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Li, Y.

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A tonoplast P$_{3B}$-ATPase mediates fusion of two types of vacuoles in petal cells*

Marianna Faraco$^{1,2,3,6}$, Yanbang Li$^{1,2,6}$, Shuangjiang Li$^{1,2,6}$, Cornelis Spelt$^{1,2}$, Gian Pietro Di Sansebastiano$^{3}$, Lara Reale$^{4}$, Francesco Ferranti$^{4}$, Walter Verweij$^{2,5}$, Ronald Koes$^{1,2,7}$ and Francesca M. Quattrocchio$^{1,2,7}$

$^{1}$Plant Development and (Epi)Genetics, Swammerdam Institute for Life Science, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
$^{2}$Department of Molecular Cell Biology, section Genetics, VU-University, de Boelelaan 1087, 1081HV Amsterdam, The Netherlands
$^{3}$Department of Biotechnology and Environmental Sciences, University of Salento, via Monteroni - Centro Ecotekne, 73100 Lecce, Italy
$^{4}$Department of Agricultural, Food and Environmental Sciences, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy.
$^{5}$Present address: ENZA Zaden. Haling 1E, 1602 DB Enkhuizen, The Netherlands
$^{6}$co-first authors.

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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 type of vacuoles remained unknown. PH1 and PH5 are tonoplast P-ATPases that form a heteromeric pump which hyper-acidifies the central vacuole 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. The traffic of proteins from vacuolinos to the central vacuole is impaired by miss-expression 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.

**Keywords**

Multiple vacuoles, membrane fusion, SNARE complex, membrane recognition, tethering factors.

**Highlights**

- Multiple vacuoles coexist and exchange material in plant petal cell
- 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
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 and 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 MYB transcription factors respectively that interact with 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 P3A-ATPase proton-pump that interacts with the P3B-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 which 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 hours 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 hours in vacuole-like structures (“vacuolinos”, Italian for “small vacuoles”) with a diameter of 1 to 15 µm, but was absent from the tonoplast of the CV containing the anthocyanins (Figure 1A). Only 48 hours after transformation PH5-GFP reached the CV. PH1-GFPi (GFP into a predicted cytoplasmic loops, 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.

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,
independently from *AN1, AN11, PH3* and *PH4* (Figure S1B-C). 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 RFP fusions of PhSYP22 and PhSYP51, like PH5-GFP, localized on the membrane of vacuolinos 24 hours after agroinfiltration of intact petals or transformation of petal protoplasts, and reached the tonoplast of the CV 48hrs after transformation (Figure 1A,B).

The heterologous proteins AtSYP52 (Uemura et al., 2004) and AtKCO1 (Czempinski et al., 2002) from Arabidopsis (Figure S2A,B) 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 (Figure 1A and C), 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 hours 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 epidermis (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 hours and no vacuolino-like structures are 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 in epidermal protoplasts from rose (*Rosa hybrida*) petals (Figure S4A), and transiently expressed PH5-GFP localized after ~ 24 hrs, to these compartments before reaching the CV after ~48 hours (Figure 1E). Because rose (*Rosids*) and petunia (*Asterids*) belong to divergent clades of Eudicots, this suggests that vacuolinos are wide spread 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).
Figure 1. Sorting of vacuolar proteins in leaves and petals of Petunia

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

(B) Confocal image of 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 marker RFP-SYP122, or RFP-PhSYP51, 24 and 48 hours after transformation. Cells from the petal epidermis contain a CV with anthocyanins (blue); cells derived from the mesophyll have no anthocyanins (see also Figure S2 A-B).
Vacuolino

(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 hours 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 hours after transformation.

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

In all panels: GFP fluorescence in green, RFP in red, anthocyanin and chloroplast in blue. Size bars, 20 µm.

