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Chapter 1

General introduction

Yanbang Li, Francesca Quattrocchio and Ronald Koes

Plant vacuoles

All plant cells display a large set of organelles with specific functions, which are generally very similar among cell types and species. Golgi and ER compartments, for example, are indeed very constant among different cell types, as well as mitochondria and chloroplasts. However, vacuoles, which are by far the largest compartments in plant cells, show a broad diversification in shape, dimensions, content and function among species and tissues.

Vacuoles are present in yeast and many unicellular and multicellular algae. Within plants a large central vacuole is observed in a broad range of phylogenetic groups including green algae, mosses, ferns and higher plants. These plant groups are nowadays thought to have evolved independently from unicellular flagellates. In this scenario, the central vacuole might have evolved several times independently in the different groups of Viridiplantae (Becker, 2007).

Plant vacuoles are multifunctional organelles essential for plant development (Marty, 1999) and are often referred to as plant lysosomes. Although vacuoles are morphologically different from mammalian lysosomes, they both play an important role in the degradation of cellular components, which is performed mainly by soluble hydrolases. Moreover, lysosomes and vacuoles also play roles in protein storage, cytosolic ion homeostasis, the sequestration of secondary metabolites and toxic compounds and the maintenance of turgor pressure and cell shape (Marty, 1999; Ferguson, 2015).

The ability of lysosomes and vacuoles to fulfill their functions is dependent on the proteins in their lumen and the transporters in their membranes, such as the tonoplast in plants. Plant vacuoles are however different from mammalian lysosomes – which are essentially lytic organelles (Yamada *et al.*, 2010) – in that plant cells can contain simultaneously different vacuoles with different functions. This is not unique to plants as some protists contain contractile vacuoles that coexist with lytic vacuoles in the same cell (Becker, 2007), but the evolution of vacuoles has continued in higher plants with the acquisition of a series of new functions that accompanied the specialization of different cell types. The distinct vacuolar functions are mirrored by the large variety of proteins that reside in this organelle. In particular, a multitude of transporters on the tonoplast define the traffic of molecules to and from the cytoplasm, resulting in distinct compositions of the vacuolar lumen. These transporters are therefore major contributors in the definition of the identity and function of the different vacuolar types.

The analysis of the proteome from the vacuole of *Arabidopsis thaliana* leaves and cell cultures (Szponarski *et al.*, 2004; Sazuka *et al.*, 2004; Shimaoka *et al.*, 2004; Carter *et al.*, 2004) revealed that besides the expected membrane proteins, such as vacuolar H⁺-adenosinetriphosphatase (V-ATPase), H⁺-pyrophosphatase (PPase) and TIPs, several more proteins crowd this membrane, most of which have unknown or unexpected functions. This list is probably largely incomplete, as it derives from only a few specific cell types of a specific species, and indeed, similar works revealed additional proteins residing on the tonoplast in barley leaves (Endler *et al.*, 2006).

The evolution of vacuoles, characterized by the appearance of new functions, has been marked by the recruitment of new proteins to the vacuole, often through the acquisition of new sorting signals

in the sequence of existing proteins (e.g. Li *et al.*, 2016). The new proteins have conferred vacuoles the capacity to participate in different detoxification processes (heavy metals, salt and other toxic molecules) as well as in the accumulation of valuable compounds (e.g. pigments and other secondary metabolites).

Here we will give, through examples, a short overview of the current ideas about vacuolar functions and vacuolar diversification (also inside a single cell). Although the current knowledge is far from being complete, it does underline the extreme eclectic nature of this plant cell organelle.

The vacuoles as detoxification organ of the cell

One of the functions of vacuoles is that of dynamically storing toxic/valuable molecules that need fine-tuning to avoid negative effects due to too high or too low cytoplasmic concentrations. Tolerant species can grow on media or soils containing high concentrations of salt or other toxic compounds, which is in part due to their sequestration in the vacuoles (Lv *et al.*, 2012). The choice of hyper-accumulation in the vacuole to cope with the presence of toxic molecules in the environment, or to create a stock of scarce compounds is a common strategy in higher plants.

