The genetics of vacuoles

Biogenesis and function in plant cells

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Chapter 6

General discussion

Vacuoles and lysosomes are related organelles that play pivotal roles in coordinating and maintaining fundamental functions of the endomembrane system. Hence, great efforts have been made by researchers world-wide to elucidate the mechanisms underlying their origins, biogenesis and biological function over last several decades. Previous findings unveiled several aspects of the machinery of vacuole/lysosome mediated biological processes, in the meanwhile, however, has raised a series of new issues to be addressed as well. As widely conserved organelles, vacuoles/lysosomes of different organisms or specialized cell-types within an organism have many features in common, just like other organelles, e.g. mitochondria or chloroplasts. However, the endomembrane system and vacuoles in particular have diverged enormously between species, and cell types within a species, with regard to size, morphology content and physiological functions. It is widely accepted that cells may contain besides vacuoles /lysosomes, additional vacuole/lysosome related organelles (LROs) that coexist with central vacuole/lysosome and perform distinct functions.

The biogenesis of lysosome and vacuoles, and endomembrane compartments relies on the trafficking of membrane vesicles that deliver both membrane materials and proteins, as well as soluble proteins in their lumen. The immense number of vesicles leaving from one membrane compartments on their way to specific target membranes implies that cells must have developed mechanisms to ensure that vesicles leave in time, contain specific proteins and membrane “cargo” and deliver these at the correct target membrane, being for example, the plasma membrane, an endosome, lysosome, vacuole or other membrane compartments. The key players (proteins) involved in these mechanisms could be identified by classical forward genetic screens for mutants with defects in trafficking pathways. While this approach was very successful to study trafficking in yeast, it met little success in higher organisms, as defects in endomembrane trafficking usually cause severe pleiotropic effects, such as severe malfunctioning of the organism (diseases), or

death (lethality). Hence, much of what is known about endomembrane trafficking in higher organism relies on biochemical work.

Genes involved in the synthesis of anthocyanin pigments have been successfully used as natural reporter genes, since the days of Gregor Mendel, to study elementary genetic process, like the inheritance of traits, transposition, RNA interference and gene regulation, to mention a few. Quite unexpectedly (flower) pigmentation mutants proved to be useful to address also questions in basic cell biology, related to the biogenesis of vacuoles and their functional differentiation. Previous work on so called *ph* mutants in petunia – which have a bluish flower color due to a failure to acidify the vacuoles where the anthocyanin pigments are stored – lead to the identification of a new proton P-ATPase pump, encoded by the *PH1* and *PH5* genes, that is involved in the hyperacidification of the vacuole in epidermal petals cells and a new pathway, including a novel endomembrane compartments called vacuolinos. Proteins (and presumably membranes) are delivered to the central vacuole by vacuolinos. Perhaps most important of all, it was found that the *ph3* and *ph4* mutations affect genes encoding transcription factors that act together with transcription factors of the anthocyanin pathway (ANTHOCYANIN11, AN11, and AN1/PH6) in a complex (MBWW) that drives the expression of downstream genes needed for hyperacidification of the central vacuole (PH1 and PH5) and unknown genes required for the formation of vacuolinos. As *an1*, *an11*, *ph3* and *ph4* mutants have no defects in growth or development, other than a change in flower color, this suggested that colored flowers might be an excellent tool to study long standing questions in basic cell biology and vacuole biology in particular.

The abovementioned findings raise a range of new questions, some of which are addressed in this thesis. Why do some cells use the newly discovered P-ATPase pump encoded by PH1 and PH5 to acidify their vacuoles, instead of the well-known

ubiquitous V-ATPase pump? What is the function of vacuolinos and how are they made?

Hyperacidification of the central vacuole

It was long believed that V-ATPases are the primary proton pumps residing on tonoplast that are responsible for the acidification of the lumen of vacuoles. The H^+/ATP coupling ratio (that is, number of H^+ transported per ATP hydrolyzed) of V-ATPase varies, depending on the conditions within the cell between 1.75 (the lowest value ever reported) to about 4. This implies that, based on thermodynamic considerations, V-ATPases can acidify the vacuolar lumen at best down to pH = 3, when the pumps works in it "lowest gear" (H^+/ATP = 1.75) and without any inhibition of pumping kinetics (ref[1, 2]).

