An ancient RAB5 governs the formation of additional vacuoles and cell shape in petunia petals


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An ancient RAB5 governs the formation of additional vacuoles and cell shape in petunia petals

Highlights

- Flowering plants contain three ancient classes of canonical RAB5 proteins
- RAB5s display divergent transcription regulation and intracellular localization
- RAB5a is specifically required to form vacuolinos from small endosomal precursors
- The others, RAB5a1 and RAB5a2, cannot functionally replace RAB5a

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In brief
Flower petals contain small vacuolar compartments, named vacuolinos, that are intermediate stations for protein trafficking to the central vacuole. Li et al. report that petals express three distinct canonical RAB5 GTPases originating from ancient duplications and that one of these directs the formation of vacuolinos from small endosomal precursors.
An ancient RAB5 governs the formation of additional vacuoles and cell shape in petunia petals

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SUMMARY

Homologous (“canonical”) RAB5 proteins regulate endosomal trafficking to lysosomes in animals and to the central vacuole in plants. Epidermal petal cells contain small vacuoles (vacuolinos) that serve as intermediate stations for proteins on their way to the central vacuole. Here, we show that transcription factors required for vacuolino formation in petunia induce expression of RAB5a. RAB5a defines a previously unrecognized clade of canonical RAB5s that is evolutionarily and functionally distinct from ARA7-type RAB5s, which act in trafficking to the vacuole. Loss of RAB5a reduces cell height and abolishes vacuolino formation, which cannot be rescued by the ARA7 homologs, whereas constitutive RAB5a (over)expression alters the conical cell shape and promotes homotypic vacuolino fusion, resulting in oversized vacuolinos. These findings provide a rare example of how gene duplication and neofunctionalization increased the complexity of membrane trafficking during evolution and suggest a mechanism by which cells may form multiple vacuoles with distinct content and function.

INTRODUCTION

In all eukaryotes, membrane traffic is an essential process that delivers proteins and other compounds to distinct cellular compartments by membrane vesicles that bud off from donor membranes to fuse with specific target compartments. SNARE proteins and RAB GTPases are major factors in determining the identity of vesicles/endosomes and their fusion with membranes from specific target compartments. SNAREs, RABs, and other trafficking regulators are encoded by gene families that existed in early eukaryotes. Since the separation of animals and plants, these families have expanded by independent gene duplications in both lineages, which is thought to have been important for the increased tissue specificity and complexity of trafficking systems during evolution. However, specific examples supporting this idea remain scarce.

Secretion is a major trafficking pathway that delivers proteins to the plasma membrane or, when the protein has a specific vacuolar sorting domain, to the vacuole or lysosome. Although many plant cells contain a single vacuole, several cell types possess multiple vacuoles with different content and, apparently, different functions (Epimashko et al., 2004; Fleurat-Lessard et al., 1997; Frigerio et al., 2008; Otegui et al., 2005; Paris et al., 1996). Cells in the epidermis of flower petals are dedicated to attracting animal pollinators by visual (color) and/or chemical (scent) cues and by providing a landing site. In petunia and roses, these cells contain a large central vacuole (CV) filled with anthocyanin pigments, as well as numerous small vacuolar compartments, called vacuolinos (Figure 1A), that lack anthocyanins (Faraco et al., 2017).

In petunia flowers, a complex of transcription factors (MYB-bHLH-WD40, MBW), which consists of the MYB protein ANTHOCYANIN2, the bHLH protein AN1, and the WD40 protein AN11, drives the expression of genes involved in anthocyanin synthesis in late stages of bud development, prior to opening of the flower (de Vetten et al., 1997; Quattrocchio et al., 1999, 2006; Spelt et al., 2000). Slightly later, when the bud has reached its maximum size and the flower opens, a related MYB-bHLH-WD40-WRKY (MBWW) complex, consisting of the MYB protein PH4, AN1, AN11, and the WRKY factor PH3, activates a (partially) distinct set of target genes, which drive the hyper-acidification of the CVs in colored cells of the petal epidermis to confer a red-violet flower color (Quattrocchio et al., 2006; Spelt et al., 2000; Verweij et al., 2016) and the formation of vacuolinos (Faraco et al., 2017). The major target genes involved in vacuolar acidification, namely, PH1 and PH5, encode two interacting P-ATPases that reside in the CV membrane (tonoplast) and act as a proton pump (Faraco et al., 2014; Verweij et al., 2008).
However, no MBWW-regulated genes involved in the formation of vacuolinos have been identified thus far. Vacuolinos represent an intermediate station for membrane proteins, like PH1, PH5, KCO1, vacuolar SNAREs, and soluble proteins, such as Aleu green fluorescent protein (Aleu-GFP), on their way to the CV (Figure 1A). In transient expression assays, these proteins accumulate first in vacuolinos, after about 24 h, before they arrive at the CV, after approximately 48 h (Faraco et al., 2017). In epidermal petal cells of an1, ph3, and ph4 mutants, which lack vacuolinos, proteins move “directly” (within 24 h) to the CV, as they do in wild-type petal mesophyll and leaf cells (Faraco et al., 2017; Figure 1A). The MBWW target gene PH1 is essential for the trafficking from vacuolinos to the CV, as in ph1 mutant petals vacuolar proteins accumulate in (enlarged) vacuolinos but do not move on to the CV (Faraco et al., 2017). This suggests that both the formation of vacuolinos and the transport from vacuolinos to the CV require a largely unknown set of factors that are encoded by MBWW-regulated genes.

GTPTases of the RAB family are regulators of endomembrane trafficking in all eukaryotes (Uemura and Ueda, 2014; Wan- dinger-Ness and Zerial, 2014). They cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound form that is attached to the membrane where they recruit specific effectors (Barr and Lambright, 2010; Langemeyer et al., 2018; Müller and Goody, 2018). Arabidopsis possesses two “canonical” RAB5s, namely, RHA1 and ARA7 (also called RABF2a and RABF2b), that are orthologous to metazoan RAB5s and a more distant relative, ARA6 (also called RABF1), belonging to a plant-specific clade of RAB5s (Rutherford and Moore, 2002; Vernoud et al., 2003). ARA7, RHA1, and ARA6 are converted from the GDP-bound to the GTP-bound form by the same activator, the Guanine nucleotide Exchange Factor (GEF) VPS9a (Fukuda et al., 2013; Goh et al., 2017; Sunada et al., 2016) and, to a lesser extent, the redundant paralog VPS9b (Nielsen and Thordal-Christensen, 2018). Yet, they have different functions. ARA7 and RHA1 localize to nearly identical populations of prevacuolar compartments (PVCs)/multivesicular bodies (MVBs) and are involved in vacuolar trafficking in leaf and root cells (Bottanelli et al., 2012; Kotzer et al., 2004; Lee et al., 2004; Sohn et al., 2003) and to specific defense structures (encasements) in pathogen-infected cells (Nielsen et al., 2017), whereas ARA6 resides on a subpopulation of endosomes partially overlapping with ARA7/RHA1 positive ones and functions mainly in the traffic between PVCs/MVBs and the plasma membrane (Ebine et al., 2011; Ueda et al., 2004).

In this study, we found that petals from an opening petunia flower express three canonical RAB5 genes, namely, RAB5a, RAB5a1, and RAB5a2 (Figure 1B; Figure S1A). RAB5a is expressed primarily in petals and in the vasculature of leaves and stems and is strongly reduced in an1, ph3, and ph4 mutant petals (Figures 1B–1D; Figure S1B), suggesting a role in the vacuolino pathway. RAB5a1 and RAB5a2, by contrast, are not controlled by AN1, PH3, or PH4 (Figures 1B and 1C) and are expressed in a broad range of tissues (Figure 1D).

