Chapter 4

Identification of AN1, PH4 and PH3 target genes through an mRNA profiling approach

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Abstract
Proton gradients are the endocellular forces required to drive the transport of ions and other micro- and macromolecules across membranes. In petunia, mutations in ANTHOCYANIN 1, (AN1), AN2, and AN11 and in PH1-PH7 affect the pH of the vacuolar lumen. Because AN1, AN2, AN11 and PH2, PH3, PH4 and PH6 encode regulatory proteins, they are likely to control transcription of genes involved in an unknown vacuolar pH-regulating pathway. In an mRNA-profiling assay (micro-array and cDNA-AFLP) we isolated of a set of transcripts, which expression is decreased in an1, ph3 and ph4 mutants. All identified target genes are unaffected in ph2 mutants, suggesting that PH2 operates in a different pH-regulating pathway or that PH2 modifies proteins at post-translational level. Transgenic plants in which the expression of two of these target genes was reduced by RNA interference showed blue flowers and increased vacuolar pH in the petal epidermis, demonstrating the role of these genes in acidification of the vacuolar lumen. MAC9F1 shows similarity to proteins with unknown function whereas MACF55 encodes a proton pump. As none of the already described regulators of vacuolar pH (vacuolar-ATPases, pyrophosphatases and enzymes for synthesis of organic acids) are present in this collection of target genes, it is possible that a novel acidification pathway involving not yet described structural genes is responsible for pH regulation of the lumen of anthocyanin accumulating vacuoles in flower petals.
Chapter 4

Introduction

Proton gradients are the major force driving the transport of ions (Cl\(^-\), Ca\(^{2+}\), Na\(^+\), K\(^+\)) and bigger organic molecules (like sugars and hormones) between different cell compartments (Gaxiola et al., 2002; Maeshima et al., 2001). Two types of proton pumps, vacuolar ATPases (v-ATPase) and pyrophosphatases (PPase) are involved in proton transport from the cytoplasm into the vacuole in plant cells. Because mutations in PPases or in sub-units of v-ATPases often result in abnormal development or lethality, the exact contribution of each of these proteins to the acidification process in each compartment is difficult to assess. In petunia petal epidermal cells vacuolar acidification is regulated by the transcription factors ANTOCYANIN1 (AN1, bHLH protein; Spelt et al., 2000;), AN2 (MYB protein; Quattrocchio et al., 1999), AN11 (WD40 protein; de Vetten et al., 1997) and PH4 (MYB protein; Quattrocchio et al., 2006; Chapter 2). Mutant flowers (an1, an2, an11 and ph4) fail to acidify the vacuolar lumen in which anthocyanins are accumulated, resulting in a more bluish flower color (van Houwelingen et al., 1998). Double mutants do not show an additional pH shift suggesting that all these factors operate in one pathway. Because AN1, AN2, AN11 and PH4 genes encode transcriptional regulators and not enzymes or ion-pumps, we hypothesize that they activate a set of structural genes that encode proteins involved in vacuolar acidification. The finding that PH3 encodes a WRKY protein and functions downstream of AN1, AN2, AN11 and PH4 in the vacuolar acidification pathway, did not help any further in this direction since PH3 is also a transcription regulator (Chapter 3). Also the cloning of PH2, which encodes a protein kinase (Spelt, Quattrocchio and Koes unpublished data) did not contribute to unravel the mechanism of vacuolar acidification. To identify target genes of the transcription regulators AN1, AN2, AN11, PH4 and PH3 we performed mRNA profiling experiments. A petunia micro-array hybridization resulted in 10 ESTs that showed a strongly decreased expression in an1 flowers compared to wild-type flowers. Because the micro-array consisted only of a small set of ESTs (~1100 ESTs; Verdonk et al., 2003) we also performed cDNA-Amplified-Fragment-Length-Polymorphism analysis (cDNA-AFLP; Vos et al., 1995) in which we compared transcripts expression patterns from wild type, an1, ph3, ph4 and ph5 petal tissue. From the ~10,000 amplified cDNA fragments we selected 20 fragments, which showed a decreased expression in an1, ph3 and ph4 mutant petals. The expression of the differentials was unaffected in ph2 and
Identification of AN1, PH4 and PH3 target genes

