Vacuolar proton pumping: more than the sum of its parts?

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Petunia flower colour is dependent on vacuolar pH and is therefore used to study acidification mechanisms. Recently, it was shown that the concerted action of two tonoplast-localised P3-ATPases is required to hyperacidify vacuoles of petunia petal epidermis cells. Here we discuss how steep cross-tonoplast pH gradients may be established in specific cells.

The acidity of plant vacuoles varies between plant species, organs, and cell types. In morning glory (Ipomoea tricolor), when the slightly acidic vacuoles of flowers become neutral, a shift from a purple to the characteristic blue flower colour can be observed. By contrast, petal epidermis cells of petunia (Petunia hybrida) contain acidic vacuoles with a pH around 5, which causes the colour-giving anthocyanins in the vacuoles to appear red. In CAM plants or fruits such as lemon (Citrus) or non-ripe grape berries (Vitis) the vacuolar pH can reach values as low as 2 or 3. Two types of proton pumps, a H+ATPase and a H+-PPase have originally been shown to reside at the vacuolar membrane [1]. Besides acidification of the vacuolar lumen, proton pumping also generates a transmembrane gradient in electric potential, $\Delta E_m$. Both gradients, $\Delta pH$ and the $\Delta E_m$, are exploited to accumulate solutes within the vacuole. While $\Delta E_m$ regulates channels that mediate vacuolar ionic uptake, $\Delta pH$ drives cation/proton, anion/proton and sugar/proton antiporters [2]. Besides its role in vacuolar acidification, the V-PPase also functions during postembryonic heterotrophic growth through hydrolysing inorganic pyrophosphate (PPi) to reduce PPi contents and support gluconeogenesis [3]. Until recently, it was thought that V-ATPases and V-PPases at the tonoplast are exclusively responsible for generating the vacuolar pH. However, recent studies comparing the pH of a vacuolar V-ATPase double mutant (vha-a2/vha-a3) with that of a V-PPase mutant suggested that yet another component was involved in the generation of vacuolar pH [4]. Possibly, the activity of a trans-Golgi network/early endosomes V-ATPase also contributes to vacuolar acidification.

Petunia flowers can be used to study vascular acidification processes

Petunia (Petunia hybrida) flowers are an ideal model system to study vacuolar pH and the genes and proteins involved in its generation. Anthocyanins accumulate in the vacuole of flower petal cells, and the colour of these anthocyanins is dependent on–among other factors–vacuolar pH. In petunia a shift in vacuolar pH causes a change in flower colour. Mutants that display a flower colour different to the red-flowering wild type are a useful tool to analyse gene function in vacuolar pH maintenance. Overall, seven pH-mutants defective in petunia flower colour and pH have been identified and termed ph1 to ph7 [5]. Within the past decade the team led by Francesca Quattrocchio at Amsterdam University has been successful in identifying several genes that are affected in the respective mutants, using transposon-tagging strategies. Some of the genes such as PH3, PH4 and PH6 are transcriptional regulators. In 2008 the team identified the mutant ph5 to be defective in a gene encoding a proton pump [5]. PH5 localises to the tonoplast and belongs to the H+-P$_{3\alpha}$-ATPase subfamily, which finds homologs in the AHA family of Arabidopsis (Arabidopsis thaliana) plasma membrane H+-ATPases. Three lines of evidence led the researchers to conclude that PH5 is responsible for the transport of protons into the vacuole: (i) PH5 expression was able to rescue a yeast mutant unable to grow on acidic medium; (ii) the difference in flower colour in ph5 mutants was not caused by altered anthocyanin accumulation; and (iii) over-expression of a PH5 transgene in a ph5 mutant background rescued the mutants’ petal pH and colour phenotype. Interestingly, PH5 overexpression in the background of other pH-mutants such as those of the transcriptional regulators ph3, ph4 and ph6 could not rescue the pH and flower colour phenotype of those mutants. Considering that PH5 expression is regulated by PH3, PH4 and PH6, the authors reasoned that these regulators may control yet another component, which, in addition to PH5, was necessary for vacuolar acidification.

The P$_{3\alpha}$-ATPase, PH1, is the second component required to rescue petunia pH-mutants

In a recent study the Quattrocchio group reports the identification of PH1 as an interactor of PH5, necessary for hyperacidification of petal vacuoles [6]. They obtained a transposon-tagged ph1 mutant line and were able to identify the gene sequence associated with ph1 to code for a P$_{3\alpha}$-ATPase. Evidence from procaroyts suggests that ATPases of this type are involved in Mg$^{2+}$ uptake. A first indication that PH1 was the missing component in PH5-driven vacuolar acidification came from gene expression analysis. PH1 mRNA expression paralleled that of PH5 in a spatio temporal manner. Like PH5, mRNA expression of PH1 was under the control of the transcription factors PH3 and PH4 and the transcriptional regulators PH6 and AN11. While a 35S-driven PH5-GFP
construct localised to the tonoplast in the petal epidermis of petunia, only an internally GFP-tagged 35S:PH1-GFPi construct localised to the tonoplast.

In light of the facts that PH1 and PH5 both encode P$_{\text{m}}$-ATPases, are both under parallel transcriptional control, and overlap in their intracellular localisation, the authors hypothesised that PH1 and PH5 acted in concert in vacuolar petal acidification. In fact, when overexpressed on their own, neither PH5, nor PH1 could rescue the colour and pH phenotype of the ph3 mutant. Only when PH1 and PH5 were co-expressed the mutant’s flower colour reverted back to red, and its petal extract pH decreased from approximately 5.7 to 5.3 (Figure 1). An elegant way to show that this mutant rescue was due to the acidification of petal cell vacuoles was the authors’ co-expression of the Na$^+$/H$^+$ and K$^+$/H$^+$ antiporter NHX1, which had been shown to reduce cross-tonoplast pH gradients [7]. The PH1/PH5-mediated rescue of the ph3 mutant colour and petal extract pH was lost when NHX1 was also expressed; as a result the flower colour reverted back from red to the pale-rose of the ph3 mutant, even though PH1 and PH5 were expressed.

