Chapter 5

The flower color gene *PH7* encodes a monosaccharide sugar transporter that mediates vacuolino formation and subsequent vacuolino-to-vacuole transport via distinct protein domains

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Abstract

Six out of seven PH loci mediating petunia flower color were successfully identified and characterized, but till short, PH7, the last PH locus, remained mysterious. Flowers of ph7 mutants look like wild type flowers when just open, but later assume a bluish color, similar to that of other ph mutants. By combining transposon tagging and RNAseq, we successfully identified the PH7 gene, encoding a monosaccharide sugar transporter closely related to EARLY RESPONSIVE TO DEHYDRATION-LIKE 6 (ERDL6), an Arabidopsis glucose/H+ symporter. Impairments of PH7 function by independent mutations disrupts the formation or fusion of vacuolinos (additional vacuole) with the central vacuole, suggesting a dual role of PH7 in the vacuolino pathway. The identification of PH7 opens the way to unravel how sugar transporters contribute to endomembrane compartment biogenesis and definition of flower color.
Introduction

Pigmentation has been used for more than a century as a visible marker to study a multitude of processes. Gregor Mendel and Thomas Hunt Morgan used amongst other pigmentation mutants in respectively pea and fruit flies to uncover the basic principle underlying the inheritance of traits. Starting in the 1940s with the seminal work of Barbara McClintock, unstable pigmentation mutants in maize were crucial to discover and understand transposition. In the late 1980s transgenic experiments with flower color genes in petunia provided the first indications for a novel mechanism regulating gene expression, which was at the time called “co-suppression” and now known as RNA interference\(^1\)\(^-\)\(^3\).

In the last decade we have used flower pigmentation mutants to gain insight in fundamental cell biological processes, in particular vacuole biology. Genetic analyses revealed that the strong blue petal color of some petunia varieties is due to mutations in one of six loci\(^4\),\(^5\). As those mutations increased the pH of petal extracts, most likely by driving acidification of the vacuoles, the corresponding loci were dubbed *PH1* to *PH6* (ref\(^4\),\(^5\)). Molecular analyses revealed that PH3 and PH4 are transcription factors of the WRKY and MYB family respectively\(^6\),\(^7\), that interact with the BHLH protein ANTHOCYANIN1 (AN1) and the WD40 protein AN11 to form a complex (dubbed MBWW)\(^8\). This complex is very similar to the one driving anthocyanin synthesis from which it differs only for the MYB component (PH4 instead of AN2). Analysis of *ph6* mutants revealed that they do not define a new locus (*PH6*), but are specific alleles of *AN1* that lost the capacity to drive vacuolar acidification, but retained the capacity to drive synthesis of anthocyanin pigments\(^5\),\(^9\). *PH1* and *PH5* are downstream genes that are activated by MBWW and encode two distinct P-ATPases that together form a powerful hetero-dimeric proton pump, which resides in the vacuolar membrane (tonoplast) and hyperacidify the vacuolar lumen\(^10\),\(^11\). In petunia this pathway is primarily active in colored cells in the
epidermis of petals and seeds, while in Citrus species it is (also) active in fruits where it drives the extreme acidification (down to pH=2) of vacuoles in juice sacs (Chapter 2 in this thesis, ref\(^4\)).

Vacuoles/lysosomes were initially considered as a storage organelle and endpoint of the endocytic pathway, but are nowadays seen as regulatory hub for many cellular activities\(^{13-15}\). Some cell types, contain multiple vacuoles which may have distinct functions to help plant adapting to various environmental stimuli\(^{16-22}\). Epidermal cells in petunia and rose petals possess besides the large central vacuole (CV) where anthocyanin pigments are stored, numerous smaller vacuolar compartments called vacuolinos\(^22\). These vacuolinos are intermediate stations for proteins trafficking to the CV\(^22\). In transient expression assays, GFP-fusions of vacuolar proteins, such as PH1 and PH5, appear in epidermal petals cells first on vacuolinos (20-24 hours after transformation) before they reach the central vacuole (after some 40 hours). Mutations such as an1, ph3 and ph4 cripple the MBWW transcription factor complex and abolish the formation of vacuolinos\(^22\). In the petal epidermis of these mutants, vacuolar proteins can still reach CV, now travelling along a faster and more direct pathway, by which they reach the CV within 24 hours, without passing through vacuolinos. The latter pathway is highly similar to vacuolar trafficking pathway(s) that operate in other cell types lacking vacuolinos, such as leaf and petal mesophyll cells. This finding suggests the vacuolino pathway provides a rare opportunity to deploy genetic approaches for the study of intercellular membrane and protein trafficking. Analysis of several MBWW target genes, indeed uncovered new factors that are involved in either the formation of vacuolinos, such as the small GTPase RAB5a (Chapter 4 in this thesis), or in the trafficking from vacuolinos to the CV, like the P\(_{3B}\)-ATPase PH1 (ref\(^{22}\)).

