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Chapter 6

General discussion

Studies of the multiple functions of vacuoles have been conducted in fungi, and among those *Saccharomyces cerevisiae* proved a particular useful model system to dissect the genetics and physiology of vacuoles in protein sorting, ion homeostasis, stress response and autophagy (Li & Kane, 2009). The studies in yeast revealed that vacuoles are distinct from other cellular compartments by the acidity, the concentration of lytic enzymes in their lumen, their role in detoxification of a range of compounds, the capacity to react to changes in the conditions outside the cells and the specific lipid composition of their membranes.

Compared to animal lysosomes, the vacuoles of fungi have a larger number of functions, which are mirrored by their complex proteome. Some of these proteins, such as members of the SNARE family, are key players in orchestrating the fusion of prevacuolar compartments and vacuoles or the fission of vacuoles, thereby controlling vacuolar morphology and vacuolar segregation during cell division. Plant vacuoles are very similar to those of yeast, but they are further evolved acquiring multiple new functions. Along with the differentiation and specialization of the host cell, their vacuoles may also differentiate, becoming specialized to store specific proteins (Jiang *et al.*, 2001), compartmentalize and display pigments (Verweij *et al.*, 2008; Faraco *et al.*, 2014), control cell shape and turgor (Leckie *et al.*, 1998), keep aggressive enzymes apart from other cellular components (Jiang *et al.*, 2001), detoxify salt, heavy metals and other molecules coming from the (polluted) environment or produced by the cell and many more similarly specialized functions (Marty, 1999; Eisenach *et al.*, 2015).

It was long thought that plant cells have a single vacuole, and until today that is how a “typical” plant cells is depicted in most textbooks. This idea was overthrown in the 1990s, with reports that some cells may have multiple vacuoles with a different protein contents and thus different functions. Paris *et al* (1996) reported in a particularly influential paper that (some) cells in root tips of barley seedlings contained multiple vacuole like structures, which could be distinguished by the different Tonoplast Intrinsic Proteins (TIPs) in their membranes. Although later studies casted doubt on these early findings it is by now well established that some highly specialized cells may indeed contain multiple vacuoles with different ion and/or protein content (Jiang *et al.*, 2001). There is at present no clear consensus as to how widespread or rare cells with multiple (distinct) vacuoles are.

Due to the large array of different vacuoles in plant cells, it is impossible to identify a single model system for the study of these organelles. Rather, different plant cell types from different species provide insights in different functions of vacuolar specialization. In spite of the enormous and very evident functional variation of vacuoles from different species or cell types, researchers tend to “merge” the data from experiments with a broad range of species, organs and cells types as if they are all the same. The results presented in this thesis show that this to some extent a blunderbuss approach that is full of potential caveats, unless one carefully considers the evolutionary and /or functional relation between the species, genotypes, cell-types and any marker proteins that were used in different studies.

In this thesis, several aspects of the eclectic and chameleonic nature of vacuoles are described by studies that shed new light on the abovementioned peculiarities of these plant organelles. **Chapter 2** provides new insights in the role that vacuoles play in conferring (hyper)tolerance to heavy metals, and in particular to Cu excess, in a plant species (*Silene vulgaris*) that is able to survive on highly polluted soils.

Chapter 3 shows how ancient plasma membrane P-ATPase proton pumps have acquired vacuolar localization conferring a new function to the vacuoles of petal epidermis and several other cell types. The hyper-acidification of the vacuolar lumen that these proteins control is at the basis of, for instance, the color displayed by the petals of several plant species.

In **Chapter 4** the protein sorting pathway to the vacuole of petal epidermal cells is analyzed. It describes the discovery of a novel type of vacuolar compartment, named vacuolino, which in epidermal petal cells coexists with the central vacuole where anthocyanin pigments are stored. Analysis of flower color mutant revealed identified several transcription factors and downstream genes that are essential for (i) the formation of vacuolinos, (ii) and the trafficking of proteins to vacuolinos, or (iii) from vacuolinos to the central vacuole (Chapter 4 and 5).

