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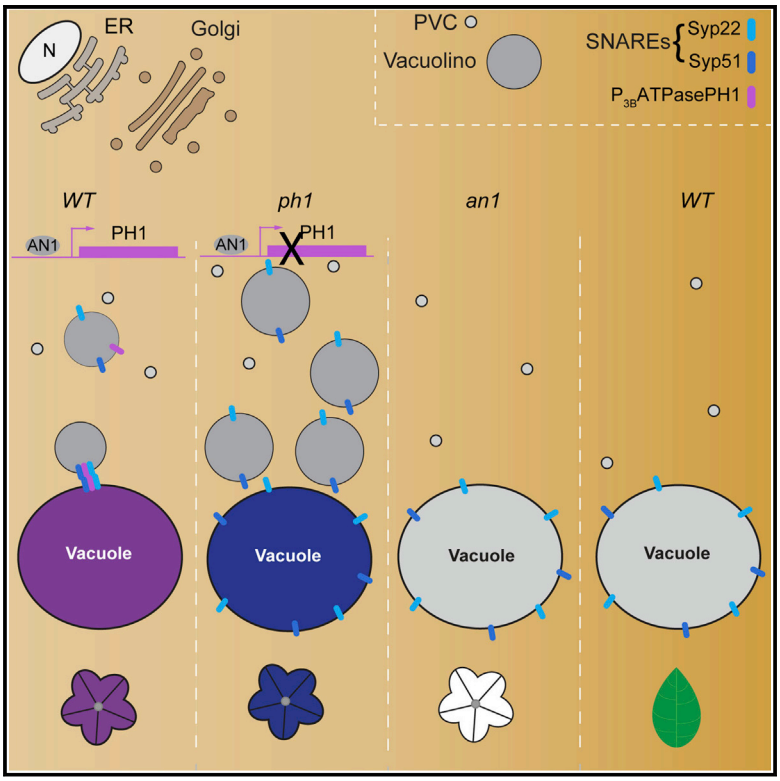
Report

A Tonoplast P_{3B}-ATPase Mediates Fusion of Two Types of Vacuoles in Petal Cells

Graphical Abstract



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In Brief

Faraco et al. describe vacuolinos, small vacuoles coexisting with the CV in petal epidermal cells. Vacuolino formation is co-regulated with pigmentation and vacuolar acidification. Vacuolar proteins traffic through vacuolinos, and their delivery to the CV requires balanced expression of vacuolar SNAREs and P-ATPases interacting on the tonoplast.

Highlights

- Multiple vacuoles coexist and exchange material in plant petal cells
- The presence of additional vacuoles is controlled by regulators of pigmentation
- The P-ATPases and vacuolar SNAREs interact with each other
- Vacuolar P-ATPases and SNAREs control protein traffic from one vacuole to the other

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Report

A Tonoplast P_{3B}-ATPase Mediates Fusion of Two Types of Vacuoles in Petal Cells

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SUMMARY

It is known that plant cells can contain multiple distinct vacuoles; however, the abundance of multivacuolar cells and the mechanisms underlying vacuolar differentiation and communication among different types of vacuoles remain unknown. PH1 and PH5 are tonoplast P-ATPases that form a heteromeric pump that hyper-acidifies the central vacuole (CV) of epidermal cells in petunia petals. Here, we show that the sorting of this pump and other vacuolar proteins to the CV involves transit through small vacuoles: vacuolinos. Vacuolino formation is controlled by transcription factors regulating pigment synthesis and transcription of *PH1* and *PH5*. Trafficking of proteins from vacuolinos to the central vacuole is impaired by misexpression of vacuolar SNAREs as well as mutants for the PH1 component of the PH1-PH5 pump. The finding that PH1-PH5 and these SNAREs interact strongly suggests that structural tonoplast proteins can act as tethering factors in the recognition of different vacuolar types.

INTRODUCTION

In *Petunia hybrida* and many other species, epidermal petal cells display color to attract pollinators (Galliot et al., 2006). Color depends on synthesis, chemical modification, vacuolar sequestration of anthocyanins and co-pigments (e.g., flavonols), and the pH of the vacuolar lumen (Koes et al., 2005). Petunia mutants with blue petals define seven loci (*PH1* to *PH7*) required for vacuolar acidification in epidermal petal cells (Quattrocchio et al., 2006). *PH3* and *PH4* encode WRKY and myeloblastosis (MYB) transcription factors, respectively, that interact with basic-helix-loop-helix (bHLH) and WD40 transcription activators encoded by *ANTHOCYANIN1* (*AN1*) and *AN11* to activate expression of ~40 genes (unpublished results), including *PH1* and *PH5*. PH5 is a tonoplast P_{3A}-ATPase proton pump that interacts with the P_{3B}-ATPase PH1 to acidify the vacuolar lumen (Faraco et al., 2014; Li et al., 2016; Verweij et al., 2008).