Several minerals are known as plant micronutrients, being necessary for the plant life, but becoming toxic when present at too high concentration. Among these are iron, zinc, copper and manganese, which are essential for all living organisms but toxic at slightly higher concentrations. Their cellular concentrations are tightly regulated by membrane transporters, which mediate the uptake and release into the cytoplasm or sequestration in the vacuoles. Thus, vacuoles function as reservoir for the homeostasis of metals in the cell (Bashir *et al.*, 2016). In plant cells, different families of tonoplast transporters, such as ferroportin (FPN), VACUOLAR IRON TRANSPORTER (VIT) and VIT-like proteins, translocate Fe (and possibly also Mn and Zn) ions into the vacuole, while a separate type of membrane proteins, such as Natural resistance macrophage proteins 3 (NRAMP3) and NRAMP4, release Fe, Mn and Cd from the vacuole into the cytoplasm, when their concentration drops (Pottier *et al.*, 2015). Similarly metal transporter1 (MTP1), MTP3 and Heavy metal ATPase 3 (HMA3) contribute to sequester Zn (and probably also Cd) in excess to the vacuolar lumen, while Zrt/Irt-like protein 1 (ZIP1) remobilizes Zn and Mn from the vacuole to the cytoplasm when required (Milner *et al.*, 2013). Several more of these transporters have been shown to play similar roles for the sequestration and release of different metal ions in different plant tissues and organs, together maintaining a constant level of micronutrients available to fulfill their different cellular functions.

All these ions are necessary for the cell life, however, the growth of the plant on soils rich in heavy metals is strongly inhibited and only some plants have developed an efficient mechanism for the detoxification of large concentrations of these molecules. One example of the important role played by vacuoles in detoxification mechanisms is offered by the strategy of copper (Cu) tolerance in plants.

Copper is an essential micronutrient for all living organisms as a cofactor for fundamental metabolic processes such as respiration, photosynthesis, oxidative stress resistance, ethylene signaling and pigmentation (Puig *et al.*, 2007; Burkhead *et al.*, 2009; Lutsenko, 2010). Copper cycles between two oxidation states, Cu^{1+} and Cu^{2+} . Due to its redox properties, copper can be strongly toxic when

present in excess (Hänsch & Mendel, 2009). Consequently, the concentration of free copper in the cytosol must be carefully regulated. In plant cells, the vacuole serves as an intermediary store of Cu to avoid toxic levels of Cu in the cytoplasm, while Cu stored in the vacuole can be remobilized under Cu limiting conditions (Huang *et al.*, 2016).

In *Arabidopsis*, Cu remobilization from vacuole to the cytoplasm is mediated by one member of the CTR-like high-affinity copper transporter (COPT) proteins, COPT5, which is localized in the tonoplast. Under copper deficiency *copt5* mutants showed defects in growth and photosynthetic activity (Klaumann *et al.*, 2011; Garcia-Molina *et al.*, 2011). In addition, the vacuoles of the *copt5* mutant contained more copper than those of wild-type plants (Klaumann *et al.*, 2011). Together, these results indicate that COPT5 plays an important role in copper reallocation from the vacuole to the cytoplasm.

Interestingly, in *Arabidopsis* the protein involved in sequestering Cu into the vacuole under excess conditions is still unknown. Four *Arabidopsis* Heavy metal ATPases, which use ATP to pump their substrate across different membranes against the electrochemical gradient (Moller *et al.*, 1996), have been implicated in Cu homeostasis (Woeste & Kieber, 2000; Shikanai *et al.*, 2003; Andrés-Colás *et al.*, 2006; Kobayashi *et al.*, 2008). AtHMA6 (PAA1) and AtHMA8 (PAA2) were shown to transport Cu into the chloroplasts (Shikanai *et al.*, 2003; Niyogi *et al.*, 2005). RESPONSIVE-TO-ANTAGONIST1 (RAN1/AtHMA7) has been proposed to deliver Cu to ethylene receptors, even though the subcellular localization of RAN1 still remains unknown (Hirayama *et al.*, 1999; Woeste & Kieber, 2000). AtHMA5 is in *Arabidopsis* encoded by a single gene, which is mainly expressed in roots, and its expression is specifically and strongly up-regulated in the whole plants by excess of Cu (Andrés-Colás *et al.*, 2006). *athma5* mutants are hypersensitive to Cu compared to wild-type plants and accumulate high levels of Cu in root and shoot, indicating that this transporter is important for the capability of the plant to cope with increased Cu in the environment.