Hyperacidification of the vacuolar lumen by P-ATPase in specialized cells

While V-ATPases and pyrophosphatases (PPases) were long thought to be the only proton transporters responsible for the acidification of endomembrane compartments in plants (Sze *et al.*, 1999; Maeshima, 2000), it was recently shown that two P-ATPases recruited from the plasma membrane by the acquisition of a vacuolar localization signal (PH5) and from the bacterial strategy for Mg²⁺ homeostasis (PH1) are responsible for the hyper-acidification of vacuoles in specialized cell types (Verweij *et al.*, 2008; Faraco *et al.*, 2014). The dimeric pump consisting of PH1 and PH5 is able to transport protons against an increasing proton gradient resulting in the hyper-acidification of the vacuolar lumen.

The first plant cells in which these transporters have been identified are those in the epidermis of petunia petals. The vacuoles of these cells are specialized to accumulate and display the desired color of anthocyanin pigments in order to attract of pollinating animals that ensure the reproduction of the plant. The strong acidification of central vacuole of these cells is necessary for determine the red color of the anthocyanin pigments that are stored there. In so-called *ph* mutants either the core components of this pump, the P-ATPases PH1 and PH5 are lost, or their expression is strongly reduced (as in *ph3* and *ph4* mutants), resulting in a less acidic vacuolar lumen and a more bluish flower color. Such *ph* mutants are perfectly viable in greenhouse condition – and as ornamental varieties decorate many gardens, balconies, and Amsterdam bridges – they are not found in natural habitats, presumably because the blue-colored flowers are less successful in attracting pollinators.

Detailed phylogenetic analysis revealed that *PH1* and *PH5* appeared during evolution already in early seed plants, predating the appearance of flowers and anthocyanins pigments. *PH5* derived by vertical evolution from plasma membrane P-ATPase proton pumps by the acquisition of a vacuolar sorting sequence in its N-terminus, whereas the appearance of *PH1* homologs apparently involved

horizontal transfer between plants and fungi. PH1 and PH5 homologs are today found in a broad range of Angiosperms, including numerous self-pollinating or wind-pollinated species, which usually have uncolored petals, inconspicuous petals, or no petals at all. These observations strongly suggest that the function of PH1 and PH5 and the generation of a steep pH gradient across the tonoplast are not limited to flower pigmentation and pollinator attraction. Indeed, genetic data indicate that PH1 and PH5 homologs are in citrus varieties (lemons, oranges, pomelos etc.) needed to drive the extreme hyperacidification of vacuoles (reaching pH2) in fruit juice cells (Strazzer et al, manuscript in preparation). Detailed expression studies in tobacco of P-ATPase proton pumps revealed that tobacco expresses its *PH5* homolog in a number of different tissues, including meristematic zones of stems and roots (Oufattole *et al.*, 2000). This suggests a role for vacuolar hyperacidification in developmental programs that shape the plant architecture, which might be clarified by further analysis of gain and loss of function mutants in which *PH1* and/or *PH5* expression is altered.

Vacuolar role in heavy metal tolerance

Since plants are immobile their only way to cope with adverse environmental conditions is the establishment of mechanisms that enable it to withstand such conditions. For example, plants growing on polluted soils containing high concentrations of (toxic) metals have developed several mechanisms to deal with that, either by extruding metal ions or sequestering them, by binding to certain proteins or transferring toxic ions to the vacuole.

