies were restreaked in fresh selective medium for further selection. Plasmid DNA was rescued from the AH109 cells using the QIAprep Spin Miniprep kit (Qiagen), reintroduced into AH109 cells and the interaction with Y187 cells expressing the GAL4 BD-binding domain-RABE.1d[QL Δ CC] fusion protein re-tested in SD agar minimal medium lacking W, L, A and H. The confirmed interactors were sequenced using the primer 5'ADLD (5'-CTATTCGATGATGAAGATACCCACCAACCCA-3') and the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems Ltd, Warrington, UK).

Yeast two-hybrid interaction by one-on-one test

GAL4 BD pAS2.1 vectors were transformed into *S. cerevisiae* Y187 and AH109 strains, whereas the GAL4 AD pACT2 vectors were transformed into the AH109 strain, as described above. AH109 cells expressing a GAL4 BD fusion protein were tested for autonomous activation before the interaction tests. Y187 cells transformed with a pAS2.1 vector and AH109 cells transformed with a pACT2 vector were mated on YPDA agar plates for 24 hours at 25°C, and the diploids were selected in SD agar medium lacking W and L. Interactions were assayed in SD agar medium lacking W, L, A and H. Y187 cells transformed with pAS2.1 or with pAS2.1-GUS and AH109 cells transformed with pACT2.1 or pACT2.1-GUS were used as negative controls for the interaction tests. None of the proteins tested interacted with the empty plasmids or with GUS fused to either the GAL4 BD or to the GAL4 AD (data not shown).

Expression and purification of GST fusion proteins

The coding sequences of RAB-E1d [QL], RAB-E1d [SN], RAB-B1b [QL] and RAB-B1b [SN] were sub-cloned from pENTR207 plasmid into a Gateway derivative of pGEX-4T-1 cloning vector (Ketelaar et al., 2004), in frame with the N-terminal glutathione S-transferase (GST) gene. The cloning vector pET-42 (Novagen, Merck Chemical Ltd., Nottingham, UK) was used to express the GST only protein. The recombinant proteins were expressed in *E. coli* strain BL21 DE3 pLys Rosetta2 cells (Novagen). Cultures were grown to OD₆₀₀ 0.6 and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 3 hours at 28°C. The cells were collected by centrifugation, resuspended in 10 ml ice-cold 1 \times PBS buffer containing Protease Inhibitor Cocktail Complete, Mini, EDTA-free (Roche) and sonicated on ice. GST-fusion proteins were purified with glutathione-Sepharose 4B (Amersham Biosciences), following the manufacturer's instructions. The quality and amount of purified GST fusion proteins were assessed by SDS-PAGE, by running 15 μ l of beads suspension along with 5 and 10 μ g of bovine serum albumin (BSA) protein.

In vitro transcription-translation (TnT) reaction

One microgram of pGEM-PIP5K2, pGEM-PIP5K2 MORN or pGEM-PIP5K2 Δ MORN plasmid was used as the template in the in vitro coupled transcription-translation reaction, using the TnT T7 Coupled Reticulocyte Lysate system (Promega UK, Southampton, UK) and Redivue L-[³⁵S]methionine (Amersham Biosciences/GE Healthcare, Chalfont St. Giles, UK). The reaction mix was prepared following the manufacturer's instructions and incubated for 90 minutes at 30°C. To check the reaction efficiency, 5 μ l of the reaction mix were run on a 12% acrylamide gel. The gel was fixed for 30 minutes in 50% methanol and 10% acetic acid, incubated for 5 minutes in gel drying solution (7% methanol, 7% acetic acid, 1% glycerol) and dried for 36 hours between two cellophane sheets (Sigma-Aldrich UK, Gillingham, Dorset, UK), before being exposed in a Phoros FX Plus Molecular Imager-S phosphorimager system [BioRad Laboratories (UK), Hemel-Hempstead, UK] using a low-energy screen. The reaction using the pGEM-PIP5K2 Δ MORN plasmid as a template never yielded a product, so it was not possible to use the ³⁵S-PIP5K2 Δ MORN as a negative control in the pull-down assays.

In vitro pull-down assay

Ten micrograms of GST protein (GST only, GST-RAB-E1d [QL], GST-RAB-E1d [SN], GST-RAB-B1b [QL] or GST-RAB-B1b [SN]) bound to glutathione-Sepharose 4B beads and 10 μ l TnT reaction mix (³⁵S-PIP5K2 or ³⁵S-PIP5K2 MORN) were incubated in 200 μ l binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 100 μ g/ml BSA) for 90 minutes at 25°C, with gentle shaking. As an additional negative control, the same amount of empty glutathione-Sepharose 4B beads was assayed for their capacity to bind the ³⁵S-labelled proteins. Following the incubation, the reaction mix was centrifuged and the beads were washed six times with 1 ml binding buffer, resuspended in 30 μ l of SDS-PAGE loading buffer and run on a 12% acrylamide gel, along with 1.5 μ l TnT reaction mix (input sample). The gel was fixed, dried and exposed as above. After the exposure, the gel was rehydrated and stained with Coomassie blue to confirm the equal loading of the GST proteins (data not shown).