**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 central vacuole we used the RFP- and 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, PiγTIP and PiδTIP), 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 hours, 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 within 24 hours on the vacuole 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 after 24 hours in vacuolinos, which in many of these TIP-RFP expressing cells appeared very small (<1-3 µm), and reached the CV after 48 hours in only a limited number of cells (2 out of 7 cells co-expressing AtδTIP, 4 out of 15 cell co-expressing AtγTIP, and 0 out of 15 cells co-expressing AtαTIP). This might be due to adverse effects of the aquaporin activity of TIP proteins, 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 nor in the CV. Petal mesophyll cells, which lack vacuolinos, contain TIP-RFP positive PSV-like similar to the epidermal cells, altogether indicating that the PSV-like structures labeled by the analyzed TIP proteins are distinct from vacuolinos (Figure 1G, H).
**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 hours and vacuolinos were never seen (Figure 2A and Figure S4B,C), 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, as they are absent in *ph3* and *ph4* mutants (Figure 2B, C). This implies that *AN1, PH3* and *PH4* are required for the formation of vacuolinos, explaining why these small vacuoles are specific for 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; Verweij et al., 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, as transiently expressed GFP-PhSYP51 localized in epidermal cells of *ph3 35S:PH1 35S:PH5* petals within 24 hours in the CV and vacuolinos were 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 (Figure 2A-C). 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.
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 hours after agroinfiltration (GFP-PhSYP51). Wild type and ph5 cells show vacuolinos, while 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 asterisk). V, vacuolinos (arrow heads); CV, central vacuole, CW cell wall.

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

Scale bars, 20 µm.
PH1 is necessary for traffic of proteins from the vacuolinos to the CV

Because for *phl* mutants no isogenic *PH1* controls are available, we analyzed *phl* mutants in two different genetic backgrounds. In both GFP-PhSYP51 and PH5-GFP localized on vacuolinos in epidermal petal cells 24 hours after agroinfection, but did not reach the CV after 48 hours or more and remained instead on vacuolinos, which had increased in size and number (Figure 3A). We observed the same in *phl* petal epidermis protoplasts transiently expressing GFP-PhSYP22 or PH5-GFP, whereas in mesophyll protoplasts, vacuolar proteins moved within 24 hours to the CV (Figure 3B), as in 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 hours. We confirmed that the trafficking defect in epidermal cells is caused by *phl*, by generating isogenic *phl<sup>123</sup>* 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 and Figure S4E). Light and electron microscopy confirmed that the tips of *phl* epidermal petal cells are crowded by vacuolinos (Figure 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 *phl* petals. In these cells PH5-GFP localized after 24 and 48 hours 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, over-expression of these vacuolar SNAREs impairs, or at least strongly delays, the fusion of the vacuolinos to the CV, just like *phl*.

We tested whether PH1 might interact with PhSYP22 and PhSYP51 by a split-ubiquitin-based yeast 2-hybrid system (Obrdlik et al., 2004). Co-expression of PH1-Cub (fusion to C-terminal part of Ubiquitin) and Nub-PhSYP22 weakly activated the *HIS* and *lacZ* reporter genes, while 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 in 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 for the already reported interactions of PH5 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.
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\textsuperscript{V23} plant, 48 hours after agroinfiltration with a GFP-PhSYP51 or PH5-GFP (see Figure S3B-S3D).

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

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

(E) Confocal micrographs of wild-type epidermal petal cells 48 hours after agroinfection with PH5-GFP, RFP-AtSYP122 and PhSYP51 or PhSYP22. Scale bars are 5 and 1 \(\mu\)m in (D) and 20 \(\mu\)m in all other panels.

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) in petunia petal protoplasts and marked transformed cells by co-expressing RFP-AtSYP122. The nYFP was inserted in a cytoplasmic loop of PH1 (PH1-cYFP\textsubscript{i}) (Faraco et al., 2014). Expression of PH1-cYFP\textsubscript{i} 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-cYFP\textsubscript{i} we observed YFP fluorescence on the tonoplast of the CV (Figure 4A and 4B (Faraco et al., 2014)). Co-expression of nYFP-PhSYP51 with PH1-cYFP\textsubscript{i} 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 Y2H assay. All interactions were detectable in pigmented epidermal as well as mesophyll protoplasts (Figure 4A, Figure S5G). Co-expression of nYFP-PhSYP51 with cYFP-PhSYP51 or cYFP-PhSYP22 did not result in YFP fluorescence, indicating that self assembly of nYFP and cYFP was negligible (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.

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 bar) or not (white bar) (See Figure S5). (C) cYFP-PhSYP51 and cYFP-PhSYP22 do not interact with nYFP-PhSYP51 (negative control). Scale bars, 10 \(\mu\)m.