Thus, to create an even more acidic vacuolar lumen (pH = 2), as *Citrus* fruit, the V-ATPase would have to (i) pump proton without any kinetic inhibition and (ii) adjust the H^+/ATP coupling ratio to lower than 1.75. However, none of these prerequisites seems to be ever met in vivo. Growing evidence showed that V-ATPase is under kinetic regulation[3], and can be disassembled or reassembled in response to intracellular or extracellular stimulations[4]. In petunia, ectopic expression of the vacuolar-proton pumping P-ATPase complex PH1-PH5, results in hyperacidification of the vacuole (due to PH1PH5 activity) which is accompanied by the strong down regulation of the vacuolar V-ATPase pump[5]. Thus, it seems there is no way that V-ATPase can get in vivo anywhere near the theoretically maximum proton gradient.

Interestingly, decades before the identification of the P-ATPase PH1 and PH5, several biochemical studies on the lemon juice sac cells detected the activity of a peculiar tonoplast H^+-ATPase, which is vanadate-sensitive, but bafilomycin A1-insensitive, and which exhibits a much lower H^+/ATP coupling ratio than V-

ATPase[2, 6-8]. All the characteristics of the novel tonoplast H^+-ATPase are compatible with P-ATPase proton pump, however, people did not draw that conclusion, as all the H^+ P-ATPases were believed to reside on the plasma membrane at the time.

Chapter 2 shows that in *Citrus* juice sac cells, the P-ATPase formed by CitPH1 and CitPH5, is the dominant proton pump that is responsible for the extreme acidification of the vacuolar lumen (pH = 2). The expression of *CitPH1* and *CitPH5* in sour *Citrus* fruits, and the abolishment of their expression in "sweet" (non-sour) fruits is associated with mutation in the components of the MBWW transcription factor complex. These findings extended our knowledge on the function of PH1 and PH5, showing that it is not only involved in the specification of flower color, but also plays pivotal a role in determining fruit taste. A recent report showed that the PH1 and PH5 homologs in grapevine berries are also involved in vacuolar hyper-acidification, and regulated by the MBW(W) complex that is homologous to petunia one[9], which opens a way to develop a reliable molecular markers for improving fruit traits. In petunia, expression of *PH1* and *PH5* is not limited to the petal epidermal cells where the anthocyanin are stored, and expression of these two genes has been detected in other tissues and cell types as well (unpublished data). Whether the expression of PH1 and PH5 in these tissue is associated with a specific function remains to be explored.

Genes involved in the formation of vacuolinos and trafficking from vacuolinos to central vacuole

Many proteins are synthesized newly at the rough ER, and are subsequently sorted and delivered to their destination organelles through a variety of pathways. One of these is the well-studied secretory pathway by which proteins traffic via the Golgi apparatus, TGN to the plasma membrane (PM), or – if they have a vacuolar sorting sequence- to, PVCs/MVBs and finally the CV. Proteins residing at the PM are for degradation transported to CV by the endocytic pathway. As intermediate

compartments on the way of proteins from Golgi/TGN or PM to central vacuole (CV), PVCs/MVBs play pivotal roles in integrating the secretary and endocytic pathways. In the extensively studied "canonical pathway" to the central vacuole, which was primarily studied in leaf cells, the biogenesis of PVCs/MVBs and their subsequent fusion with CV are mediated by a set of proteins including two RAB GTPases (RAB5 and RAB7), the ESCRT machinery, SNAREs, and the tether complexes COVERT and HOPS. As the vacuolar sorting receptors (VSRs) involved in the sorting of soluble proteins to the vacuole, have been demonstrated to predominantly localize on PVCs/MVBs, VSRs, and few other proteins are widely used as the marker for identifying PVCs[10].

Chapter 4 presents the discovery of a novel plant GTPase, RAB5a, which represents an ancient clade of conventional plant RAB5s that was previously overlooked and shows that RAB5a expression is activated by the MBWW complex and necessary to mediate the fusion of a subpopulation of PVCs/MVBs to form vacuolinos. RAB5a localizes to vacuolinos and a subpopulation of PVCs/MVBs that is distinct the subpopulations of PVCs/PVC-like compartments that are labeled by the RAB5a paralogs RAB5a1 or RAB5a2. In addition, also two different members of VSR family, BP80 and VSR2, were shown to localize to distinct populations of small compartments (puncta) that overlap only partially. All these findings suggest that PVCs/MVBs are quite heterogeneous even within a single cell. The divergence of PVCs/MVBs into distinct types with different protein content, suggests that they operate in distinct trafficking pathways to the CV to boost the efficiency of proteins trafficking to CV, and/or to increase possibilities to regulate the trafficking of specific (sets of) proteins to the CV by which plant cells can adapt to different environmental conditions.

