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Vacuolino, a novel endomebrane compartment involved in the trafficking of proteins to the vacuole in epidermal petal cells of petunia flowers.


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SUMMARY
The lytic vacuole is the main vacuole of the plant cell and is usually indicated as the central vacuole (CV). This compartment occupies up to over 90% of the cell volume and its lumen is more acidic than the surrounding cytoplasm. In the epidermal cells of flower petals the CV specialized to display the color due to anthocyanin pigments accumulation. Here we show that these cells contain, besides the central vacuole, several smaller vacuoles on which tonoplast proteins are sorted before reaching the CV. The small vacuole, here named vacuolino, is absent in mutants for the transcription factors PH3 and PH4 (involved in vacuolar pH regulation). The vacuolino requires the presence of the PH1 P3B-ATPase to fuse to the CV and is altered by the overexpression of vacuolar SNAREs SYP51 and SYP22. We show that the vacuolino is specific for petal epidermal cells, is not required for correct vacuolar acidification and is part of a novel protein sorting pathway. The availability of mutants that control the biogenesis and/or the fusion of vacuolinos with the CV, provides a formidable chance to study the mechanisms of vacuolar dynamics.

INTRODUCTION
The vacuole is a multifunctional organelle that is essential for plant cell life as demonstrated by the lethality of mutations that block its biogenesis (Rojo et al., 2001). Some cell types contain multiple vacuoles with distinct functions, such as lytic vacuoles with lysosome-like properties and protein storage vacuoles (Paris et al., 1996; Fleurat-Lessard et al., 1997; Frigerio et al., 2008). However the mechanisms regulating the co-existence of multiple vacuoles in one cell and the sorting of proteins to the correct vacuolar types, remain unclear (Zouhar and Rojo, 2009). In most plant cells, the lytic vacuole is the main vacuole and occupies more than 90% of the total volume. Because of its dimensions, it pushes the other organelles and the cytoplasm to the periphery of the cell in close proximity to the plasma membrane, facilitating gas exchange and other physiological processes. It is usually indicated as the central vacuole (CV). The pH value of the lumen of the CV is typically around 5, but can be as low as 2 in specialized tissues of citrus species (Muller and Taiz, 2002; Martinoia et al., 2007).

Vacuoles are the largest H+ storage compartments in the plant cell and the accumulation of protons in the vacuoles is, in most tissues, dependent on two types of tonoplast transporters: the complex pumping system V-ATPase, consisting of some 25 subunits, and the single-peptide H+-pyrophosphatase pump (H+-PPase) (Maeshima, 2001). The resulting electrochemical gradient across the tonoplast mediates the movement of other ions, such as K+, Cl-, Na+ and more complex molecules between the vacuole and the cytoplasm, and is important for the homeostasis of a variety of compounds in the different compartments (Shiratake and Martinoia, 2007) and the control of turgor (Schroeder et al., 2001).

In Petunia hybrida epidermal petal cells are specialized to display color, which is necessary to attract pollinators (Galliot et al., 2006). The coloration of petals depends on the synthesis of anthocyanin pigments and their stable accumulation in the CV. The final color is determined by the chemical modifications of the anthocyanin molecules, the presence of co-pigments (e.g. flavonols) and the pH of the vacuolar lumen (Koes et al., 2005). That is, anthocyanins behave as pH indicators and
display a reddish color in an acidic environmental or a bluish color at higher pH (Yoshida et al., 2003).

By mutations causing a blue flower color, seven so-called PH loci have been identified in petunia that are required for vacuolar acidification in epidermal petal cells (Quattrocchio et al., 2006). The loci PH3 and PH4 encode transcription factors of the WRKY and MYB family respectively, that act in concert with regulatory genes of the anthocyanin pathway, such as ANTHOCYANIN1 (AN1) and AN11 to activate expression of > 10 subordinate genes (Verweij, 2007). Among these target genes are PH1 and PH5, which encode two novel P-ATPases. PH5 is a member of the plant-specific family of P_{3A}\text{-ATPase} proton-pumps, while PH1 is highly similar to bacterial P_{3B}\text{-ATPases}, which can transport Mg\text{^{2+}} (see Chapter IV). PH5 diverged from other P_{3A}\text{-ATPases}, as it resides in the tonoplast, whereas most, if not all, other P_{3A}\text{-ATPases localize in the plasma membrane. (Verweij et al., 2008b)}

Here we show that in epidermal petal cells PH5 and other vacuolar proteins, such as the vacuolar SNAREs PhSYP22 and PhSYP51, are sorted to the tonoplast via a pathway a novel intermediate endomembrane compartment, which we designated vacuolino, because it resembles a small vacuole. The formation of vacuolinos is specific for epidermal petal cells and is genetically controlled by the very same set of transcription regulators that control the expression of PH5 and PH1 (AN1, PH3 and PH4). The tonoplast of this small compartment is labeled by the petunia vacuolar SNAREs PhSYP22 and PhSYP51, which, in combination with PH1 are required for the subsequent fusion of the vacuolinos with the central vacuole.

This is, to our knowledge, the first time that the genetic control of the production of an intracellular compartment and the physiology of its membranes is defined by well-characterized mutants.

RESULTS.

PH5-GFP characterizes a new small vacuolar compartment: the vacuolino.

PH5 is, unlike all other characterized P_{3A}\text{-ATPases}, localized on the tonoplast (Verweij et al., 2008b). In plants expressing a constitutive 35S:PH5-GFP transgene most of the PH5-GFP protein resides in the tonoplast in every analyzed cell type. To study the pathway by which PH5 is targeted to the tonoplast, we transformed cells in wild type petals and leaves by agro-infection and we followed the localization of PH5-GFP at various time points. In epidermal leaf cells PH5-GFP appeared on the tonoplast within 24 hours after infection (Fig. 1A). In epidermal petal cells, however, PH5-GFP was found after 24 hours on small vacuolar structures with a diameter of 2 to 15 µm, but was absent from the tonoplast of the large CV that contains the anthocyanins (Fig. 1B). Only after 48 hours PH5-GFP arrived at the CV tonoplast and exhibited a similar distribution pattern as in epidermal petal cells of stable transformants expressing 35S:PH5-GFP (Fig.1C) (Verweij et al., 2008b).