In this paper we analyzed ph7 mutants and identified the PH7 gene. The first ph7 allele was identified more than 20 years ago as a flower color mutant which petals
acquire at late developmental stages the bluish color typical for \textit{ph} mutants\textsuperscript{23}. As this mutant complemented in test crosses mutations in all previously known \textit{ph} genes (\textit{ph1} to \textit{ph6/an1}), it was assumed to define a new locus (\textit{PH7}). However, thus far no attempts were made to identify the \textit{PH7} locus and its gene product. Here we show that \textit{PH7} is required during (\textit{i}) the formation of vacuolinos and (\textit{ii}) the subsequent trafficking of proteins from vacuolinos to the CV and present evidence that the contribution of \textit{PH7} to both processes can be separated genetically. We identified the \textit{PH7} gene by RNA-seq analysis of petals from an unstable transposon-tagged allele and stable \textit{ph7} allele and found that it encodes monosaccharide transporter homologous to ERD6L from Arabidopsis. Although the mechanism by which \textit{PH7} facilitates membrane trafficking remains to be solved, these findings underline the potential power of the vacuolino system to uncover essential components of membrane trafficking pathways that were not identified by other, more conventional, strategies.

\textbf{Results}

\textbf{Characterization of the \textit{ph7} phenotype}

The mutations \textit{ph1} to \textit{ph6} reduce the acidification of vacuoles in petal cells. In lines such as R27 and R176, which accumulate cyanidin 3-glycosides, the mutations \textit{ph1} to \textit{ph6} cause a change of the color of petals from red to a greyish/bluish color, which is immediately evident after bud opening. Two mutations in \textit{PH7} (\textit{ph7}\textsuperscript{R146} and \textit{ph7}\textsuperscript{R173}) arose in the line W138, which is fully isogenic to R176 and contains a large set of extremely active transposon elements. Petals of \textit{ph7}\textsuperscript{R146} and \textit{ph7}\textsuperscript{R173} mutants by contrast, have a normal red color that is indistinguishable from wild type (\textit{PH7}) up to two days after opening (DAO) of the bud (Fig. 1a), when they gradually acquire the greyish color typical for \textit{ph} mutants over the next 4/5 days. Five days after opening (flower stage 8), \textit{ph7}\textsuperscript{R146} and \textit{ph7}\textsuperscript{R173} petals develop blue/grey patches, and subsequently turn almost entire grey/blue at 7 days after opening (stage 9), while \textit{PH7} petals remain red (Fig. 1a). Both \textit{ph7} alleles arose spontaneously in progeny of line W138 (ref\textsuperscript{23} and unpublished data), which derives from the red
(cyanidin) line R27 after the insertion of a copy of the transposon \( dTPH1 \) (defective Transposon Petunia hybrida 1) in the \( AN1 \) locus. W138 contains an high number of copies of \( dTPH1 \) and presents high transposition activity\(^\text{23} \). Petals of \( ph7^{R146} \) contain frequently small spots and large sectors that do not turn grey/blue as the flower ages but keep their normal red color, suggesting that \( ph7^{R146} \) is an unstable mutant caused by a transposon insertion. However, in \( ph7^{R173} \) petals we never observed such somatic reversions.

Figure 1. Flower color phenotype of \( ph7 \) mutants and a related \( PH7 \) line (R176). (a) Flower phenotype of the \( PH7^{R176} \) (line R176), and \( ph7^{R173} \) (line R173) and \( ph7^{R146} \) (line R146) mutants. (b) Genetic instability of \( ph7^{R146} \). Note that most of the petal tissue in this open flower started bluing, while some sectors (marked with white arrows) retained their normal red color, due to somatic reversions of the mutations. DAO= days after opening.