To examine the relationship of these petunia proteins with the canonical Arabidopsis RAB5s (ARA7 and RHA1) and AtARA6, which belongs to a distinct plant-specific clade of RAB5-like proteins, we retrieved highly similar proteins from genome databases and performed phylogenetic analyses (Figure 1E). These analyses revealed that the petunia protein PhARA6 is the apparent ortholog of ARA6, which in this and other analyses is more related to RAB22 than to RAB5s, whereas RAB5a, RAB5a1, and RAB5a2 are canonical RAB5s that are co-orthologs of animal RAB5s (Figure 1E). The canonical plant RAB5s proved more diverse than previously thought. RAB5a2 is apparently orthologous to ARA7 and RHA1 from Arabidopsis, whereas RAB5a1 and RAB5a represent two distinct clades. These three clades existed already in early angiosperms because distantly related Eudicot species belonging to Asterids

**RESULTS**

Identification of three canonical RAB5 homologs in petunia

Examination of RNA sequencing (RNA-seq) data indicated that petunia petals express three canonical RAB5 genes, namely, RAB5a, RAB5a1, and RAB5a2 (Figure 1B; Figure S1A). RAB5a is expressed primarily in petals and in the vasculature of leaves and stems and is strongly reduced in an1, ph3, and ph4 mutant petals (Figures 1B–1D; Figure S1B), suggesting a role in the vacuolino pathway. RAB5a1 and RAB5a2, by contrast, are not controlled by AN1, PH3, or PH4 (Figures 1B and 1C) and are expressed in a broad range of tissues (Figure 1D).

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Figure 2. RAB5 proteins bind the same GEF but localize in different cell compartments

(A) Yeast two-hybrid assays indicate the interaction between GAL4BD fusions of WT and mutant versions of RAB5s and a GAL4AD fusion of PhVPS9a. (B–E) Split YFP assays confirming the interaction of RAB5 proteins with PhVPS9a in cells from petunia petal epidermis. Transformed cells are marked by (co-)expression of the plasma membrane protein RFP-SYP122. (B) Negative control showing that reassociation of nYFP and cYFP moieties fused to non-interacting proteins (RAB5a T24N) is negligible (n = 29 cells). (C) Positive control showing that PhVPS9a oligomerizes, like AtVPS9a (n = 21 cells). (D) PhVPS9a interacts with RAB5a T24N (n = 61 cells) but not, or much less, with RAB5a (n = 23 cells) or RAB5a Q69L (n = 19 cells). (E) Interaction of Petunia RAB5 homologs and Arabidopsis RAB5 (AtARA7) with PhVPS9a (n = 13 to 33 cells).

(F–I) Confocal micrographs of WT petal protoplasts co-expressing RFP-RAB5a and PH5-GFP representing two types of cells with enlarged vacuolinos (top) or normal-sized vacuolinos (bottom). Yellow arrowheads indicate co-localization; green arrowheads indicate the absence of co-localization. Co-localization, or lack thereof, in the micrographs is quantified in the scatterplots on the right (r_p, Pearson’s r; r_s, Spearman’s r).

(legend continued on next page)
(e.g., petunia and *Solanum lycopersicum*), Rosids (e.g., *Vitis vinifera* and *Medicago truncatula*), and Ranunculales (*Aquilegia caerulea*), as well as monocot species (e.g., *Oryza sativa* and *Ananas comosus*), all possess homologs of RAB5a, RAB5a1, and RHA1/ARA7/RAB5a2 (Figure 1E). Thus, the absence of RAB5a and RAB5a1 homologs in *Arabidopsis* and related Brassicaceae is most likely due to gene loss in the lineage that gave rise to these species.

In early diverging plant lineages, such as the bryophytes *Physcomitrella patens* and *Sphagnum fallax*; the liverwort *Marchantia polymorpha*; the algae *Klebsormidium flaccidum*, a Charophyte; and *Chlamydomonas reinhardtii* and *Volvox carteri*, two Chlorophytes, no such diversification of RAB5s is found, as these proteins cluster in a single clade. Surprisingly, this clade is more similar to the angiosperm RAB5a homologs than to RAB5a1 or RHA1/ARA7/RAB5a2 homologs. The process by which daughters of a gene duplication accumulate sequence changes at uneven rates is known as “asymmetric evolution” and is at least in some cases associated with the acquisition of new molecular functions by the faster diverging daughters (Holland et al., 2017). This suggests that the molecular function of the angiosperm RAB5a homologs (e.g., in terms of the effectors on which they act) may be more similar to that of their progenitors in basal plants, whereas the more diverged homologs from the RHA1/ARA7/RAB5a2 and RAB5a1 clades acquired distinct (molecular) functions that may involve distinct effectors.

**All three petunia RAB5 homologs interact with the GEF PhVPS9**

To assess the functional differences and similarities between the petunia RAB5s, we analyzed their interaction with a candidate GEF activator(s) (Herman et al., 2019). All known GEFs that activate RAB5a share a conserved domain (VPS9) (Carney et al., 2006). Although animals, fungi, and non-flowering plants can have up to five types of VPS9-domain-containing proteins, flowering plants have only one type (Herman et al., 2018; Letunic et al., 2021) that is encoded by two closely related and functionally redundant paralogs in *Arabidopsis* (AtVPS9a and AtVPS9b) (Goh et al., 2007; Nielsen et al., 2017; Nielsen and Thordal-Christensen, 2018) or a single gene in petunia (*Phvps9*) and many other species (Figure S1C). In GAL4-based yeast two-hybrid assays, we observed little or no interaction between PhVPS9 and GAL4AD and GAL4BD fusions of various RAB5s (Figure 2A), which was expected because in yeast RABs are thought to be mostly in their active GTP-bound form (Goh et al., 2007). Therefore, we examined interactions of PhVPS9 with the GDP-fixed mutants RAB5aT24N, RAB5a1S24N, RAB5a2S24N, and PhARA6S46N, which cannot be converted into the active GTP-bound form (Carney et al., 2006), and we found positive interactions in all cases (Figure 2A). The GTP-fixed mutant RAB5aQ69L, by contrast, could not bind PhVPS9 (Figure 2A).

To examine these interactions in petunia petal cells, we performed bimolecular fluorescence complementation (BiFC), using split-yellow fluorescent protein (YFP) assays. In protoplasts originating from the petal epidermis or petal mesophyll, which can be distinguished by the presence or absence of anthocyanin pigments in their CVs, respectively (Faraco et al., 2011), co-expression of nYFP-RAB5aT24N and cYFP-RAB5aT24N fusions did not result in noticeable YFP fluorescence (Figure 2B; Figure S1D), indicating that spontaneous reconstitution of YFP is negligible in these cells. Oligomerization of nYFP-PhVPS9 and cYFP-PhVPS9 was readily detectable (Figure 2C; Figure S1E), consistent with previous findings at AtVPS9a (Sunada et al., 2016). In line with the yeast two-hybrid results, we observed that cYFP-PhVPS9 interacted with GDP-fixed nYFP-RAB5aT24N in cells from the petal epidermis and the mesophyll, but not, or much less, with GTP-fixed nYFP-RAB5aQ69L or wild-type nYFP-RAB5a (Figure 2D; Figure S1F). We obtained essentially similar results with native and GDP-fixed forms of RAB5a1 (Figure 2E; Figure S1G), whereas RAB5a2, its paralog AtARA7, and PhARA6 interacted with PhVPS9 in both their GDP-fixed and the wild-type form (Figure 2E; Figure S1G). These results indicate that the three distinct canonical RAB5s, as well as PhARA6, can bind to and may potentially compete for the same GEF activator PhVPS9.

**Intracellular localization of RAB5s**

We compared the intracellular localization of the three petunia RAB5s by using fusions to fluorescent proteins (FPs). A considerable number of FP fusion proteins undergo cleavage in plant cells because of which the separated moieties containing the FP and the tagged protein may localize to different compartments or undergo differential turnover (Quattrocchio et al., 2013). In such cases, the FP fluorescence does not reliably report the whereabouts of the tagged protein, even when expression of the FP fusion protein efficiently complements a mutant phenotype (Quattrocchio et al., 2013). Therefore, we analyzed GFP-RAB5a, GFP-RAB5a1, and GFP-RAB5a2 by immuno-blot analysis and found that these fusion proteins remain intact in petal cells (Figure S2A). Together with the finding that GFP-RAB5a can functionally substitute for native RAB5a in the rescue of vacuolinos in protoplasts and transgenic plants (see below), this indicates that GFP-RAB5a fluorescence reliably reports RAB5a trafficking and localization.