\(ph5\) mutant petals, indicating that the pH change does not affect gene expression. These findings also suggest that PH2 modifies proteins in the pH pathway or that PH2 performs a function in a different pH-regulating pathway. PH5 encodes a proton pump, which presumably acidifies vacuoles (Chapter 5). To verify whether the identified target genes are involved in vacuolar acidification, we used RNA interference (RNAi), and for two genes this resulted in plants having blue flowers and increased vacuolar pH suggesting a role for these genes in vacuolar acidification.

**Results**

**Micro-Array Analysis**

To isolate structural genes of the vacuolar acidification pathway we hybridized a micro-array containing \(\sim 1100\) petunia ESTs (most from petal tissue; Verdonk et al., 2003) with labeled cDNA from R27 (wild type) and W225 (\(an1\)) petal tissue. The hybridization signal of 10 ESTs was more than 7-fold lower in \(an1\) mutant compared to the wild type and 10 ESTs were more than 3-fold upregulated in \(an1\) mutants. Because of the expectation that genes in the pH-regulating pathway are controlled by AN1, we decided to proceed with the ESTs, which are downregulated in \(an1\) mutants and renamed these ESTs **Micro-Array Clones**, or MACs.

**Anthocyanin structural genes function as internal controls of the micro-array hybridization**

Among the 10 ESTs that were down regulated in \(an1\) petals, 4 were known anthocyanin biosynthesis genes. **ANTHOCYANIN 9** (\(AN9\), encodes a GLUTATHIONE S-TRANSFERASE; Alfenito et al., 1998) was 27-fold lower expressed in \(an1\) petals compared to wild type petals. Three other genes **DIHYDROFLAVONOL 4-REDUCTASE** (\(DFR\); Huits et al., 1994), **RHAMNOSYL TRANSFERASE** (\(RT\); Kroon et al., 1994), **CYTOCHROME b5** (\(CYTb5\); de Vetten et al., 1999) were respectively 15-, 9- and 7-fold downregulated in \(an1\) petals. These “internal controls” made us confident that the outcome of the micro-array hybridization was reliable. The 5 other strongly reduced ESTs represent an interesting new class of genes, which might function in the vacuolar acidification pathway.
Identification of genes involved in vacuolar acidification by cDNA-AFLP

Because only a relatively small set of ESTs was available on the micro-array, more mRNAs were analyzed by cDNA-AFLP display. By this approach we compared mRNA expression profiles in wild-type petals compared to an1, ph3, ph4 and ph5 mutant petals. In total we analyzed ~10,000 mRNA fragments of which the majority did not show any relevant intensity difference between the wild types and the mutants. However, 76 transcripts, that we named cDNA-AFLP-Clones (CACs), showed differential expression in one or more ph mutants. Figure 1A shows parts of cDNA-AFLP gels with examples of fragments for each pattern of differential expression. We classified the differential fragments in 6 main groups: 1) downregulated in an1, ph3 and ph4, 2) downregulated in an1, 3) downregulated in ph3. The CACs of other two groups were upregulated in 4) an1, ph3 and ph4, 5) upregulated in...
Identification of AN1, PH4 and PH3 target genes

ph3. Group 6 contains the remaining CACs, which showed different expression regulation (Figure 1A).