Discussion
It is long known that plant cells can contain multiple vacuoles that can differ in size, protein or ion content and function, but is unclear how common (or rare) multivacuolar cells are in plants (Frigerio et al., 2008), as they were 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 as 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, 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 from a canonical ubiquitous default vacuolar trafficking pathway operating in other tissues and becoming evident in epidermal petal cells 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), 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 independent from proton pumping activity of the PH1-PH5 complex, as 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<sup>42209</sup> and ph5<sup>5169</sup>, accumulating PH5 transcripts for a truncated protein (Verweij et al., 2008). Alternatively, the function of PH5 in the SNARE complex could be redundant, as PH5 is one of 10-12 P<sub>3A</sub>-ATPases, while PH1 is the only P<sub>3B</sub>-ATPase protein in petunia 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 miss-regulated in these mutants, suggests 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 traffic directly to the CV also in epidermal petal cells. This mutation probably affects a protein that operates at, or close to, the point of bifurcation between 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 towards the CV.
Materials and methods

Plant material
All *Petunia hybrida* lines (from the Amsterdam petunia collection) were grown in greenhouse: temperature 19°C / 30°C (min/max), a minimum of 16 hours of light in all seasons.

Lines W225 (*an1H*225), R149 (*ph4H*2153) and R159 (*ph5H*2006) derive from R27 (wild type), by transposon insertion and excisions. We used the (transformable) F1 hybrids M1xV30 (wild type), R143xR144 (*ph3H*49/B2267FP) and V69xR159 (*ph5H*2006/V69), and a homozygous *ph1H*23/V23 individual of the F2 cross V23xV30 or a *ph1H*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; Verweij et al., 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., 2005; Karimi et al., 2002) 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: AtKCO1-GFP (Assaad et al., 2004; Czempinski et al., 2002; Faraco et al., 2014; Fluckiger et al., 2003; Verweij et al., 2008). Aleu-GFP(Asaad 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 primers in Table S1, and recombined into the 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.
Split-Ubiquitin assay.
Split-Ubiquitin assays were performed as described (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; #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 24h, washed four times for 15 min each in 75 mM cacodylate buffer, pH 7.2, post-fixed in 1% (w/v) OsO₄ for 90 min., dehydrated in increasing concentrations of ethanol and included in resin (Epon, 2-dodecenylsuccinic anhydride, and methyladnic anhydride mixture). Semi-thin sections (1–2 µm) were cut by ultramicrotome (OmU2, Reichert, Heidelberg) equipped with a glass blade, stained with toluidine blue and observed under a light microscope (DMLB, Leica, Wetzlar, Germany). Transmission Electron Microscopy of petal cells was performed as previously reported (Verweij et al., 2008)

Accession numbers
Sequences of the Petunia hybrida vacuolar SNAREs PhSYP22 (KY196467) and PhSYP51 (KY196466) are deposited in Genbank, those of PiαTIP (Peinf101Scf02502g03029.1), PiδTIP (Peinf101Scf02095g01012.1) and PiγTIP (Peinf101Scf00487g13016.1) are available at: https://solgenomics.net/
Acknowledgements

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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. K.S. generated constructs and performed Y2H analysis. L.R. and F.F. performed light and electron microscopy.
References


Figure S1. Characterization of PhSYP22 and PhSYP51, Related to Figure 1

(A) Phylogenetic tree of SNARE proteins from different plant species: Ph, Petunia hybrida; Pa, Petunia axillaris (Bombarely et al., 2016); At, Arabidopsis thaliana; Sc, Saccharomyces cerevisiae; St, Solanum tuberosum; Os, Oryza sativa; Sl, Solanum lycopersicum; Nt, Nicotiana tabacum; Cr, Capsella rubella; Bn, Brassica napus. The two petunia vacuolar SNAREs PhSYP22 and PhSYP51 are marked in red. Sequence alignments were generated with MUSCLE and phylogenetic trees were constructed with Maximum likelihood (PhyML) after curation of the alignments by the G-blocks tool, and then rendered by TreeDyn online (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi). Branch support is expressed as percentage of 300 bootstraps.

(B) RT–PCR analysis of PhSYP22 and PhSYP51 mRNA in wild type petunia (line V30) in different tissues and different developmental stages. “Throat” is the part of the corolla at the junction of the petal limb and the tube.

(C) RT–PCR analysis of PhSYP22 and PhSYP51 mRNA in petals of opening flower buds (stage 5) from different genotypes. GAPDH (GLYCOLALDEHYDE 3-PHOSPHATE DEHYDROGENASE) was used as a constitutively expressed control. None of the two genes seems to be regulated by any of the regulatory genes involved in petal pigmentation or vacuolar acidification.