We previously reported that in agroinfected epidermal petal cells, as well as protoplasts isolated from the same tissue, the lytic vacuolar marker ALEU-GFP accumulates 24 hrs after transformation in the lumen of some small compartments that are distinct from the central vacuole and reaches after 48 hrs the lumen central vacuole.

However, when expressed in epidermal leaf cells ALEU-GFP reaches the lumen of the central vacuole already after 24 hrs without labeling of small vacuolar compartments (see chapter III, and
These observations suggest that the small compartments marked by ALEU-GFP, are the same as those containing PH5-GFP within 24 hrs of accumulation, before these proteins reach the tonoplast of the CV.

The small vacuolar compartments in epidermal petal cells are distinct from the much smaller “prevacuolar compartments” (PVCs) with a diameter ranging from 100 to 250 nm (Paris et al., 1997; Marty, 1999). PVCs punctate structures and small peripheral vacuoles are both clearly labeled by ALEU-GFP, therefore, we decided to call the new vacuole-like organelle vacuolino.
**Figure 2. Transient expression of GFP-PhSYP51 and RFP-PhSYP22 in petal cells.**
(A) GFP-PhSYP51 in petal epidermis and (B) in protoplast from petal epidermis; (C) RFP-PhSYP22 in petal cells and (D) in petal protoplast. In blue the anthocyanins, in green GFP-PhSYP51 and in red RFP-PhSYP22 fluorescence. Scale bars: 20µm.

**Different vacuolar proteins localize on the membrane of the vacuolino.**

To determine how other vacuolar proteins that have no obvious role in flower pigmentation are sorted in epidermal petal cells, we identified petunia homologs of the vacuolar SNARE proteins AtSYP22 and AtSYP51 from Arabidopsis, and named the petunia homologs PhSYP22 (containing the Qa-SNARE domain) and PhSYP51 (containing the Qc-SNARE domain) (Lipka et al., 2007). The PhSYP51 sequence is equally similar to the AtSYP51 and AtSYP52 (Supplemental Fig. 1A) suggesting that the latter are paralogs that duplicated after the separation of the Arabidopsis and petunia lineage and that PhSYP51 can been seen as the homolog of both. In petunia PhSYP22 and PhSYP51 are expressed in
all plant parts (Supplemental Fig. 1B) and their expression is not affected by mutations in any of the regulatory genes that activate structural anthocyanin genes and/or genes involved in vacuolar acidification (Supplemental Fig. 1C).

To study the traffic and localization of these SNAREs, we expressed GFP or RFP tagged fusion proteins in intact petal tissue and in petal protoplasts. These experiments revealed that GFP-PhSYP51 (Fig. 2A and B) and GFP-PhSYP22 (Fig. 2C and D) follow a similar protein sorting pathway as PH5-GFP. That is, GFP-PhSYP51 and GFP-PhSYP22 localize on the tonoplast of vacuolino–like structures 24 hrs after transformation and are found on tonoplast of the CV after 48 hrs. We confirmed by Western blot that both GFP-PhSYP22 and GFP-PhSYP51 fusions remain membrane bound and largely intact when expressed in plant cells (Supplemental Fig.1D).

The sorting of these SNAREs via the vacuolino structures is tissue and cell type specific, like that of PH5-GFP and ALEU-GFP. When expressed in epidermal leaf cells or petal mesophyll protoplasts, GFP and RFP tagged fusions of PhSYP22 and PhSYP51 appeared on the large CV within 24 hrs and vacuolino-like structures were not observed (Fig. 3).

To determine whether the vacuolinos labelled by PH5-GFP, ALEU-GFP, GFP-PhSYP22 and GFP-PhSYP51 are all the same compartments or constitute a heterogeneous population, we co-localized these fusion proteins in agroinfected intact petals, as well as in petal protoplasts. PH5-CHERRY, RFP-PhSYP22 and RFP-PhSYP51 (Fig. 4A, 4B and 4C respectively) all localize on the membrane of compartments that are marked by ALEU-GFP in their lumen after 24 hours of co-expression.
Furthermore, agroinfiltration of intact petals with RFP-PhSYP22 and GFP-PhSYP51 showed that these proteins co-localize on the membrane of the vacuolinos in epidermal petal cells (Fig. 4D).

Figure 4. Transient expression of different markers in wild type petal protoplast or wild type intact petals tissue. (A) PH5-cherry and ALEU-GFP (B) RFP-PhSYP22 and ALEU-GFP, (C) RFP-PhSYP51 and ALEU-GFP, (D) RFP-PhSYP22 and GFP-PhSYP51. The insets in panel D are enlargements of indicated vacuolinos. Anthocyanins are visible in blue, the GFP in green and the RFP in red. Scale bars: 20µm.

These results show that all proteins that reach the central vacuole, even the heterologous proteins AtSYP51 and AtKCO1 from Arabidopsis (Supplemental Fig.2), follow a sorting pathway that includes their (transitory) presence on the membrane, or in the case of ALEU-GFP the lumen, of the vacuolino. The presence of all these vacuolar markers on the vacuolino confirms the vacuolar nature of this compartment.
The formation of vacuolinos requires PH3 and PH4, but not PH5