Silene vulgaris is a “champion” metallophyte that can withstand particularly high concentrations of toxic metals, like zinc, cadmium and copper. Because of this (rare) character it is a prominent species on (polluted) soils with high metal concentrations. *Silene* ecotypes originating from unpolluted sites show in general less tolerance for heavy metals than the hypertolerant ecotypes from polluted sites, suggesting that heavy metal tolerance evolved repeatedly. **Chapter 3** shows that a *Silene vulgaris* population at a polluted site became hypertolerant by increasing the expression of two Heavy Metal ATPase5 (HMA5) proteins, which are copper transporting P-ATPase transmembrane transporters belonging to the 1B subfamily. One of these, SvHMA5I resides on the tonoplast of the central vacuole, the other (HMA5II) is on the membranes of the ER and gets reallocated to the plasma membrane by vesicles transport when the cells are exposed to high concentration of Cu. If the situation gets even more critical, HMA5II is finally internalized to the central vacuole (Li *et al.*, 2017). The co-expression of these two transporters confers strong tolerance to Cu when expressed in transgenic *Arabidopsis*, probably by the combination of sequestration (hyperaccumulation) in the vacuole and transport to other plant parts. Analysis of Cu levels in tissues overexpressing HMA5I and HMA5II alone or in combination would tell to which extent the activity of HMA5I in the vacuole contributes to the tolerant phenotype. Transport of Cu to the vacuolar lumen by HMA5I, not only contributes to the protect the cells from toxic Cu effects, but also builds a Cu reservoir that can be used by the plant in absence of this essential microelement.

The Cu-regulated reallocation of HMA5II appears a very deeply conserved mechanism, as related Cu transporters in mammals are reallocated in a very similar way upon exposure to excess copper (Petris *et al.*, 1996; Roelofsen *et al.*, 2000; Ke *et al.*, 2006). The evolution of this mechanism might have occurred in common ancestors of plants and animals. Alternatively, this could have evolved independently multiple times, being an example of convergent evolution. In either case, the deep conservation of this complex reallocation mechanism, points towards an optimization of the function of vacuole/lysosome compartments as final destination of the Cu transporter and possibly of the metal itself.

Multiple vacuoles in plant cells

It was long thought that plants cells contain a single vacuole, and textbooks continue to depict a “typical plants cell” like that. However, over the last 20 years several studies provided evidence that at least some cells can have multiple vacuoles, which can be distinguished by differences in ion content, pH of their lumen and/or the presence of different proteins. The notion of plant cells with multiple distinct vacuoles fitted nicely with the discovery that plants possess multiple distinct protein trafficking pathways to the vacuole (Vitale & Raikhel, 1999; Park, 2004). Although there is now strong evidence for coexistence of multiple vacuoles with distinct content in some highly specialized cells, such as mesophyll cells of ice plants which are adapted to a salt rich environment (Epimashko *et al.*, 2004) and motor cells in *Mimosa pudica* involved in rapid leaf movements (Fleurat-Lessard *et al.*, 1997), it is debated whether multivacuolar cells are “the rule or exception” (Frigerio *et al.*, 2008).

Specific issues that complicate this debate are that vacuoles often differentiate along with the host cell, because of which during certain developmental changes cells may briefly contain two kinds of vacuoles, associated with the cellular status before and after the developmental switch. This might explain why data on vacuoles with different protein content in germinating seeds are not always reproducible. Another issue is that the vacuolar identity is often inferred via the localization of heterologous proteins, without considering that these may be mislocalized, or that “very similar” proteins are used in different studies without properly establishing whether they are true orthologs and/or experimental confirmation that “high similarity” is associated with “functional equivalence/interchangeability”. Furthermore, the use of a broad range of cell-types from different species, as if they are all the same, is a recurrent source of erroneous conceptions.

While studying the localization of vacuolar proteins in petunia revealed, as a complete surprise, that these proteins move in epidermal petal cells via an usual pathway, which involves a novel vacuole-like compartment that we dubbed “vacuolino”. **Chapter 4** shows that vacuolinos are vacuolar compartments that coexist with the central vacuole and are an ‘intermediate station’ where proteins briefly reside on their way to the large central vacuole. Vacuolinos are present in petal epidermal cells, which contain anthocyanin pigments, but absent from the underlying unpigmented mesophyll cells. These mesophyll cells directly sort vacuolar proteins to the central vacuole, without a stop on other vacuolar structures, following a pathway that is very similar to the well studied “canonical” pathway

that operates in leaf cells. Vacuolinos are not specific for petunia, but are also present in the epidermis of rose petals (Faraco *et al.*, 2017; this thesis Chapter 4). As roses and petunia belong to respectively Rosids and Asterids, which are the two main clades in eudicots, they are wide spread, at least among eudicots species. It is interesting to analyze whether they are also present in monocots to define if their evolutionary origin is linked with the appearance of Angiosperms.

