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2011

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Povero, G. (2011). *Physiological and genetic control of anthocyanin pigmentation in different species.*

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Chapter 7

Gene ‘swapping’ for the study of alterations generating pigmentation patterns in plants

with Cornelis Spelt, Simona Cardellicchio, Ronald Koes and Francesca Quattrocchio

Abstract

The genetic mechanism generating variation among organisms is still a matter of animated debate in evolutionary biology. Anthocyanin pigmentation in plants is a good model to attempt the definition of the genetic changes generating new patterns. In this chapter, we studied anthocyanin ‘master’ regulators -at the top of the hierarchy in the regulatory network- from different plant species, to assess whether changes in cis-elements in the regulatory region of such genes, rather than changes in their regulators -one level up in the regulatory cascade- are the reason of the appearance of new pigmentation patterns. For this purpose, we “swapped” genomic fragments encoding for anthocyanin MYB regulators, among different species. The activity of the transgenes was then monitored through the activity of the GUS reporter gene contained in the constructs. The display of different pigmentation patterns is coupled with different expression of the MYB genes and the ectopic expression of the same MYBs results in ectopic anthocyanin accumulation. Therefore we expect that changes in the expression of the MYB regulators are at the basis of the acquisition of new pigmentation phenotypes. If the different expression patterns are the result of altered cis-regulatory elements, each gene should maintain its own expression in a heterologous host, but when it higher-level regulators are responsible for the variation, the transgene will mimic the expression pattern of the endogenous gene of the host.

The preliminary results reported here suggest that the second scenario is the most likely one, although additional characterisation of the transgenic plants needs to be carried out.

Introduction

The study of the evolution of development (EvoDevo), aims to understand how DNA sequence changes have directed the evolution of morphological diversity. The central question is how alterations in deeply conserved “master genes” or toolkit genes can have generated during evolution the enormous variety of shapes, colours, and structures that can be observed in nature. The HOX genes of animals provide a typical example of this paradox, as they encode deeply conserved (and often interchangeable) transcription factors that direct the development of body segments with widely different morphologies in distinct species. Therefore, it is thought that the main source of morphological variation comes from alterations in the expression of the conserved toolkit genes, rather than in differences in encoded proteins.

According to the current genetic theory, which is mostly based on work in animals, form evolves largely by altering the expression patterns of functionally conserved proteins. However, whether such changes occur primarily via alterations in cis-regulatory elements or trans-acting regulatory proteins, like transcription factors, is subject of fierce debate (Carroll, 2008).

In the plant kingdom, considerable variation can be observed, for example in the architecture of the plant body, the size and shape of organs, but also metabolite accumulation, and pigmentation patterns (chapter 2; Martin et al., 2010). Cis-acting motifs could play a crucial role also in plants, regardless of the changes in the products encoded by genes. In other words, the action of evolution in plants could be dependent on variation in cis-acting regulatory elements rather than the products that the genes encode. Unfortunately, supporting evidences for this evolutionary mechanism need to

be found, since it is difficult to assess if the evolution of cis-acting motifs has major influence than the changes in the proteins encoded by those genes (Martin et al., 2010). It should also be considered that the expression of a particular gene can result from alterations either in its cis-regulatory sequences and /or alterations in the deployment and function of the transcription factors that control the gene's expression (Gompel et al., 2005).

In this work, we focused on the differentiation of pigmentation patterns, since pigmentation represents a good model to study the genetic changes that generate new patterns in plants, with the advantage that variations can just easily identified by eye (see also chapter 2). In particular, we studied the specification of anthocyanins, which are important and ubiquitous molecules that provide the red/purple/blue colours familiar in many flowers and fruits (Holton and Cornish, 1995). Anthocyanin biosynthesis is crucial for the attraction of pollinators and seed dispersers, for the signalling of fruit quality, and protection against predators and other stresses (Willson and Whelan, 1990; Smillie and Hetherington, 1999; Neill and Gould, 2003). Anthocyanin pigmentation patterns and their specification in different species are described in detail in chapter 2.