Mutations in the regulators PH3 and PH4 block the expression of the genes that encode the two vacuolar pumps PH1 and PH5, resulting in high vacuolar pH in petals (Verweij et al., 2008b) and Chapter IV). To understand whether these mutations affect the sorting of vacuolar proteins, we transiently expressed several vacuolar markers in petals of ph3 and ph4 mutants. In wild type petals, agroinfiltration with constructs driving expression of the vacuolar markers GFP-PhSYP51, GFP-PhSYP22, PH5-GFP and ALEU-GFP labels exclusively vacuolinos after 24 hrs of transient expression (Fig. 5A). After longer expression periods (48 hours) these markers are distributed on both the main tonoplast and vacuolinos (Fig. 1C and Fig. 5B) even if, at this stage these typical structures cannot be distinguished from CV invaginations and tabulations if not characterized by anthocyanins accumulation. However, in epidermal cells from ph3 (Fig. 5C) and ph4 (Fig. 5E) mutant petals, GFP-PhSYP51 arrived at the main tonoplast already within 24 hours and vacuolinos were never visible. The same was seen when other GFP-tagged vacuolar markers, like GFP-PhSYP22 and PH5-GFP, were expressed in ph3 and ph4 petals (Supplemental Fig. 3) This implies that PH3 and PH4 are essential for the formation of vacuolinos.
To investigate whether this is an effect of the block in vacuolar acidification that occurs in *ph3* and *ph4* mutant petals or another defect, we infiltrated *ph5* mutant petals with GFP-PhSYSP51 and observed that 24 hrs after transformation the GFP signal resides in *vacuolinos* (Fig. 5G) and reaches the central vacuole only after 48 hrs, just as in wild type (Fig. 5B). Since *PH5* mutations do not affect the sorting of proteins to the *vacuolinos*, we conclude that the absence of *vacuolinos* in *ph3* and *ph4* mutants is not merely the consequence of failure in vacuolar acidification.

**Epidermal cells from wild type petals contain structures resembling *vacuolinos***

So far we showed the *vacuolinos* only in cells that were transformed with GFP marker genes. To exclude the possibility of observing artifact related to transformation or the expression of GFP fusion proteins, we examined several populations of freshly prepared petal protoplasts of the wild type line R27 by microscopy. In a large number of these cells, we observed transparent bodies that were not always easy to reveal by imaging methods, but were clearly distinct from the anthocyanin containing central vacuole (Fig. 6 A-C, arrows).

Next, we examined semi-thin sections (1-2 mm) of petals from wild type, *ph1, ph3* and *ph4* flowers. Light microscopic analysis shows the presence of sub-cellular compartments with a diameter between 1 and 10 µm which seemed squeezed against the plasma membrane by the large volume of the CV in epidermal petal cells of wild type petals. We believe that these structures correspond to the *vacuolinos*, because they are absent in *ph3* and *ph4* mutants and present in excess in *ph1* petals (Fig. 6 D-G).

*Figure 6: Vacuolinos in untransformed cells.*
(A) Light micrograph of freshly prepared protoplasts: the vacuolar volume looks divided into different sub-compartments. (B) Confocal picture: the small compartments do not contain anthocyanin fluorescence. (C) Merged of pictures in A and B: the small compartments, (indicated by arrows) are not connected to the lumen of the central vacuole. Size bar 20µm. (D-G) light microphotographs of semi-thin sections of petunia petals. *Vacuolinos* are visible in *wild type* (D) and *ph1* mutant petals (E) while they are absent in *ph3* (F) and *ph4* (G) mutants.

*Vacuolinos* are not required for vacuolar acidification.
Transcript profile analysis of ph3, ph4 and an1 mutants revealed that PH1, PH5 and at least ten other genes are strongly down-regulated in all three mutants. We have recently shown (see Chapter V) that the combined expression of PH1 and PH5 from the cauliflower mosaic virus 35S promoter of PH1 and PH5 is sufficient to restore vacuolar acidification and the wild type (reddish) petal color in ph3, and ph4 mutants (and vacuolar acidification in an1), suggesting that the other target genes have distinct functions.

To assess whether forced expression of PH1 and PH5 also restores the formation of vacuolinos, we agro-infiltrated petals from ph3 lines expressing 35S:PH1 and/or 35S:PH5 with 35S:GFP-PhSYP51 and analyzed the localization of the GFP-PhSYP51 protein after 24 hours. We found that in these cells GFP-PhSYP51 already arrived at the central vacuole within 24 hours after transformation, and vacuolinos were not seen at all in any of these lines (Fig. 7). These results imply: (i) that vacuolinos are not required for vacuolar acidification, (ii) that PH1 and PH5 expression does not restore the formation of vacuolinos, and, hence, (iii) that the formation of vacuolinos depends on target genes of PH3, PH4 and AN1 that are distinct from PH1 and PH5.

![Image](image_url)

**Figure 7: Transient expression of GFP-PhSYP51 in transgenic ph3 petals expressing PH1 and/or PH5.** Images were taken 24 hours after agroinfiltration A) ph3 35S:PH5 petal epidermis; B) ph3 35S:PH1 petal epidermis; C) ph3 35S:PH1 35S:PH5 petal epidermis. Vacuolinos were visualized via the expression of a agroinfected –PhSYP51 transgene (green staining). Anthocyanins are seen in blue. The formation of vacuolinos is not restored by the expression of the transgenes. Scale bars: 20µm

The fusion of vacuolinos to the main vacuole requires PH1.

To assess whether PH1 is required for sorting of vacuolar proteins to the vacuolinos, we followed the localization of GFP-PhSYP51 in epidermal cells from ph1 petals. We observed that 24hrs hours after agroinfiltration, GFP-PhSYP51 localized on the membrane of the vacuolinos as in wild type. However, in contrast to what we observed in wild type petals, (Fig. 8A), in ph1 cells GFP does not reach the tonoplast of the central vacuole, not even after 48 hrs. At the same time vacuolinos increase in number and volume (Fig. 8B). This observation suggests that the PH1 protein is involved in the fusion of the membrane of the vacuolinos to the tonoplast of the central vacuole. Interestingly, over expression of PH1-GFP (Chapter V) results in smaller vacuolinos without affecting the targeting to the tonoplast of the central vacuole (Fig. 8C). It is tempting to propose that high abundance of PH1 protein results in faster fusion of the vacuolinos to the CV, which is the contrary of what observed in ph1 mutants (smaller vacuolinos may indicate they have a shorter life).
Over-expression of vacuolar SNAREs affects the sorting of PH5-GFP.