The next surprise was that mutations in the regulatory genes *ANTHOCYANIN1 (AN1)*, *PH3* and *PH4*, which abolish the synthesis of anthocyanins and the hyperacidification of the central vacuole, also abolish the formation of vacuolinos. In *an1*, *ph3* and *ph4* mutant epidermal petals cells vacuolar proteins are no longer sorted via vacuolinos, but now reach the central vacuole directly via a pathway resembling the canonical pathway active in petals mesophyll or leaves. AN1, PH3 and PH4 are part of a so-called WD-repeat-bHLH-MYB-WRKY (WMBW) transcription factor complex suggesting that among their downstream targets should be a set of genes involved in the genesis of and trafficking of proteins and membranes via vacuolinos. These findings imply that vacuolinos provide an excellent model system to elucidate cellular mechanisms of protein sorting, formation of membrane compartment and interaction between different vacuolar types by forward and reverse genetic approaches, even when the function of these compartments in the petal epidermis is at present unknown.

Chapters 4 and 5 provide examples of how protein and membrane trafficking along the vacuolino pathway can provide a powerful model system to identify novel proteins involved in the formation of endomembrane compartments and or membrane recognition and fusion by genetic approaches. It is well established role that the interaction between SNARE proteins in the membranes of membrane vesicle (vSNARES) and the target membrane (tSNARES) is a key step in the recognition between both membrane and their subsequent fusion (Sztul & Lupashin, 2006). It is long known some SNAREs may interact with transmembrane transporters, thereby regulating activity of the transporter, both in animals and plants (Naren *et al.*, 1997; Deken *et al.*, 2000; Quick, 2006). However, little or no evidence exists that such transporter-SNARE interactions have a role in membrane recognition and fusion. **Chapter 4** now presents strong genetic evidence that PH1, a P-ATPase transporter protein that can interact with vacuolar SNARES, is essential for the trafficking of proteins from vacuolinos to the central vacuole, most likely by mediating the fusion of both membranes.

Chapter 5 describes how additional components of the vacuolinos pathway can be identified by characterization of genes that are misregulated in the transcription factor mutants *an1*, *ph3* and *ph4*. RNAseq analysis identified some 148 genes that were down regulated in all three mutants. Reverse genetic experiments with three such target genes revealed that mutations or RNA interference mediated knock down of one of these genes (NorfA, encoding a long non-coding RNA) cause no obvious defects, whereas in mutants for the other two genes, PAT1 and CAC12.3 vacuolinos are absent. In *pat1* petal epidermis vacuolar proteins are sorted directly to the vacuole, just as in mesophyll cells, whereas in *cac12.3*, the vacuolar markers remain stuck in “punctae” without reaching vacuolinos or the central vacuole. These phenotypes indicate that *PAT1* and *CAC12.3* are active in the vacuolinos pathway and

that they are required for two distinct steps of the sorting to the vacuole. *PAT1* acts presumably at the point in which sorting to the vacuole through the vacuolinos pathway or the canonical pathway bifurcate. *PAT1* encodes a phospholipase and this suggests that specific phospholipids are required for the formation of vacuolinos and could be a distinct character that diversifies them from the central vacuole. We can at this point only speculate on how an enzyme that modifies phospholipids can be crucial for the building of a vacuolar compartment, but the study of the defects of the *pat1* mutant will be crucial in unraveling this process. The *cac12.3* mutant apparently blocks the vacuolino pathway after the point of separation from the canonical pathway. Proteins on their way to the vacuole at this point cannot shift to the canonical pathway and therefore cannot reach the final destination. The protein encoded by *CAC12.3* has little or none homology to proteins of known function. On the other hand, the identification of the nature of the punctuated structures on which vacuolar proteins are blocked in *cac12.3* mutants will be possible by the use of markers for specific membrane compartments. This will be helpful to identify early compartments along the pathway, which could be precursors of the vacuolinos.