To assess the localization of RAB5a in wild-type (M1xV30) petal cells, we transiently co-expressed RFP-RAB5a (red fluorescent protein-RAB5a) with PH5-GFP (Figure 2F; Figure S2B),...
which marks the tonoplast and vacuolino membrane, or Aleu-GFP (Figure S2C), which marks the lumen of the CV and vacuolinos (Faraco et al., 2017). In protoplasts from the petal epidermis, RFP-RAB5α accumulated in the cytoplasm, the membrane of vacuolinos, and smaller compartments (puncta) that also contain PH5-GFP and Aleu-GFP and might represent PVCs (Figure 2F; Figure S2C). However, RFP-RAB5α was not seen in all PH5-GFP-positive puncta (see green arrowhead in the lower cell in Figure 2F), suggesting that these represent a partially distinct population (or maturation stages) of compartments. Cytoplasmic RFP-RAB5α is likely to be in the (inactive) GDP-bound state, whereas membrane-bound RFP-RAB5α is in the (active) GTP-bound state (Barr and Lambricht, 2010; Müller and Goody, 2018). Also in petal mesophyll protoplasts, which lack anthocyanins and vacuolinos, RFP-RAB5α localized in puncta, partially overlapping with the puncta marked by PH5-GFP (Figure S2B).

To compare the subcellular localization of RAB5α, RAB5α1, and RAB5α2 proteins, we transiently co-expressed fusion proteins tagged with RFP or GFP in wild-type (M1xV30) petal protoplasts and assessed colocalization by Pearson’s r and Spearman’s correlation analysis (Figures 2G and 2H; Figures S2D and S2E). GFP-RAB5α and RFP-RAB5α accumulated in similar patterns in epidermal cells and in mesophyll cells too, but the fluorescence of RFP-RAB5α was considerably weaker than that of GFP-RAB5α (Figure S2D). GFP-RAB5α1 localized in epidermal cells in the cytoplasm and small punctate compartments that do not overlap with RFP-RAB5α-positive puncta or vacuolinos (Figure 2G). RFP-RAB5α2 localized to the cytoplasm and to punctate compartments that are distinct from puncta containing GFP-RAB5α (Figure 2H). Interestingly, also, GFP-RAB5α1 and RFP-RAB5α2 labeled in epidermal petal cell distinct populations of puncta, with little or no overlap (Figure S2G). In petal mesophyll cells, which lack vacuolinos, the same RAB5α, RAB5α1, and RAB5α2 fusions also accumulated in the cytoplasm and distinct populations of puncta (Figures S2E and S2F). Taken together, our results show that the localization of RAB5α partially overlaps with that of PH5 and hardly, if at all, with RAB5α1 and RAB5α2 (Figure 2I).

Next, we examined the identity of the GFP-RAB5α-positive puncta by using markers for the endoplasmic reticulum (ER) (RFP-KDEL; Fluckiger et al., 2003), cis-Golgi (ERD2-RFP; Silva-Alvim et al., 2016), trans-Golgi network/early endosomes (TGN/EE) (RFP-LeRAB11; Rahman et al., 2008), and PVCs/MVBs (CHERRY-BP80; De Benedictis et al., 2013). In epidermal petal cells, we observed no co-localization of GFP-RAB5α with ERD2-RFP, RFP-LeRAB11, or RFP-KDEL (Figures S3A, S3B, and S4A). GFP-RAB5α-positive puncta partially overlapped with CHERRY-BP80-positive compartments (Figure S3C), suggesting that they are a subpopulation of PVC-like compartments. Furthermore, GFP-RAB5α localized with CHERRY-BP80 in vacuolinos (Figure S3C). Interestingly, CHERRY-BP80, or at least its FP-tag, seems to become soluble in vacuolinos and to accumulate in the vacuolino lumen rather than the membrane (Figure S3C). In petal mesophyll cells, where RAB5α is normally not expressed, we observed partial co-localization of GFP-RAB5α with CHERRY-BP80 (Figure S3C) and with RFP-KDEL (Figure S4A), indicating that in mesophyll cells, part of GFP-RAB5α is localized in or close to the ER and to PVCs. It is unlikely that the association of RAB5α with the ER is caused by the absence of vacuolinos, as we found no co-localization of GFP-RAB5α and RFP-KDEL in epidermal cells from ph3 and ph4 mutant petals, which lack vacuolinos (Figures S4B and S5). As RAB5α is normally not expressed in the petal mesophyll, the association of GFP-RAB5α with the ER in these cells has no apparent biological relevance and was not investigated further.

In Arabidopsis cells, ATAR6 and ATARA7-positive PVCs/MVBs become labeled within 30 min after addition of the

**Figure 3. Constitutive RAB5α expression blocks vacuolino-to-CV transport and alters cell shape**

(A) Transient expression of PH5-GFP labels vacuolinos with normal size (24 h) and reaches tonoplast (48 h) in WT (M1xV30) petal protoplasts. The number of epidermal cells imaged at 24 h (n.epi-24h) and 48 h (n.epi-48h) is 97 and 33, respectively; mesophyll cells imaged at 24 h (n.mes-24h) and 48 h (n.mes-48h) is 26 and 15, respectively. Scale bars, 10 μm.

(B) Percentage of cells with enlarged vacuolinos and/or tonoplast localization of PH5-GFP after transient expression of RAB5α, RAB5α1, and RAB5α2 for 48 h. Normal-sized vacuolinos (1–10 μm); T+NV, cells with PH5-GFP on both tonoplast and normal-sized vacuolinos; T+EV, cells with PH5-GFP on both tonoplast and enlarged vacuolino (such cells were not observed).

(C) Transient expression of RAB5α in WT (M1xV30) petal protoplasts induces the formation of enlarged vacuolinos (n.epi-24h = 19, n.mes-24h = 3, n.epi-48h = 32, and n.mes-48h = 10 cells). Scale bars, 10 μm.

(D) Flower phenotype and pH value of the petal extracts (n = 6 flowers) of WT and transgenic RAB5αOE plants (bars, 1 cm).

(E) Light micrographs of epidermal cells from WT and RAB5αOE petals. Red, anthocyanins; scale bars, 20 μm.

(F) Confocal micrograph of protoplasts from RAB5αOE petals, transiently expressing PH5-GFP and RFP-SYP122 (n.epi-24h = 50, n.mes-24h = 11, n.epi-48h = 15, and n.mes-48h = 3 cells). Scale bars, 10 μm.

(G and H) Confocal images of petals from transgenic WT (M1xV30) plants expressing 3SS:PH5-GFP (PH5-GFPOE) (G) or 3SS:GFP-RAB5α (GFP-RAB5αOE) (H), 2 x Enlargement refers to the portion of the image contained in the white dashed square. Bars, 10 μm.

(I) Light micrographs and transmission electron micrographs (TEMs) of petals sections and environmental scanning electron micrographs (ESEMs) of the surface of WT, RAB5αOE, and RAB5αOE petals. Scale bars for light microscopy are 20 μm, for TEM are 5 μm, and for ESEM are 50 μm. Red arrows mark the tip of the cells, and black arrow marks ILVs.

(J) EM micrograph depicting how height, basal width, and apical width (width at 4 μm below the cell tip) of epidermal cells were measured.

(K) Dimensions of epidermal cells from different lines (height and basal width, n > 100 cells; apical width, n > 80 cells). Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5 x interquartile range; diamonds, outliers. Statistical significance was assessed by Student’s t test/Mann-Whitney rank sum test: ***p < 0.001; NS, not significant.

(L) Simplified model of the effect of altered RAB5α expression on dimensions and shape of petal epidermal cells of the same genotypes as above.

**Transgenic plants** produced large and/or multiple vacuolinos and showed an altered cell phenotype (Figures 2J and 3J). In petal mesophyll cells, which lack anthocyanins and vacuolinos, RFP-RAB5α localized in puncta, partially overlapping with puncta marked by PH5-GFP (Figure S2B).