To test whether over-expression of vacuolar SNAREs can influence the sorting of PH5-GFP, we over-expressed PhSYP22 or PhSYP51. We built single-plasmid constructs for the simultaneous expression of either PhSYP51 or PhSYP22 together with the PH5-GFP fusion. These multi-gene constructs were used to ensure that after agroinfection any cell expressing PH5-GFP also expresses the SNARE construct.

After transient co-transformation of petal epidermis with these multigenic constructs together with 35S:RFP-AtSYP122 as control marker, we observed that PH5-GFP was only visible on the membrane of the vacuolinos in the presence of either PhSYP51 (Fig. 9B) or PhSYP22 (Fig. 9C) 48 hours after transformation and little fluorescence reached the tonoplast of the central vacuole. Thus, over-expression of either of these two vacuolar SNAREs phenocopies the effect of a ph1 loss of function mutation and impairs, or at least strongly delays, the fusion of the vacuolinos to the CV. The over-expression of either of the two SNAREs did not affect the sorting of AtSYP122 to the plasma membrane, indicating that the delay in sorting is specific for the pathway involving vacuolinos.
Knock down of \textit{PhSYP51} expression via RNAi results in persistence of \textit{vacuolinos} and anthocyanin accumulation in their lumen.

As changes in the expression of vacuolar SNAREs affect the fate of \textit{vacuolinos}, we have generated transgenic petunia plants in which the steady state transcript level of \textit{PhSYP51} was knocked down by RNA interference (RNAi) to about 20\% of the level in the untransformed controls (Fig. 10A).
Light microscopy of petals of the untransformed control (wild type hybrid M1xV30) showed that the epidermal petal cells contain a single large central vacuole colored by accumulation of anthocyanins, as in all wild type lines (Fig. 10B, D). The petals of the transgenic plants with severe silencing of $\text{PhSYP51}$ expression ($\text{PhSYP51}^{\text{RNAi-25}}$ and $\text{PhSYP51}^{\text{RNAi-26}}$) contained patches of cells in which we could identify a multitude of smaller vacuoles containing anthocyanins besides the central vacuole, (Fig. 10C, E). It is known that silencing of gene expression via RNAi results in a mosaic of regions where the target gene is completely silenced and areas gene expression is not affected (Stam et al., 1997), which can explain why we observed this vacuole phenotype only in certain patches of the flowers.

The phenotype of these transgenic lines supports the idea that SYP51 (and related proteins) have a role in the fusion of vacuolinos with the CV, except that the small vacuolar compartments observed in the $\text{SYP51}^{\text{RNAi}}$ flowers are colored by anthocyanins, whereas the vacuolinos observed in transient expression assays do not (see Discussion).

**PH1 and PH5 can interact with the SNAREs PhSYP22 and PhSYP51.**

The observation that both PH1 and the SNARES PhSYP22 and PhSYP51 are required for the fusion of the vacuolinos to the central vacuole, prompted us to test whether PhSYP22 and PhSYP51 interact with the PH1 protein to mediate the docking or the fusion of the membranes. Therefore we have assayed the
physical interaction of the SNAREs and PH1 by in vivo bimolecular fluorescence complementation (BiFC) (Kerppola, 2009).

For this purpose, PH1 and the SNAREs were expressed as fusions to the complementary N- and C-terminal parts of YFP and combinations of these constructs were transformed in petunia petal protoplasts to assess whether YFP fluorescence was restored. We fused the N-or C-terminal part of split YFP to the N-termini of the SNAREs, because cytosolic exposition of the fluorophore is required for fluorescence. Similarly, for PH1 we only produced the N-terminal fusion as we had previously observed that the C-terminal fusion to GFP does not result in fluorescence (Chapter V). The empty vectors expressing the two halves of the split-YFP were used as a negative control.

When YFP$^N$-PH1 was expressed alone or in combination with the empty vectors expressing YFP$^C$, no fluorescence was detected. Also no fluorescence was seen when both halves of YFP were co-expressed from the empty vectors (not shown). After co-transformation of YFP$^N$-PH1 and either YFP$^C$-PhSYP51 or YFP$^C$-PhSYP22 fluorescence was detected in protoplasts from the petal epidermis, which can be recognized by the anthocyanin presence in the central vacuole (Fig. 11A-C)), while no fluorescence was ever detected in white cell, which originate from the mesophyll. This finding indicates that PH1 can physically interact with PhSYP51 and PhSYP22.

Figure 11. Interaction of PH1 and vacuolar SNAREs.
Bimolecular fluorescence complementation in wild type petal protoplasts. 

(A-C) Co-expression of YFP\textsuperscript{N}-PhSYP22 and YFP\textsuperscript{C}-PhSY51. In both cases the expression of the two constructs results in a fluorescent signal indicating interaction. 

The same results were obtained when the YFP\textsuperscript{N}-PhSY51 or YFP\textsuperscript{C}-PhSYP22 were co-expressed with YFP\textsuperscript{N}-PH5. (data not shown) indicating that the two SNAREs can also interact with PH5.

To confirm these results by an independent method, we used a yeast 2 hybrid system based on the restoration of “split ubiquitin” which is designed to assess interaction between membrane proteins (Snider et al., 2010). Therefore we expressed fusions of PH1, PH5, PhSYP51 and PhSYP22 to the N-terminal part of ubiquitin (Nub) or the C-terminal part (Cub) and assayed the activity of the HIS3 and LacZ reporter genes. In preliminary experiments we could detect interactions in cells co-expressing PH1-Cub and either Nub-PhSYP22 or Nub-PhSYP51 (Fig. 1D-E). Even stronger interaction was detected in yeast cells expressing PH5-Cub and either Nub-PhSYP22 or Nub-PhSYP51 (data not shown). Yeast cells containing Cub or Nub alone, or one of our fusions in combination with an empty Nub or Cub vector did not activate the HIS3 or LacZ reporters, or to a very low extent only. Hence these results indicate that PH1 and PH5 can also interact with PhSYP22 and PhSYP51 in yeast cells, which lends further support that these proteins interact in vivo, and may act in the docking or fusion of vacuolinos with the central vacuole.