The identification of a novel RAB5a that is widely present in the plant kingdom, and the characterization of its function in the vacuolino formation, reveal an unexpected

complexity of the plant RAB5 family. It is widely accepted that alterations in RAB GTPases was a major factor for the evolutionary divergence of trafficking pathways. Indeed, RAB5a and RAB5a1 or RAB5a2 are not functionally interchangeable in the biogenesis of vacuolino. To date, the function of RAB5a1 in intracellular trafficking remains largely elusive. Although the function of RAB5a2 in petunia has not been analyzed, the fact that it is orthologous to the Arabidopsis ARA7/RHA1 may suggest for it a potential role in the endosomal pathway. Comparative analysis of the function of RAB5a, RAB5a1 and RAB5a2 would provide a good opportunity to unravel how alterations with a RAB protein enabled the evolution of distinct intracellular trafficking pathways.

Previous studies, using a variety of different systems, ranging from yeast to mammalian neurons, lead to the discovery of a range of deeply conserved proteins, including RABs, SNAREs, ESCRTs, COVERT and HOPS, that are specifically involved in common steps of endomembrane trafficking pathways, like budding of vesicles, or their tethering and fusion with target membranes.

The biogenesis of vacuolino and the subsequent contact of vacuolinos with the central vacuole is activated by the MBWW transcription factor complex. By comparing the transcriptomes of wild type petals and mutants in which individual component of the MBWW complex was mutated, some 31 MBWW regulated genes were identified, among which should be several genes encoding proteins involved in the vacuolino pathway. Except for RAB5a, none of these MBWW regulated genes encode proteins of “usual suspects”, that are known to be involved in membrane trafficking, like RABs, SNAREs, ESCRTs, COVERT and HOPS. Thus, it is likely that the vacuolino pathway depends on a combination of more general factors that are expressed ubiquitously and involved in intracellular trafficking, with a bunch of factors encoded by the MBWW complex regulated genes. A good example supporting this idea is that all three RAB5s in petunia interact with a GEF VPS9a,

suggesting that although RAB5a is involved in vacuolino biogenesis, it shares a common activator with other RAB5s that are responsible for distinct steps in endomembrane trafficking. While the formation of vacuolinos through fusions of a subset of PVCs/MVBs is regulated by RAB5a, it is possible that the formation of these PVC/MVB-like precursors of vacuolinos is mediated by a MBWW independent pathway. To fully unravel the vacuolino biogenesis pathway and understand how vacuolinos can coexist with the CV, it would be worthwhile to broaden the range of candidate genes that is studied, instead of focusing on the MBWW target genes alone.

Chapter 5 shows that the putative glucose/H^+ symporter, PH7, a type of proteins not previously associated with membrane trafficking, is essential for at least two distinct processes of vacuolino pathway: (i) the biogenesis of vacuolinos and (ii) subsequent transport from vacuolinos to the CV. This adds to the short but growing list of reports, which suggest that some membrane transporters may have additional ('moonlighting') functions in membrane trafficking. In this list are the P_{3B}-ATPase PH1 (ref[5, 11]), the V-ATPase proton pump in mammals[12-15] and the cation/H^+ exchangers (CHX) of yeast[16-21].

The apparent dual role of the above-mentioned proteins in the transmembrane transport of small molecules (ions, glucose) and the trafficking of membranes, raised the question to what extent these two roles are mechanistically linked. Previous studies on the yeast V-ATPase revealed that low vacuolar pH, instead of the physical presence of Vph1p, is necessary in mediating homotypic vacuole fusion[13].

In case of the P_{3B}-ATPase PH1 from petunia, by contrast, its role in trafficking from vacuolinos to the CV and the boosting proton pumping activity of PH5, seems genetically separable. The $PH1^{Rev1}$ allele identified as a partial revertant among progeny of an unstable transposon insertion mutants, encodes a protein with 2 extra amino acids in the N terminus, which can drive vacuolar acidification and confer a

red flower color, indicating that vacuolar acidification is restored, while failed to rescue the fusion of vacuolino with CV (unpublished data). Whether PH7 mediating vacuolino pathway relies on its glucose/H^+ symporter activity or not would be an interesting topic for future research.

By interacting with SNAREs, PH1 plays a crucial role in the vacuolino and CV fusion. It is widely accepted that membrane transporters would function as a scaffold for proteins to form a complex that subsequently participates in cellular processes. In this scenario, further studies screening PH7 interacting proteins, such as SNAREs, would be helpful in understanding the functional role of PH7 in regulating vacuolino interactions with the CVs.

Biological function of vacuolinos

Although vacuolinos provide an attractive, genetically amenable, system to study membrane trafficking, their biological function remains mysterious. Whereas impairment of the biogenesis of PSVs or CV generally leads to embryo lethality, complete abolishment of vacuolino pathway (mutation in any components of the MBWW complex) does not cause any obvious defects in growth or development. This is one reason why vacuolinos are a nice system to study trafficking processes, but at the same time raises the question regarding the function of these compartments.