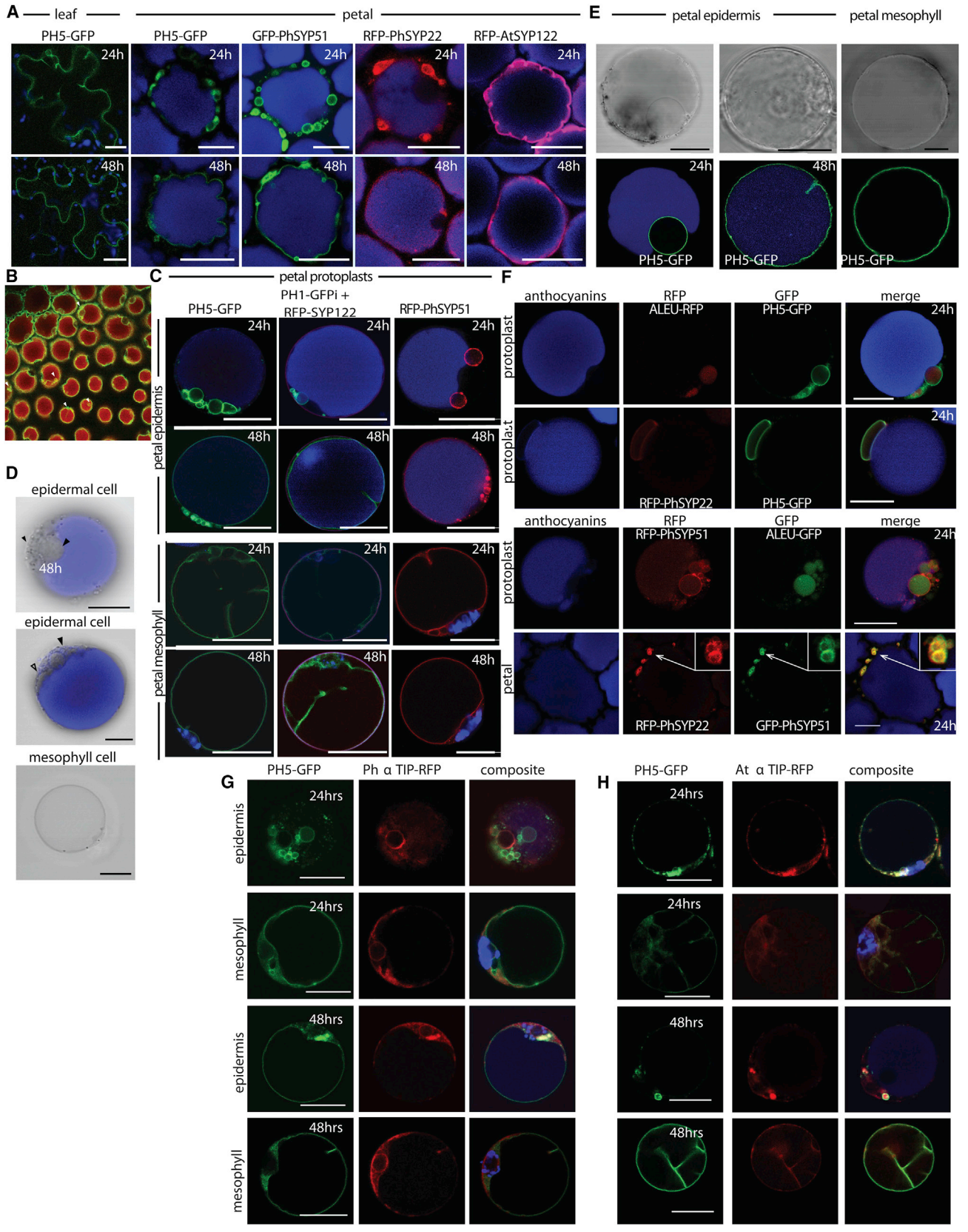
In plants constitutively expressing *35S:PH5-GFP*, most of PH5-GFP resides in the tonoplast of the central vacuole (CV) in all cell types (Verweij et al., 2008). By studying the cellular localization of the components of the PH1-PH5 pump, we found a peculiar compartment that represent a station of transit for protein directed to the vacuole in petal epidermal cells: vacuolino. This compartment, and the collection of mutants affecting its formation or fusion to the CV, provide access to the regulation of formation of multiple vacuoles and the coexistence of distinct vacuoles in a single cell.

RESULTS

Vacuolar Proteins Are Sorted through Vacuolinos to the CV in Epidermal Petal Cells

In agroinfiltrated petunia leaves, PH5-GFP localized after 24 hr on the tonoplast of epidermal cells (Figure 1A) by the "canonical" pathway described in a variety of tissues and species. In epidermal petal cells, however, PH5-GFP localized within 24 hr in vacuole-like structures ("vacuolinos," Italian for "small vacuoles") with a diameter of 1–15 μm but was absent from the tonoplast of the CV containing the anthocyanins (Figure 1A). Only 48 hr after transformation did PH5-GFP reach the CV. PH1-GFPi (GFP in a predicted cytoplasmic loop; Faraco et al., 2014) moved in a similar way (Figure 1A).

Within the petal epidermis of transgenic plants expressing the same *35S:PH5-GFP*, transgene expression remained lower than that of the PH5 endogene, both at mRNA and protein level, probably explaining why 35S:PH5-GFP rescued the *ph5* flower color only partially (Verweij et al., 2008). Epidermal petal cells of these plants also contain vacuolinos marked by PH5-GFP (Figure 1B), indicating that sorting through vacuolinos is the natural pathway by which proteins reach the CV and not the result of jamming of the sorting system or other artifacts.



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To investigate whether this trafficking pathway was specific for PH5 and PH1, we studied the sorting of PhSYP22 and PhSYP51 (petunia orthologs of, respectively, the vacuolar Qa-SNARE paralogs AtSYP21 and AtSYP22 (Uemura et al., 2004) and the Qc-SNARE paralogs AtSYP51 and AtSYP52 from *Arabidopsis* (De Benedictis et al., 2013; Figure S1A). Both are expressed in all plant parts independent from *AN1*, *AN11*, *PH3*, and *PH4* (Figures S1B and S1C). In plant cells, GFP-PhSYP22 and GFP-PhSYP51 fusions remain largely intact and membrane-bound (Figure S1D); therefore, they are reliable localization markers. In epidermal petal cells, GFP and red fluorescent protein (RFP) fusions of PhSYP22 and PhSYP51, like PH5-GFP, localized on the membrane of vacuolinos 24 hr after agroinfiltration of intact petals or transformation of petal protoplasts and reached the tonoplast of the CV 48 hr after transformation (Figures 1A and 1B).

The heterologous proteins AtSYP52 (Uemura et al., 2004) and AtKCO1 (Czempinski et al., 2002) from *Arabidopsis* (Figures S2A and S2B) and the soluble protein ALEU-GFP (Di Sansebastiano et al., 2001) also trafficked via the vacuolinos in petal epidermal cells. ALEU-GFP, PhSYP51, and PhSYP22 labeled, in addition, some punctate structures (Figures 1A and 1C), probably prevacuolar compartments (PVCs), which have a diameter ranging from 100–250 nm (Marty, 1999; Paris et al., 1996), or endosomes en route to vacuolinos. However, the plasma membrane marker RFP-AtSYP122 (Assaad et al., 2004) reached the cell outer membrane within 24 hr after transformation (Figure 1A), indicating that the transit via vacuolinos is specific for vacuolar proteins.