\( ph7^{R146} \) and \( ph7^{R173} \) impair the vacuolino pathway at different points

The phenotype of \( ph7 \) mutant flowers suggests a role for \( PH7 \) in maintaining vacuolar pH in petals of open flowers. To understand the mechanism by which petal color is prevented from bluing, we investigated the effects of different \( ph7 \) alleles on the trafficking of PH5-GFP to vacuolinos and, subsequently the CV, and the trafficking of the plasma membrane marker RFP-SYP122b (ref\(^\text{22} \)) in protoplasts isolated from \( PH7^{R176} \), \( ph7^{R146} \) and \( ph7^{R173} \) petals (Fig. 2a-c). In \( PH7^{R176} \) protoplasts originating from the petal epidermis (which can be recognized from the presence of anthocyanins in the vacuolar lumen), PH5-GFP resided on the membrane of
vacuolinos 24 hours after transfection, and reached the tonoplast of the CV after 48 hours (Fig. 2a), consistent with our previous findings. In the epidermal cells of ph7R146 petals, PH5-GFP resided 24 hours after transformation, on small punctate compartments, instead of vacuolinos, where it remained stuck for the next 24 hours while little or no PH5-GFP was delivered to CV after 48 hours (Fig. 2b). This defect in PH5-GFP trafficking is very similar to that observed in rab5a mutants (Chapter 4 in this thesis), and indicates that PH7 is required for the formation of vacuolinos.

By contrast, in epidermal cells from ph7R173 petals, PH5-GFP localized after 24 hours on vacuolinos, which were often enlarged. However, PH5-GFP did not move on to the CV in the next 24 hours, as it does in wild type petals, but remained instead on the membrane of these enlarged vacuolinos. (Fig. 2c). This defect in PH5-GFP trafficking is very similar to that observed in ph1 and 35S:RAB5a petal epidermal cells, and indicates that PH7 is required (together with PH1) for the traffic of proteins from vacuolinos to the vacuole. The protein fusion RFP-SYP122 reaches the plasma membrane within 24 hours in both wild type and ph7 petal epidermal cells, indicating that PH7 has no effect on the traffic of proteins to the plasma membrane.

In protoplast from petal mesophyll (recognizable from the lack of anthocyanins in the CV lumen) of PH7R176, ph7R146 and ph7R173 petal, the proteins PH5-GFP and RFP-SYP122 traffic within 24 hours to CV tonoplast and plasma membrane, respectively (Fig. 2a-c).

Together these findings indicate that PH7 is needed for (i) the formation of vacuolinos and (ii) subsequent trafficking of proteins from vacuolinos to the CV, and that PH7 facilitates both processes though distinct domains (and mechanisms) that can be separated genetically.
Figure 2. Vacuolino formation and fusion to the CV is blocked in \( \text{ph}^7_{\text{R146}} \) and \( \text{ph}^7_{\text{R173}} \) mutant petal epidermal cells, respectively. (a) Confocal micrographs of \( \text{PH}^7_{\text{R176}} \) petal protoplasts transiently expressing PH5-GFP and RFP-SYP122. (b) and (c) Confocal images of transiently expressed PH5-GFP and RFP-SYP122 in \( \text{ph}^7_{\text{R146}} \) and \( \text{ph}^7_{\text{R173}} \) mutant petal protoplasts, respectively. Scale bars =10 \( \mu \)m in all panels.

Table 1. Overview of samples used for RNA-seq experiment

<table>
<thead>
<tr>
<th>Lines</th>
<th>Tissue</th>
<th>Developmental stage*</th>
<th>Biological replicates</th>
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<td>stage 4-6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>petal limb</td>
<td>stage 7</td>
<td>2</td>
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<tr>
<td>Stable mutant (ph7(^{R173}))</td>
<td>petal limb</td>
<td>stage 4-6</td>
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<td></td>
<td>petal limb</td>
<td>stage 7</td>
<td>2</td>
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<tr>
<td>Unstable mutant (ph7(^{R146}))</td>
<td>petal limb</td>
<td>stage 4-6</td>
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<tr>
<td></td>
<td>petal limb</td>
<td>stage 7</td>
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*stage 4-6: RNA was isolated from stage 4/5 and stage 6 independently, and pooled in equal amount (of total RNA) to generate one mixed sample. Developmental stages are defined as follows: stage 4/5, maximum length bud, about to open; stage 6: open flower prior to anthesis: stage 7, open flowers, post anthesis.