(D) Western blot analysis of protein fractions (M; membrane fraction, S; soluble fraction) obtained from GFP-PhSYP22 and GFP-PhSYP51 expressing petal protoplasts of wild type. Proteins were detected using an anti-GFP serum. Arrows indicate free GFP, which is in low amount and only visible in the soluble fraction.
Figure S2. Intracellular trafficking of AtKCO1, PhSYP51 and TIP proteins from the α-, γ- and δ-subfamilies, Related to Figure1
(A) GFP-AtSYP52 localization in epidermal cells of intact petals 24 and 48 hours after agroinfiltration. Fluorescence of GFP-AtSYP52 is shown in green and anthocyanin autofluorescence in blue.
(B) AtKCO1-GFP localization in leaf and petal epidermis 24 hours after agroinfiltration. In leaf epidermis GFP signal is on the tonoplast, while in the petal epidermal cells it is on the membrane of vacuolinos. AtKCO1-GFP fluorescence is shown in green and of anthocyanins in blue.
(C) Phylogenetic tree of α, γ and δ TIP proteins from different species (At: Arabidopsis thaliana, Pi: Petunia inflata, Sl: Solanum lycopersicum, St: Solanum tuberosum). Sequence alignments were generated with MUSCLE and phylogenetic trees were constructed with Maximum likelihood (PhyML). After curation of the alignments by G-blocks tool, rendered by TreeDyn on line (http://phylogeny.lirmm.fr/phylo.cgi/index.cgi). Branch support is the percentage of 300 bootstraps.
(D,E) Localization of FP-tagged TIPs from Arabidopsis and petunia in petunia protoplasts from petal epidermis and petal mesophyll. RFP-KDEL was co-expressed to mark the ER. Fluorescence of Arabidopsis TIP-YFP fusions is shown in green, that of RFP-KDEL and petunia TIP-RFP fusions in red and of anthocyanins and chlorophyll in blue. The rightmost panel (yellow frame) shows an enlargement of the marked part in the leftmost image taken with lower laser intensity to better distinguish the membranes surrounding small brightly fluorescent compartments. The composite panel shows the merged image from the red (RFP), green (YFP) and blue (anthocyanin and chloroplasts) channels. Scale bars equal 20 µm in all panels.
**Figure S3.** RFP fusions of γ and δ TIPs from Arabidopsis and petunia do not co-localize with PH5-GFP and affect its sorting to the vacuole in epidermal petal cells, Related to Figure1.

PH5-GFP was cotransformed with Arabidopsis or petunia RFP-fusions of TIPs from the α, γ and δ families. Fluorescence of PH5-GFP is shown in green, that of TIP-RFP fusions in red and of anthocyanins and chlorophyll in blue. The composite panel shows the merged image from the red, green and blue channel. Scale bars equal 20 µm in all panels.
Figure S4. Vacuolinos formation and fusion, Related to Figures 1, 3 and 4

(A) Light and confocal micrographs of freshly prepared rose petal protoplasts. Vacuolinos are visible and the CV is recognizable from the anthocyanins in the lumen (blue fluorescence).
(B) Localization of PH5-GFP in epidermal cells of an1, ph3, ph4 or ph1 petals 24 hours after agroinfiltration.
(C) PH1-GFPi in petal protoplasts from ph3 and ph4 petals 24 hours after transformation.
(D) GFP-PhSYP22 in ph1 protoplasts shows that the fusion protein is stuck on the membrane of the vacuolinos in epidermal pigmented cells, while it normally reaches the tonoplast of the CV in uncolored mesophyll cells (white arrowhead).
(E) Localization of PH5-GFP in petunia protoplasts from petals of a ph1V23 mutant and a derived transgenic line transformed with the 35S:PH1 construct. Magenta arrowheads indicate protoplasts derived from the petal epidermis, orange arrowheads indicate mesophyll protoplasts. Scale bars equal 20 µm in all panels in A, B and C.
(F) Split ubiquitin based Yeast 2 Hybrid assay. Cells expressing different combinations of Nub (N-terminal part of Ubiquitin) and Cub (C terminal part of ubiquitin) fusions were grown on distinct media to select for interactions. The addition of 75 µM methionine lowers the strong background given by the PH5-Cub construct.
(G) Bimolecular fluorescence complementation in protoplasts of petunia petals shows that all interactions are detectable in colored cells from the epidermis as well as in white cells from the other cell layers. RFP-AtSYP122 marks the plasma membrane and serves as marker of transformation. Scale bars are 10 µm.
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1 F indicates a forward orientation of the primer, relative to the orientation of the gene, and R reverse orientation.