**Figure 1.** Petunia flower colour and pH phenotype of the ph3 mutant is restored by the constitutive co-expression of the P-ATPases PH1 and PH5. (A) In the ph3 mutant PH3-regulated genes such as PH1 and PH5 are not expressed, resulting in a pale-rose flower phenotype and an increase in vacuolar pH compared to wild type petunia flowers. (B) The flower and vacuolar pH phenotype of ph3 mutants is only rescued when 35S:PH1 and 35S:PH5 are co-expressed. The additional introduction of the Na$^+$/H$^+$ and K$^+$/H$^+$ antiporter NHX1 restores the ph3 mutant phenotype. Reproduced, with permission, and modified from [8]. (C) Multiple protein sequence alignment of the putative transmembrane domain (TM) 5 and 6 of AtAHA2 according to the UniProt database including the conserved aspartate residue (D684 in AtAHA2) between the Arabidopsis thaliana AHA2 and AHA10 (homolog of PH5), PH5 and PH1 of Petunia hybrid, and the bacterial P$_{\text{m}}$-ATPases, Mgta and Mgtb of Salmonella typhimurium. Asterisks indicate sequence identity, dots the degree of similarity. The alignment was performed using ClustalW.
Hyperacidification of petunia petal cell vacuoles relies on both P₇-ATPases

The final and crucial question addressed in the study was: What constituted the concerted action of PH1 and PH5 in vacuolar acidification on a mechanistic level? To answer this question the authors carried out two different protein interaction studies using Bimolecular Fluorescence Complementation and Split-Ubiquitin-System assays. Both approaches yielded that PH1 and PH5 were able to interact with one another. Furthermore, the authors applied the patch-clamp technique to whole-vacuoles of petunia leaf cells. Clamping the membrane potential at 0 mV the team could observe ATP-dependent outward currents, arising from proton-transport from the cytosol to the vacuolar lumen. The vast majority of this current was sensitive to bafilomycin, a highly specific inhibitor of V-ATPases. However, in vacuoles expressing PH5, half of the ATP-dependent current was sensitive to bafilomycin, whereas the other half was sensitive to vanadate, an inhibitor of P-ATPases such as PH5. This indicated that PH5 was transporting protons to the vacuolar lumen. Surprisingly, vacuoles expressing PH1 did not display any vanadate-sensitive current, but when PH5 and PH1 were co-expressed the vanadate-sensitive current nearly doubled, when compared to vacuoles expressing PH5 only. This indicates that, although PH1 does not seem to have proton-transport activity, it enhances the PH5-mediated current. How such an enhancement may work on a mechanistic level, however, remains elusive.

PH1 displays sequence similarity to bacterial P₃₈-ATPases such as MgtA and MgtB, which are required for Mg²⁺ uptake. Such transporter systems had previously been thought absent in plants but when the researchers carried out phylogenetic analysis they found homologues in other species including Vitis vinifera, the berries of which also require vacuolar hyperacidification. Although the authors hypothesised that PH1 might boost PH5-mediated H⁺ pumping by dissipating ΔEm through Mg²⁺ import to the cytosol, they found no evidence for this and reasoned that such an import was energetically unfavourable.

Concluding remarks and outlook

The authors of this review found that PH1 is lacking an aspartate (D) residue that is conserved in Mg²⁺-, Ca²⁺-, Na⁺/K⁺-, H⁺/K⁺-, and H⁺-ATPases such as the prokaryotic PH1-homologues and PH5, and which has been proposed to be essential for cation binding and translocation [8]. In PH1 this residue is replaced by an asparagine (N) (Figure 1). When the corresponding Asp 684 of the Arabidopsis H⁺-ATPase AHA2 was mutated to Asn (D684N), the enzyme still displayed ATP hydrolysis activity but did no longer show coupling to H⁺ transport [8]. The stoichiometry of H⁺ transported to ATP hydrolysed (n) for V-ATPases typically has a value of 2 but depends on vacuolar pH. With steeper cross-tonoplast pH gradients (ΔpH) the value of n decreases [9], because, from a thermodynamic standpoint, transport against a larger concentration gradient requires more energy through ATP hydrolysis. In fact, H⁺ P-ATPases are essential for the energisation of even steeper gradients as they operate at coupling ratios of 1H⁺/ATP [10]. Early work from the Taiz laboratory uncovered the existence of vanadate-sensitive cross-tonoplast H⁺ transport in lemon fruit [11]. This vanadate-sensitive transport may indicate the existence of vacuolar P-type ATPases in lemon, which, in light of the findings presented here, may be homologous to that of PH5 or PH1/PH5. Taken together, we speculate that when PH1 and PH5 interact, this might decrease the H⁺/ATP stoichiometry to hypothetically 0.5H⁺/ATP allowing for hyperacidification. To test this hypothesis, initially one may generate the mutation N782D in PH1 in order to determine if proton-pumping activity is restored. Furthermore, it would be valuable to determine the H⁺/ATP coupling ratio of the interacting PH1–PH5.

In conclusion, the study described above has not only identified a missing component in petunia flower colour and pH regulation, but it may point to entirely new mechanisms of proton-pumping via P-ATPases.

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References