**DISCUSSION**

The plant vacuole fulfills a multitude of functions that are essential for viability. Consequently, mutations in genes affecting vacuole biogenesis, like *vacuoleless* in Arabidopsis (Rojo et al., 2001), result in severe developmental defects or lethality which underlines the importance of these organelles, but also complicates the genetic analysis of the biogenesis and physiology of vacuoles. Although most plant cells contain one single vacuole, some cells have multiple types of specialized vacuoles with different functions, such as lytic and protein storage vacuoles (Vitale and Raikhel, 1999). Here we describe the identification of a novel pathway by which proteins traffic to the vacuole and demonstrate that plants possess multiple pathways, that are controlled by distinct genes, by which membrane vesicles and proteins traffic to the vacuole.

Our data show that in epidermal cells of petunia petals vacuolar proteins traffic to the central vacuole via a pathway that involves their (temporary) localization in vacuolinos, a novel endomembrane compartment that is specific for epidermal petal cells and coexists with the central vacuole that contains the anthocyanins (Faraco et al., 2011). Vacuolinos have a diameter ranging from 1 to 10 micrometers and are therefore much larger than pre-vacuolar compartments, which are 100 to 250 nm in size (Paris et al., 1997; Marty, 1999). Vacuolinos resemble true vacuoles for their protein content, except that they are several times smaller than the central vacuole, which occupies most of cell volume in epidermal petal cells.
Figure 12. Model of the two coexisting pathways of vacuolar protein sorting in petal epidermal cells. The ‘vacuolinos pathway’ is controlled by the AN1, PH3 and PH4 petal specific regulators, while the ‘canonical pathway’ is independent from them and active in all cell types.

Figure 12 shows a model of the sorting pathways by which proteins reach the vacuole central vacuole. We propose that two distinct pathways exist that are not necessarily active in the same cells. One is the “canonical” pathway by which vacuolar proteins move relatively quickly (within 24 hrs) to the central vacuole, via the Golgi and small (100-250 nm) prevacuolar compartments (PVC) that are thought to fuse with the large vacuole (Hanton et al., 2007). This pathway is active in many cell-types and has been observed in many studies. In contrast, the “vacuolino pathway” that we describe here, is cell-specific, because it operates in petunia only in epidermal petal cells, and is relatively slow, as it takes 48 hrs for a transiently expressed marker to become fully localized on the tonoplast of the central vacuole. The distinguishing features of this pathway are the involvement of a vacuolino as an intermediate compartment and the genetic control of the distinct steps. The data show that vacuolar proteins first appear on vacuolinos, which might originate from the Golgi or directly from the endoplasmic reticulum (ER) and indicate that the vacuolinos subsequently fuse with the central vacuole to deliver their membrane and proteins therein.

Interestingly, the vacuolino pathway is activated by transcription regulators encoded by PH3, PH4 and AN1, presumably in conjunction with AN11 and AN2, that are important for the pigmentation of the flower. As the expression of PH4 and PH3 is essentially limited to the petal limb epidermis, this explains why the vacuolino pathway is cell-type specific and does not operate in petal mesophyll or leaf cells, where these regulators are not expressed (Quattrocchio et al., 2006). In epidermal cells of ph3, ph4 and an1 petals the vacuolinos pathway is inactive, but vacuolar proteins still reach the vacuole via a distinct route that resembles the canonical pathway seen in other cells. However, in epidermal cells of wild type petals, the bulk of the vacuolar proteins moves via the vacuolino route instead of the canonical pathway, as 24 hrs after transformation little or no protein has reached the vacuole. That might be because the vacuolino route is more efficient, at least at the key-step where the two pathways diverge, or because AN1, PH3 and PH4 repress the expression of genes that operate the canonical pathway. The transcription regulators PH3 and PH4 are required in conjunction with AN1, AN11 and
AN2 to activate the transcription of >10 distinct target genes, including *PH1* and *PH5*. *PH1* and *PH5* are both necessary and sufficient to acidify the central vacuole and to confer a reddish flower color (Chapter V), but seem not involved in the formation of *vacuolinos*, as the *vacuolinos* do form in *ph5* mutants and because the formation of *vacuolinos* is not restored in *ph3* mutants that constitutively express *PH1* and *PH5*. Thus, the formation of *vacuolinos* apparently relies on distinct target genes of *PH3* and *PH4*.

We favor a model in which the *vacuolinos* are an intermediate compartment that delivers proteins to the central vacuole through fusion, rather than a final destination. Such a fusion model is conceptually similar to that of the canonical pathway where PVCs fuse to the vacuole and is consistent with the finding that alterations in the expression of *PH1* or the SNARES *PhSYP22* and *PhSYP51* result in over-accumulation of *vacuolinos* and a failure of proteins to reach the central vacuole. It appears that fusion depends not only on the presence or absence of these proteins, but also on their proportional abundance, because over expression of *PhSYP51* or *PhSYP22* causes essentially the same defect as elimination of *PH1*. That is in both situations vacuolar proteins remain “stuck” in *vacuolinos* and do not, or with a large delay, reach the central vacuole, and *vacuolinos* over accumulate (see Fig. 8B). It is noteworthy that over-expression of the Arabidopsis homolog of *PhSYP22* (*AtSYP21*) in tobacco protoplasts blocks the targeting of vacuolar proteins via the canonical pathway in a similar way, as they remained trapped in PVCs, apparently because the fusion of PVCs to vacuole was impaired (Foresti et al., 2006).

As membrane fusion is thought to be initiated by interactions between proteins in the membrane, which aids in recognition and selection of the target membrane, excess of one of the components of such a complex may titrate away other partners and result in non-fusogenic protein complexes (Varlamov et al., 2004). *PH1* could act, for example, as a tether for the approaching membranes of the *vacuolino* and the central vacuole to facilitate the formation of a SNARE complex that leads to fusion. Tethering factors have been described as “molecular bridges” that determine the specificity of the fusion and are able to interact with SNAREs (Sztul and Lupashin, 2006). Our data suggest that *PH1* has all these characteristics, as it essential for fusion of *vacuolinos* and the vacuole and because it can interact directly with the *PHSYP22* and *PHSYP51*. Interestingly, the defect caused by over expression of *PH1* results in a defects that seems the opposite of the defect caused by its elimination, as it decreases the size of the *vacuolinos*, which can be explained by precocious fusion to the vacuole resulting in a shorter lifetime during which the *vacuolinos* can “grow”.