Identification of the PH7 gene by RNA-seq analysis of stable and unstable mutants

To identify the \( \text{PH7} \) gene, we used RNA-seq analysis and compared the transcriptomes of petals from wild type (\( \text{PH7} \)), stable mutant (\( \text{ph}^7_{\text{R173}} \)) and unstable mutants (\( \text{ph}^7_{\text{R146}} \)). For each genotype, we analyzed two biological replicates of petals at two different developmental stages (Table 1). We obtained about 20 million reads (2\( \times \)150 bp paired-end reads) for each sample, which were further analyzed by bioinformatics (Fig. 3). Given that \( \text{ph}^7_{\text{R146}} \) is somatically unstable (Fig. 1), we assumed that \( \text{ph}^7_{\text{R146}} \) was inactivated by the insertion of a \( d\text{TPH1} \) transposon, like most other mutations that arose in the progenitor line W138 (ref \(^{24}\)). Within each data set from \( \text{ph}^7_{\text{R146}} \) we found some 2000 reads (out of 20 million) containing the 12-bp terminal inverted repeat (TIR) of \( d\text{TPH1} \) (Fig. 3). By comparing 20 bp of flanking sequence from each of these reads, we identified 21 distinct \( d\text{TPH1} \) insertions that were present in all samples from the \( \text{ph}^7_{\text{R146}} \) mutant, but absent in all samples of \( \text{PH}^7_{\text{R176}} \) wild type and \( \text{ph}^7_{\text{R173}} \) stable mutant (Fig. 3). Next, we deduced from the petunia transcriptome for each of these 21 \( d\text{TPH1} \) flanking (nearly) full size sequences of the corresponding mRNAs.

We used a pool of all raw reads from all samples of the same genotype for \textit{de novo} transcriptome assemblies with Trinity, and obtained full-length transcriptomes for all three genotypes (Fig. 3), and extracted for all 21 transcripts (near) full size
mRNA sequences from the PH7R176, ph7R173 and ph7R146 transcriptomes. Finally, by aligning for each of the 21 mRNAs, the sequences from PH7R176, ph7R173 and ph7R146 petals, we found one transcript that was interrupted by 8-bp insertion in the stable ph7R173 mutant, while the same transcript contains a dTPH1 insertion in ph7R146 unstable mutant. Furthermore, we found that abundance of this transcript (judged from the number of RNA-seq reads) was down regulated in both ph7R173 and ph7R146 mutant compared to the wild type PH7R176 (Supplementary Fig. 1). Thus, the gene from which this transcript originates – corresponding to Peaxi162Scf00064g00532.1 in the Petunia axillaris genome25 – is likely to be at the PH7 locus (Fig. 4a). To further verify whether mutations in the P. hybrida homolog of Peaxi162Scf00064g00532.1 are responsible for the ph7 mutant phenotype, we used PCR to analyses DNA isolated from individual plants of the F2 families V2009 and V2011 segregating for PH7 and the unstable allele ph7R146 allele. As shown in Fig. 4b, all PH7+/+ plants yielded as expected a clear fragment of 554 bp, whereas ph7 m/m plants yielded mainly a fragment that is about 300 bp (dTPH1 transposon is 283 bp) larger, while the ph7 m/+ heterozygotes yielded both fragments.

Given that the identified gene contains an insertion of an active dTPH1 transposon in the unstable mutant ph7R146 and an 8-bp insertion in the stable mutants ph7R173 and isogenetically linked to PH7, we concluded that the gene is identical to PH7.
**Figure 3 Schematic diagram of the workflows for the identification of the PH7 locus.** The terminal inverted repeat of dTPh1 transposon used for selecting out reads containing dTPh1 insertion from raw reads are CAGGGCGGAGC and GCTCCGCCCTG.
Figure 4. Characterization of the \textit{PH7} gene and mutant alleles. (a) Schematic diagram of the \textit{PH7} wild type gene and the mutant alleles \textit{ph7}^{R173} and \textit{ph7}^{R146}. Exons: black rectangles; intron: black lines; \textit{dTph1} transposon: grey triangle. The 8-bp insertion in the \textit{ph7}^{R173} allele, is indicated in blue italics font. Underlined positions indicate the target site duplication (TSD) originated from the insertion of the transposon (b) PCR analysis of \textit{PH7} in the F2 families V2009 and V2011, segregating for \textit{PH7} and the unstable allele \textit{ph7}^{R146}. Homozygosity (\textit{PH7}/\textit{PH7}) or heterozygosity (\textit{PH7}/\textit{ph7}^{R146}) was determined by analysis of F3 progeny from each plant.