Sequencing of 6 fragments of group 2 (down in an1 mutants) revealed that 2 encoded known enzymes of the anthocyanin pathway: CAC4.5 and CAC13.2 encode ANTHOCYANIN 5-O-GLUCOSYLTRANSFERASE (5-GT) and ANTHOCYANIN 3-O-GLUCOSYLTRANSFERASE (3-GT), respectively. Both proteins have been shown to be involved in anthocyanin biosynthesis (Gerats et al., 1985; Yamazaki et al., 2002). The 4 others did not show homology with known proteins from the anthocyanin pathway. Other AN1-regulated genes like DFR, RT and PH3 (Chapter 3) were not detected, which can be explained by the fact that the cDNAs of these genes do not contain EcoRI restriction sites and therefore can not be amplified with the EcoRI adaptors specific primers used in the cDNA-AFLP. Although PH3 contains two EcoRI sites in the 5'-UTR (-45 and -67) and a MseI restriction site at nucleotide 196 (it should generate a product of 241 bp), this fragment was never detected. This may be because an mRNA fragment of another gene that is not controlled by AN1, PH3 and PH4 masks this fragment.

Expression pattern of CAC and MAC in mutants is confirmed by RT-PCR analysis

Because we previously showed that PH3 expression is controlled by AN1 and PH4 (Chapter 3), we focused on the 20 CACs that were down-regulated in an1, ph4 and ph3, assuming that these are the best candidates for genes of the pH pathway. From the MACs that are down-regulated in an1 mutants we have choosen the 3 ESTs for which reliable sequence data was available, for further analysis.

To confirm the expression regulation of the identified target genes we designed gene-specific primers and performed RT-PCR analysis in the different mutants. cDNAs from petals of developmental stage 5, 6 and 7 (from the same pooled petal tissue used in the cDNA-AFLP analysis) were used as template. For CAC7.5, CAC13.10, CAC14.2, CAC16.1, CAC16.2, CAC16.5, CAC4.4 and CAC12.3 we confirmed that their mRNA is reduced in an1, ph3 and ph4 mutant petals (Figure 2A). The CAC8.9, CAC10.6, CAC14.3 and CAC5.6 mRNA levels show similar intensity in wild type and mutant indicating that these fragments are false positive transcripts or that the cloned fragment was dissimilar from the cDNA-AFLP band. mRNAs of CAC6.6, CAC7.0, CAC7.4, CAC8.8, CAC12.1 and CAC13.4 were
hardly detectable by RT-PCR and therefore we could not confirm whether these derive from AN1, PH3 and PH4 controlled mRNAs. 

*MACF55, MAC9F1* and *MAC1D2* were identified based on the lower expression levels in *an1* petals. The RT-PCR analysis revealed that their expression is also regulated by AN1, PH4 and PH3, identical to the confirmed *CACs* from group 1. This suggests that *MACF55, MAC9F1* and *MAC1D2* may be involved in vacuolar acidification rather than anthocyanin biosynthesis.

**Figure 2. RT-PCR analysis of several differentials from group 1.** A) Confirmation of the genetic control of some differentials in petal tissue of flowers at stages 5, 6 and 7 in different mutant backgrounds. *GAPDH* was used as internal control. B) Expression levels of some differentials in petal (flower development stage 5,6 and 7), tube, anthers, pistil (stigma and style) sepal, stem, leaf and root tissue. C) mRNA levels in petal tissue of *an1* complemented with 35S:AN1 (first panel), 35S:AN1 fused to the rat glucocorticoid receptor (35S:AN1-GR, last panel) and an untransformed (none) control plant. Multiple flowers were harvested on time point’s 0, 2 and 24 hrs after treated with DEX, DEX/CHX of CHX.

**CAC and MAC tissue specificity**

To determine the tissue specificity of the identified target genes, we analyzed by RT-PCR their expression in different tissue (petal, tube, anther, pistil, sepal, stem, leaf and root) of the wild type-line R27. The results show that *MACF55* and *CAC16.5* are exclusively
expressed in petal and tube tissue (Figure 2B), supporting their possible involvement in vacuolar acidification since PH3, PH4 and PH5 show the same tissue specificity (Chapter 3, 2 and 5, respectively). CAC16.2 and MAC9F1 are expressed in every tissue of the plant although their mRNA levels in petals and tubes are 5-to 10-folds higher when compared to other plant tissues. This suggests that some of the target genes (e.g. MAC9F1 and CAC16.2) are in the petals under the control of AN1, PH4 and PH3, while their transcription is activated in other tissues by distinct regulators.