The finding that *PhSYP22* and *PhSYP51* can also interact with *PH5*, while *ph5* mutants do not have a clear defect in the fusion of vacuole and *vacuolinos*, may be because this interaction is not involved for fusion but some other process (vacuolar acidification?), or may simply reflect that the role of *PH5* in the fusion process is redundant. Because also *PH1* and *PH5* are capable of forming complexes, it is possible that the complex involved in fusion is large and involves multiple SNARE molecules that interact with a *PH1-PH5* complex. Although interactions between SNARE proteins and membrane pumps have been observed before (Honsbein et al., 2009), to our knowledge no evidence was obtained for a role of such complexes in membrane fusion.
We also considered whether alternative models, in which vacuolinos are not an intermediate compartments for vacuolar proteins but an alternative final destination. One difficulty with such models is that it needs to assume that PH3 and PH4 activate not one, but two novel pathways that are both distinct from the canonical pathway and deliver proteins to either the vacuolinos or to the vacuole. In such a model the PH3/PH4-dependent route that goes directly to the vacuole, without passing through vacuolinos, must be rather slow as it takes it takes 48 hrs after the introduction of a gene construct for the encodes protein to reach the vacuole, whereas the canonical pathway delivers proteins to the vacuole within 24 hrs. This time difference is even more dramatic if one considers that a major part of the first 24 hrs after transformation is required to first synthesize the protein via transcription and translation. In this model a second PH3/PH4 dependent pathway delivers proteins within 24 hrs to the vacuolinos where they remain and do not move on to the vacuole. The major problem of such a model is that it cannot easily explain the phenotypes cause by mutation and/or over expression of PH1 or the SNARES. For example, to explain why in ph1 mutants proteins end up exclusively in vacuolinos, one has to assume that PH1 specifically represses the pathway to the vacuolino, which is difficult to fit role of PH3 and PH4 as activators of this route as well as the expression of its repressor PH1. Furthermore, it is in this way also difficult to explain why vacuolinos increase in size and in number in ph1 mutants, or why the up- or down-regulation of SYP22 or SYP51, which interact with PH1, would eliminate both PH3/PH4-dependent sorting pathways and to make the proteins move to the vacuole within 24 hrs by the canonical pathway.

Currently the biological function of the vacuolino and the vacuolino pathway is unknown. Although our data suggest a role for vacuolinos in the sorting of vacuolar proteins, and via their fusion to the vacuole in the formation of this organelle, these functions are apparently redundant with those of the canonical pathway. That is, proteins can still reach the vacuole in ph3 and ph4 petal cells by a pathway that resembles the canonical pathway in that it delivers proteins to the vacuole within 24 hrs after transformation, does not involve vacuolinos and is PH3/PH4-independent. This can explain why epidermal cells of ph1, ph3, ph4 and an1 petals contain a normal looking large vacuole that accumulates anthocyanins, despite the defects in the formation of vacuolinos or their fusion to the vacuole. Because AN1, PH3 and PH4 are expressed in relatively late stages of flower development, when the bud expands and opens, it is likely that during flower development petal cells rely during early stages primarily on the canonical pathway for the biogenesis of the vacuole and the trafficking of vacuolar proteins, and switch to the vacuolino pathway during later stages. Hence, vacuolinos may have a specific role during the last phase of petal maturation, when cell expansion drives the unfolding of the petals, acidification of the vacuolar lumen takes place (Quattrocchio et al., 2006) and the display of color and/or the emission of volatiles can start to attract pollinators. Studies in entirely different systems, previously suggested that the expansion of some cells may be driven by the expansion of by specific a subtype of vacuoles (Fleurat-Lessard et al., 1997; Fluckiger et al., 2003). However, flowers of ph3 and ph4 mutants, which lack the vacuolino pathway, contain normal amounts of pigments and do not show obvious defects in bud opening, indicating that vacuolinos are not essential for these processes. Furthermore, ph3 35S:PH1 35S:PH5 epidermal petals cells lack vacuolinos (Fig. 7), but
nevertheless have a normal wild type flower color and vacuolar pH, implying that vacuolinos are not essential for vacuolar acidification either.

It is likely that vacuolar proteins, such as membrane transporters, are already active in vacuolinos before they reach the central vacuole, however, as the life-time of vacuolinos is probably short, when compared to that of the large central vacuole, the activity of proteins while they are in vacuolinos may have little impact on the large vacuole where they are active over a much longer period. That explains, for example, why the vacuolinos that we observed in transient assays are not colored, as they had insufficient time to accumulate substantial amounts of anthocyanin, whereas the vacuolino-like structures that we observed in the petals of transgenic plants with reduced PhSYP51 expression have accumulated over a longer period and, hence, have sufficient time to sequester substantial amounts of anthocyanin.

The finding that the biogenesis of these small vacuoles is under the control of well-known regulatory genes, such as AN1, PH3 and PH4, opens the way to identify the target genes required for the formation of such vacuoles by straightforward reverse-genetic methods. The analysis of these genes by knock out mutations as well as reconstruction experiments in ph3, ph4 and an1 are likely to uncover novel aspects of the mechanisms by which vacuolinos are formed and fuse with the vacuole.
EXPERIMENTAL PROCEDURES.

Plant material, growth conditions and agroinfiltration procedures

All *Petunia hybrida* lines used in this work derive from the Amsterdam petunia collection and were grown under normal greenhouse conditions: temperature between 19°C and 30°C min/max), cycle of a minimum of 16 hours of light in all seasons.

For transient expression assays via agroinfiltration, isolation of protoplasts and generation of transgenic plants we used petals and leaves from different petunia lines and hybrids of the following lines: R27 (WT), M1xV30 (hybrid between two wild type lines), R169 (*ph*3 mutant), V74 (*ph*4 mutant), V30xV23 (hybrid of a wild type and a *ph*1 line, in the F2 it is possible to select for isogenic PH1 and *ph*1 plants), V69xR159 (hybrid between two *ph*5 lines).