In rice, the protein involved in the transport of Cu into vacuoles has been recently characterized. OsHMA4, the rice homolog of AtHMA5, was shown to localize at the tonoplast to sequester Cu into root vacuoles avoiding in this way toxicity under high Cu concentration. *OsHMA4* is expressed in most plant organs and is strongly induced in the roots by high Cu concentrations, but not in the shoots (Huang *et al.*, 2016). The *oshma4* mutant is, similar to *Arabidopsis athma5* mutants, hypersensitive to Cu excess as compared to wild-type. Rice contains a second AtHMA5 homolog, named OsHMA5, which has also been reported to be involved in copper homeostasis. OsHMA5 localizes to the plasma membrane (PM) and is primarily expressed in the roots at the vegetative growth stage, where it is strongly and specifically induced by Cu excess. Mutation in *OsHMA5* results in increased Cu levels in the roots but decreased levels in the shoots. Moreover, unlike *athma5* mutants, *OsHMA5* knockout did not affect Cu tolerance compared to wild-type. All these findings suggest that OsHMA5 is involved in the root-to-shoot translocation of copper (Deng *et al.*, 2013).

We recently showed that most plant species have two types of HMA5 transporters. HMA5I located on the tonoplast of the central vacuole, and HMA5II, including AtHMA5, on the ER. A

mechanism of reallocation upon exposure of the cells to high copper concentration is a characteristics of the plant HMA5II transporter (Li *et al.*, 2017 and this thesis Chapter 2). Under extremely high Cu concentrations HMA5II gets delivered to the plasma membrane where it is thought to translocate excess copper out of the cell. From the plasma membrane HMA5II is internalized and degraded in the vacuole. The combined action of HMA5I and HMA5II is at the basis of Cu hyper-tolerance in plants like *Silene vulgaris*, which adapted to grow on heavily Cu polluted soils.

In human and mice cells, Cu transport from the lysosomal lumen to the cytoplasm is mediated by a copper transporter localized on late endosomes and lysosomes, copper transporter 2 (CTR2), which is a COPT5 homolog. Overexpression of *CTR2* in human cells results in increased copper levels in the cytosol (van den Berghe *et al.*, 2007). In addition, *ctr2* mutant mice showed increased Cu concentrations in lysosome-like compartments (Ohrvik *et al.*, 2013). This indicates that CTR2 (like COPT5 in plants) is involved in the translocation of copper from the storage location (lysosomes). The similarity in the mechanism of copper homeostasis in plant and animal cells goes even further as shown by the presence in mammalian cells of ATP7A and ATP7B, which are AtHMA5 and AtRAN1 homologs. Mutations in these transporters are responsible for Menkes' and Wilson's diseases, respectively, both associated with cellular copper metabolism disorders (de Bie *et al.*, 2007). ATP7A is located in the trans-Golgi network (TGN) when the cells are grown at not toxic level of copper. When exposed to increasing Cu level, ATP7A moves from the TGN to the plasma membrane to translocate excess copper out of the cell (Petris *et al.*, 1996). In the pigmented cells of wild-type mouse melanocyte cell lines, ATP7A localizes to the TGN as in other cell type, but is here also detected within mature melanosomes (Setty *et al.*, 2008), which are lysosome-related organelle within which melanin pigments are synthesized and stored (Marks & Seabra, 2001). ATP7A imports Cu into melanosomes to maintain the activity of tyrosinase (Setty *et al.*, 2008), a key cuproenzyme in melanin biosynthesis (Marks & Seabra, 2001). ATP7B is highly expressed in liver and kidney (Bull *et al.*, 1993) where, at basal copper level, ATP7B resides in the TGN of hepatocytes. At higher copper levels, ATP7B redistributes from the TGN directly to the lysosomes. Here ATP7B sequesters Cu into the lysosome lumen to transiently store Cu excess. Further Cu increase over a threshold value includes the apical exocytosis of lysosomes contain ATP7B with subsequent delivery of the Cu transporter to the apical surface of hepatocytes and the release of Cu into the biliary space (Polishchuk *et al.*, 2014).