**Target genes are directly regulated by AN1**

In previous experiments we have shown that AN1 is a direct activator of DFR transcription and does not require any intermediate factors (Spelt et al., 2000). Since all newly identified target genes are also transcriptionally regulated by AN1, we wanted to know whether this regulation is direct. We therefore, used an an1 mutant that constitutively expresses (from the 35S Cauliflower Mosaic virus promoter) AN1 fused to the ligand-binding domain of the rat glucocorticoid receptor (GR; 35S:AN1-GR). Dexametasone (DEX) treatment releases the AN1-GR protein from the HSP90 protein allowing it to enter the nucleus and activate target genes. an1 flowers harboring 35S:AN1-GR and treated with DEX turned red due to induction of anthocyanin production, demonstrating that the fusion protein was fully functional (Spelt et al., 2000). We measured the expression level of MACF55 and MAC9F1 in a quantitative PCR with gene-specific primers. Figure 2C shows that in an1 petals (middle panel) no target gene transcripts are detected, which is consistence with our data. In an1 petals, which are fully complemented by 35S:AN1 (left panel), the mRNA of the target genes reach wild-type levels, independently from the treatments with DEX or cyclohexamide (CHX, a translation inhibitor). The last panel represents an1 petals, expressing the 35S:AN1-GR trans gene. Treatment with DEX, already after 2 hrs, induces MAC9F1 and MACF55 transcription. When the flower buds are treated with both DEX and CHX, transcripts are detectable 2 hrs after treatment while 24 hrs after treatment they become undetectable. Treatment with only CHX did not trigger transcription of MAC9F1 and MACF55. Because it is essential to know whether translation is completely blocked after 2 hrs, plants that harbor 35S:AN1-GR and pDFR-GUS were tested for GUS enzyme activity after 0, 2 and 24 hrs. No GUS activity was observed 2 hrs after treatments (while the GUS transcript is clearly present), indicating that the translation is completely blocked...
(Spelt et al., 2000). These results show that at least MAC55 and MAC9F1 are directly activated by AN1 without requiring synthesis of intermediate factors.

Some differential fragments share sequence homology with known genes

Each group of target genes contains a majority of CACs for which the sequence did not show any relevant homology to known genes. In most cases this could be due to the small size of the fragment, or to the fact that some bands could not be re-amplified from gel, or the PCR product did not produce reliable sequence data. From the 76 CACs a small number showed high similarity with proteins with an unknown, putative or hypothetical function from other organisms.

To obtain more information about the function of the different MACs and CACs that are down-regulated in an1, ph3 and ph4, similarity searches (BLASTx) were conducted with the available sequence data. Because cDNA-AFLP generates generally short fragments, more sequence data for CAC7.5, CAC12.3, CAC13.10, CAC16.2, CAC16.5, MAC55, MAC9F1 and MAC1D2 were obtained by screening R27 petal specific cDNA library. Figure 3 shows the level of homology of the identified target genes with proteins available in the gene bank.

The MAC9F1 protein contains two domains that are strongly conserved in proteins from rice, tomato and Arabidopsis (Figure 4). In Arabidopsis, only two annotated proteins contain these conserved domains indicating that this type of proteins represents a very small protein family. Although no further information can be deduced from this, the high level of

<table>
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<th>blastX score</th>
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<td>114</td>
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<td>-</td>
</tr>
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<td>7.5</td>
<td>543</td>
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<td>-</td>
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<td>12.3</td>
<td>845</td>
<td>3'-5' exonuclease containing protein</td>
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<td>13.10</td>
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<td>Putative protein (Arabidopsis)</td>
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<td>No open reading frame</td>
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<td>87</td>
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<td>-</td>
</tr>
<tr>
<td>16.2</td>
<td>1405</td>
<td>Identical to CAC14.2</td>
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<tr>
<td>16.5</td>
<td>1538</td>
<td>Cysteine proteinase (Anthurium)</td>
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</tbody>
</table>

MAC

| F55 | 2850 | P-ATPase AHA10 (Arabidopsis) | 0.0 |
| 9F1 | 789 | Hypothetical protein At2g17710 | 2.00^-16 |
| 1D2 | 151 | P70 protein | 7.00^64 |

Figure 3. Similarity of MACs and CACs with proteins from other plant species. The available sequence data was used to perform a BLASTx search. Only the first hit with the e-value is shown.
conservation of these domains suggests that they define a new class of proteins with an, yet, unknown function.