Biological function of vacuolinos?

Petal epidermal cells of several flowering plants have been shown to have a papillar structure. These cell shape is important for the definition of the color of the petals and the attraction of pollinators, as shown by snapdragon mutants in which the papillar shape is compromised (Noda *et al.*, 1994). Mutants in regulatory genes controlling the same pathway in different species, including petunia, also result in loss of papillar cell shape in petals (Baumann *et al.*, 2007; Di Stilio *et al.*, 2009). It is tempting to propose that the vacuolinos, which crowd the tip of the papillae, play a role in the shaping of these cells. This is supported by the observation that the height of the papillae is reduced by about 30% in mutants for the WMBW complex in which vacuolinos are lost (unpublished results). Because the basis of the cell cannot expand in the presence of extra vacuoles, which increase turgor, due to the resistance opposed by the neighboring cells, the tip of each cell is the only part that can expand, and this result in the positioning of vacuolino in the apical portion an the growth of the papilla. In this scenario vacuolinos would be part of the pollination syndrome of flowering plants that have increased their chances of getting numerous offspring by specializing their petals for the attraction of pollinators. This hypothesis is at the moment being tested in experiments where bees are offered wild type petunia flowers next to isogenic mutants for different genes hat control vacuolinos formation, and the number of bees visits to the different genotypes is evaluated. These types of experiments have already been used to identify other genetic factors involved in the petunia domestication syndrome (Hoballah *et al.*, 2007; Dell’Olivo *et al.*, 2011; Dell’Olivo & Kuhlemeier, 2013). The outcome of these trials, together with accurate measures of the turgor and dimension of wild type and vacuolinos-less mutants, will tell us whether indeed these small vacuoles are an adaptation of the petals to the interaction with pollinating animals.

Targets of the WMBW complex in different species and different tissues

Transcription regulating WMBW complex(es) are conserved in the plant kingdom. They were initially discovered for their role in activating anthocyanin and proanthocyanidin (tannin) synthesis in a both mono (maize) and dicot species (petunia, Antirrhinum, Arabidopsis). Further studies, primarily in *Arabidopsis* and petunia revealed that the same or closely related WMBW complexes (differing with regard to the MYB partners) regulate a plethora of seemingly unrelated processes, which (at first sight) appear restricted to much smaller groups of species. In *Arabidopsis* WMBW complexes activate, amongst others, also the formation of mucilage in the seed, the formation of hairs (trichomes) on leaves and stems and the differentiation of non-hair cell (atrachoblasts) in the root epidermis. This role of (W)MBW complexes seems limited to *Arabidopsis* and related species, but is non-existent in petunia or maize (Koes *et al.*, 2005; Ramsay & Glover, 2005). In petunia petals the WMBW complex AN11-AN1-PH4-PH3 was to regulate acidity of the central vacuole (Verweij *et al.*, 2008; Faraco *et al.*, 2014), the stability of anthocyanins therein (de Vlaming *et al.*, 1982; Quattrocchio *et al.*, 2006) and the formation of vacuolinos (Faraco *et al.*, 2017; this thesis Chapter 4). Since blue flowering *ph* mutants are unique for petunia and not identified in other species it appeared initially that these processes are limited to petunia and some related species. However, the subsequent findings that downstream genes involved in vacuolar acidification (PH1 and PH5) are widespread among angiosperms (Li *et al.*, 2016; this thesis Chapter 3) and that rose petals also contains vacuolinos-like compartments (Faraco *et al.*, 2017; this thesis Chapter 4), indicates that these processes are more widespread among angiosperms than initially thought. Hence they may be operational in *Arabidopsis* too, raising the question whether they are in any way linked to well-known WMBW-regulated process like trichome development.