**To compare the subcellular localization of RAB5α, RAB5α1, and RAB5α2 proteins, we transiently co-expressed fusion proteins tagged with RFP or GFP in wild-type (M1xV30) petal protoplasts and assessed colocalization by Pearson’s r and Spearman’s correlation analysis (Figures 2G and 2H; Figures S2D and S2E). GFP-RAB5α and RFP-RAB5α accumulated in similar patterns in epidermal cells and in mesophyll cells too, but the fluorescence of RFP-RAB5α was considerably weaker than that of GFP-RAB5α (Figure S2D). GFP-RAB5α1 localized in epidermal cells in the cytoplasm and small punctate compartments that do not overlap with RFP-RAB5α-positive puncta or vacuolinos (Figure 2G). RFP-RAB5α2 localized to the cytoplasm and to punctate compartments that are distinct from puncta containing GFP-RAB5α (Figure 2H). Interestingly, also, GFP-RAB5α1 and RFP-RAB5α2 labeled in epidermal petal cell distinct populations of puncta, with little or no overlap (Figure S2G). In petal mesophyll cells, which lack vacuolinos, the same RAB5α, RAB5α1, and RAB5α2 fusions also accumulated in the cytoplasm and distinct populations of puncta (Figures S2E and S2F). Taken together, our results show that the localization of RAB5α partially overlaps with that of PH5 and hardly, if at all, with RAB5α1 and RAB5α2 (Figure 2I).
endocytic tracker FM4-64, indicating that they are intermediates in an endocytic pathway (Ueda et al., 2001, 2004). In petunia protoplasts from the petal mesophyll and the epidermis, FM-64 is also rapidly endocytosed and after 10 min labels numerous endosomes that are distinct from the GFP-RAB5a-positive punctate compartments and vacuolinos (Figure S5). After 1 h, the punctate FM-64-positive and GFP-RAB5a-positive compartments remained separate populations, whereas co-localization of FM-64 and GFP-RAB5a became evident on vacuolinos, which increased further in time (Figure SS).

These findings suggest that (1) the RAB5a-positive puncta in epidermal petals cells are PVC-like compartments, which are distinct from the PVC/MVBs involved in endocytic pathway or the “direct” AtARF7 (RAB5a2)-dependent pathway to the CV and (2) that vacuolinos may be the station in epidermal petal cells where the endocytic pathway and vacuolino pathway merge.

**Effects of constitutive RAB5a expression**

We noticed that vacuolinos labeled by transiently expressed GFP-RAB5a were often much larger than those in cells expressing other GFP-tagged proteins. To further investigate this finding, we transiently co-expressed native RAB5a with PH5-GFP from the 35S promoter in wild-type (M1xV30) petal protoplasts. When expressed alone in epidermal petal cells, PH5-GFP localized 24 h after transformation on vacuolinos with a normal size (1–10 μm in diameter) and reached the CV after 48 h in ~35% of the cells (Figures 3A and 3B), as described previously (Faraco et al., 2017). However, when co-expressed in epidermal petal cells with 3SS:RAB5a, PH5-GFP localized after 24 h in vacuolinos, which were in many cells much larger than those in cells expressing PH5-GFP alone (Figure 3C). After 48 h, PH5-GFP reached the CV in only 4% of the epidermal cells, which had vacuolinos of a normal size (1–10 μm), whereas PH5-GFP did not reach the CV in in any of the cells with enlarged vacuolinos (>10 μm) (Figures 3B and 3C). In the same cells, the sorting of the plasma membrane marker RFP-SYP122 was not affected by 3SS:RAB5a (Figure 3C). In addition, 3SS:RAB5a had no noticeable effect on the trafficking of PH5-GFP to the CV in petal mesophyll cells either (Figure 3C). This result indicates that constitutive RAB5a (over)expression from the 3SS promoter affects only the pathway from vacuolinos to the CV. Interestingly, 3SS:RAB5a2 also resulted in cells with enlarged vacuolinos and blocked transport to the CV, although with lower frequency (26.5%) than 3SS:RAB5a (40%), whereas 3SS:RAB5aI had little or no effect (Figure 3B).

Next, we generated 40 transgenic wild-type (M1xV30) plants containing 3SS:RAB5a and analyzed the phenotype of the six plants (RAB5aOE) that (over)expressed RAB5a mRNA (Figure S6A). When RAB5aOE flowers opened, most of the petal tissue had a blue-violet color and acquired its normal red-violet color only at later stages (Figure 3D; Figure S6B). The blue-violet flower color is typical of ph mutants and is caused by the reduced acidity of the CV, where anthocyanin pigments reside, due to impaired expression of the proton pumping PH1-PH5 complex in the tonoplast (Faraco et al., 2014; Verweij et al., 2008). Due to the reduced acidity of the CV (and other PH1-PH5-containing compartments, like vacuolinos), the acidity of crude homogenates from ph mutant petals is also reduced (de Vlam et al., 1983; Faraco et al., 2017; Verweij et al., 2008). The homogenate pH of blue-violet RAB5aOE petals, by contrast, was similar to that of wild-type petals (Figure 3D), suggesting that reduced acidity of the CV is compensated by increased acidity of other compartments, most likely the vacuolinos where PH5 is now upheld.

Epidermal cells of wild-type petals contain numerous vacuolinos, which accumulate preferentially in the conical tip (Faraco et al., 2017), whereas those from RAB5aOE petals contain a lower number of vacuolinos that are increased in size, thereby pushing the anthocyanin-containing CV down to leave the conical cell tip essentially colorless (Figures 3E, 3H, and 3I). In transmission electron micrographs (TEMs), we observed intraluminal vesicles (ILVs) in sections of all four enlarged vacuolinos analyzed (Figure 3I) and at a much lower frequency in vacuolinos (2 vacuolinos out of 37, with 1 or 2 ILVs). In addition, the tip of epidermal cells in RAB5aOE petals had a rounder shape, associated with increased cell width at 4 μm below the tip, as compared to the more pointed tips of wild-type cells (Figures 3I–3L). In protoplasts from the RA-

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Figure 5. The rab5a\textsuperscript{2043} mutation blocks vacuolino formation and delivery of proteins to the central vacuole in epidermal petal cells

(A) Confocal micrographs of transiently expressed Aleu-GFP in RAB5a\textsuperscript{+/+} and rab5a\textsuperscript{2043} petal protoplasts. The number of epidermal RAB5a\textsuperscript{+/+} cells imaged after 24h (n\textsubscript{epi-24h}) and 48h (n\textsubscript{epi-48h}) is 35 and 28, respectively. For rab5a\textsuperscript{2043}, n\textsubscript{epi-24h} = 48, and n\textsubscript{epi-48h} = 53 cells.

(B) Transient expression of 35S:RAB5a or GFP-RAB5a in protoplasts from petals of the rab5a\textsuperscript{2043} mutant. For 35S:RAB5a, n\textsubscript{epi-24h} = 72 and n\textsubscript{epi-48h} = 51 cells; and for GFP-RAB5a, n\textsubscript{epi-24h} = 34 and n\textsubscript{epi-48h} = 27 cells.

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after 24 h in the (enlarged) vacuolinos, but little or no PH5-GFP arrived at the CV after 48 h (Figure 3F), similar to wild-type cells transiently expressing 35S::RAB5a. Also in stably transformed plants expressing 3SS::GFP-RAB5a, vacuolinos in most of the petal epidermis were much larger than vacuolinos in control plants expressing 3SS::PH5-GFP (Figures 3G and 3H). The prolonged presence of PH5-GFP (Figure 3F), and endogenous PH1 and PH5, on vacuolinos and the reduced trafficking to the CV will delay the acidification of the CV, explaining the blue-violet petal color when the flower opens, while also enhancing the acidification of vacuolinos, explaining why the overall acidity of RAB5aGFP petals is not altered.