We succeeded in isolating the full-length cDNA of MACF55, which gives extremely high similarity (e-value of 0.0) with the proton pumping ATPase AHA10 from Arabidopsis (Baxter et al., 2005). In Chapter 5 MACF55 is characterized in more detail.

The full-length cDNA of CAC16.5 consists of 1538 nucleotides and shows high similarity (e-value 2.00e⁻¹⁴⁷) to a cysteine proteinase from Anthurium. Cysteine proteinases are involved in maturation of vacuolar proteins but recently the activity as transcription factor of one of such a protein has been reported, opening new possibilities for the function of this family of proteinases (Matarasso et al., 2005).

Fragments CAC16.2 and CAC14.2 were amplified with different primer combinations (MseI+GT / EcoRI+TA and MseI+CC / EcoRI+TA, respectively) but turned out to derive from the same transcript. The 1368 bp cDNA we have isolated seems to be the full-size transcript of CAC14.2/CAC16.2 since this length corresponds with the size of the hybridizing band in a Northern blot (not shown). The CAC14.2/CAC16.2 sequence does not

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Figure 4. Protein alignment of MAC9F1 with the homologous proteins from tomato (EST SGN-E545014), Arabidopsis (accession number At2g17710) and rice (accession number OS0084K11.10). Amino acids colored gray and outlined indicate identical and only gray colored amino acids appear similar. The alignment calculation was performed in the freely available program ClustalW (http://crick.genes.nig.ac.jp/homology/clustalw-e.shtml) and alignments were made in AAA seqVu1.0.1.
show any homology to known sequences. An interesting observation is that CAC16.2/CAC14.2 transcript is not translatable into a long protein, but seems to contain 2 Open Reading Frames (ORF) of 153 bp (short ORF1) and 174 bp (short ORF2). Transient expression experiments in which ORF1 cDNA (including its leader) was fused to GFP, did show GFP signal in petunia petal cells indicating that ORF1 can be translated and it results in a stable protein. Whether ORF1 and ORF2 have a function in pH regulation or CAC16.2/CAC14.2 represents some kind of regulatory RNA has to be investigated.

MAC1D2 shows homology with P70, which is a myosin heavy chain protein and is thought to be part of the cellular motor involved in intra-cellular transport of organelles and vesicles (Sellers et al., 1999). Whether transport of organelles and/or vesicles is required for vacuolar acidification and if MAC1D2 is involved in these processes remains to be analyzed.

CAC12.3 shows relatively high similarity with a 3'-5'-exonuclease domain containing protein of Arabidopsis. This information does not immediately suggest a function of CAC12.3 in vacuolar pH regulation. CAC13.10 shares little homology with a putative protein from Arabidopsis and it remains therefore a question whether and how this gene is involved in vacuolar pH regulation. CAC4.4, CAC7.5 and CAC16.1 are very small fragments and database searches did not provide any information. More sequence of these genes has to be gained in order to get indications of the function of the encoded proteins.

Gene silencing of the new PH genes by RNA interference

To determine whether the identified AN1, PH4 and PH3 target genes are required for vacuolar pH regulation, we used RNA interference (RNAi) to silence the corresponding endogenous gene. Inverted repeat (Jorgensen et al., 1996) constructs were made with the fragments isolated by cDNA-AFLP or micro-array assay. The length of the fragments used in inverted repeat constructs varied from 70 bp up to 499 bp, which has been shown to be sufficient to produce dsRNA to trigger silencing of the endogenous gene (Elbashir et al., 2001). We used wild type petunia plants (hybrid between lines M1 and V30) to generate stable transformants expressing the inverted repeat constructs. In total we made inverted repeat constructs of 11 target genes (Figure 5).