In some cases a WMBW complex may activate in different tissues seeming unrelated phenotypic processes through the very same target genes. For example, the AN1-AN11-PH3-PH4 complex activates in petals *PH5*, encoding the proton pump required for vacuolar hyper-acidification, and this results in reddish color of the petals (Verweij *et al.*, 2008). In *Arabidopsis*, the *PH5* homolog *AHA10* is expressed in seeds under the control of the *Arabidopsis* transcription factors TTG2, TTG1, TT8 and GLABRA2 (GL2) (homologs of the above mentioned petunia regulators) and results in the accumulation of tannins and the production of mucilage (Xu *et al.*, 2014; Appelhagen *et al.*, 2015). In this case, conserved transcription factors form a complex that activates the same target structural gene, to induce different cellular programs.

Alternatively, it is possible that homologous WMBW complexes may have different mutant phenotypes in two species, because they acquired completely or partially different sets of target genes during evolution. Transgenic experiments in which one or more WMBW components were swapped between species suggested that the transcription factor proteins themselves are functionally highly conserved and functionally interchangeable (Lloyd *et al.*, 1994; Quattrocchio *et al.*, 1998; Carey *et al.*, 2004), suggesting that the functional diversification of these complexes during evolution relied mostly on changes in cis-regulatory elements within downstream target genes. A recent example concerns the WRKY component of the complex, which is encoded by the *TTG2* gene in *Arabidopsis* and by *PH3*

gene in petunia (Johnson *et al.*, 2002; Verweij *et al.*, 2016). *Petunia ph3* mutants show a shift of petal color as compared to wild-type controls, which is due to the failure to hyper-acidify the central vacuole in petal epidermal cells. Instead, *ttg2 Arabidopsis* mutants, show aberrant development of leaf trichomes, which is never observed in petunia *ph3* plants. Despite the different *ttg2* and *ph3* phenotypes, the expression of TTG2 in petunia *ph3* mutant fully recovered the color and pH phenotype of the petals, again suggesting that the diverse functions of WMBW complexes of distinct species does not result from changes in the transcription factors themselves (Verweij *et al.*, 2016).

Because it is unclear whether also other targets of the WMBW complex are the same in different organ/tissues, or rather, different sets of genes got under the control of the same transcription regulators, we have compared the results of the RNAseq in petals of wild-type and *ph3*, *ph4* and *an1* petunia mutants to those obtained for similar analysis in *Arabidopsis* mutants for components of the WMBW complex (Morohashi & Grotewold, 2009; Gao *et al.*, 2017). **Chapter 5** shows that only a limited number of the WMBW regulated genes are similar in leaves of *Arabidopsis* (induction of trichomes) and petals of petunia (vacuolar hyperacidification, control of anthocyanin biosynthesis, vacuolino pathway). The most striking (rare) similarities are that WMBW complexes in *Arabidopsis* leaves and petunia petals both activate GL2, encoding a homeodomain transcription activator (Rerie *et al.*, 1994), and a similar set of inhibitory R3 MYB proteins (Schellmann *et al.*, 2002; Kurata *et al.*, 2005; Digiuni *et al.*, 2008; Zhao *et al.*, 2008; Wester *et al.*, 2009). However, there is surprisingly little similarity between the repertoires of “structural” genes that are regulated by MBW complexes in *Arabidopsis* leaves and those in petunia petals. This may suggest that the functional differences of *Arabidopsis* and petunia MBW complexes are due to cis-regulatory changes in a large numbers of downstream target genes. However, because this analysis involves a comparison between species (*Arabidopsis* vs petunia), but inevitably, also between tissues (petals vs leaves), it cannot be excluded that WMBW complexes in concert with tissue-specific transcription factors, also contribute to similar pathways. That remained unknown so far.

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