To approach the study of the mechanisms that generated pigmentation patterns, we need to identify the genes representing the best candidates for "master regulators". Such regulators are genes (see also chapter 2) that, if mutated in activity or expression pattern can give rise to completely new patterns of accumulation of the final product. It is known that transcription factors belonging to the MYB class, together with basic helix-loop-helix (bHLH) and WD40 proteins, form a complex (MBW complex) that regulates the expression of anthocyanin biosynthetic genes (Koes et al., 2005). Among these anthocyanin regulators, the MYB genes look like the best candidate master regulators, according to the following considerations:

- R2R3-MYB transcription factors belonging to the PhAN2 clade regulate anthocyanin biosynthesis in all analyzed plant species (Allan et al., 2008), and even small changes in these proteins can have a strong effect on phenotype (Schwinn et al., 2006);
- there is much experimental evidence that the expression of one such MYB gene alone is enough to trigger pigmentation in virtually any tissue of different species (see chapters 5 and 6 of this thesis);
- MYB proteins are functionally extremely conserved and interchangeable even among very distantly related species (e.g. the PhAN2 protein is functionally interchangeable with C1 from maize (Quattrocchio et al., 1998; Quattrocchio et al., 1999).

Genes encoding for bHLH and WD40 factors controlling anthocyanin biosynthesis would be a less good choice because i) although bHLH are expressed exclusively in pigmented tissues and have been shown to directly activate transcription of structural anthocyanin genes, they operate downstream of the MYB genes (Spelt et al., 2000). Indeed, MYBs activate transcription of bHLHs, as we also showed in chapter 6. On the other hand, ii) the WD40 regulators have been shown to be ubiquitously expressed (Koes et al., 2005) and cannot be involved in determining accumulation patterns which are tissue or organ specific. Therefore bHLH and WD40 surely are not the master genes we are looking for.

It has been long thought that only the combined expression of the bHLH and the MYB factors can induce pigmentation in tissues that do not accumulate anthocyanins, as shown for example by Butelli et al. (2008). Therefore, it would seem necessary that changes in cis-element in the HLH gene as well as in the MYB gene must have occurred, in order to generate new pigmentation patterns. Alternatively, changes in the regulatory network upstream the HLH and the MYB might result in new pigmentation phenotypes. In both cases it would be necessary to make changes in at least two

regulatory pathways in a coordinate way (to ectopically express the MYB in one tissue and the HLH in another would not lead to any new pigmentation pattern at all, according to this view).

However, in chapter 5, 6, and in the experiments presented here, we show that in different species anthocyanin MYB regulators activate transcription of the bHLH gene, and that consequently are alone able to give strong pigmentation in almost all plant tissues. As a consequence of this observation, it becomes clear that the only change of the expression pattern of the MYB gene is sufficient to activate the pathway in a new tissue (i.e to create novel pattern). Starting from these considerations, it can be supposed that during evolution new pigmentation patterns could have resulted from i) changes in cis-elements in the promoter region of MYBs (high in the hierarchy of the network activating anthocyanin biosynthesis), which result in dramatic changes in expression domain, or ii) modifications in the expression of the next level of regulators, like transcription factors necessary for the expression of the MYB genes. These two options/theories can be also proposed for the study of pathways involved in formation of other patterns, but for the moment too few studies are available, and we cannot define which of these mechanisms is at the basis of (most of) the variation observed.

In this work, we chose to “swap” selected MYB genes (*PhAN2* and its homologues involved in anthocyanin production in different species) among petunia, tomato, and Arabidopsis, all species displaying different pigmentation patterns. We monitored the expression of the transgenes studying the activity of the GUS reporter gene introduced in each construct. GUS analyses revealed for each gene if cis-regulatory sequences drive or not different expression in the host. This could allow to assess whether changes in cis-elements in the regulatory region, rather than changes in their regulators (one level up in the regulatory cascade) could have brought to the appearance of new pigmentation patterns.