Transient expression expression assays

Agroinfiltration of intact petals and leaves was performed as previously described in (Verweij et al., 2008a). Preparation of petunia leaf and petal protoplasts as well as transformation, were done as described in Chapter IV.

Isolation of petunia vacuolar SNAREs and preparation of expression constructs

The petunia PhSYP51 sequence was identified by searching the Non-Redundant protein database and the EST (tBLASTn algorithm) databases of GenBank with the Arabidopsis AtSYP51 protein sequence as a query. A search identified one EST from *Petunia hybrida* (EST886476). Based on this sequence we designed several primers (Table 2) to amplify the full length sequence from petal cDNA of the petunia wild type line R27.

The full size PhSYP51 cDNA sequence, amplified with primers #4655 (5'\-CACCATGGCTTCTTCTTCTGGA-3', containing CACC TOPO binding site) and #4675 (5'\-CACCATGGCTTCTTCTTCTGGA-3') was recombined in pENTR/D-TOPO® (Invitrogen) to yield a pENTRY clone. This plasmid was recombined by a LR Clonase (Invitrogen) with several destination vectors (Table 1, Plant System Biology, Gent University, Belgium) to give all the expression clones used in further experiments.

A BLAST search with the amino acid sequence of AtSYP22 did not yield any petunia EST, but instead identified an EST from *Nicotiana tabacum* (AGN_RNC129xb11f1.ab1). Based on this tobacco sequence, we designed primers (Table 3) to isolate amplify the petunia homolog PhSYP22 sequence. This was cloned in a pENTRY vector that was used to produce a series of expression constructs as described above for PhSYP51.

<table>
<thead>
<tr>
<th>Table 1 Destination vector used in LR recombination.</th>
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<tbody>
<tr>
<td>vector</td>
</tr>
<tr>
<td>pK7WGF2</td>
</tr>
<tr>
<td>pH7WGR2</td>
</tr>
<tr>
<td>pE-SPYCE</td>
</tr>
<tr>
<td>pE-SPYNE</td>
</tr>
<tr>
<td>pK7GW1WG2</td>
</tr>
</tbody>
</table>
**Table 2:** Primer sused to amplify and to recombine the full length *PhSYP51* or *PhSYP22* cDNA in Gateway vectors.

<table>
<thead>
<tr>
<th>name</th>
<th>gene</th>
<th>sequence 5'-3'</th>
<th>ori</th>
</tr>
</thead>
<tbody>
<tr>
<td>4520</td>
<td><em>PhSYP51</em></td>
<td>CATCGCCGATCAA AAAAGAGGT</td>
<td>F</td>
</tr>
<tr>
<td>4521</td>
<td><em>PhSYP51</em></td>
<td>ATGGCTTCTTCTTGGAGACTCATGGGATCCAGG</td>
<td>F</td>
</tr>
<tr>
<td>4522</td>
<td><em>PhSYP51</em></td>
<td>AGCTTCCGAAAGAAGCCT</td>
<td>R</td>
</tr>
<tr>
<td>4523</td>
<td><em>PhSYP51</em></td>
<td>TGGCTTGCAAAAGCAGACTCAT</td>
<td>R</td>
</tr>
<tr>
<td>4533</td>
<td><em>PhSYP51</em></td>
<td>TTCTGCGGTTGCTGGTCTGCA</td>
<td>F</td>
</tr>
<tr>
<td>4555</td>
<td><em>PhSYP51</em></td>
<td>CACCATGCTTCTTCTGGA</td>
<td>F</td>
</tr>
<tr>
<td>4566</td>
<td><em>PhSYP51</em></td>
<td>CACCGGTCTTGACAATTCTGG</td>
<td>F</td>
</tr>
<tr>
<td>4557</td>
<td><em>PhSYP51</em></td>
<td>TTATCCACCCCTTGGTGTC</td>
<td>F</td>
</tr>
<tr>
<td>4658</td>
<td><em>PhSYP51</em></td>
<td>TTACATATACTTAAACCAACATCCA</td>
<td>R</td>
</tr>
<tr>
<td>4675</td>
<td><em>PhSYP51</em></td>
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<td>R</td>
</tr>
<tr>
<td>4670</td>
<td><em>PhSYP22</em></td>
<td>ATGAGCTTTCAAGATCTAGATACGTG</td>
<td>F</td>
</tr>
<tr>
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<td><em>PhSYP22</em></td>
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<td>F</td>
</tr>
<tr>
<td>4672</td>
<td><em>PhSYP22</em></td>
<td>CGTTCCTCTATAATAGCTTCAATTAAAG</td>
<td>R</td>
</tr>
<tr>
<td>4673</td>
<td><em>PhSYP22</em></td>
<td>AGCCATGATCGATATATGGTTTC</td>
<td>F</td>
</tr>
<tr>
<td>4674</td>
<td><em>PhSYP22</em></td>
<td>AGTTGAGCATGAGAATTCCTCA</td>
<td>R</td>
</tr>
<tr>
<td>4794</td>
<td><em>PhSYP22</em></td>
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<td>F</td>
</tr>
<tr>
<td>4798</td>
<td><em>PhSYP22</em></td>
<td>GGGGACAAGTTTTAGTACAAAAAGCAGGCTTTATG</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGCTTCAAGATCTA</td>
<td>F</td>
</tr>
<tr>
<td>4799</td>
<td><em>PhSYP22</em></td>
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<tr>
<td></td>
<td></td>
<td>GATAACAGAAGGAG</td>
<td>F</td>
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<tr>
<td>4800</td>
<td><em>PhSYP22</em></td>
<td>GGGGACAACCTTTGTAAAGAAAGCTGGTGAAGAT</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAATTCGATCTTTGG</td>
<td>F</td>
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<tr>
<td>4863</td>
<td><em>PhSYP22</em></td>
<td>GGGGACAACCTTTGTAAAGAAAGCTGGTGAAGAT</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGAATTCGATCTTTGG</td>
<td>F</td>
</tr>
</tbody>
</table>