Therefore, Cu supply into the lumen of lysosomes/vacuoles is a common features of Cu P_{1B}-ATPases in both plants and mammalian cells mechanism of homeostasis of Cu concentration in the cytoplasm.

Vacuolar sequestration and pH regulation

Vacuoles are the largest H⁺ storage compartments in the plant cell. The proton gradient built across the tonoplast is a large energy source that drives the transport of a plethora of compounds by secondary transporters (symporters, antiporters) and channels.

It was long thought that the accumulation of protons in the plant vacuoles is mainly controlled by two types of proton pumps, the V-PPase and the vacuolar V-ATPase (Drozdowicz & Rea, 2001). V-ATPases are multisubunit proton pumps that are found in plants, animals and fungi and consist of two sub-complexes, the peripheral V₁ complex responsible for ATP hydrolysis and the membrane-integral V₀ complex, which is a proto-conducting channel (Sze *et al.*, 2002). The V-PPase, by contrast, is a much simpler plants-specific proton pump, which consists of a single polypeptide and uses PPI as energy source to pump protons across the tonoplast (Maeshima, 2000). Due to their different energy sources, it was generally assumed that the combined activity of the two pumps can create the proton gradient and the membrane potential necessary to transport compounds against their concentration or electrochemical gradient (Martinoia *et al.*, 2007; Viotti *et al.*, 2013). It has been reported that the activity of these two proton pumps is increased in cucumber (Kabała *et al.*, 2010; Kabala *et al.*, 2013, 2014) and grape (Martins *et al.*, 2012; Leng *et al.*, 2015) upon exposure to high concentrations of heavy metals, suggesting that this would help the plant to power the detoxification machinery in order to cope with this type of stress.

Activities of V-ATPase and V-PPase were promoted in cucumber roots exposed to 10 μM Cu for 6 days, although, the transcription level as well as protein levels of V-ATPase subunit and V-PPase were not affected under excess Cu in comparison to the control (Kabala *et al.*, 2013). Also in Cabernet Sauvignon Berry cells cultivated in medium with high copper concentration, the activity of V-PPase were not affected compared with the control, while the magnitude of the H⁺ gradient generated by V-ATPase is decreased (Martins *et al.*, 2012). Based on the RNAseq analysis on the young third and fourth leaves of grape from the stem apex, one V-ATPase subunit appears to be up-regulated under excess Cu condition. Thus, the effect of excess Cu on the activity of vacuolar proton pumps still remained unclear, opening the possibility the other type of proton transporters contribute to the detoxification of heavy metals in plants.

A vacuolar hyper-acidification mechanism revealed by petunia petal epidermal cells

Vacuoles in plant cells also function as storage of several valuable molecules, including pigments like tannins, anthocyanins and betalains. The mechanism of transport of most of these molecules to the vacuolar lumen is still (partially) unclear, with the only exception of tannins. The (precursors of) tannin molecules are translocated into the vacuole by a multidrug and toxin efflux (MATE) type of membrane transporter (TRANSPARENT TESTA 12, TT12), which consumes the proton gradient exchanging H⁺ for proanthocyanidin molecules (Debeaujon *et al.*, 2001; Marinova *et al.*, 2007). The proton gradient, required to energize TT12 in the seed coat of *Arabidopsis* is built by AHA10, a P_{3A}-ATPase localized on the tonoplast (Debeaujon *et al.*, 2001). The mechanism of accumulation of proanthocyanidins is

probably very old, as this class of pigments is wide spread in the plant kingdom, also among ancient plants like ferns. With the appearance of anthocyanins in higher plants, new mechanisms of sequestration showed up, as suggested by the fact that mutations that affect the expression of the homolog of AHA10 in petunia (PH5) result in a drop of acidification of the vacuolar lumen, but do not affect the accumulation of anthocyanin molecules in the central vacuole (Quattrocchio *et al.*, 2006; Verweij *et al.*, 2008, 2016). This indicates that anthocyanin sequestration to the vacuolar lumen is not dependent from a large proton gradient and suggesting that it relies on a transporter that is distinct from the MATE-type tannin transporter.