Of the 84 regenerated plants, only 44 plants harbored the constructs. The remaining plants were possibly escapes or represented a recombination event in which the inverted repeat
construct was lost. Some plants transformed with MACF55 and MAC9F1 inverted repeat constructs showed substantial silencing of the endogenous gene and had blue flowers (Figure 6 and Chapter 5). Plants that contained an inverted repeat construct but did not show reduction of their endogenous transcripts, obviously, did not show a mutant phenotype (Figure 5). For the moment it is unclear why most of the inverted repeat constructs did not trigger silencing. Possibly no (or not sufficient) small interfering RNAs (siRNA) were produced and therefore degradation of the endogenous transcripts did not take place. However, this suggestion is not experimentally supported.

<table>
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<th>number of transformants</th>
<th>silenced plants</th>
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<td>287 bp</td>
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</table>

Figure 5. Transformation data of the CAC and MAC inverted repeat constructs transformed into wild type D2228 (hybrid between M1 and V30). For the RNAi construct fragments from 70 bp up to 499 bp were used. From all transgenic plants only 9 in total showed a blue flower phenotype.

Figure 6. Phenotype and genetic analysis of transgenic plants containing RNAi constructs. A) Phenotypes of flowers from plants transformed with MAC9F1, MACF55 inverted repeat or empty vector (control). B) Expression levels in petal tissue of endogenous genes MAC9F1 and MACF55 in RNAi silenced plants. GAPDH was used as internal control.
Discussion

To understand the process of vacuolar acidification in petunia petals we set out to isolate AN1, PH4 and PH3 target genes by micro-array hybridization and cDNA-AFLP transcript-profiling assays. This approach yielded at least 11 target genes, which expression was confirmed to be reduced in an1, ph4 and ph3 mutants. The fact that we found in both micro-array hybridization and cDNA-AFLP screen known genes of the anthocyanin biosynthesis pathway, indicates that our results are reliable and that the identified target genes are good candidates to further investigate the mechanism of vacuolar acidification in petal cells.

Because PH3 expression requires AN1 and PH4 (Chapter 3), we hypothesized that PH3 induces the expression of the target genes since their expression is dependent on AN1, PH4 and PH3 (Figure 2A). However, in AN1-GR plants we could show that transcription of MACF55, MAC9F1 and PH3 (and possibly more target genes) are directly controlled by AN1 without the need of intermediate protein. Probably PH3 functions parallel with AN1 in the activation of the target genes.

The fact that the expression of the target genes is not reduced in ph2 and ph5 petals indicates that the downregulation is not a secondary effect of the increased vacuolar pH. Moreover, the blue flower phenotype of the MACF55 and MAC9F1 silenced plants proves that the pH shift is a consequence of the reduced transcripts.

PH2 encodes a kinase (unpublished data) and mutations in PH2 do not affect the expression of the AN1, PH4 and PH3 target genes, suggesting that PH2 controls the vacuolar pH in a different way or that PH2 modifies proteins in this vacuolar acidification pathway. Whether these are proteins in the AN1, PH4 and PH3 controlled pathway (e.g. MACF55) or proteins in another pH regulating pathway is unknown. We cannot exclude the second possibility because double mutants with PH2 are not available at the moment.

The only two pumping systems that have been shown to contribute to vacuolar acidification in plant cells are v-ATPases and PPases. It was previously found that expression of v-ATPases (subunits A and B) and PPases is unaffected in an1 and ph4 mutants (Quattrocchio et al., 2006). This is consistent with the findings that none of the AN1, PH4 and PH3 target genes encode (subunits of) these proteins. Although our transcript analysis is not complete, the similarity with other (known) genes indicates that we identified a novel vacuolar acidification pathway involving a new vacuolar pump (MACF55, Chapter 5). The observations that mutations in MACF55 results in the same vacuolar pH shift detected in all an and ph mutants,
together with the fact that MACF55 encodes a proton pump, suggests that MACF55 acidifies anthocyanin containing vacuoles. The AN1, PH3 and PH4 target genes that we identified in this study, might regulate MACF55 activity or are involved in another, yet unknown, mechanism leading to vacuolar acidification. Unfortunately, similarity searches in the public database of NCBI did not give useful information for most target genes, and therefore it is unclear how they are involved in vacuolar acidification. Further studies will be necessary to define the precise function of each of these genes in the construction of the pH gradient across the vacuolar membrane. Nevertheless, the isolation of the proton pump MACF55 is a first fundamental setup to unravel the mechanism of vacuolar acidification in petunia petal cells.