F indicates a “forward” orientation of the primer, relative to the orientation of the gene, and R, reverse orientation.
**Split-Ubiquitin assay.**

Split-Ubiquitin assay was performed as described in (Grefen et al., 2009) and in Chapter V. Flanking sequences B1 and B2 were added to SNAREs by PCR using the following primers: 

- **#5750**
  
  \[(ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGAGCTTTCCTCGAGATCTAATGAGC)\]

- **#5751**
  
  \[(TCCGCCACCACCAACCACCATTTTGTACAAAAGCTGGGTATGATAACAATAAGGGAGACCGAT)\]

- **#5752**
  
  \[(ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGAGCTTTCCTCGAGATCTAATGAGC)\]

- **#5753**
  
  \[(TCCGCCACCACCAACCACCATTTTGTACAAAAGCTGGGTATGATAACAATAAGGGAGACCGAT)\]

Confocal microscopy

Protoplasts and intact tissue were observed by fluorescence microscopy in their culture medium at different times after transformation. They were examined with a confocal laser-microscope (LSM Pascal Zeiss). The excitation wavelength was 488nm, GFP was detected with the filter set for FITC (505-530 nm), RFP with a 560-615 nm filter set, while chlorophyll epifluorescence was detected with the filter set for TRITC (>650nm) and eliminated.

**RNA interference.**

The full length *PhSYP51* cDNA was recombined by Gateway LR recombination, (Invitrogen) in pK7GW1WG2(I) to yield an inverted repeat construct for expression from the 35S promoter (35S:SYP51RNAi), which was transformed by Agrobacterium-mediated leaf disk transformation (Quattrocchio et al., 2006) in the wild type petunia hybrid M1xV30, to generate stable transformants.

**Light microscopy of petal semi-thin sections**

To obtain the semi-thin sections, small pieces of the petal limb were fixed in 3% (w/v) glutaraldehyde in 0.075 M phosphate buffer, pH 7.2, for 24h. Then samples were washed four times for 15 min each in 0.075 M phosphate buffer, pH 7.2 and post-fixed in 1% (w/v) OsO4 for 90 min. At this stage, samples were dehydrated in increasing concentrations of ethanol and then included in resin (Epon, 2-dodecenylsuccinic anhydride, and methylnadic anhydride mixture). Semi-thin sections (1–2 mm) were cut with an ultramicrotome (OmU2, Reichert, Heidelberg) equipped with a glass blade, stained with toluidine blue and observed under a light microscope (DMLB, Leica, Wetzlar, Germany).
Figure S1: Characterization of PhSYP22 and PhSYP51.

(A) Phylogenetic tree of different SNARE proteins. Ph, Petunia hybrida (PhSYP51, PhSYP22); At, Arabidopsis thaliana (SYP51:Q9SA23.1, SYP52: Q94KK7.1, SYP21: Q39233.1, SYP22: P93654.1, SYP61: AEE30983.1, SYP121: Q9ZSD4.1, SYP122: Q9SVC2.1, SYP123: Q9ZQZ8.1, SYP71: AEE74807.1, SYP72: AEE78017.1, SYP73:AEE80207.1); Sc, Saccharomyces cerevisiae (VAM7:CAA96928.1, TLG1: Q03322.1, Syn8p: NP_009388.2, Vam3p: NP_014749.1, Pep12p: AAB38370.1; St, Solanum tuberosum (PEN1:AAU04512.1); Os, Oryza sativa (Os06g0590500: NP_001057967.1, Os08g0277900: NP_001061436.1, hypothetical protein OsJ_26713: EEE68383.1, Os02g0119400: NP_001045701.1, AC105363_8 Hypothetical protein: AAM52319.1.

(B) RT–PCR analysis of PhSYP22 and PhSYP51 mRNA in different tissues in increasing stages of development (indicated by triangles) of wild type plants.

(C) RT–PCR analysis of PhSYP22 and PhSYP51 in petals of opening flower buds (stage5) from different genotypes. GAPDH (GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE) was used as a constitutively expressed control.

(D) Western blot analysis of protein fractions (M; membrane fraction, S; soluble fraction,) obtained from GFP-PhSYP22 and GFP-expressing protoplasts of wild type (wt). Proteins were detected using an anti-GFP serum; Arrows indicate free GFP just visible in the soluble fraction.
Figure S2. Localization of GFP-AtSYP52 and AtKCO-GFP in epidermal cells of petunia leaves or petals
GFP-AtSYP52 signal is visible in green and anthocyanins in blue. (A) GFP-AtSYP52 in epidermal petal cells, 24 hours after agroinfiltration. Fluorescence is seen on the vacuolino. (B) GFP-AtSYP52 in wild type epidermal petal cells 48 hours after agroinfiltration: fluorescence is visible on the tonoplast of the central vacuole.
(C) AtKCO-GFP in epidermal leaf cell, 24 hours after agroinfiltration. Fluorescence is now detectable on the tonoplast of the central vacuole. (D) AtKCO-GFP in wild-type petals 24 hours after agroinfiltration. Fluorescence is on the vacuolino. Size bars 20µm.

Figure S3. Localization of PH5_GFP and GFP-PhSYPP 22 in different cell types an genotypes
(A-D) PH5-GFP localization in epidermal petals cells of agroinfected flowers from an1 (A), ph3 (B), ph4 (C) and ph1 (D) mutants. 

(D-E). Localization of GFP-PhSYP22 petunia petal protoplasts. (E) GFP-PhSYP22 in wild type petal protoplast 24 hours after transfection. Fluorescence is detectable on the vacuolinos. (D) GFP-PhSYP22 in ph1 petals 48 hours after transformation. Fluorescence is still localized on the vacuolino in colored protoplast originating from the epidermis (top), while in the uncolred protoplast from mesophyll cells protoplast GFP-PhSYP22 reside in the tonoplast of the central vacuole. Size bars 20µm.
REFERENCES