Our preliminary results suggest that the differences observed among the species analyzed likely reside in higher level regulators, since the transgenes often mimicked the expression pattern of the endogenous gene. The approach carried out in this study can elucidate the origin of diversification of pigmentation patterns, and could be extended to other evolutionary studies.

Results and Discussion

MYB homologous genes activate the anthocyanin pathway in different species

As described before, R2R3-MYB transcription factors belonging to the PhAN2 clade regulate anthocyanin production in many plants (Allan et al., 2008). If ectopically expressed in the original species or in heterologous hosts, these genes are sufficient to trigger pigmentation of virtually any tissue, indicating that the encoded proteins are functionally interchangeable among distantly related species and act as “master regulators” of the anthocyanin pathway in most, if not all species (see also chapter 2).

In chapter 5 and 6 of this thesis we described the effect of ectopic expression (driven by the *35SCaMV* promoter) of MYB genes, like *SlANT1* and *SlAN2* from tomato, and *PhAN2* and *PhAN4* of petunia in the homologous host species, and demonstrate that this results in the activation of the entire anthocyanin pathway in the whole plant. We focused for example on *PhAN4*, a gene that stimulates anthocyanin production in petunia, and is required for the colouration of the anthers (Spelt et al., 2000). We expressed *PhAN4* in petunia under the *35SCaMV* promoter, and this resulted in the production of anthocyanins not only in anthers, but also in petals and leaves (chapter 6).

Here we show the effect of ectopic expression of *PhAN4* in another species, such as tomato. We chose a wild type tomato accession (‘MicroTom’, which bears anthocyaninless fruits),

transforming it -via *Agrobacterium*- with the construct *35S:GFP:PhAN4*, described in chapter 6. Twelve transgenic lines were generated, most of them showing a strong anthocyanin-phenotype in almost all tissues (Figure 1A-1C). Intense purple pigments were easily visible in flower and fruit starting from the first stages of development (Figure 1A-B-C). Furthermore, synthesis of anthocyanins was stimulated in vegetative tissues, especially in leaf veins (Figure 1A-C). The presence of the GFP protein in the construct allowed us to localize PhAN4 in the nuclei of leaves, flowers, and fruits (Figure 4D). This result confirms that the ectopic expression of only a specific R2R3 MYBs, such as PhAN4, is sufficient to trigger anthocyanin production in normally acyanic tissues, also in a heterologous host (in this case tomato).

Given that PhAN4 acts as a 'master regulator' of anthocyanin genes that activates transcription of the bHLH gene *AN1* and the MYB gene *MYBb1*, and the structural genes, like *DFR* and many others, we hypothesized that changes in the expression patterns of such MYB genes, by alterations in either cis- or trans-regulatory elements, were the major factor responsible for the appearance of new pigmentation patterns during evolution. To test this idea we transformed MicroTom tomato plants with a construct containing the entire *PhAN4* gene with its own ~3kb regulatory region (construct named *PhAN4transgene*, also described in chapter 6), to see if it was active in the host species, and affected the pigmentation pattern in tomato. Phenotypic observations and analysis carried out on transformed plants showed that *PhAN4transgene* was not expressed in transgenic tomatoes, and, consequently, that the target genes for the synthesis of anthocyanins were not activated (data not shown). This suggests that the genomic fragment used was not sufficient for the activation of the anthocyanin pathway in tomato, although the same construct drives anthocyanin deposition in anthers of petunia. It is possible that the expression of some other tomato endogenous regulators is required to get anthocyanin gene expression and pigment production.