The building of a strong pH gradient across the membrane of the central vacuole is not only important to drive transport processes. The color of anthocyanin molecules accumulated in the vacuole of epidermal cells of flowers depends on the metals ions, the presence of copigments and the pH of the vacuolar lumen (Koes *et al.*, 2005). In all species analyzed to date, the expression of the structural anthocyanin genes encoding the enzymes of the pathway is regulated by WD40 (tryptophan-aspartic acid (W-D) dipeptide repeat), bHLH (basic Helix-Loop-Helix) and MYB regulators of transcription (Koes *et al.*, 2005). Recently it was shown that a fourth protein (PH3, a WRKY transcription factor) participates to the regulation of anthocyanins structural genes as an intensifier. These proteins interact with each other forming a WMBW transcription complex, which was strongly conserved during evolution and regulates pigmentation, and other epidermal cell differentiation programs, in many different plant species (Verweij *et al.*, 2016).

In petunia, the structural genes involved in anthocyanin biosynthesis are controlled by the bHLH protein ANTHOCYANIN1 (AN1), the WD-repeat protein AN11 and the MYB protein AN2 (Spelt *et al.*, 2002; Quattrocchio *et al.*, 2006). The epidermal cells of petals from wild-type petunia flowers show a lower vacuolar pH than green tissues (e.g. leaves), which is coupled to reddish flower color. Mutations in seven loci, *PH1* to *PH7*, result in blue flower color, and a higher pH value for petal homogenates, similar to that of other plant organs, like leaves (Quattrocchio *et al.*, 2006; Verweij *et al.*, 2008, 2016; Faraco *et al.*, 2014). The shift of color towards blue in these mutants is due to the pH indicator behavior of the anthocyanin molecules stored in the lumen of the central vacuole of the petal epidermal cells. *PH4* encodes a MYB transcription factor, that, similarly to AN2, can interact with the other components of the WMBW complex to regulate the transcription of at least 40 downstream genes (Spelt *et al.*, 2002; Quattrocchio *et al.*, 2006; Verweij *et al.*, 2016; this thesis Chapter 5). Among these genes are *PH1* and *PH5* that encode two P-ATPase type pumps residing on the tonoplast. Co-expression of PH1 and PH5 is sufficient to rescue vacuolar acidification in *ph3*, *ph4* and *an1* petals (Verweij *et al.*, 2008; Faraco *et al.*, 2014). PH5 encodes a P_{3A}-ATPase that can pump protons into the vacuolar lumen. PH1 encodes a member of the P_{3B}-ATPase proteins, which were firstly identified as facilitators of Mg²⁺ import in bacteria (Smith & Maguire, 1998). In petunia, PH1 has no H⁺ transport activity on its own but can interact with PH5 on the tonoplast and promote PH5 H⁺ transporting activity (Faraco *et al.*, 2014).

The presence of PH1 and PH5 in the genome of several plant species indicates that vacuolar hyper-acidification has made its come up in the plant kingdom before Angiosperms appeared and became a widespread phenomenon among all classes of plants (Li *et al.*, 2016; this thesis Chapter 3).

The genetic and electrophysiological demonstration that these two pumps are required for the hyper-acidification of the vacuolar lumen (Faraco *et al.*, 2014) has definitely shown that specialized vacuoles can differently regulate the pH in their lumen thanks to the presence of different proton transporters which are able to generate larger proton gradient than those built by the combination of PPases and V-ATPases.