**Effects of the loss of RAB5a function**

To further assess the function of RAB5a, we obtained 2 transformant lines (out of 15 transformants) in which RAB5a was about 3-fold downregulated by RNAi (Figures S6D and S6E). In addition, we identified among the progeny of line W138 a mutant allele, rab5aX2043, in which a dTPH1 transposon disrupts the coding sequence 252 bp downstream of the ATG (Figure S6F; Figure 4A) and strongly reduces the amount of RAB5a transcripts, without affecting RAB5a1 and RAB5a2 mRNA levels (Figure 4B). Petals from rab5aX2043 homozygotes had a similar color as RAB5aX4/4 siblings in the same background (W138) (Figure 4C), but the epidermal cells lacked vacuolinos. Furthermore, the height and width of the cells, measured at the base of the cell and 4 μm below the tip, were reduced (Figures 4D–4G). Downregulation of RAB5a by RNAi also reduced the height of epidermal cells but had, in contrast to rab5aX2042, little or no effect on the width at the cell base and significantly increased the width at 4 μm below the tip (Figures 3I–3L). The latter might be an effect from the reduced cell height because the width at 4 μm below the tip now measures the width of "main body" of the cell rather than the tip. The different effect of rab5aX2042 and RAB5aRNAi on (apical) cell width might stem from the somewhat different shape of the epidermal cells in the W138 (rab5aX2043) and M1xV30 (RAB5aRNAi) genetic background (compare the scanning electron micrographs [SEMs] in Figures 3I and 4D) and/or the strength of the mutation (knockout versus knockdown).

To investigate how rab5aX2043 affects the sorting of vacuolar proteins, we transiently expressed PH5-GFP or Aleu-GFP in petal protoplasts isolated from rab5aX2043 homozygotes. In this genetic background (W138), PH5-GFP and Aleu-GFP reached vacuolinos within 24 h after transformation and the CV after 48 h in RAB5aX4/4 petal cells (Figures 4G and 5A), as previously observed for wild-type cells in the M1xV30 genetic background (Faraco et al., 2017). However, in epidermal petal cells of rab5aX2043 siblings, PH5-GFP and Aleu-GFP labeled 24 h after transformation punctate structures instead of vacuolinos, and only a small fraction reached the CV 48 h after transformation (Figures 4G and 5A). In petal mesophyll cells from rab5aX2043 mutant and RAB5aX4/4 siblings, PH5-GFP and Aleu-GFP reached the CV directly within 24 h after transformation, as in the M1xV30 wild type (Figures 4G and 5A; Figure S6K). We obtained similar results in the RAB5aRNAi knockdown line (Figure S6G) and in lines containing the rab5aX2042 allele in a distinct genetic background accumulating malvidin anthocyanin (Figures S6H and S6I). Thus, rab5aX2043 specifically affects the vacuolin pathway in epidermal petal cells and has no effect on the direct pathway to the CV that is active in other cells.

When we co-transformed rab5aX2043 petal protoplasts with 3SS::RAB5a along with 3SS::PH5-GFP, we observed that transient RAB5a expression promptly restored the sorting of PH5-GFP to vacuolinos within 24 h after transformation (Figure 5B; Figure S6J). Transient expression of GFP::RAB5a rescued vacuolo formation with similar efficiency (Figure 5B). Some of these rescued cells contained enlarged vacuolinos and showed a delayed delivery of PH5-GFP to the CV, similar to wild-type M1xV30 petal cells expressing 3SS::RAB5a or 3SS::GFP::RAB5a (Figures 3B, 3F, and 3H). The expression of 3SS::RAB5a or 3SS::GFP::RAB5a had no effect on the delivery of PH5-GFP to reach CV in rab5aX2043 petal mesophyll cells (Figure S6L). Co-transformation of 3SS::RAB5a1 or 3SS::RAB5a2 did not rescue PH5-GFP trafficking by vacuolinos (Figure 5C), in contrast to 3SS::RAB5a and 3SS::GFP::RAB5a, indicating that RAB5a1 and RAB5a2 are functionally distinct from RAB5aA.

Mutation of PH1 blocks trafficking from vacuolinos to the CV and results in enlarged vacuolinos (Faraco et al., 2017). To better position the function of RAB5a in the vacuolin pathway, we transiently expressed PH5-GFP in petal protoplasts from the rab5a ph1 double mutant (Figure 5D). In epidermal protoplasts from rab5a ph1 petals, PH5-GFP remains stuck on puncta, where it is still visible 48 h after transformation (as in rab5a cells), instead of labeling vacuolinos and after 48 h the CV (as in wild-type) or enlarged vacuolinos (as in ph1). Transient expression of RAB5a in rab5a ph1 petal epidermis protoplasts restored the presence of vacuolinos (rescue of the rab5a defect) but did not rescue the defect in trafficking to the CV caused by ph1 (Figure 5D). This finding indicates that RAB5a acts upstream of PH1 in the vacuolin pathway.

To characterize the PH5-GFP-positive puncta in RAB5a-deficient epidermal petal cells and thereby gain insight into the origin of vacuolinos, we co-transformed 3SS::RAB5aRNAi petal protoplasts with constructs expressing PH5-GFP and RFP- or CHERRY-tagged markers for different cell compartments (Figure 6;
Figure 6. PH5-GFP-positive puncta accumulating in RAB5aRNAi petal epidermis are a sub-population of PVCs

(A–D) Confocal pictures of protoplasts derived from the RAB5a knockdown mutant (RAB5aRNAi) transiently expressing PH5-GFP together with PVC/MVB marker CHERRY-BP80 (A). Confocal pictures of protoplasts derived from the RAB5a knockdown mutant transiently expressing PH5-GFP together with RFP-KDEL, a marker for the ER (B); ERD2-RFP, a marker for cis-Golgi membranes (C); and RFP-LeRAB11, a marker for the TGN/EE membranes (D). The scatterplots on the right show a correlation of localization patterns, which are expressed as $r_p$ and $r_s$ correlation coefficients. Autofluorescence of anthocyanins is shown in blue, fluorescence of RFP in red, and fluorescence of GFP in green. Size bars, 10 μm in all panels; 3x enlargement shows the region marked by the white dashed

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In leaf cells, PVCs are MVBs that become enlarged PVCs (ePVCs) by homotypic fusions after treatment with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin (Tse et al., 2004; Wang et al., 2009; Zheng et al., 2014). To study if and how wortmannin affects the vacuolino pathway, we transiently expressed CHERRY-BP80 and GFP-RAB5a or PH5-GFP in petal protoplasts. In these cells, GFP-RAB5a and PH5-GFP-labeled vacuolinos were observed (Figures 6B–6E; Figures S7B–S7D). However, we did observe (partial) co-localization of PH5-GFP with RFP-KDEL, indicating that none of the FP-tagged (E) Quantification of $r_p$ and $r_s$ colocalization coefficients between PH5-GFP and different organelle markers (n = 11 to 15 cells). See also Figure S7.

 supporting the idea that these puncta resemble PVC-like compartments.

DISCUSSION

Previous findings indicated that the MBWW transcription factor complex PH4–AN1–AN11–PH3 activates a tissue-specific trafficking pathway to the CV by vacuolinos (Faraco et al., 2017). Here, we further characterized this pathway and identified the first MBWW target gene, namely, RAB5a, that is involved in the formation of vacuolinos. RAB5a represents an ancient previously unrecognized clade of “canonical” RAB5s that diverged from the well-studied RHA1/ARA7 homologs by changes in its transcriptional regulation, intracellular localization, and biological function, providing a rare example in support of the hypothesis that the diversification and the increased complexity of membrane trafficking during evolution was facilitated by duplication and neo-functionalization of genes encoding key factors like RAB GTPases and SNAREs (Dacks and Field, 2007; Dacks et al., 2008; Sanderfoot, 2007; Schlacht et al., 2014).