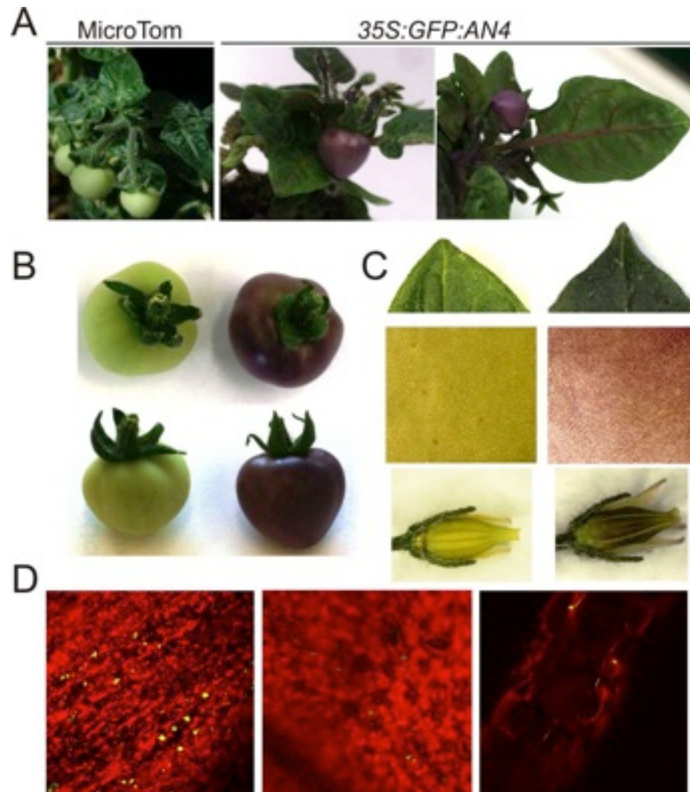

Figure 1. Anthocyanin accumulation in *35S:GFP-PhAN4* tomato plants.

(A) Strong anthocyanin-phenotype of transgenic *35S:GFP:PhAN4* plants compared to wild type plants (MicroTom line) lacking the transgene. Anthocyanins are visible in all plant parts, especially in fruits and leaf veins.
(B) Detail of fruits at green stage of development of a transgenic *35S:GFP:AN4* line (left) compared to wild type (right).
(C) Micrographs of leaf sections, fruit skin, and young flowers comparing MicroTom (left) to *35S:GFP:AN4* tissues (right).
(D) Confocal microscope images showing the location of PhAN4-GFP in the nuclei of cells of young flower (left image), leaf (middle), and root (right image). GFP fluorescence is seen as green staining, autofluroescence of anthocyanins in red.

Choice of the MYB genes for the swaps

The MYB gene *PhAN2* and its homologous were selected from petunia and Arabidopsis, and transferred in petunia and tomato, in order to analyze different pigmentation patterns driven by genes that did not yet diverge so much from each other. Indeed, we focused on the species *Petunia hybrida* and *Solanum lycopersicum*, both belonging to the *Solanaceae* family. In *P. hybrida* petals and anthers are usually pigmented, whereas anthocyanins are hardly present in the fruit and vegetative parts. In *S. lycopersicum*, common wild type ecotypes (red-fruited domesticated tomatoes) accumulate anthocyanins only in vegetative tissues, but not in flowers and fruits (Gonzali et al., 2009). We also extended our analysis to *Arabidopsis thaliana*, which displays unpigmented flowers and siliques, whereas anthocyanins can be produced in vegetative tissues, especially under stress conditions (see chapter 2). The "*AN2*-like" MYB genes chosen for this study were: *PhAN2*, required for petal pigmentation in petunia (Quattrocchio et al., 1999), *PhAN4*, involved in the colouration of the anthers in petunia (Spelt et al., 2000) (chapter 6) and*AtPAP1*, required for anthocyanin accumulation in vegetative tissues of Arabidopsis (Borevitz et al., 2000).

Swap experiments revealing the activity of the transgene in the host plants

In order to study the expression patterns driven by the selected MYB genes *PhAN2*, *PhAN4*, and *AtPAP1*, we prepared constructs containing their entire genomic fragments including promoter, coding sequence with introns and exons, and a fragment of 500 to 1000 bp at the 3' end. To be able to monitor the expression of the regulators directly, we inserted the GUS coding sequence into these genes in frame with the MYB coding sequence to obtain the constructs *pAN2:GUS-AN2, pAN4:GUS-AN4, pPAP1:GUS-PAP1,* which express a fusion of GUS and the respective MYB proteins. As a positive control we used *pDFR:-GUS*, in which the promoter of the petunia *DFR* gene drives the expression of GUS.