Vacuolinos Are Petal Epidermis-Specific and Exist in Different Plant Species

Petal protoplasts are a mixture of epidermal cells (with anthocyanin-containing vacuoles) and mesophyll cells (unpigmented; Faraco et al., 2011). In epidermal protoplasts, PH5-GFP (and other vacuolar proteins) mimics the trafficking in infiltrated petals (Figure 1C). In mesophyll cells, vacuolar proteins reached the CV within 24 hr, and no vacuolino-like structures were seen, indicating that the sorting via vacuolinos is cell type-specific (Figure 1C).

In freshly prepared petal protoplasts, bodies distinct from the central vacuole are visible (Figure 1D), indicating that vacuolinos are present before the introduction of transgenes. Similar structures are also found in epidermal protoplasts from rose (*Rosa hybrida*) petals (Figure S4A), and transiently expressed PH5-GFP localized after ~24 hr to these compartments before reaching the CV after ~48 hr (Figure 1E). Because roses (rosids) and petunias (asterids) belong to divergent clades of eudicots, this suggests that vacuolinos are widespread, at least among dicots.

Co-localization experiments in petunia epidermal petal cells and derived protoplasts showed that all vacuolar protein fusions co-localize in the same small compartment(s), indicating that vacuolinos constitute a homogeneous population (Figure 1F).

TIPs Are Not Sorted to Vacuolinos or the CV in Petal Cells

Tonoplast intrinsic proteins (TIPs) have been observed on different types of vacuoles (Jauh et al., 1999; Paris et al., 1996). To test whether TIPs differentially mark vacuolinos and the central vacuole, we used RFP and yellow fluorescent protein (YFP) fusions for α, δ, and γ TIPs from *Arabidopsis* that were previously used by others (Hunter et al., 2007) as well as fusions of homologous TIPs from petunia (PiαTIP, Peinf101Scf02502 g03029.1; PiγTIP, Peinf101Scf00487 g13016.1; PiδTIP, Peinf101Scf02095 g01012.1), identified via searches of the petunia genome (Bombarely et al., 2016) and phylogenetic analyses (Figure S2C). In petal protoplasts, the *Arabidopsis* TIPs (AtTIPs) marked the endoplasmic reticulum (ER) of epidermal and mesophyll cells after 24 and 48 hr, colocalizing with the ER marker GFP-KDEL, also on bright small vacuole-like compartments (2–3 μm). These also contained KDEL-RFP (Figure S2D) and may be similar to the αTIP-positive PSV-like compartments observed in leaf cells (Park et al., 2004). The petunia TIPs (PiTIPs) colocalized with their *Arabidopsis* homologs in the ER and the PVC-like compartments but reduced the number of these PSV-like structures substantially.

To address whether the AtTIP-positive compartments were (a subclass of) vacuolinos, we co-expressed TIP-RFP fusions and PH5-GFP. In mesophyll cells, PH5-GFP was on the vacuole within 24 hr with little or no overlap with the TIPs. In epidermal petal cells, expression of the TIP-RFP fusions affected the sorting of PH5-GFP in a major fraction of the cells. In most cells, PH5-GFP resided in vacuolinos after 24 hr, which, in many of these TIP-RFP-expressing cells, appeared to be very small

Figure 1. Sorting of Vacuolar Proteins in Leaves and Petals of Petunias

(A) Confocal images of epidermal cells from agroinfected leaves and petals expressing fluorescent fusions of vacuolar (PH5, PhSYP51, and PhSYP22; see also Figure S1) and plasma membrane (AtSYP122) proteins 24 and 48 hr after agroinfiltration. Epifluorescence of chloroplasts and anthocyanins is shown in blue and RFP and GFP fluorescence in red and green, respectively.

(B) Confocal image of the petal epidermis of a 35S:PH5-GFP plant. Where the top of the conical cells is imaged, vacuolinos are visible, marked by GFP fluorescence (white arrowheads). Anthocyanins are shown in red.

(C) Confocal micrographs of petal protoplasts expressing PH5-GFP, a combination of PH1-GFPi, and the plasma membrane markers RFP-SYP122 or RFP-PhSYP51 24 and 48 hr after transformation. Cells from the petal epidermis contain a CV with anthocyanins (blue); cells derived from the mesophyll have no anthocyanins (see also Figures S2A and S2B).

(D) Merged light and confocal micrographs of freshly prepared petunia petal protoplasts. Epidermal cells contain a CV filled with anthocyanins (blue) and small (5–10 μm) vacuolar compartments (vacuolinos) lacking anthocyanin (arrowheads). Mesophyll cells have no vacuolinos (see also Figure S4A).