Sorting of proteins to vacuoles

Although proteins are all synthesized on ribosomes, their final destination within the cell can be different. Different sorting pathways are entered by the mature proteins depending on specific signals contained in their sequence, or on the absence of those. The default secretion pathway seems to be the sorting to the plasma membrane via vesicles and consequent delivery of the vesicle content and membranes by fusion. The pathway that sorts vacuolar proteins is a variation on this theme and involves traffic of vesicles to the vacuole. Vacuolar sorting signals found in the sequence for instance of the barley proaleurin (a soluble protein accumulated in the vacuolar lumen of the aleurone) bind to vacuolar sorting receptors like the bean BP80 and the *Arabidopsis* vacuolar sorting receptor (VSR) (Kirsch *et al.*, 1994; Paris *et al.*, 1997; Saint-Jean *et al.*, 2010). Several homologs of these genes are present in every plant species, suggesting a certain degree of specificity of the receptors. The secretion of large amount of storage proteins in *vsr1* mutants, and the localization of VSRs in the Golgi and in prevacuoles, indicate that these receptors redirect proteins to the vacuolar pathway, halting their sorting to the plasma membrane for secretion. Prevacuoles later mature and fuse with the central vacuole finally delivering the proteins to their final destination.

It has been shown that the inositol transporter INT1 (Schneider *et al.*, 2008), the tonoplast monosaccharide transporters TMT1 and TMT2 (Wormit *et al.*, 2006), the sucrose transporter SUC4 (Schulz *et al.*, 2011), PH5 (Li *et al.*, 2016; this thesis Chapter 3) and HMA5 (Li *et al.*, 2017; this thesis Chapter 2) are in the tonoplast, but have plasma membrane localized paralogs. This strongly supports the hypothesis that several vacuolar transporters have evolved from plasma membrane proteins, by acquiring a vacuolar sorting signal. A dileucine motif (LXXXLL) was shown to be necessary for the vacuolar localization of several membrane proteins like for instance the monosaccharide transporter ESL1 (Yamada *et al.*, 2010), the type III SUT sucrose transporters (Yamada *et al.*, 2010), HMA5I (Li *et al.* 2017; this thesis Chapter 2) and PH5/AHA10 (Li *et al.*, 2016; this thesis Chapter 3). The truncation of the dileucine motif from the N-terminus was shown to result in the localization of these proteins to the plasma membrane, while its addition to proteins normally sorted to the plasma membrane gives vacuolar localization (Li *et al.*, 2016; this thesis Chapter 3). The acquisition of such sequence by an existing plasma membrane transporter, results in the recruitment of the protein to the vacuolar membrane and the acquisition of a new function for the vacuole.

It is long known that plant cells can contain multiple vacuoles, such as the lytic vacuole (LV) and the protein storage vacuole (PSV), that can differ in size, the set of proteins in their lumens or on their membranes, in particular, the class of aquaporins residing on the tonoplast (Paris *et al.*, 1996; Jiang *et al.*, 2000; Luu & Maurel, 2005; Frigerio *et al.*, 2008). Twenty years after the first description of the presence of multiple vacuolar types in a single cell (Paris *et al.*, 1996), however, the number of reports about distinct vacuoles in one cell remains low, suggesting that this situation is exceptional and related to very specialized cell types. Till now, the knowledge about the mechanisms underlying vacuolar differentiation and protein trafficking to the correct vacuole type remains limited (Zouhar & Rojo, 2009). In particular, it is intriguing to ask how the different vacuolar functions are kept apart for compartments that coexist in the same cells and how the traffic of different membranes, metabolites, ions and other compounds is sorted to distinct vacuoles.

The study of the sorting of PH5, and other vacuolar proteins, to the central vacuole of petal epidermal cells in petunia, showed that these proteins are first delivered to small vacuoles, vacuolinos, and only later to the central vacuole. This suggests the presence of two vacuolar types in petal epidermal cells, which exchange proteins and content (Faraco *et al.*, 2017, this thesis Chapter 4). The same WMBW complexes of transcription factors that regulates anthocyanin accumulation and vacuolar acidification, was shown to control the formation of vacuolinos which coexist with the central vacuole in petal epidermal cells (Faraco *et al.*, 2017, this thesis Chapter 4).