We used the petunia line W115, which has unpigmented petals and anthers due to inactive *an2* and *an4* alleles, and wild type tomato (MicroTom accession) for the transformation with the above mentioned constructs. After transformation, GUS assays on transgenic plants allowed us to follow the promoter activity in the transgenics, independently from the production of anthocyanins. The results of the swaps showing the detection of GUS activity are summarized in Figure 2 (transformation in petunia) and Figure 3 (transformation in tomato).

In all transformed petunia plants GUS activity was detected in specific tissues of the flower, such as anther and petal (Figure 2A-B-C), but not in other organs of the plant (data not shown). In particular, GUS colouration was observed in anthers and petals of petunia W115 lines containing *pPAP1:GUS-PAP1* (Figure 2A-B-C) and, to a lesser extent, in petals of the *pAN2:GUS-AN2* transformants (Figure 2B). The construct *pDFR:GUS* worked properly as positive control for this experiment (Figure 2A). It should be pointed out that a significant "background" GUS staining (see "Protocol A" of the paragraph "GUS staining" in material and methods) was seen in the anthers of all petunia lines, including the untransformed plants (negative control; Figure 2A). This "background

effect" was avoided (Figure 1C) using another system for the GUS staining (see 'Protocol B' of the paragraph 'GUS staining' in material and methods).