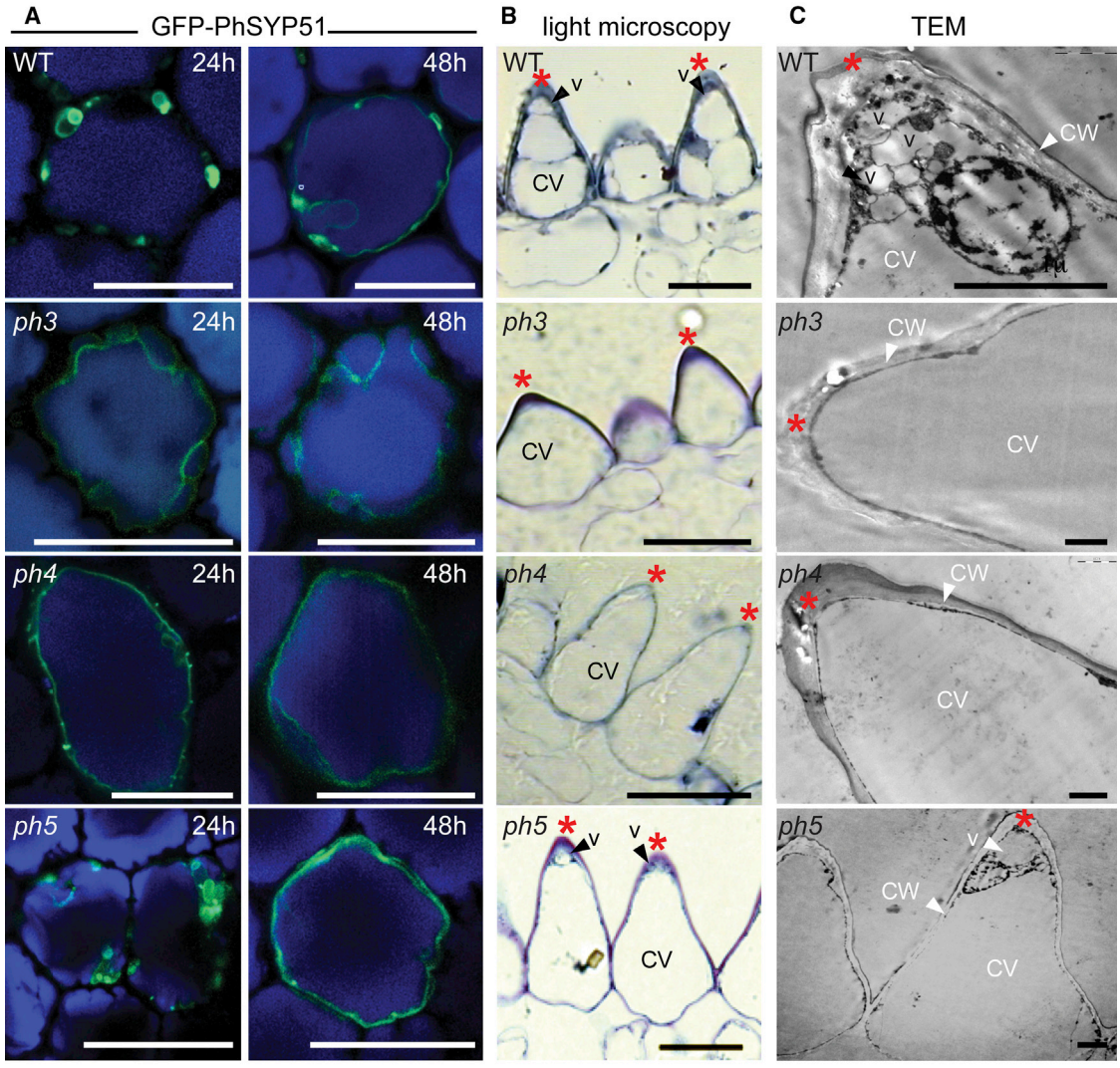
(E) Confocal (bottom) and light micrographs (top) of protoplasts from rose petals 24 hr after transformation with a PH5-GFP construct (see also Figure S4A).

(F) Vacuolar proteins co-localize in vacuolinos of epidermal petal protoplasts and epidermal cells from agroinfected petals. The insets are enlargements of the area indicated by the arrowheads.

(G) Confocal microphotographs of petal protoplasts expressing PH5-GFP and petunia αTIP-RFP 24 or 48 hr after transformation.

(H) Confocal microphotographs of petal protoplasts expressing PH5-GFP and *Arabidopsis* αTIP-RFP (see also Figures S2D and S3).

(A–H) GFP fluorescence is shown in green, RFP in red, and anthocyanin and chloroplast in blue. Scale bars, 20 μm.



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(<1–3 μm), and reached the CV after 48 hr in only a limited number of cells (2 of 7 cells co-expressing AtδTIIΠ, 4 of 15 cell co-expressing AtγTIIΠ, and 0 of 15 cells co-expressing AtαTIIΠ). This might be due to adverse effects of the aquaporin activity of TIPs, as observed previously (Oufattole et al., 2005), or indicate of a role for TIPs in trafficking from vacuolinos to the CV.

Although PH5-GFP and TIP-RFP patterns suggest co-localization in (some) small vacuolino-like structures, we observed no co-localization in vacuolinos of normal size or in the CV. Petal mesophyll cells, which lack vacuolinos, contain TIP-RFP-positive PSV-like structures similar to those observed in epidermal cells. This indicates that the PSV-like structures labeled by the analyzed TIPs are distinct from vacuolinos (Figures 1G and 1H).

an1, *ph3*, and *ph4* Mutations Abolish Vacuolino Formation but *ph1* and *ph5* Do Not

We also investigated vacuolar trafficking in *ph* mutant petals. Mutations in *PH3* and *PH4* block expression of the tonoplast pumps PH1 and PH5, affecting vacuolar acidity and petal color (Verweij et al., 2008). After agroinfiltration of *ph3* and *ph4* petals or transformation of *ph3* and *ph4* petal protoplasts, GFP-SYP51, PH5-GFP, and PH1-GFPi moved in epidermal cells to the CV within 24 hr, and vacuolinos were never seen (Figure 2A; Figures S4B and S4C), resembling the canonical sorting of vacuolar proteins in leaf and petal mesophyll cells. Light and electron microscopy revealed that epidermal cells from wild-type petals contain, within the conical tip, numerous membrane compartments (1–10 μm in size) squeezed between the plasma membrane and the CV. These structures correspond to the vacuolinos because they are absent in *ph3* and *ph4* mutants (Figures 2B and 2C). This implies that *AN1*, *PH3*, and *PH4* are required for the formation of vacuolinos, explaining why these small vacuoles are specific to epidermal petal cells.