\textbf{PH7 encodes a member of the monosaccharide sugar transporter superfamily}

The \textit{PH7} transcript encodes a protein of 492 amino acids, which shows 80\% sequence identity to the \textit{Arabidopsis} ERD6-like 6 (AtERDL6)\textsuperscript{26, 27} that belongs to the ERD6-like subfamily of monosaccharide sugar transporters (MSTs) superfamily. Sequence alignments of PaPH7 and its paralog PaPH7-like, AtSTP10 (belonging to the sugar transporter subfamily of \textit{Arabidopsis})\textsuperscript{28, 29}, several members of the \textit{Arabidopsis} ERD6-like subfamily\textsuperscript{30-32} (including AtERD6, AtESL1 and AtSPF1), and GLUT8, the closest homolog of \textit{PH7} in \textit{Homo sapiens}\textsuperscript{33}, indicated that sugar binding sites, residues functioning as proton donor/acceptor pairs, and a conserved signature motif typical of MSTs proteins are all conserved in \textit{PH7} (Fig. 5). Furthermore, a potential dileucine-based motif is recognizable in the N terminal region of PaPH7 suggesting its localization at the vacuolar membrane.

To gain insights into the evolutionary relationships between \textit{PH7} and other members of MSTs superfamily, we constructed phylogenetic trees on the basis of all the
protein sequences of monosaccharide sugar transporter (MSTs) superfamily from *Petunia axillaris* and *Arabidopsis thaliana* (Fig. 6a). The MST superfamily in both *Petunia* and *Arabidopsis* comprises seven clades which comprise: (i) the tonoplast sugar transporters (TST)/tonoplast monosaccharide transporters (TMT) \(^{34-39}\), (ii) the vacuolar glucose transporters (VGT)\(^{40}\), (iii) the inositol transporters (INT)\(^{41-44}\), (iv) the polyol/monosaccharide transporters (PMTs)\(^{45-47}\), (v) the plastid glucose translocators (pGlcT)/suppressors of G protein beta1(SGB1)\(^{48, 49}\), (vi) the early response to dehydration 6 (ERD6)-like subfamily\(^{26, 27, 30-32, 50}\) and (vii) the sugar transporters (STP)\(^{29, 51-54}\). PaPH7 as well as PaPH7-like belong to the ERD6-like subfamily clade.

To investigate the distribution of PH7 and homologs in the plant kingdom, we built a phylogenetic tree containing sequences of PH7 and putative homologs from basal plants (green algae, mosses, and fern) and higher plants (angiosperms). The tree in Fig. 6b shows that the PH7 clade (ERD6-like4/6 clade) is ancient and originating before basal plants and Angiosperms separated. This is supported by the finding of multiple PH7 orthologs in basal plants e.g. green algae (*Klebsormidium flaccidum* and *Chara braunii*). However, while basal plants only have one or two paralogs of PH7, three or four present in several species of higher plant, such as *Oryza sativa*, *Petunia axillaris*, *Solanum lycopersicum* and *Medicago truncatula*, indicating an amplification of PH7 gene numbers in the genome, probably followed by diversification to meet specific requirements and new functions.
Figure 5. Multiple sequence alignment of PaPH7 and members of the Arabidopsis ERD6-like subfamily, STP subfamily, and the Homo sapiens homolog GLUT8. Sequences from Petunia axillaris, PaPH7 (Peaxi162Scf00064g00532.1) and PaPH7-like (Peaxi162Scf00931g00029.1); from
Arabidopsis thaliana, AtERD6 (AT1G08930.1), AtESL1 (AT1G08920.2), AtSFP1 (AT5G27350.1), AtESL6 (AT1G75220.1) and AtSTP10 (AT3G19940.1); from Homo sapiens, HsGLUT8 (ENST00000373371.7). Conserved residues are shaded with gray-scale (50% < identity <75%) and black-scale (identity >75%). Sugar binding residues are highlighted in red. Residues functioning as proton donor/acceptor pair are highlighted in green. MSTs signature motifs are highlighted in purple. The dileucine-based motifs (D/E)XXXL(L/I) of HsGLUT8 and LXXXLL of AtESL1 are marked by sky blue rectangle. The potential dileucine-based motif of PaPH7 is marked by sky blue dashed rectangle.
Figure 6. PH7 belongs to the ERD6-like subfamily of the monosaccharide sugar transporters (MSTs) superfamily. (a) Phylogenetic analysis of PH7 and other monosaccharide sugar transporters (MSTs) from Petunia and Arabidopsis. (b) Phylogenetic analysis of PH7 homologs from different plant species; the pGlcT subfamily of Petunia and Arabidopsis is present as outgroup of PH7 group; one
paralog is present in *Klebsormidium flaccidum* and *Chara braunii* of green algae, *Sphagnum fallax* of moss, and *Selaginella moellendorffii* of fern, respectively; two paralogs exist in both *Physcomitrella patens* and *Marchantia polymorpha* of moss. Protein sequences were aligned with MUSCLE and subsequently cured by the G-blocks tool. The resulting alignment was used in the construction of the phylogenetic tree by Maximum likelihood method (PhyML) with 300 bootstraps. Branch support is indicated as percentage of 300 bootstraps if $\geq 50\%$. Red arrows point the Petunia PH7.