The activation of the vacuolino pathway and the acidification of lumen of CV are controlled by the same MBWW transcriptional factor complex, suggesting that vacuolinos might have a role in the acidification of CV lumen. However, the finding that expression of PH1 and PH5 from a (constitutive) viral promoter was sufficient to rescue vacuolar acidity and the flower color of an *mbww* mutants (*ph3*), indicated that vacuolinos are not essential for vacuolar acidification[5].

Mutations that affect the shape of epidermal petals cell, affect their optical features and thereby the color that is perceived[22, 23] and that leads to the attraction of distinct pollinators that may ultimately result in speciation. We observed that genetic changes that disrupt vacuolinos formation, such as mutations affecting MBWW function or constitutive expression of RAB5a from the 35S promoter, affect the width and height of epidermal petals cells (**chapter 4** in this thesis) and measurable changes in optical features (unpublished data). Although that does not result in color changes that can be easily distinguished by the human eye, they might be (more) visible for pollinators like bees. Hence, it would be interesting to investigate whether vacuolinos contribute to the attraction of pollinating animals in *Petunia* and to what extent they affect pollinator preference. Ongoing analyses of the vacuolino pathway, in addition to *RAB5a* and *PH7* described in this thesis **(Chapter 4** and **Chapter 5** in this thesis), has at present identified another 7 genes that are also involved in different steps of the vacuolino pathway[11, 24], which provide plenty of vacuolino mutants and isogenic wild types to test their attractiveness for pollinators in different tests including bees choice experiment[25, 26] and physical measurement of optical properties of flowers[27]. These types of analysis have been developed and widely used and would hopefully help us to draw a conclusion on the possible functional role of vacuolino on the regulation of pollinator preference and therefore its possible contribution to the development of different pollination syndromes.

Plant vacuoles are described as multifunctional compartments, which are divided into two functionally distinct types: protein storage vacuoles (PSV) and lytic vacuole (LV). The PSV mainly serves as compartment for protein storage, while LV functions as a degradative organelle for proteins and cargoes. In particular cell type or at defined developmental stages, the PSV coexists with the LV or even the PSV converts into LV within a single cell. This suggests that the functional differentiation of multiple types of vacuole is cell type and developmental stage dependent. The appearance of multiple types of functionally distinct vacuoles in one

cell has enriched the complexity of subcellular compartmentation and is likely an efficient way to improve the efficiency of cells in the control of different cellular processes, which subsequently allow plants to deal with various environmental stimuli and to meet specific requirements during development.

In *Petunia*, the coexistence of vacuolinos with the anthocyanin containing central vacuole (CV) is only detectable in petal epidermal cells starting from bud stage 6 (defined as totally opened flower with closed anthers) (unpublished data). In other words, the vacuolino biogenesis pathway is off during early stages of the flower bud life, indicating that this process is in petal cells developmentally regulated. This observation raises the question of which vacuole, vacuolino or CV, serves as lytic vacuole and performs degradative functions.

Chapter 3 shows that fluorescence protein (FP)-tagged FA(FA-GFP or FA-RFP) gets likely trapped in the lumen of vacuolino, instead of reaching the tonoplast of the CV, in petal epidermal cells of wild type petals which do not display color fading. Similarly, in **Chapter 4** the FP-tagged vacuolar sorting receptors BP80 and VSR2, which have been proved to attach on the membrane of PVCs/MVBs in vacuolino absent cells[28, 29], are shown stuck in the lumen of vacuolino. These findings suggest that vacuolinos may act as a "gatekeeper" or a sorting station that prevents specific proteins from reaching the central vacuole. The FP-tag is for BP80 and VSR2 fused to the N-terminus. Therefore, when the FP tagged proteins reside on the vacuolino membrane, the FP tags are on the luminal side of vacuolino membrane and the presence of fluorescent signal in the vacuolino lumen could simply be the consequence of cleavage of the FP-tag. Whether this is the case, remains to be explored. This can however not be the case for the FA-FP fusion protein, as the FP tag in this case is on the cytoplasmic side of the vacuolino membrane. Cleavage of the FP-tag would result in the detection of fluorescent signal in cytoplasm instead of in vacuolinos lumen. Further researches, e.g. western blotting of the FA-FP, FP-

BP80 and FP-VSR2 fusion proteins, would help to illustrate the nature of the fluorescent signal detected in the vacuolinos lumen and would enrich our understanding of the role of vacuolinos as "gatekeeper" in the choice of proteins that can reach the CV.

One is tempted to suggest that in petal epidermal cells, the vacuolino, instead of the anthocyanin containing CV, is the lytic vacuole which function as the degradative organelle to halt certain proteins on their way to the CV.

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