Among the ~40 genes regulated by the AN1-PH4-PH3 complex are *PH1* and *PH5* (Faraco et al., 2014; Verweij et al., 2008, 2016). Co-expression of PH1 and PH5 is sufficient to restore vacuolar acidification and (reddish) petal color in *ph3* and *ph4* mutants (Faraco et al., 2014). However, it did not rescue the formation of and the protein sorting via vacuolinos because transiently expressed GFP-PhSYP51 localized in epidermal cells of *ph3 35S:PH1 35S:PH5* petals within 24 hr in the CV and vacuolinos was not seen (Figure 2D), as in *ph3* petals. Furthermore, a *ph5* loss-of-function mutation did not abolish the formation of vacuolinos or the sorting of transiently expressed GFP-PhSYP51 to these compartments (Figures 2A–2C). These findings imply that the hyper-acidification of the CV or precursor compartments by the PH1/PH5 pump is neither necessary nor sufficient for the formation of and protein trafficking via vacuolinos.

PH1 Is Necessary for Traffic of Proteins from the Vacuolinos to the CV

Because no isogenic *PH1* controls are available for *ph1* mutants, we analyzed *ph1* mutants in two different genetic backgrounds. In both backgrounds, GFP-PhSYP51 and PH5-GFP localized on vacuolinos in epidermal petal cells 24 hr after agroinfection, but did not reach the CV after 48 hr or more and remained instead on vacuolinos, which had increased in size and number (Figure 3A). We observed the same in *ph1* petal epidermis protoplasts transiently expressing GFP-PhSYP22 or PH5-GFP, whereas, in mesophyll protoplasts, vacuolar proteins moved to the CV within 24 hr (Figure 3B), as in the wild-type (Figure 1B). This implies that PH1 is essential for the trafficking of proteins from vacuolinos to the CV in epidermal petal cells but not for the "direct" transport that, in other cells, delivers proteins to the CV within 24 hr. We confirmed that the trafficking defect in epidermal cells is caused by *ph1* by generating isogenic $ph1^{V23}$ lines with a *35S:PH1* transgene. *35S:PH1* rescued color and vacuolar acidification in the petal epidermis, similar to previous results (Faraco et al., 2014), as well as the trafficking of PH5-GFP to the CV (Figure 3B; Figure S4E). Light and electron microscopy confirmed that the tips of *ph1* epidermal petal cells were crowded by vacuolinos (Figures 3C and 3D). This indicates that PH1 is involved in trafficking from the vacuolinos to the CV, possibly by mediating the fusion of the two types of vacuoles.

Membrane fusion relies on SNARE complexes that bridge the membranes. Excess of one of the components of such a complex may titrate away other partners, resulting in non-fusogenic complexes (Burian et al., 2016; Di Sansebastiano, 2013; Weimbs et al., 2003). Wild-type petals agroinfiltrated with a single plasmid co-expressing PH5-GFP with either PhSYP51 or PhSYP22 phenocopied *ph1* petals. In these cells, PH5-GFP localized after 24 and 48 hr in the vacuolinos, whereas little or none reached the tonoplast of the CV (Figure 3E). In the same cells, trafficking of RFP-AtSYP122 to the plasma membrane was not affected. Thus, overexpression of these vacuolar SNAREs impairs, or at least strongly delays, fusion of the vacuolinos to the CV, just like *ph1*.

We tested whether PH1 might interact with PhSYP22 and PhSYP51 by a split ubiquitin-based yeast two-hybrid system (Obrdlik et al., 2004). Co-expression of PH1-Cub (fusion to the C-terminal part of ubiquitin) and Nub-PhSYP22 weakly activated the *HIS* and *lacZ* reporter genes, whereas co-expression of PH1-Cub and Nub-PhSYP51 resulted in a stronger response (Figure S5F). Expression of PH5-Cub alone gave some background growth, reduced by addition of methionine to the medium. Co-expression of PH5-Cub and Nub-SYP122 or Nub-SYP51 under the same conditions induced activity of the *LacZ* reporter. The same holds true for the already reported interactions of PH5

Figure 2. Vacuolinos Are Absent in *ph3* and *ph4* Mutants

(A) Confocal images of epidermal cells from isogenic wild-type, *ph3*, *ph4*, and *ph5* petals 24 and 48 hr after agroinfiltration (GFP-PhSYP51). Wild-type and *ph5* cells show vacuolinos whereas the other mutants do not (see also Figure S3A).

(B and C) Light (B) and electron micrographs (C) of isogenic wild-type and mutant petals. In wild-type and *ph5* epidermal cells, small vacuolar structures fill the tip of the conical cells (red asterisks). V, vacuolinos (arrowheads); CV, central vacuole; CW, cell wall.

(D) Forced expression of PH1 and PH5 in transgenic *ph3* does not rescues the formation of vacuolinos. Top: confocal images of epidermal cells 24 hr after agroinfection with a GFP-PhSYP51 construct. Bottom: light micrographs.

Scale bars, 20 μm.