**Discussion**

*PH* genes in petunia (*PH1* to *PH7*) were originally identified via mutants displaying bluish petal color and reduced acidity of the central vacuole in (epidermal) petal cells, where anthocyanins are stored. *PH3, PH4* and *PH6/AN1* are expressed in only a few specific cell types, like the epidermis of petals and seeds, where they drive vacuolar acidification by activating expression of *PH1* and *PH5*, which encode a P$_{3B}$-ATPase and P$_{3A}$-ATPase respectively. *PH1* and *PH5* are the components of a powerful proton pump in the tonoplast which (hyper)acidify the vacuolar lumen. Here we report an initial characterization of *PH7* and show that it encodes a putative H$^+/sugar$ symporter homologous to ERD6-like from Arabidopsis. The analysis of two *ph7* mutants revealed that it is involved in the formation of vacuolinos and in the transport of proteins (and presumably membranes) from vacuolinos to the central vacuole.

The hyper-acidification of the CV in petal epidermal cells sets in at the very last stage of bud development, just before opening. This matches with the temporal expression patterns of *PH1* and *PH5* (ref$^{10,11}$) and the moment in which the color phenotype of *ph1* to *ph6* mutants becomes evident and is consistent with the idea that the proton-pumping activity of the PH1-PH5 complex is responsible for generation of the steep proton gradient across the tonoplast resulting in hyperacidification of the vacuolar lumen$^{10}$. The *ph7* phenotype, by contrast, becomes evident much later (around 3 days after bud opening), suggesting that PH7 has a role in maintaining vacuolar acidity, rather than generating it.
Several types of MSTs were shown to act as $H^+$ antiporters or symporters, and consequently affect $H^+$ homeostasis directly\textsuperscript{55,56}. In all cases protons are translocated down the proton gradient, which is from the lumen of the vacuole to the cytosol, to drive either import of sugars into the vacuole via antiporters of the TMT subfamily\textsuperscript{57}, or the export of sugars via symporters, like ERDL6 (ref\textsuperscript{27}). Given that PH7 is highly similar and probably homologous to ERDL6, it is likely to act as an $H^+/sugar$ symporter and consequently to reduce vacuolar acidity. This is at odds with the $ph7$ phenotype – which indicates the PH7 promotes (the maintenance of) vacuolar acidity –, PH7 must affect $H^+$ homeostasis (also) in a different way, possibly via its role in the vacuolino trafficking pathway.

Vacuolar proteins like PH1 and PH5 can reach the CV in at least two ways. In cells lacking vacuolinos (cells in leaves or petal mesophyll) they move ‘directly’ to the CV via a ‘canonical pathway’ that is PH7-independent and highly similar to the well-studied pathway(s) that operate(s) in a variety of plant tissues and species. Thus, in young flower buds, when vacuolinos biogenesis is not yet induced, vacuolar proteins most likely travel to the CV via the canonical pathway. When the flower bud opens, the vacuolino pathway becomes active. Hence it is conceivable that in opening flowers, sufficient PH1 and PH5 arrived at the tonoplast via the vacuolino-independent canonical pathway to reduce the pH of the vacuolar lumen sufficiently to confer a red petal color. As the flower matures the maintenance of PH1 and PH5 levels at the CV relies on continued delivery of PH1 and PH5, which at this stage requires the PH7-dependent transport along the vacuolino pathway. We propose that, as a consequence of a block in the vacuolino pathway, the concentration of vacuolar proteins, among which PH1 and PH5, diminishes because of turnover and lack of replacement. This would impair the maintenance of the steep proton gradient across the CV tonoplast and result in a less acidified vacuolar lumen.
The trafficking of membranes, vesicles and proteins is orchestrated by range of proteins and protein complexes, like for example coat proteins, SNAREs, RABs and their effectors, each covering specific roles in budding of vesicles, recognition, tethering and fusion with target membranes. Several studies revealed that besides this extensively studied core machinery, also proteins functioning in transmembrane transport are involved. For instance, the P$_{3B}$-ATPase$^{10,22}$, the vacuolar-type H$^+$-ATPase (V-ATPase)$^{58-61}$ and cation/H$^+$ exchangers (CHX)$^{62-67}$ have been shown to participate in various endosomal trafficking processes in plants, yeast and mammals. Arabidopsis nhx5 nhx6 double mutants, affecting endosome localized Na$^+/H^+$ exchangers (NHEs), result in smaller and more numerous protein storage vacuole (PSV) in hypocotyl cells of *Arabidopsis* embryos$^{62,63}$. Similarly, NHX1, an NHE in *Saccharomyces cerevisiae*, mediates the fusion of multi-vesicular bodies (MVB) to the vacuole in a pH- and monovalent cation gradient-dependent manner$^{64-67}$. Even in the case of V-ATPases, the best studied example, it remains controversial is needed in membrane trafficking is for it its proton pump activity by acidification of the endosome or rather by its (activity-independent) role as a docking station for protein-protein interactions, or both$^{59,68}$.