Expression and purification of 6xHis fusion proteins

The coding sequence of PIP5K2 was amplified from the pENTR207-PIP5K2 plasmid using primers PIPK2-EcoRI-Fa (5'-AAAGAATTCATGCGTGAACCGCTTGTAGC-3'; EcoRI site underlined) and PIPK2-XhoI-R (5'-AAACTCGAGTTAGCCGCTTCGATGAAGAT-3'; XhoI site underlined) and cloned between the EcoRI and XhoI sites of the expression vector pET28 (Novagen), in frame with the 6xHis coding sequence. *E. coli* strain BL21 DE3 pLys Rosetta2 cells (Novagen) transformed with the plasmid were grown until OD₆₀₀ 0.6 and induced by 1 mM IPTG medium for 3 hours at 30°C. The cells were collected by centrifugation, resuspended in 10 ml ice-cold extraction buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 5 mM β -mercaptoethanol) containing protease inhibitors [5 ng/ml leupeptin, 5 ng/ml pepstatin A, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 ng/ml aprotinin and 10 ng/ml N- α -(p-toluene sulfonyl)-L-arginine methylester (TAME)] and sonicated on ice. The His-tagged proteins were purified with Ni-NTA resin (Qiagen) and a gradient of imidazole. Upon elution from the resin and change of buffer to the phosphorylation assay buffer, the concentration of the fusion proteins was assessed using Bradford reagent (BioRad Laboratories) and its integrity analysed by SDS-PAGE.

In vitro phosphorylation assay

The lipid substrates tested were phosphatidic acid (PA; Sigma-Aldrich UK, Gillingham, Dorset, UK), phosphatidylinositol 3-phosphate (PtdIns(3)P) and phosphatidylinositol 4-phosphate (PtdIns(4)P; both from Calbiochem/GE Healthcare), phosphatidylinositol 5-phosphate (PtdIns(5)P; Echelon Biosciences, Salt Lake City, UT) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂; Sigma-Aldrich UK). Ten micrograms of 6xHis-tagged PIP5K2 fusion protein were resuspended in 50 μ l of phosphorylation assay buffer containing 50 mM HEPES pH 7.4, 2 mM MgCl₂, 10 mM 2-glycerophosphate, 2 mM DTT, 200 mM NaCl, 2% (w/v) sodium cholate, 5 mM α -cyclodextrin (Sigma-Aldrich UK), 5 μ g lipid substrate, 0.2 μ M ATP and 10 μ Ci of [γ -³²P]ATP (Amersham/GE Healthcare). The reaction was incubated for 10 minutes at 25°C and stopped with 1 ml methanol-chloroform (1:1 ratio), 50 μ g bovine brain extract Folch Fraction I (Sigma-Aldrich UK) and 0.5 ml 1.2 M HCl. The organic phase was washed twice with methanol-HCl (1:1 ratio), dried using a nitrogen stream and the lipids resuspended in 30 μ l of chloroform. The lipids were applied to a freshly heat activated Partisil LK6D TLC plate (Whatman/GE Healthcare), eluted using a mixture of chloroform:methanol:H₂O:NH₄OH (15N) (90:90:20:7 ratio) and the signal was detected by exposing an X-ray film overnight or quantified using a Typhoon 9400 imager (GE Healthcare). To test the effect of RAB-E1d on PIP5K2 activity towards PtdIns(4)P, phosphorylation assays were performed as above but 2.5 mM GTP and 10 μ g of GST or GST-RAB-E.1d proteins were also included in the reaction mixture. The assays were performed three times independently.

Transient expression of fluorescent proteins in tobacco leaves

The cDNA of PIP5K2, MORN domains or catalytic domain (Δ MORN) were sub-cloned from the pENTR207 vector into the Gateway destination binary vector pB7WGF2 (Karimi et al., 2002), to obtain a N-terminal GFP-fusion protein, or pEarleyGate104, to obtain a N-terminal YFP-fusion protein (Earley et al., 2006). YFP-RAB-E1d and the S29N mutant form were as described (Zheng et al., 2005). YFP-RAB-B1b was a kind gift from Ulla Neumann and Chris Hawes (Oxford Brookes University, Oxford, UK). The fluorescent markers used were the plasma-membrane marker pVKHEn6-PMA4-GFP (Lefebvre et al., 2004), the cytosolic marker pVKHEn6-HAVenus (Zheng et al., 2005) and the Golgi markers pVKH-N-ST-YFP (Brandizzi et al., 2002) and ST-RFP (Renna et al., 2005). The fluorescent proteins were transiently expressed in *Nicotiana tabacum* (cv Petit Havana SR1) plants as described before (Zheng et al., 2005). The leaf lower epidermal cells were inoculated with an *Agrobacterium* cell suspension at OD₆₀₀ 0.05 for all constructs except pB7WGF2-PIP5K2, which was infiltrated at OD₆₀₀ 0.3. At 36 hours after infiltration, pieces of infected leaf were sampled randomly, mounted in water and the lower epidermis imaged using a Zeiss LSM 510 META laser scanning microscope and a Plan-Neofluar 25 \times 0.8 immersion corrected DIC lens or a C-Apochromat 40 \times 1.2 NA water corrected lens. For single GFP fluorescence analysis, the 488 nm excitation line of a 25 mW argon ion laser and a 505-530 nm band-pass filter were used.