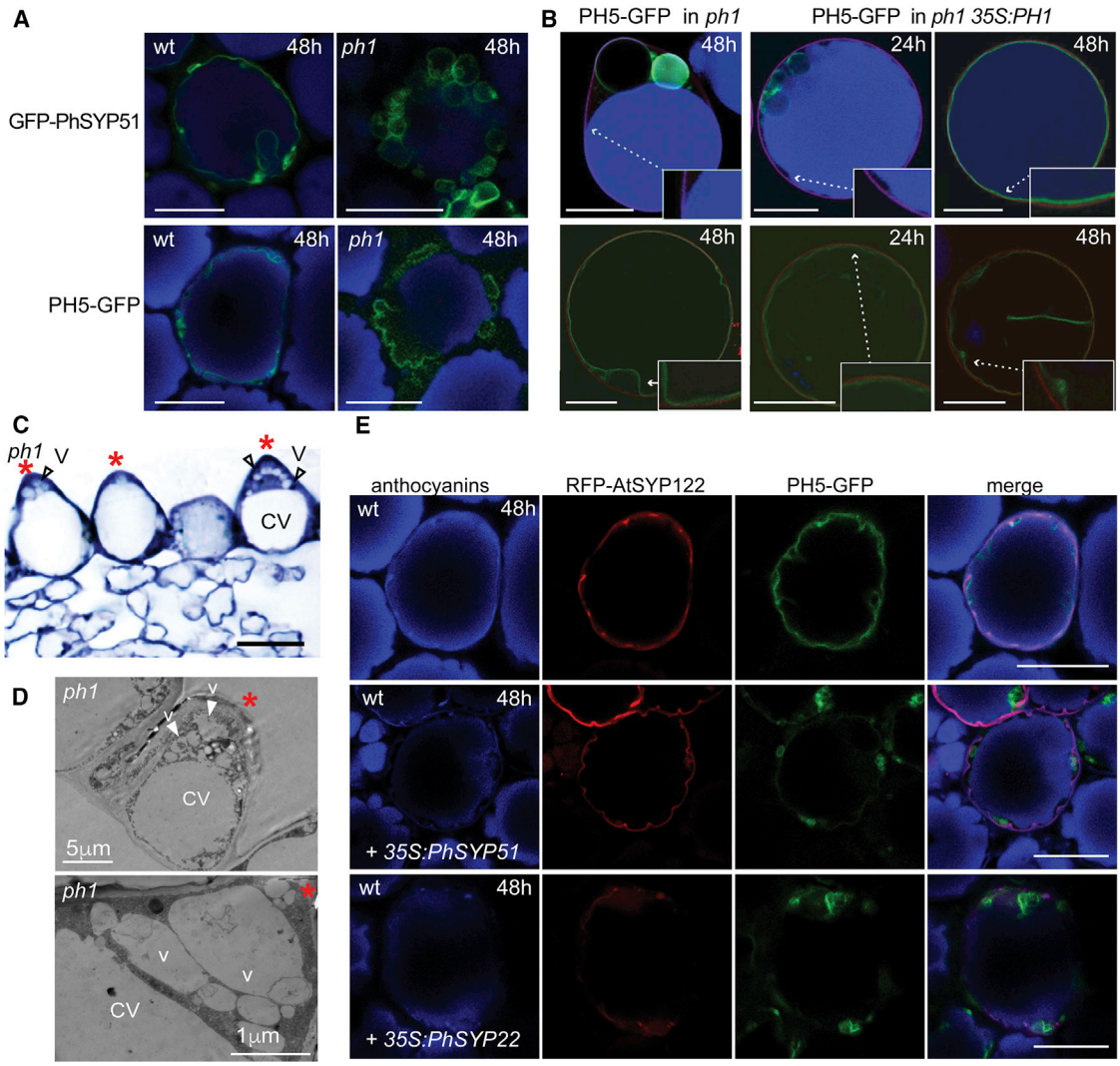

Figure 3. Mutation of *PH1* and Expression of PhSYP51 or PhSYP22 Block Trafficking from Vacuolinos to the CV

(A) Confocal images of epidermal petal cells from a wild-type and a *ph1*^{V23} plant 48 hr after agroinfiltration with GFP-PhSYP51 or PH5-GFP (see also Figures S3B–S3D).

(B) Protoplasts originating from the petal epidermis (top) and mesophyll (bottom) of a *ph1*^{V23} and complemented transgenic clone expressing *35S:PH1* 48 hr after transformation with a PH5-GFP construct. The insets show enlargements of the regions indicated by the arrows (see also Figure S3E).

(C and D) Light (C) and electron (D) micrographs showing epidermal cells from *ph1*^{V23} petals. V, vacuolinos (arrowheads); CV, vacuole. The tips of the conical cells are marked with red asterisks.

(E) Confocal micrographs of wild-type epidermal petal cells 48 hr after agroinfection with PH5-GFP, RFP-ATSYP122, and PhSYP51 or PhSYP22.

Scale bars are 5 and 1 μm in (D) and 20 μm in all other panels.

with PH1 and with itself (Faraco et al., 2014) and the dimerization of AtKAT1 (Obrdlik et al., 2004). Yeast cells expressing Cub or Nub alone or in combination with one of the aforementioned fusions did not significantly activate the reporter.

To study these interactions in planta, we used bimolecular fluorescence complementation (BiFC) (Kerppola, 2009). We expressed PH1, PH5, and the SNAREs as fusions to the N- or C-terminal parts of YFP (nYFP and cYFP, respectively) in petunia petal protoplasts and marked transformed cells by co-expressing RFP-AtSYP122. The nYFP was inserted in a cytoplasmic loop of PH1 (PH1-cYFPi) (Faraco et al., 2014). Expression of PH1-cYFPi alone or in combination with nYFP yielded no YFP fluorescence (Faraco et al., 2014). In nearly all cells co-expressing PH5-nYFP with either PH5-cYFP or PH1-cYFPi, we observed YFP fluorescence on the tonoplast of the CV (Figures 4A and 4B; Faraco et al., 2014). Co-expression of nYFP-PhSYP51 with PH1-cYFPi or PH5-cYFP resulted in YFP fluorescence on the tonoplast, confirming the interaction of PhSYP51 with both PH1 and PH5 (Figure 4). Co-expression of cYFP-PhSYP22 with PH5-nYFP or PH1-nYFPi (Figure 4) resulted in less bright fluorescence than that of PH1xPhSYP51 and PH5xSYP51, suggesting a weaker interaction, as in the yeast two-hybrid (Y2H) assay. All interactions were detectable in pigmented epidermal as well as mesophyll protoplasts (Figure 4A; Figure S5G). Co-expression