Our findings suggest a model (Figure 7C) in which vacuolinos originate from the RAB5a-mediated fusion of a sub-population of PVCs to form larger structures, which also receive (FM4–64 positive) membrane material from the plasma membrane by distinct RAB5a-negative endosomes. In epidermal protoplasts from wild-type petals, we could indeed observe fusion events among PH5-GFP-labeled vacuolinos (Video S1). As RAB5a is necessary to send proteins to vacuolinos but not sufficient to rescue $p_h 4$ (not shown), we infer that other MBWW-regulated factors are involved, of which some act before RAB5a in the vacuolino pathway, at or close to the point where it diverges from the ubiquitous direct pathway(s) to the CV. Thus, in $a n 1$, $p_h 3$, and $p_h 4$ mutants, the vacuolino pathway is blocked at an early point from which proteins can be redirected into the direct pathway to the CV, whereas in rab5a mutants, proteins are upheld at a later step in the vacuolino pathway, after a “point of no return” (in small PVC-like compartments) from where they cannot be redirected anymore.

The vacuolino pathway displays striking differences and similarities with trafficking pathways to (incipient) vacuoles in leaf, root, and/or suspension cells (Cui et al., 2020; Figure 7C). The most distinguishing feature is that the formation of vacuolinos and subsequent vacuolino-to-CV transport require MBWW-target genes like RAB5a and PH1 that are inactive in leaves and roots (and petal mesophyll) or even missing in Arabidopsis. Yet, there are also striking similarities at the microscopic level. In root cortex cells, vacuoles form de novo from multivesicular PVCs that fuse into small vacuoles (~1 μm) that grow by additional fusions into a large CV (Cui et al., 2019). The vacuolino pathway also generates de novo new secondary vacuoles, but this happens in a late stage of petal development, long after these cells form the large CV. Whether or not these vacuolino precursors are multivesicular is currently unknown. Vacuolinos do contain ILVs at low numbers and at much higher numbers in enlarged vacuolinos in RAB5a$^{OE}$ cells. It is, however, unclear whether these ILVs originate from MVB precursors or the internalization of vesicles from the vacuolino membrane, by micro-autophagy, as part of a degradation process (Yang et al., 2020), or both.

The rapid enlargement of the RAB5-positive compartments after wortmannin treatment indicates that PI3K negatively regulates their homotypic fusion, as it does for PVCs/MVBs in root and leaf cells (Cui et al., 2019; Tse et al., 2004; Wang et al., 2009; Zheng et al., 2014). Expression of the constitutively active (GTP-fixed) ARA7$^{Q69L}$ in leaf and suspension cells also triggered the formation of ePVCs/MVBs (Jia et al., 2013; Kotzer et al., 2004). This (superficially) resembles events along the vacuolino pathway on two points. The first point is the formation of square at a 3-fold larger magnification. Yellow arrowheads indicate co-localization; red and green arrowheads indicate the absence of co-localization. Abbreviation: ER, endoplasmic retilium.
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vacuolinos through fusions of the small punctate precursors, except that this process is mediated by (native) RAB5a, which is lacking in Arabidopsis. The second point is the formation of enlarged vacuolinos and in cells expressing RAB5a or RAB5a2 from the 35S promoter, which is correlated with and possibly a consequence of the reduced (or delayed) trafficking from vacuolinos to the CV. However, as the last process requires the P35S\(^{ATPase PH1}\), which is lacking in Arabidopsis, the similarities at a microscopic level are likely to rest on (partially) different molecular mechanisms.

The formation of enlarged vacuolinos by RAB5a\(^{OE}\) shows that a cell may generate multiple vacuoles with distinct function, content, and/or size solely by regulating the temporal expression of RAB5s without a need for specific sorting domains that send proteins to one vacuole or the other. Epidermal petal cells in young petunia flower buds already contain a CV, which during further development to stage 5 (maximum size bud), accumulates, amongst others, anthocyanins. Expression of MBWW genes and their targets, such as RAB5a, sets in later when anthocyanin synthesis is ceasing, because of which the newly formed vacuolinos are largely devoid of anthocyanins and other proteins that are synthesized only in earlier stages. Upon prolonged 35S-driven RAB5a expression, the vacuolinos become enlarged, in some cells to a similar or larger size than the CV, becoming essentially an additional anthocyanin-less (central) vacuole that co-exists with the older anthocyanin-containing CV.

Although vacuolinos are an attractive model with which to study membrane trafficking by genetic approaches, their biological function remains to be solved. Vacuolinos may affect optical features of the colored petal cells, which are important for the attraction of pollinators, by excluding the anthocyanin-containing CV from the conical tip and by altering cell dimensions. In Arabidopsis petals, the orientation of cortical microtubules is important to give epidermal cells the correct domed shape (Ren et al., 2017), and the formation of vacuolinos might add to shaping epidermal cells, possibly by providing mechanical force. However, vacuolinos may also exist in other cell types, suggesting additional functions, as RAB5a is also expressed in the vasculature of petunia leaves and in Citrus fruits. In the latter, AN1 drives expression of PH1 and PH5 to hyperacidify the CV of juice cells (Strazzer et al., 2019), as well as a RAB5 gene (Cs1g14330/orange1.1g029103m) (Huang et al., 2016) that is homologous to petunia RAB5a (Figure 1B). Whether or not vacuolinos may serve as a sorting station, to prevent selected proteins from reaching the CV, is currently under investigation. Our findings reported here and elsewhere (Faraco et al., 2017) suggest that further analysis of flower color mutants and additional AN1-PH3-PH4 regulated genes is likely to uncover additional factors and mechanisms involved in membrane trafficking that could not be predicted by other approaches.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109749.

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AUTHOR CONTRIBUTIONS

S.L. performed most of the experimental work. P.S., M.B., Y.L., C.S., E.M.-C., and B.L. helped in some experiments. L.R. and M.C. performed the light microscopy, TEM, and ESEM analysis. M.V. isolated the transposon insertion mutant rab5aX2043, and F.M.Q. and R.K. conceived this project. S.L., F.M.Q., and R.K. wrote the paper.

DECLARATION OF INTERESTS

Authors declare to have no competing financial interests.

REFERENCES


# STAR★METHODS

## KEY RESOURCES TABLE

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<tr>
<td>3SS:GFP-RAB5a in P. hybrida M1xV30</td>
<td>This study</td>
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<tr>
<td>3SS: RAB5a^{RNAi} in P. hybrida M1xV30</td>
<td>This study</td>
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<tr>
<td>3SS: PH5-GFP in P. hybrida M1xV30</td>
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<td>proRAB5a:GFP-GUS in P. hybrida M1xV30</td>
<td>This study</td>
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<td><strong>Saccharomyces cerevisiae PJ69</strong></td>
<td>James et al., 1996</td>
<td>N/A</td>
</tr>
</tbody>
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## Oligonucleotides

**Please see Table S2**

### N/A

## Software and algorithms

**MUSCLE**

Dereeper et al., 2008

http://www.atgc-montpellier.fr/phyml/

**PHYML**

Dereeper et al., 2008

http://www.atgc-montpellier.fr/phyml/

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Requests for further information and/or resources should be directed to and will be fulfilled by the lead contact, Ronald Koes (ronald.koes@uva.nl).

Materials availability
All materials generated in this study are available from the lead contact.

Data and code availability
Sequence data generated in this study were deposited in the National Center for Biotechnology Information (NCBI) under Bioproject Genbank: PRJNA753066 (RNAseq data) and accession numbers Genbank: MH986793 (RAB5a mRNA), Genbank: MH986794 (RAB5a1 mRNA), Genbank: MH986795 (RAB5a2 mRNA), Genbank: MK896357 (PhARA6 mRNA), and Genbank: MK896358 (PhVPS9 mRNA).

This paper does not report original code.

Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The wild-type P. hybrida accessions used for plant and protoplast transformation were the F1 hybrid M1xV30 or a homozygous AN1+ revertant of the inbred line P. hybrida W138 that was maintained in line R175. The transposon insertion mutant rab5aX2043 was isolated and maintained in the AN1+ revertant W138 background. For the expression analyses in Figure 1 we used the inbred P. hybrida lines R175 (wt), R162 (ph4), and R167 (ph3), which were all in an AN1+ revertant W138 genetic background (Quattrocchio et al., 2006; Verweij et al., 2016) and line W225 (an1) containing a stable recessive an1 allele derived from an1W138.

