Summary

Flowers display a large pallet of different colors determined by the accumulation of pigment in petal cells. A largely diffused class of pigments is the one of the anthocyanins, which are responsible for red and purple colors in many plant species. In petunia, anthocyanins are synthesized in epidermal layer of the flower petal mainly to attract pollinators (e.g., bees and butterflies). Additional factors influencing the flower color are flavonols, metal ions and the pH value in the vacuole. The vacuole is the largest compartment of the plant cell (~90% of the volume) in which these pigments and co-pigments are stored. Because anthocyanins have the capacity to function as natural pH indicators, flowers are reddish when the vacuolar pH is low (acidic) and blue when the pH is high (nearly neutral). Petunia flowers produce anthocyanins early during flower development when the flower bud expands. Soon after the moment that the bud reaches the maximum length the flower will open. Around this moment, the pH of the vacuolar lumen decreases by a process called “vacuolar acidification”.

In plant cells, three types of pumps are known, which pump proton across membranes. One is the vacuolar-type H⁺-ATPase (v-ATPases), a complex consisting of at least 26 proteins, and uses ATP as energy source. The pyrophosphatase (PPases) is a single sub-unit protein and uses pyrophosphate as energy source. The third one is the P-ATPase, which is used to pump protons across the plasma membrane. This protein functions similarly to the Na⁺/H⁺ exchanger in animals and creates an electro-chemical gradient, which is used for ion transport by sym- and antiporters. Mutations in PPase or v-ATPase sub-units often result in lethality or developmental defect, indicating the importance of the pH gradients in the internal compartments of the cells. Research on proton pumping proteins is therefore restricted to biochemical and electrophysiological approaches as mutants are not available.

Fortunately, in petunia seven mutants (ph1 to ph7) have been identified in which the pathway that leads to the acidification of the vacuole is affected. The mutant plants are vital and do not show any severe developmental defects although the flowers are more bluish compared to the ones of wild type plants. Analysis of the anthocyanin content in the corolla indicates that these mutations only affect vacuolar pH and not anthocyanin composition. Regulatory proteins ANTHOCYANIN 1 (AN1), AN2 and AN11 control besides genes in
the anthocyanin biosynthesis also unknown genes in the vacuolar acidification pathway. This was in first instance overseen since an mutants are depleted from anthocyanins and therefore no color shift could be observed. The crude petal extract of an mutants, however, showed an increased pH value of about 1 pH unit. The goal of the project was to get insights in the molecular mechanism of the vacuolar acidification pathway by cloning transposon tagged PH genes and AN1 target genes, which are involved in this process. 

**Chapter 1** reviews the current knowledge about the regulation of anthocyanin synthesis in several model plant species and compares it to the regulation of other apparently unrelated processes: root and leaf hair formation, mucilage production in seeds and seed coat coloration in *Arabidopsis*. All these processes involve a similar protein complex consisting of bHLH, WD40 and MYB transcription regulators. It was shown that the petunia bHLH protein AN1 induces besides the anthocyanin producing enzymes, also an unknown vacuolar acidification pathway.

**Chapter 2** reports the cloning and molecular analysis of the PH4 gene. We cloned PH4 two times independently via (1) transposon tagged mutants and (2) by a yeast two-hybrid as interactor of AN1. ph4 mutants are characterized by an increased pH of the crude petal extract and bluish flowers, indicating that PH4 is required for vacuolar acidification. The data presented in this chapter shed light on how AN1 regulates two separated processes in the same petal cells, by interacting with different MYB partners. The interaction with AN2 results in anthocyanin biosynthesis whiles the recruitment of PH4 (also a MYB protein) activates genes in the vacuolar acidification pathway. Mutations in PH4 also seem to trigger a process called “fading”, a phenomenon by which flower lose coloration and get white upon aging. Little is known about this process but it requires flowers with (1) a high vacuolar pH (ph mutants), (2) a specific anthocyanin composition and (3) the presence of the dominant fading locus.

**Chapter 3** describes the cloning of PH3 from three instable ph3 mutants. Similarity searches revealed that PH3 encodes a protein belonging to the WRKY family of transcription factors that shares very high homology with TTG2 from *Arabidopsis*. TTG2 is involved in several processes like trichome morphogenesis, mucilage formation in seeds and pro-anthocyanidin production in the seed coat. By loss- and gain-of function analysis we discovered that PH3 is genetically controlled by the AN1/AN11/PH4 complex in a way similar to TTG2, which is regulated by TTG1, GL3/EGL3 and GL1. Their parallel
regulation and protein structure raised the question whether PH3 and TTG2 fulfill similar functions in the two (possibly) unrelated pathways. The results from gene-swap experiments in which we transformed \textit{35S:TTG2} into \textit{ph3} petunia mutant show that TTG2 can replace the function of PH3. Whether PH3 is able to complement the \textit{ttg2} mutation in \textit{Arabidopsis} remains to be established.

In order to identify structural proteins involved in vacuolar acidification, we identified AN1/PH3/PH4 target genes through an mRNA profiling approach, which is described in \textbf{Chapter 4}. We found 23 target genes of which 11 are confirmed to show a decreased mRNA level in \textit{an1}, \textit{ph4} and \textit{ph3} but were unaffected in \textit{ph2} and \textit{ph5} mutant petals, (proving that their expression is not affected by an increase vacuolar pH). To test whether the identified target genes are involved in vacuolar acidification, we produced RNAi constructs to silence their expression in wild type plants. For the moment, silencing of two transcripts (\textit{MACF55} and \textit{MAC9F1}) resulted in blue flowers with high pH of the flower homogenate, indicating their involvement in vacuolar acidification. MACF55 shares similarity with proteins belonging to the P\textsubscript{3A}\textsubscript{c}-ATPase proton pumping family and MAC9F1 with unknown proteins from \textit{Arabidopsis}, rice and tomato. AN1 activates \textit{MACF55} and \textit{MAC9F1} directly, without the need of intermediate proteins as shown by experiments in plants expressing an \textit{AN1-GR} construct. Because, for most genes, similarity searches did not give information of their possible function in the vacuolar acidification pathway, further studies are needed to define the precise role of each target gene in acidifying the vacuolar lumen.

\textbf{Chapter 5} shows that the cloning of \textit{PH5}, via transposon tagged mutants, yielded a genomic fragment of the \textit{MACF55} gene, which had been identified in the micro-array hybridization in Chapter 4. \textit{PH5} encodes a P\textsubscript{3A}\textsubscript{c}-ATPase type protein. Members of this class of proteins are known to serve as “engine” for the cell since they establish a chemical gradient across the plasma membrane. The \textit{PH5-GFP} fusion protein clearly showed that this protein defines a new function for this class of pumps as it localizes on the vacuolar membrane in cowpea protoplast. The experiment in which we transformed several \textit{ph} and \textit{an} mutants with a \textit{35S:PH5} construct, revealed that \textit{ph5} can be complemented by this transgene but \textit{an1}, \textit{ph3} and \textit{ph4} not. This suggests that AN1, PH4 and PH3 induce other genes required for PH5 activity. As \textit{PH5} encodes a proton pump and all \textit{ph} mutants and \textit{ph} double mutants show similar shift in pH as \textit{ph5} mutants, we propose that \textit{PH5} is the protein...
that acidifies the vacuolar lumen. This is the first member of the P$_{3A}$-ATPase family to be shown to localize on a membrane different than the plasma membrane. This finding opens larger possibilities of involvement of these pumps in different aspects of cell physiology and sets a first step to the unraveling of the mechanism at the basis of the pH homeostasis in the highly differentiated petal epidermal cells.