SUMMARY

The appearance of endomembrane system during evolution was a key feature in the transition from a prokaryotic to a eukaryotic life style. Possibly, the Last Common Eukaryotic Ancestor (LCEA) had already endomembranes providing functions similar to those of the endoplasmic reticulum, multivesicular bodies and Golgi apparatus. This early complexity of the membrane system probably relied on a preexisting richness at molecular level: a core of proteins involved in transport carrier formation, compartment specificity and membrane fusion. This group of proteins included SNARE proteins, Rab GTPases and tethering factors.

The evolution of membrane trafficking system occurred by gene duplication and diversification of the encoded proteins. It has been shown, for instance, that a basic set of 20 types of SNARE proteins is conserved in all eukaryotes, suggesting that they represent the ancestral SNARE genes present in the progenitor proto-eukaryotic cell. Flowering plant genomes display a higher number of SNARE encoding genes compared to unicellular plants and this increase in complexity might be associated with the acquisition of novel trafficking functions, such as polarized secretion. An higher number of SNARE genes could be correlated with increased specialization of different cells with the advent of multicellularity. However, although SNARE proteins themselves can drive vesicle fusions in vitro, they can cooperate with many other factors to determine material exchange and targeting specificity. In this way the same SNARE can have very different functions, depending on the set of factors with which it interacts. Proteins like Sec1/Munc18 are known to regulate conformational changes of syntaxins, α-SNAP and NSF are involved in SNARE recycling and Rab GTPases in vesicles docking.

Rab proteins belong to a large family of proteins with different localizations all involved in secretory and endocytic pathways. Comparative genomic studies suggest that the LCEA displayed most of Rab family members displayed nowadays by eukaryotes implying that complexity grew not only by increasing gene numbers, but also by the capability of SNARE to alternatively cooperate with multiple regulatory factors.

This thesis investigates different aspects of post-Golgi membrane traffic specificity in vivo to unravel how combinations of SNAREs and interacting proteins shape endomembrane compartments in different steps of cellular processes as well as in different cell-types.

In Chapter II is a short review on plant SNAREs families and functions in which most of the points just mentioned here are discussed.

In Chapter III we apply a new “in vivo” experimental approach, in which we assessed the effects caused by the transient expression dominant negative (DN) forms of different proteins on the secretion of a reporter enzyme (secRGUS). This strategy (Di Sansebastiano et al. 2007) allowed us to detect the functional specificity of two Arabidopsis plasma membrane syntaxins SYP121 and SYP122. These two proteins have very similar sequences and were, therefore, considered, to be fully functionally redundant. However, co-expression of dominant-negative forms of Rab11a and SYP121 results in a decrease of secRGUS secretion, whereas co-expression of the dominant negative forms of SYP122 of Rab11a had no effect. Furthermore, the sorting of the GFP-tagged SYP122, but not that of GFP-SYP121, is specifically altered by this dominant-negative Rab11a mutant. The differential effect of
Rab11a supports the interpretation that SYP121 and SYP122 drive, in spite of their highly similar sequences, distinct secretory events.

The secretory events occurring downstream the Golgi compartments are not completely understood and might involve more cases of functional diversification and/or specialization between species, cell types, or even developmental stages of cells and tissues than proposed by the current widely diffused general models. The traffic to the vacuole, for example, displays a high degree of cell-type dependent variability. Many SNAREs have been shown to be involved in membrane trafficking to the vacuoles in Arabidopsis, including the Qa-SNAREs SYP21 and SYP22, the Qb-SNAREs VTI11 and VTI12, and the Qc-SNAREs SYP51 and SYP52 but till now it proved difficult to understand whether this complexity represents functional redundancy or whether there is a degree of specialization among very similar proteins. To this end it becomes also interesting to compare distinct systems like different cell types or even different plant species.

Petunia hybrida petal epidermal cells offer a great opportunity for an easier, well defined and genetically approachable in vivo experimental system. Petunia is easy to transform, has a well-defined transposon system that proved very useful to identify mutants and genes by forward genetics, and, more important, a large collection of well-defined flower mutants in which endomembrane traffic is affected.

In Chapter IV we describe a simple protocol for the isolation and transformation of petal protoplast from P. hybrida and we show that petal protoplasts fully maintain the tissue-specific features of the cells in the intact tissue, such as gene expression patterns and the characteristic endomembrane organization, within the time required for transient expression analysis. This finding opened the way for the analysis of tissue specific endomembrane structures in protoplasts and at the same time warns against the widely diffused practice of using of “any protoplast” to study the sorting of membrane vesicles and proteins sorting as this can differ substantially from one to another cell type.