Our results show that the putative sugar transporter PH7 is required in at least two distinct steps along the vacuolino pathway. In petals homozygous for $ph7^{R146}$ vacuolinos are missing and transiently expressed PH5-GFP remains stuck in small punctate structures. This phenotype is very similar to that of $rab5a$ mutants, in which vacuolinos are missing and PH5-GFP, and other vacuolar proteins, also accumulate in punctate structures that represent a subset of prevacuolar compartments (PVCs) (Chapter 4 in this thesis). Assuming that the PH5-GFP positive puncta in $ph7^{R146}$ petals are the same compartments as those in $rab5a$ mutants, which remains to be established this suggests that PH7 contributes to the formation of vacuolinos by facilitating homotypic fusions of PVCs. The $ph7^{R173}$ mutation, on the other hand, blocks the vacuolino pathway at a later step, the
trafficking from vacuolinos to the CV. In \( ph7^{R173} \) petals, PH5-GFP accumulates in vacuolinos, but does not reach the CV (Fig. 2c), while vacuolinos are enlarged compare to wild type (PH7) petal epidermal cells. This phenotype strongly resembles the trafficking defect in \( ph1 \) petals\(^{22} \). Whether PH7 associates with the PH1-SNARE complex or participates in totally distinct complexes with other components to regulate the fusion of two types of vacuoles remains to be investigated.

The distinct phenotypes of \( ph7^{R146} \) and \( ph7^{R173} \) indicate that PH7 enables the formation of vacuolinos and the vacuolino to CV transport via different mechanisms, and possibly interactions with distinct partners. The \( ph7^{R146} \) mutation disrupts the coding sequence 131 bp downstream of the ATG and truncates the encoded protein before the first transmembrane domain (Supplementary Fig. 2), suggesting that \( ph7^{R146} \) is most likely a null allele. The \( ph7^{R173} \) on the other hand, is a weak (partially active) allele, supports the formation of vacuolinos, but not the vacuolino to CV traffic. The coding sequence of the \( ph7^{R173} \) is disrupted 397 bp downstream of the ATG, and might express a mutant protein truncated in the third transmembrane domain and retaining at least the first two cytosolic domains (Supplementary Fig. 2), and possibly even some of the more C terminal loops, if \( ph7^{R173} \) expresses one or more splicing variants in which exon 4 is skipped. It is unlikely that such a truncated protein retains H\(^+\)/sugar transport activity, suggesting that it is the presence of the protein (fragment) rather than its transport activity that facilitates vacuolino formation, for instance by facilitating tethering of membranes through protein-protein interactions. Analysis of the trafficking defect in \( ph7^{R146} \) \( ph7^{R173} \) trans-heterozygotes and MS analysis of PH7 proteins expressed in mutants may shed more light on this point.
Methods

Plant material

Petunia line R146 (ph7\textsuperscript{m/m}) is a transposon insertion mutant for the PH7 locus isolated from progeny of the high transposition line W138, described previously\textsuperscript{23}. Line R173 (ph7\textsuperscript{-/-}) is a stable mutant containing a footprint in the PH7 coding sequence. Line of R176 (PH7\textsuperscript{+/+} AN1\textsuperscript{+/+}) is a revertant line with a fully active AN1 allele originated from W138 (PH7\textsuperscript{+/+} an1\textsuperscript{m/m}). The V2009 and V2011 plant families generated for the scoring of the PH7 genotype came from the crossing ([R146 × R162]×W137)×[R146 × R162]. Lines R162 (ph4\textsuperscript{B3021}) and W137 (an1\textsuperscript{w137}) derived from W138 by transposon insertions and excisions and are also in the W138 background.