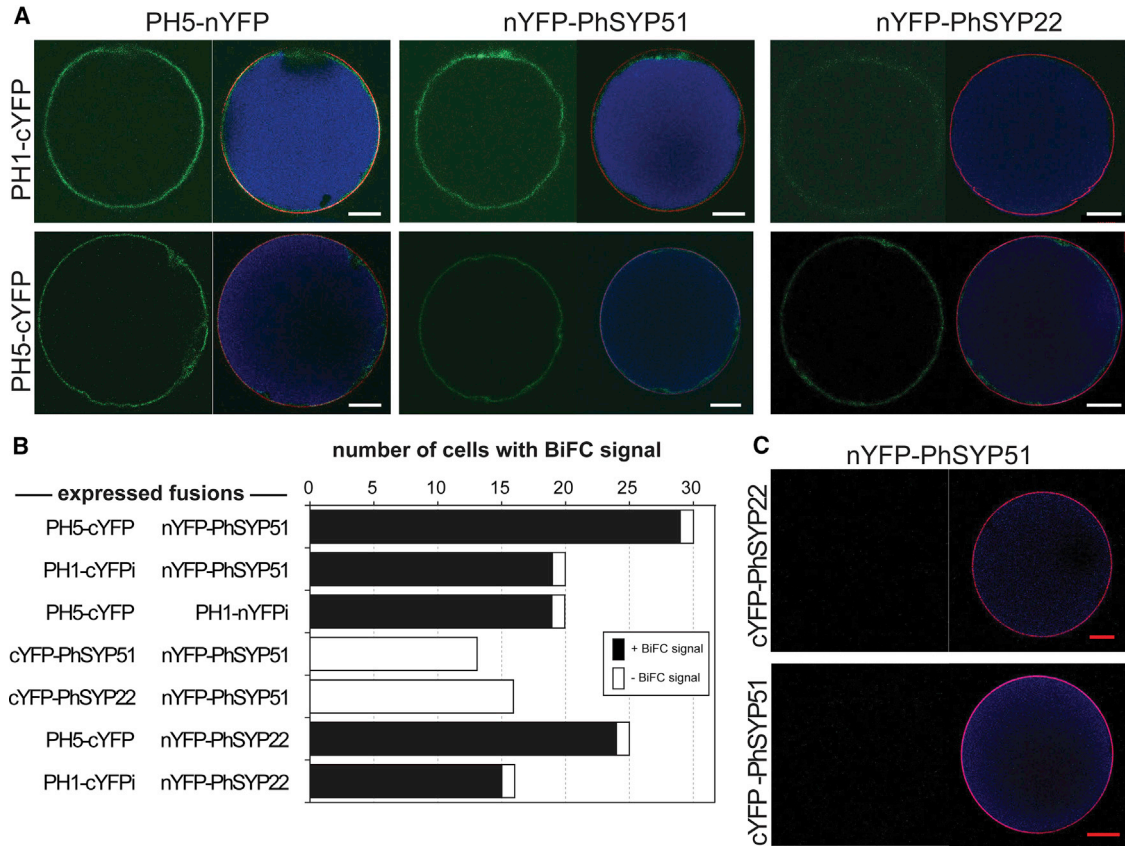

Figure 4. Bimolecular Fluorescence Complementation in Petunia Epidermal Petal Protoplasts Showing Interactions between PH1, PH5, and Vacuolar SNAREs

(A) YFP fluorescence (green) alone (left) or merged with fluorescence of RFP-AtSYP122 (red) and anthocyanins (blue) (right).

(B) number of analyzed transformed cells displaying YFP fluorescence (filled bars) or not (white bars) (see also Figure S5).

(C) cYFP-PhSYP51 and cYFP-PhSYP22 do not interact with nYFP-PhSYP51 (negative control).

Scale bars, 10 μm.

of nYFP-PhSYP51 with cYFP-PhSYP51 or cYFP-PhSYP22 did not result in YFP fluorescence, indicating that self-assembly of nYFP and cYFP was neglectable (Figure 4C). These results show that PH1 and PH5 interact with PhSYP51 and, to a lesser extent, with PhSYP22 on the membrane of the CV.

DISCUSSION

It has long been known that plant cells can contain multiple vacuoles that can differ in size, protein or ion content, and function, but it is unclear how common (or rare) multivacuolar cells are in plants (Frigerio et al., 2008) because they are only seen in protoplasts derived from unknown cell types in leaves, seeds, or root tips but not in intact plant tissues (Hunter et al., 2007; Olbrich et al., 2007), except for a few highly specialized cells types. Among these are motor cells in motile organs of some legumes (Fleurat-Lessard et al., 1997), leaf cells from a salt-stressed halophyte (Epimashko et al., 2004), senescing leaves (Otegui et al., 2005), and grape cells synthesizing anthocyanins (Gomez et al., 2011). Whether the latter are vacuolar compartments is unclear because no information is available about the proteins residing in these structures.

We demonstrated that (pigmented) epidermal cells in mature petals of diverse species contain multiple small vacuoles (vacuolinos), which differ in size and content from the large CV, and other small TIP-positive PSV-like compartments (Park et al., 2004). The vacuolinos are defined and distinguished from other small vacuoles by the transcription factors (AN1, PH3, and PH4) that govern their formation and fusion to the central vacuole. AN1, PH3, and PH4 activate a limited number (about 40) of downstream genes (Verweij, 2007); unpublished data), which seems insufficient to operate an entire trafficking pathway, considering that several of those are involved in vacuolar hyper-acidification and the stabilization of anthocyanins within the vacuole (Passeri et al., 2016; Quattrocchio et al., 2006). We presume that the vacuolino pathway is an alternative form of a canonical ubiquitous default vacuolar trafficking pathway operating in other tissues, which becomes evident in epidermal petal cells only when AN1, PH3, or PH4 is mutated.

SNAREs, Rabs, and their effectors promote membrane recognition and fusion (Stenmark, 2009; Uemura and Ueda, 2014).

Data suggest that SNARE proteins can physically and/or genetically interact with certain membrane transporters (Honsbein et al., 2009; Martin-Moutot et al., 1996; Pagel et al., 2003; Sasser and Fratti, 2014; Sasser et al., 2012; Zhang et al., 2015). Some of these interactions were shown to regulate the membrane transporter (Honsbein et al., 2009; Zhang et al., 2015), and other data report the effect of transporters on membrane fusion (Sasser et al., 2012), but evidence for a direct role in membrane recognition/fusion is lacking. PH1 is required for trafficking from vacuolinos to the CV, most likely by mediating the fusion of vacuolinos with the CV. This role of PH1 appears to be independent from the proton pumping activity of the PH1-PH5 complex because mutation of *PH5*, which is essential for electrogenic activity of PH1 (Faraco et al., 2014), does not affect the trafficking from vacuolinos to the CV. This suggests that PH1 operates as a structural component in a tethering or fusogenic complex together with SNAREs. That *ph5* did not reduce trafficking of proteins from vacuolinos to the CV, even though PH5 interacts, like PH1, with vacuolar SNAREs (Figure 2), might be explained by the nature of the *ph5* alleles used ($ph5^{A2209}$ *and* $ph5^{V69}$, accumulating *PH5* transcripts for a truncated protein; Verweij et al., 2008). Alternatively, the function of PH5 in the SNARE complex could be redundant because PH5 is one of 10–12 P_{3A}-ATPases, whereas PH1 is the only P_{3B}-ATPase protein, in petunias and other plants (Li et al., 2016). Analysis of additional *ph5* alleles or RNAi lines could solve this uncertainty.