In the endomembrane system each organelle maintains and displays several characteristics that make itself “unique” in the cell. One of these parameters is the luminal pH. The steady state pH in the lumen of organelles and vesicles is determined by the activity of proton pumps that generate an electrochemical proton gradient across the membrane and secondary transporters that consume this gradient for the transport of a wide variety of molecules. P. hybrida epidermal petal cells synthesize and accumulate anthocyanins, which are one of the major plant pigments and serve a variety eco-physiological functions. Because the color of these pigments is dependent on pH of the medium in which they are dissolved, they act as natural pH indicators of the vacuolar lumen, where the anthocyanins accumulate, and thus provide a unique tool to identify mutants in which pH homeostasis is impaired. By studying such ph mutants we discovered a new mechanism by which vacuoles are acidified and by which proteins and membrane vesicles reach the vacuole.

In Chapter V, we describe a novel mechanism for the acidification of the vacuole, that involves two P-ATPases encoded by PH1 and PH5. The phenotype of ph1 and ph5 mutants shows that both are needed for the acidification of vacuoles in epidermal petal cells. Previous work showed that PH5 encodes a P3A ATPase proton pump that resides in the tonoplast. We now isolated PH1, using a transposon-tagged allele, and found that this gene encodes a P-ATPase belonging to the 3B subfamily,
which was thought to be unique for prokaryotes. Because the two proteins physically interact, we propose that they might form heterodimers, which could represent the active proton pumping unit. Although this mechanism is active only in petal cells, the forced expression of these two pumps is sufficient to restore vacuolar acidification in ph3 and ph4 petals, which lack expression of PH1, PH5 and at least 10 other genes, and can drive vacuolar acidification also in other cell types (e.g. leaf mesophyll) where they are normally not expressed. This demonstrates that PH1 and PH5 are both necessary and sufficient to drive vacuolar acidification and suggests that both proteins form an efficient acidification unit that can translocate protons across the tonoplast independently from other cell-specific factors.

The investigation of the sorting of these two tonoplast proteins to the vacuole led us to the discovery of a novel pathway by which proteins and membranes are delivered to the tonoplast. Chapter VI describes a novel pathway by which PH1, PH5 and other vacuolar proteins traffic to the tonoplast of the central vacuole. When we used transient expression experiments to follow the localization of GFP-tagged versions of PH1 and PH5 in time, we observed, to our surprise, that both proteins accumulate at first on small vacuolar compartments, that we named vacuolinos, that later fuse to the main vacuolar compartment. Vacuolinos are small colourless endomembrane compartments which can be easily distinguished from the central vacuole based on their dimensions and the lack of anthocyanins in their lumen.

By homology with these Arabidopsis genes we identified two petunia vacuolar SNARE genes: PhSYP22 and PhSYP51 and we showed that GFP-PhSYP22 and GFP-PhSYP51 follow the same protein sorting pathway as PH5-GFP and GFP-PH1: within the first 24 hours they localize on vacuolinos membrane, and only later-on, they become visible on the tonoplast of the main vacuole containing anthocyanins. Other vacuolar markers, like Aleu-GFP, and Arabidopsis SNAREs and tonoplast transporters, also followed the same sorting route indicating that this pathway represents the major way to the tonoplast in petal epidermal cells. Curiously this vacuolino pathway appears to operate only in epidermal petal cells, because in other cells (petal mesophyll or leaf epidermis and mesophyll) the same vacuolar proteins are sorted directly to the tonoplast, without passing through vacuolinos.

Mutations in the genes PH3 and PH4, which encode transcription factors, dramatically reduce the expression in petals of >10 target genes, including PH1 and PH5, and disrupt the formation of vacuolinos. Although forced expression of PH1 and PH5 in ph3 and ph4 mutants is sufficient to restore the acidification of the vacuole, it does not rescue protein sorting via the vacuolino pathway, indicating that the formation of vacuolinos relies on target genes of PH3 and PH4 that are distinct from PH1 and PH5. Mutations in PH1 instead, as well as over expression of PhSYP22 or PhSYP51, impair the fusion of vacuolinos to the central vacuole. These observations strongly suggest that PhSYP51 and PH1 act in concert to mediate the docking or the fusion of the membranes. Preliminary results from biomolecular fluorescence complementation (BiFC or “split-YFP) analysis in epidermal petals cells and yeast two-hybrid assays using a split-Ubiquitin assays that was specifically designed to assays interactions between membrane proteins, provided direct evidence for a physical interaction between PH1 and
PhSYP51 (as well as PhSYP22). The interaction of membrane proteins to influence membranes fusion (this thesis) is a completely new aspect of SNARE function. These exciting new findings will lead in the near future to a better understanding of how cells control the sorting of proteins and vesicles to specific compartments and how different combinations of SNAREs.