Materials and Methods

Plant material and growth
All lines were grown under identical greenhouse conditions and from each line petal tissue of developmental stage 5-6 was harvested, pooled and stored at –80°C for later use. To make a transformable wild-type plants, a hybrid between line M1 and V30, was generated.

Table 1: Petunia lines used

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</tr>
<tr>
<td>V30</td>
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1) R27 is hf1- , hf2- , rt- , an4- and fl-. 2) M1 is hf1- , hf2- , hr2- and fl-. 3) V30 is fl-.  

cDNA-AFLP profile analysis
Total RNA (10 petals each samples) was isolated by using Trizol (GIBCO/BRL, Gaithersburg, MD, USA) followed by polyA’RNA isolation (polyATtract mRNA Isolation System III, Promega). Subsequently, one microgram polyA’RNA was used to synthesize cDNA (SUPERSCRIPT II RNase H <sup>+</sup> Reverse Transcriptase, Invitrogen, USA) according to the manufactures instructions. The cDNA-AFLP analysis was performed as described previously (Vos et al., 1995). We used wild-type plants and isogenic lines carrying single mutations in AN1, PH3, PH4 or PH5 (Table 1). Plants were sown and grown
simultaneously side-by-side. Samples have been harvested (for period of a few weeks), pooled and stored for total RNA isolation. Before performing the cDNA-AFLP, mRNA levels for AN1, PH3, PH4, DFR and GAPDH (control) were measured by RT-PCR to compare cDNA levels in each sample. For the production of the cDNA-AFLP patterns we used primers EcoRI+NN with MseI+NN, resulting in 256 combinations. The fragments produced with each primer combination were separated on sequencing gels. The gels were exposed overnight and visualized by a Phosphor imager (Molecular Dynamics, Sunnyvale, USA). The bands of interest were cut from gel, re-amplified, sub-cloned into pGEM-teasy (Promega) and used for sequencing analysis by Big Dye Terminator technology (Perkin-Elmer Applied Biosystems).

**Construction and hybridization of the micro-array**
The micro-array used in this experiment plus the conditions of the probe labeling and analysis was described by Verdonk et al (2003). For the hybridization R27 and W225 polyA+ RNA (developmental stage 5-6) were labeled with cyanine-3-dCTP and cyanine-5-dCTP, respectively, and used as a fluorescent probe (Amersham, Biosciences AP).

**RNAi Constructs and plant transformation**
To make the RNA interference constructs, a PCR was done with two CAC or MAC specific primers and was ligated into pGEMt-easy (Promega). Next, this insert was cut from pGEMt-easy with EcoRI and the fragments were ligated into, EcoRI linearized, pENTR-Gm entry vector (Invitrogen). The resulting entry clones were used in a LR Clonase recombination reaction (Invitrogen) with destination vector pK7GW1WG2(I) (Plant System Biology, Gent University, Belgium). The resulting RNAi constructs were analyzed by PCR and used to transform petunia (van der Meer et al., 1999). mRNA levels of the endogenous genes of interest were measured by RT-PCR when the plants were flowering.

**Gain of (nearly) full size sequence for the differential fragments**
More CAC and MAC sequence was obtained by screening a petal cDNA library made from line R27 (HybriZAP® 2.1, Stratagene) with 32P-dCTP labeled probe corresponding to the gene of interest or by a PCR on excised plasmid from the same cDNA library (R27) with gene specific and vector specific primes.
References