The viability of *an1*, *ph3*, and *ph4* plants and the low number of genes misregulated in these mutants suggest that additional factors involved in the formation of vacuolinos and/or their fusion to the CV can be identified by straightforward reverse genetic analyses. Mutations in three additional AN1-PH3-PH4-regulated genes disrupt the vacuolino pathway (unpublished data). In two of these mutants transiently expressed vacuolar proteins remain stuck in puncta and do not reach the vacuolinos or the CV, with no effect on vacuolar trafficking in leaf or petal mesophyll cells. In the third mutant, vacuolar proteins also traffic directly to the CV in epidermal petal cells. This mutation probably affects a protein that operates at or close to the point of bifurcation between the vacuolino and canonical pathway. It might act to promote entrance of vesicles into the vacuolino pathway or to inhibit their entrance into the canonical pathway toward the CV.

EXPERIMENTAL PROCEDURES

Plant Material

All *Petunia hybrida* lines (from the Amsterdam petunia collection) were grown in a greenhouse at a temperature of 19°C/30°C (minimum/maximum) and a minimum of 16 hr of light in all seasons.

Lines W225 ($an1^{W225}$), R149 ($ph4^{V2153}$), and R159 ($ph5^{A2209}$) were derived from R27 (wild-type) by transposon insertion and excisions. We used the (transformable) F1 hybrids M1xV30 (wild-type), R143xR144 ($ph3^{R49/B2267FP}$), and V69xR159 ($ph5^{A2209/V69}$) and a homozygous $ph1^{V23/V23}$ individual of the F2 cross V23xV30 or a $ph1^{L2164/R6}$. Further details on these alleles can be found elsewhere (Faraco et al., 2014; Quattrocchio et al., 2006; Spelt et al., 2000; Verweij et al., 2008, 2016).

Transient Expression Assays

Agroinfiltration of intact tissues, transient transformation of leaf and petal protoplasts (Faraco et al., 2011), and generation of transgenic plants were described previously. Petals from flowers that had just opened (stage 6) were used for protoplast preparation and agro-infection.

Gene Constructs

The full-length PhSYP51 sequence, amplified from petal RNA of line R27 with primers 4655 and 4675 (Table S1) was cloned in pENTR/D-TOPO (Invitrogen) and recombined (Gateway, Invitrogen) into specific destination vectors (Karimi et al., 2002, 2005) to give all expression clones.

Primers (Table S1) were designed to amplify the full-size cDNA of the petunia *PhSYP22* from R27 petals and produce expression constructs as described above for PhSYP51.

Phylogenetic analyses were done as previously described (Li et al., 2016).

Other constructs are described elsewhere as follows: AtKCO1-GFP (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008), Aleu-GFP (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008), RFP-AtSYP122 (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008), PH5-GFP (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008), PH1-GFPi (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008), and *Arabidopsis* TIP-GFP fusions (Hunter et al., 2007).

The coding sequences of petunia αTIP, δTIP, and γTIP were amplified from genomic DNA of *P. inflata* (line S6) using the primers listed in Table S1 and recombined into pK7RWG2,0 (Karimi et al., 2002), in which the GFP coding sequence was replaced by the RFP coding sequence. RFP fusions for *Arabidopsis* TIPs were constructed in a similar way.

The accession numbers for the sequences of PiαTIP, PiδTIP, and PiγTIP reported in this paper are Sol Genomics: Peinf101Scf02502 g03029.1, Peinf101Scf02095 g01012.1, and Peinf101Scf00487 g13016.1, respectively.

Split Ubiquitin Assay

Split ubiquitin assays were performed as described previously (Obrdlik et al., 2004). The constructs for PH1 and PH5 were described previously (Faraco et al., 2014).

B1 and B2 sequences were added to SNAREs by PCR using the following primers: 5750 and 5751 to amplify PhSYP22 and 5752 and 5753 (Table S1) to amplify PhSYP51. The PCR products were then recombined in split ubiquitin vectors (Obrdlik et al., 2004).

Confocal Microscopy

Protoplasts and intact tissue were imaged with a confocal laser microscope (Zeiss, LSM510) (Faraco et al., 2011).

Microscopy of Petal Semi-thin Sections

Petal limbs were fixed in 5% (w/v) glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.2) for 24 hr, washed four times for 15 min each in 75 mM cacodylate buffer (pH 7.2), post-fixed in 1% (w/v) OsO_4 for 90 min, dehydrated in increasing concentrations of ethanol, and included in resin (Epon, 2-dodecenylsuccinic anhydride, and methylnadic anhydride mixture). Semi-thin sections (1–2 μm) were cut by ultramicrotome (OmU2, Reichert) equipped with a glass blade, 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).

ACCESSION NUMBERS

The accession numbers for the sequences of the Petunia hybrida vacuolar SNARES PhSYP22 and PhSYP51 reported in this paper are GenBank: KY196467 and KY196466, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.076.

AUTHOR CONTRIBUTIONS

R.K. and F.M.Q. conceived and supervised the project. M.F., Y.L., R.K., and F.M.Q. wrote the paper with input from all other authors. M.F., Y.L. and S.L. performed most of the experiments. W.V. and G.P.D.S. performed the early localization studies, which revealed the existence of vacuolinos. C.S. generated constructs and performed the Y2H analysis. L.R. and F.F. performed light and electron microscopy.

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