M1×V30 (wild type) is a F1 hybrid accumulating the anthocyanin malvidin. This hybrid is used for protoplast isolation and RNAs extraction. All Petunia lines used in this work were kept in the greenhouse under normal conditions (min 19°C / max 30°C, with cycle of 16/8 hours light/dark in summer, and with cycle of additional illumination of 15/9 hours light/dark in winter).

DNA extraction and RNA isolation

Genomic DNA isolation from young leaves were performed as described before\textsuperscript{69}. For RNA-seq analysis lines we harvested petals from plants were germinated and grown side by side under the same condition in greenhouse. Total RNAs of petal limb (flower development stage 4/5, stage 6, and stage 7) of petunia line R146, R173, and R176 was extracted by a TRIzol based method. Equal amounts of RNAs of buds at stage 4/5 and stage 6 were pooled to represent an early stage sample before the ph7 mutant phenotype becomes evident. Together with RNAs from open flowers at stage 7 sample (2 biological replicates for each sample) were quality checked and libraries were constructed, and prepared for paired-ends Illumina sequencing.

RNA-seq libraries preparation

Quality and quantity of total RNAs were checked by Qubit\textsuperscript{TM} RNA HS Assay Kit with the Bioanalyzer 2100. RNA-seq libraries were constructed with the method described before\textsuperscript{70}, with minimal modification That is, 1 µg total RNAs was used to
prepare a library. Quantity of prepared library was checked by Qubit™ dsDNA HS Assay Kit with the Bioanalyzer 2100.

**Phylogenetic analysis and multiple sequence alignment**

Amino acid sequences of monosaccharide sugar transporters from *Petunia* and *Arabidopsis* were collected from Petunia Genome SGN dedicated database (http://petunia.sgn.cornell.edu/) and Phytozome databases (https://phytozome.jgi.doe.gov/). Homologs of PaPH7 in basal plants and higher plants were obtained from Phytozome databases (https://phytozome.jgi.doe.gov/), with some exceptions, *Klebsormidium flaccidum* (http://www.plantmorphogenesis.bio.titech.ac.jp/~algae_genome_project/klebsormidium/index.html) and *Chara braunii* (https://bioinformatics.psb.ugent.be/orcae/overview/Chbra). The online tool PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) was used to make phylogenetic tree\(^7\); MUSCLE and G-block were engaged in the multiple sequence alignment, and 300 bootstrap replicates were used to assess the branch support value. Multiple sequence alignments were performed with software ClustalX2 and further edited by GeneDoc.

**Constructs**

PH5-GFP and RFP-SYP122, which have been characterized as vacuolino or tonoplast marker, and plasma membrane marker respectively, were described before\(^1\).2

**Protoplast transformation and confocal microscopy**

Protoplast isolation and transformation was performed as previously described\(^1\), with slight modifications. Instead of using 0.4M sucrose protoplast isolation buffer, a 0.5M sucrose protoplast isolation buffer was applied in the protoplast isolation and transformation for line R176 (*PH7*\(^+/+\)), R146 (*ph7*\(^mm\)) and R173 (*ph7*\(^-\)). Protoplasts transformed with constructs of interest were observed and imaged with a Zeiss LSM510 confocal microscope using a 40×/1.2 water objective at 24 or 48 hours after transformation. A 488nm laser was applied for the excitation of GFP and anthocyanins, which were detected by the filters BP505-550 and LP650 respectively. RFP was excited/detected by 568nm laser and a BP585-615 filter. For each independent transformation, more than hundred cells were observed and around 20 to 50 cells were imaged.
Supplementary information

**Supplementary Figure 1.** Number of reads of PH7 and PH7-like in PH7\textsuperscript{R176} wild type, ph7\textsuperscript{R173} stable mutant and ph7\textsuperscript{R146} unstable mutant from RNA-seq data.

**Supplementary Figure 2.** The membrane topologies and transmembrane domains of PH7 were generate by using the web-accessible Protter software (http://wlab.ethz.ch/protter). The N-terminal domain marked out is (potentially) expressed in ph7\textsuperscript{R173} stable mutant, which is likely involved in the vacuolino formation.
### Supplementary Table 1. Primers used in this study

<table>
<thead>
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<th>Gene</th>
<th>Name</th>
<th>Sequence 5’-3’</th>
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</tr>
</tbody>
</table>

* F indicates a forward orientation of the primer, relative to the orientation of the gene, and R reverse orientation
Reference


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