Between the two vacuolar types, proteins and membranes are exchanged along a protein sorting pathway in which vacuolinos represent a intermediate station on the way to the central vacuole. The material exchange between the two vacuolar types is mediated by structural proteins on their tonoplast (PH1) which interact with vacuolar SNAREs and promotes membrane docking/fusion as demonstrated by the failure in delivery of vacuolar proteins from the vacuolinos to the central vacuole in *ph1* mutants. Although the function of these small vacuolar compartment is not yet clear, they represent a good model for the study of the coexistence of multiple vacuoles in plant cells and the mechanism of recognition of distinct vacuolar types that govern the assignment of specific functions to each of them (Faraco *et al.*, 2017, this thesis Chapter 4).

Analysis of the transcriptomes of wild-type and mutants lacking specific components of the WMBW transcription factor complex, brought recently to the isolation of genes that are good candidates to contribute to the formation and physiology of vacuolinos (this thesis Chapter 5). The analysis of the set of genes differentially expressed in wild-type and *an1*, *ph3* and *ph4* petals also identifies proteins unique for this vacuolar type unraveling the mechanisms defining vacuolar identity.

Conclusions

The study of vacuoles in different specialized cell types as well as different vacuoles coexisting in one single cell, revealed that vacuoles are eclectic organelles with highly variable functions that are defined by the set of proteins sorted to their membranes and lumens. The study of the proteomics of different vacuolar types will in the near future help to further understand how vacuolar specialization is achieved

and how this process is regulated during cell differentiation.

Outline of the thesis

The aim of the research described in this thesis is to shed light on the evolution, development and function of plant vacuoles by characterizing genes involved in the trafficking of proteins and membranes to the vacuole, and thereby the formation of vacuoles, or trans-membrane transporters that determining specific vacuolar functions and contribute to the functional divergence of vacuoles in distinct tissues and species.

These genes encode: i) transcription factors and structural proteins orchestrating the formation of a specific vacuolar type, ii) proteins that are involved in the mechanism of recognition between two different membrane types, the docking and the fusion of the membranes and iii) proteins localized on the vacuole which define specific vacuolar function and differentiate one vacuolar type from another.

Among the last category of genes are tonoplast pumps, which are crucial for the detoxification of molecules by vacuolar sequestration, the acidification of the vacuolar lumen and, in general, the control of the concentration of ions and other molecules in the vacuole.

The general introduction **Chapter 1** provides a brief overview of the large repertoire of vacuolar functions. This chapter also briefly reviews the mechanisms of vacuolar pH homeostasis and summarizes the current knowledge on the diversification of distinct vacuolar types coexisting within one cell.

Chapter 2 describes the role of the vacuole in copper tolerance and the mechanism by which some plants survive on highly copper polluted sites. The analysis of the expression and cellular localization of copper transporters of the HMA5 type, from a non-metallophytic species (*A. thaliana*) and a Cu-hypertolerant population of a metallophyte (*Silene vulgaris*) revealed how cells can resist high Cu concentration, by reallocating paralogous copper-transporting HMA5 proteins to distinct cellular membranes. We also report the discovery that this mechanism is conserved between plants and animals.

Chapter 3 describes the distribution in the kingdom of life of two tonoplast P-ATPase transporters, PH1 and PH5, known to be involved in vacuolar hyper-acidification and color determination in petals of flowers. The presence of these two pumps in species that do not produce flowers with colored petals suggests yet unknown functions for vacuolar hyper-acidification in other plant organs. In this chapter, the evolutionary mechanisms that recruited plasma membrane proton transporters and bacterial magnesium translocators to the tonoplast of plant cells to build a strong proton gradient across this membrane, are studied.

Chapter 4 describes the coexistence of two vacuolar types in petunia petal epidermal cells and the mechanism underlying vacuolar differentiation and communication among different type of vacuoles. The genetics of the formation of additional vacuoles (vacuolinos) and their fusion to the central vacuole is also analyzed by the study of mutants affecting these processes.

Chapter 5 presents the transcriptomic analysis of petals from mutants for three transcription factors shown in Chapter 4 to control vacuolinos formation. The comparison to wild type petals

identified candidate genes involved in the formation of vacuolinos. Based on the comparison of the target genes of conserved transcription factors in petunia and *Arabidopsis*, we formulate some ideas of how transcription factors and regulatory networks evolved.

Chapter 6 provides a general discussion of the findings presented in the thesis.

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