All Petunia plants used in this work were grown in a greenhouse under normal conditions (19°C/30°C min/max, with cycles of 16/8 hours light/dark in summer, and with cycles of additional illumination of 15/9 hours light/dark in winter).

METHOD DETAILS

Plant material
Petunia line R182 (rab5aX2043) is a transposon insertion mutant for RAB5a in the W138 line. The (transformable) F1 hybrid M1xV30 (wild-type) was used to generate stable transformants. V23 (ph1X23) is a petunia line harboring a stable ph1 allele disrupted by a 7-bp transposon footprint in the coding sequence. The rab5a/ph1 double mutant was isolated by PCR screening of the F2 progeny from the cross R182 (rab5aX2043) X V23 (ph1X23). A rab5aX2043 mutant in a malvidin background was isolated from the F2 of the cross of R182 (rab5aX2043) with the wild-type line V30. Transgenic plants expressing 35S:RAB5a, 35S:GFP-RAB5a, 35S:PH5-
GFP and plants expressing proRAB5a:GFP-GUS were obtained by *Agrobacterium tumefaciens*-mediated leaf disc transformation of the F1 hybrid M1×V30 (wild-type). RAB5aRNAi knock down lines were generated by RNA interference in the F1 hybrid M1×V30 (wild-type).

RNA extraction and quantitative real-time PCR
Total RNA was isolated from petunia tissues using the TRIzol reagent (Thermo Fisher Scientific). Quantitative real-time PCR was performed with an iTaq Universal SYBR Green kit (Bio-Rad) using primers listed in Table S1 and an Applied Biosystems QuantStudio 3 Real-Time PCR System and QuantStudio Design Analysis Software.

Genes and constructs
RAB5a coding sequence (CDS) was amplified from M1×V30 (wild-type) petal cDNAs with primer 7253 and 7254 containing attB1 and attB2 sites using Phusion® High-Fidelity DNA Polymerase; Purified RAB5a CDS was subsequently cloned into Gateway® pDONR221 donor vector as an entry clone by Gateway® BP recombination reaction (Invitrogen Clonase Gateway BP Clonase II Enzyme Mix); Finally Gateway® LR recombination reaction (Invitrogen Clonase Gateway LR Clonase II Enzyme Mix) were performed with RAB5a CDS entry clone and multiple destination vectors (Table S2) to make functional plasmids for several purpose. By using the same procedures, CDS of RAB5a1 (primer 7602 + 7603), RAB5a2 (primer 7607 + 7608), PhAR6 (primer 7577 + 7578) and PhVPS9a (primer 7508 + 7509) were amplified and subsequently subcloned into multiple destination vectors (Table S2). Promoter sequence of RAB5a (primer 7513+7514) obtained from wild-type petal gDNA with Phusion® High-Fidelity DNA Polymerase, cloned into Gateway® pDONR221 donor vector, and recombined with destination vector pKGWSF7 to drive the expression of GFP and GUS. Constitutively active (GTP-bound) and constitutive negative (GDP-bound) mutants of RAB5a were generated by PCR based site-directed mutagenesis (Landt et al., 1990). The GDP-bound mutant of RAB5a termed RAB5aT24N for example was generated as follows: forward primer (7253), containing an attB1 site, and a reverse primer (7656) containing the desired mutation (mutating amino acid T (Threonine) to N (Asparagine)) were used in a first round of PCR with Phusion® High-Fidelity DNA Polymerase. The resulting PCR product was used as forward primer together with reverse primer (7254), containing an attB2 site, in a second PCR reaction. Products of this second PCR reaction were cloned into the Gateway® pDONR207 donor, sequenced and subsequently transferred by Gateway recombination into multiple types of destination vectors for functional constructs (Table S2). For RAB5a RNA interference (RNAi), a target sequence containing partial the exon and 3′utr were amplified with primers (7854+7855), which were cloned into Gateway® pDONR221 donor vector and pK7GW/VG2 (I) binary silencing vector.PH5-GFP was identified as marker for vacuolinos membrane and tonoplast of CV (Faraco et al., 2017); RFP-SYP122 has been described as plasma membrane (PM) marker, detailed description of how these plasmids were made published before (Faraco et al., 2017). Aleu-GFP (Aleu = the first 431 bases of the CDS of the barley aleurain cDNA) was previously used as marker for the lumen of central vacuole (Di Sansebastiano et al., 2001) and vacuolino (Faraco et al., 2017). ERD2-RFP were constructed by amplifying (primers 7474 + 7475) the CDS of ERD2 cDNA of *A. thaliana* and subcloning it into the Gateway destination vector pK7GW/VG2,0 by BP and LR reactions. RFP-KDEL (Faraco et al., 2017), RFP-LeRAB11 (Rehman et al., 2008) and CHERRY:BP80 (BP80 = the transmembrane domain/cytoplasmic tail sequence of the BP80 cDNA) (De Benedictis et al., 2013; Tse et al., 2004) were described in previous works. All the primers used in this study are reported in Table S1. Vectors applied in this study are presented in Table S2.

Phylogenetic analysis and sequence alignment
Amino acid sequences of RAB5 homologs from different plant species were selected from the Petunia Genome SGN dedicated database (https://solgenomics.net/), Phytozone databases (https://phytozome-next.jgi.doe.gov/), PLAZA (https://bioinformatics.psb.ugent.be/plaza/), and Ensembl (http://www.ensembl.org/useast.ensembl.org/?redirectsrc=/www.ensembl.org%2F). Multiple sequence alignments were conducted by MUSCLE. The phylogenetic tree was produced by maximum-likelihood using the online tool PhyML 3.0 (http://www.atgc-montpellier.fr/phyml). G-blocks were applied to curate the alignment and 300 bootstrap replicates were used to assess the branch support (Dereeper et al., 2008). Amino acid sequence alignment was performed by ClustaIX version 2.1, and further edited by program GeneDoc version 2.7.

Protoplast transformation and confocal microscopy
Protoplast isolation and transformation was done as described previously with minor modifications (Faraco et al., 2011). Petal limbs from three stage-7 flowers (2 days after bud opening) were sterilized for 30 s in 0.5% hypochlorite in a rooting pot (190ml, 66x68mm, Greiner), rinsed three times in sterile water, transferred to a Petri dish (94x16 mm, with vents, Greiner Bio-One B.V.) containing 2 mL filter-sterilized TEX buffer (3.1g/L Gamborg’s B-5 basal salt mixture, 1.28mM MES, 6.8mM CaCl₂·2H₂O, 1.32mM NH₄NO₃, and 0.4M sucrose, pH 5.7, sterilize with 0.45 μm sterile syringe filter) containing 0.2% (w/v) macroyme R-10, and 0.4% (w/v) cellulose onozuka R-10, gently sliced petals using a sterile scalpel. After addition of another 8 mL digestion solution (0.2% (w/v) macroyme R-10, and 0.4% (w/v) cellulase R-10 in TEX buffer) the sliced petals were incubated overnight at room temperature in the dark. The next morning protoplasts were released by gently lifting the digested petal pieces up and down with a forceps and passed through a 100 μm nylon mesh to remove undigested tissue and other debris. After addition of 15 mL freshly prepared TEX buffer, the protoplast suspension is transferred to a 50mL centrifuge tube and spun for 10 min at 100 g in a swing out rotor with deceleration set at zero. Using a long Pasteur pipette (230 mm) connected to an electronic pipette controller by a pipette tip(1mL), a piece of rubber tube and a serological pipette(25mL), the pelleted debris and as much of the buffer was removed from under the layer of floating live protoplasts. The pro-
toplasts were washed once by adding 25 mL fresh TEX buffer, centrifugation for 10 minutes at 100 g (deceleration set at zero) and removal of the TEX buffer from under the floating protoplasts. After resuspension in 25 mL fresh TEX buffer, protoplasts were then kept on ice for 30 minutes and centrifuged again for 10 min at 100 g. After removal of the TEX buffer and resuspension in 3-5 mL (depending on the amount of protoplast) of MMM solution (0.5M Mannitol, 15mM MgCl₂, and 0.1% MES) protoplasts are ready for transformation.