Combinations of fluorescent proteins were imaged as described (Samalova et al., 2006) or by spectral deconvolution using 458 nm and 514 nm excitation wavelengths, a 458/514 primary dichroic, and emission collected over the 475-560 range of the META detector and deconvolved using fluorescence and reflection spectra acquired with the same primary dichroic. Images were processed with LSM 3.5 software (Carl Zeiss, Welwyn, UK).

Infiltration of tobacco leaves with proteasome inhibitors

Twenty four hours after infiltration with *Agrobacterium tumefaciens* GV3101, infected areas of the tobacco leaves were injected with different protease or 26S proteasome inhibitors. The injected areas were excised from the plant and floated on the same chemical solution for 24 hours in the dark, before imaging with the confocal microscope. The chemicals tested were the protease inhibitor E64-d ethyl ester (Bachem, St Helens, UK; stock, 200 mM in DMSO; infiltrate, 400 µM; float, 200 µM), and the proteasome inhibitors MG132 (stock, 100 mM in DMSO; infiltrate and float, 100 µM), ALLN (stock, 100 mM in DMSO; infiltrate and float, 100 µM) and PSI (stock, 100 mM in DMSO; infiltrate and float, 100 µM) (all from Calbiochem/GE Healthcare). The highest concentration of DMSO used in the experiments above was used to inject and float tobacco leaves, to serve as a negative control. Low-magnification and high-magnification images were acquired from 20 randomly selected regions of the leaf 24 hours and 48 hours after infiltration, using the same imaging settings for all images.

Stable transformation of *Arabidopsis* plants

The plasmids pEarleyGate104 only, pEarleyGate104-PIP5K2 and pEarleyGate104-PIP5K2 MORN domains were electroporated into *Agrobacterium tumefaciens* GV3101 cells and used to transform *Arabidopsis thaliana* Col-0 plants by the flower dip method (Clough and Bent, 1998). Transformed seeds were selected in half-strength Murashige and Skoog (MS) medium supplemented with 10 µg/ml of glucophosphate, 5 µg/ml of amphotericin, 40 µg/ml of nystatin, 200 µg/ml of carbenicillin, 2 µg/ml of timentin and 15 µg/ml of hygromycin. Seedlings were treated with brefeldin A as described previously (Teh and Moore, 2007).

Cloning of RAB-E1d [QL] by directed point mutation

Amplification was performed from plasmid pSKUE11, containing the full-length cDNA for RAB.E1d, with the primers UE11QL-F (5'-TGGACACTGCAGG-TCTCGAACGTTTCAGAACTATCAC-3'; mutation underlined) and PAR8-4 (5'-TAGCGGGATCCCTAAACGTAACAGCAAGC-3') and with the primers UEQL-R (5'-TCTGAAACGTTTCAGACCTGCAGTGTCCAAATCTG-3'; mutation underlined) and PAR8-1 (5'-AATGGATCCGCTCGAGCACCATGG-CGTTTGCAGCCGGC-3') using Accuzyme (Bioline). The PCR products were purified using QIAquick kit (Qiagen), quantified in 1% agarose gels and 10 ng of each PCR product were mixed and used as template for the second round PCR. The PCR product was cloned into pGEM T-Easy plasmid (Promega) and checked by restriction enzyme digestion and sequencing as described above.

Cloning of PIP5K2 ΔMORN

The plasmid pENTR207-PIP5K2, containing the full-length cDNA for PIP5K2, was amplified with the primers PIPK1F (5'-TTTGGGCCATGATGCGTGAACCGC-TTGT-3', *Apal* site underlined) and PIPK2BamHI_in_frame (5'-AAAGGATCCA-CGTTGGGAAACCCGCGGA-3', *Bam*HI site underlined) using PyroBest DNA polymerase (Takara). The PCR product was purified using QIAquick kit (Qiagen), cloned into pGEM T-Easy plasmid (Promega, Southampton, UK) and checked by restriction enzyme digestion and sequencing, as described before. The plasmid pGEM T-Easy-PIP5K2-1/2 was digested with *Apal* and *Bam*HI, and the restriction digestion fragment was desphosphorylated and cloned into plasmid pENTR207PIP5K2 previously digested with *Apal* and *Bam*HI restriction enzymes, to obtain the plasmid pENTR207-PIP5K2 ΔMORN. The final plasmid was sequenced to confirm that the two fragments of PIP5K2 cDNA were cloned in frame.

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