For one transformation, 20 μg plasmid DNA, 300 μL protoplast suspension and 300 μL PEG solution (0.4M Mannitol, 0.1M Ca(NO₃)₂, adjusted to pH to 6 before adding 40% PEG4000) were gently mixed in a 12 mL tube for 1 minute. After addition of 2 mL TEX buffer the suspension was gently mixed again and incubated for 1 hour at room temperature. After addition of 5 mL W5 buffer (154 mM NaCl, 125mM CaCl₂, 5 mM KCl, 5 mM Glucose, pH 5.7) and gentle mixing, protoplasts are pelleted at 100 g for 10 minutes, and resuspended in 2 mL TEX buffer and incubated for 20-48 hr before microscopic analysis.

Transfected cells were imaged with a Zeiss LSM510 confocal microscope using a 40x/1.2 water objective at 24 or 48 hours after transformation. For GFP and anthocyanins excitation we used a 488nm laser and detection filters BP505-550 and LP650 respectively. For RFP and CHERRY excitation was at 568nm and detection was achieved with a BP585-615 filter. Protoplasts isolated from petals of a stable 35S:PH5-GFP transgenic line (PH5-GFP<sup>C226</sup>) were loaded into WillCo-dish® Glass Bottom Dishes (HBST-3522) to make movies of the fusion event between vacuolinos by Andor spinning disk confocal microscopy (Nikon TI microscope body equipped with Andor spinning disk and FRAPPA unit for confocal imaging).

**FM4-64 imaging**

Protoplasts prepared for staining with FM4-64 (Synaptobre Red C2, Sigma-Aldrich®) were isolated from petals of transgenic plants expressing 35S:GFP-RAB5a. After addition of FM4-64 to a final concentration of 50 μM FM4-64 protoplasts were kept in the dark and imaged after 10 mins, 1 hour, 5 hours, and 24 hours with a Zeiss LSM510 confocal microscope using a 40x/1.2 water objective.

**Wortmannin treatment**

Protoplasts were isolated from petals of wild-type (M1xV30), <sup>rab5</sup><sup>A22043</sup> or phd mutants and transiently transformed with plasmids of interest, respectively. 24 hours after transformation, transfected protoplasts were treated with 16.5 μM (final concentration) wortmannin (LC Laboratories®) for 30 mins in the dark. After the treatment, protoplasts were centrifuged, resuspended and kept in fresh buffer for a variable period before imaging with a Zeiss LSM510 confocal microscope using a 40x/1.2 water objective.

**Yeast two hybrid and bimolecular fluorescence complementation (BiFC) assay**

Yeast two-hybrid assays were performed as reported before (Quattrocchio et al., 2006). In BiFC assays, plasmids encoding N-terminal fusions of protein of interest to nYFP and cYFP and, to mark the plasma membrane of transformed cells, RFP-SYP122 were transformed into protoplasts isolated from petunia M1xV30 (wild-type) petals. Transfected cells were imaged with a Zeiss LSM510 confocal microscope with a 40x/1.2 water objective at 24 hours after transformation. Over hundred transfected cells expressing RFP-SYP122 were observed for each independent experiment, and around 20 cells were imaged by confocal microscopy.

**Light microscopy and transmission electron microscopy of petal sections**

Semi-thin sections of petal tissue were prepared and observed as previously described (Faraco et al., 2017). Petal limbs at anthesis (stage 6) were fixated for 24 hours in in75 mM sodium cacodylate buffer (pH 7.2) containing 5% (w/v) glutaraldehyde, washed four times for 15 min each in 75 mM cacodylate buffer (pH 7.2), post-fixed for 90 min in 1% (w/v) OsO₄, dehydrated by passage through a series of stepwise increased ethanol concentrations o, and included in resin (Epon, 2-dodecenylsuccinic anhydride, and methylnadic anhydride mixture). Semi-thin sections (1–2 mm) were cut by ultramicrotome (OmU2, Reichert), stained with toluidine blue, and observed under a light microscope (DMLB, Leica Microsystems).

Transmission electron microscopy of petal cells was performed as previously reported (Verweij et al., 2008). Petal tissue from stage 6 flowers (anthesis) was fixed overnight at room temperature in 75 mM cacodylate buffer, pH 7.2 containing 5% (v/v) glutaraldehyde and post-fixed for 4 hours in 75 mM cacodylate buffer, pH 7.2 containing 1% (w/v) OsO₄ for 4 h. After dehydration in a graded ethanol series and propylene oxide, samples were embedded in Epon resin (2-dodecenylsuccinic anhydride, and methylnadicanhydride mixture). Semi-thin sections (1–2 mm) were cut by ultramicrotome (OmU2, Reichert), stained with toluidine blue, and observed under a light microscope (DMLB, Leica Microsystems).

Transmission electron microscopy of petal cells was performed as previously reported (Verweij et al., 2008). Petal tissue from stage 6 flowers (anthesis) was fixed overnight at room temperature in 75 mM cacodylate buffer, pH 7.2 containing 5% (v/v) glutaraldehyde and post-fixed for 4 hours in 75 mM cacodylate buffer, pH 7.2 containing 1% (w/v) OsO₄ for 4 h. After dehydration in a graded ethanol series and propylene oxide, samples were embedded in Epon resin (2-dodecenylsuccinic anhydride, and methylnadicanhydride mixture; Sigma-Aldrich). A pre-inclusion at room temperature in increasing concentrations of resin dissolved in propylene oxide was followed by the final inclusion in freshly prepared resin followed by the polymerization at 35°C for 12 h, 45°C for 12 h, and at 60°C for 12 h. Ultrathin sections were mounted on uncoated copper grids (200 mesh).

Cell dimension measurements were performed on the semi-thin sections of petal tissue. Height of cell was defined as the distance from the cell tip to the base of the cell. Basal width and apical width of cells were measured at the basal end of the cell and at 4 μm down from the cell tip, respectively. All the measurements have been done with CellSens image analysis software.

**Environmental scanning electron microscopy**

Petal portions were fixed in 0.075M cacodylate buffer (pH 7.2) containing 3% (w/v) glutaraldehyde. Pre-fixed samples were then washed with 0.075M cacodylate buffer (pH 7.2) three times (7 mins each time), which were post-fixed in 0.075M cacodylate buffer (pH 7.2) containing 1% (w/v) OsO₄ for 1 hour. Post-fixed samples were further washed three times (7 mins each time) in 0.075M caco-
dylate buffer (pH 7.2) and observed by ESEM (Environmental scanning electron microscopy) in wet mode (Stabentheiner et al., 2010).

GUS Staining
Flowers and leaves of petunia line transformed with proRAB5a:GFP-GUS and untransformed line (M1 × V30) were collected and immediately dipped into cold 90% acetone for 20 mins. After removal of acetone by two washes with staining buffer (100mM sodium phosphate buffer (pH 7.2), 0.1% (w/v) Triton X-100 and 10mM EDTA) tissues were transferred to staining buffer containing 2mM X-Gluc (5-Bromo-4-Chloro-3-Indoyl-Beta-D-Glucuronide). To aid penetration of substrate into the tissue, samples were put under vacuum for three times 20 minutes and further incubated at 37°C for several hours till the blue color developed and then transferred to 70% ethanol for long term storage and imaging.

Western blotting
GFP-tagged proteins prepared for western blotting were isolated from wild-type protoplasts transformed with plasmids for the expression of the different protein fusions. Protoplast isolation and transformation protocols (Faraco et al., 2011), as well as western blotting (Verweij et al., 2008) procedures were previously reported.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed using SigmaPlot 12.0. The normal distribution of values for all samples was confirmed by using Shapiro-Wilk normality test. Comparisons between two groups were performed using Student’s t test if variance was equal (Levene’s mean test) or using Mann-Whitney Rank Sum test if variance was unequal. Quantification of colocalization of FP-tagged proteins was performed with the PSC colocalization plugin (French et al., 2008) of the ImageJ.