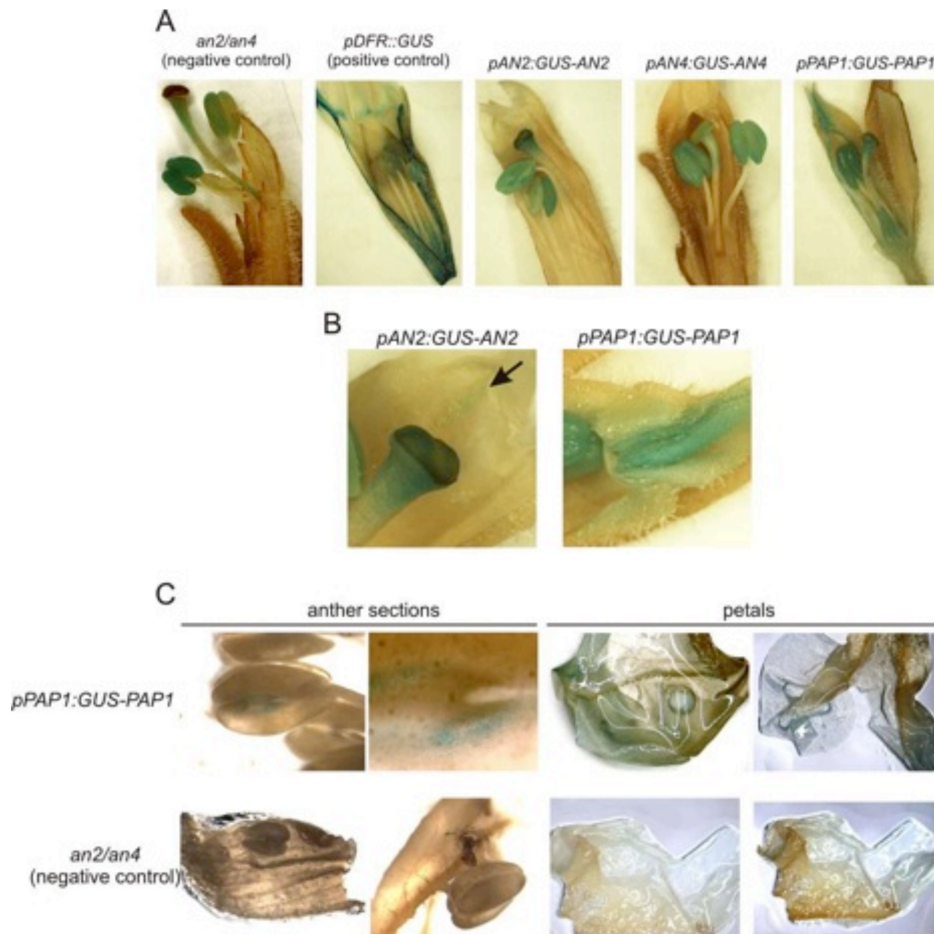

Figure 2. Expression of *pAN2:GUS-AN2*, *pAN4:GUS-AN4*, and *pPAP1:GUS-PAP1* in transgenic petunia plants. See material and methods for the different protocols of GUS staining showed in this Figure.
(A) Overview of the GUS staining in all the lines, including the negative (*an2 an4*) and positive (*pDFR:GUS*) controls.
(B) Details of petals that highlights GUS activity in flowers transformed with the chimeric constructs containing the genomic fragment *pAN2:GUS-AN2* and *pPAP1:GUS-PAP1*.
(C) GUS staining in anthers and petals of *pPAP1:GUS-PAP1* transformants in comparison with untransformed controls (*an2/an4*).

The detection of *pAN2:GUS-AN2* expression in petals and *pAN4:GUS-AN4* expression in anthers was expected, since *PhAN2* and *PhAN4* are normally expressed in these organs (Quattrocchio et al., 1999; Spelt et al., 2000). Interestingly, also *pPAP1:GUS-PAP1* was expressed in transgenic petals and anthers (Figure 1A-B-C), but not in leaves (data not shown), reflecting the pattern of the endogenous genes *PhAN2* and *PhAN4*, rather than expression pattern of *PAP1* in the original species, because in Arabidopsis the expression of *PAP1* is limited to sepals and other organs, especially vegetative tissues, but not petals (http://www.arabidopsis.org/servlets/TairObject?id=29514&type=locus). This would suggest that for this (trans-)gene the pattern of expression mimics the one of the host, meaning that the differences in pigmentation between Arabidopsis and petunia probably reside higher-level regulators of PAP1 and AN2/AN4.

In tomato, GUS background was detected in the anthers of the wild type (Figure 3A). Interestingly, all the transformants, especially the ones containing the *pPAP1:GUS-PAP1* transgene, displayed intense GUS activity in fruit sections and particularly in regions surrounding the seeds, like the placenta (Figure 2A-B). GUS activity was absent or at most very weak in the wild type (Figure 2A). It is known that the endogenous *SlAN2* gene is not expressed in the flesh of tomatoes (Povero et al., 2011), although nothing is known about its expression in seed and embryo. In chapter 6 we propose that the SlAN2 gene from cultivated tomato might be mutated in its promoter, and that therefore it is not expressed in fruits, while the *SlAN2* gene from the *Aft* accession, which is highly expressed in fruits, might represent the wild type allele. We also showed that the *SlAN2* allele from the *Aft* accession is active when introduced in normal red tomato cultivars and is able to induce anthocyanin accumulation in their fruits. This implies that the regulatory mechanisms involved in the activation of *SlAN2* in fruits are still active in the cultivars with acyanic fruits.

Interestingly, GUS activity for *pPAP1:GUS-PAP1* was detected also in tomato leaves (Figure 3C), probably mimicking the pattern of expression of the endogenous *SlAN2* gene. However, if future works will detect *SlAN2* expression in leaves and especially around the seeds, then the detection of GUS activity in our transgenic tomatoes could be explained by the mimic of the expression pattern of the *SlAN2* gene of the host.

In conclusion, these data suggest that the differences in expression patterns of these MYB regulators, and consequently pigmentation patterns in Arabidopsis, petunia, and tomato, do not result from differences in the cis-regulatory elements of these genes but from differences in the upstream higher-level regulators.

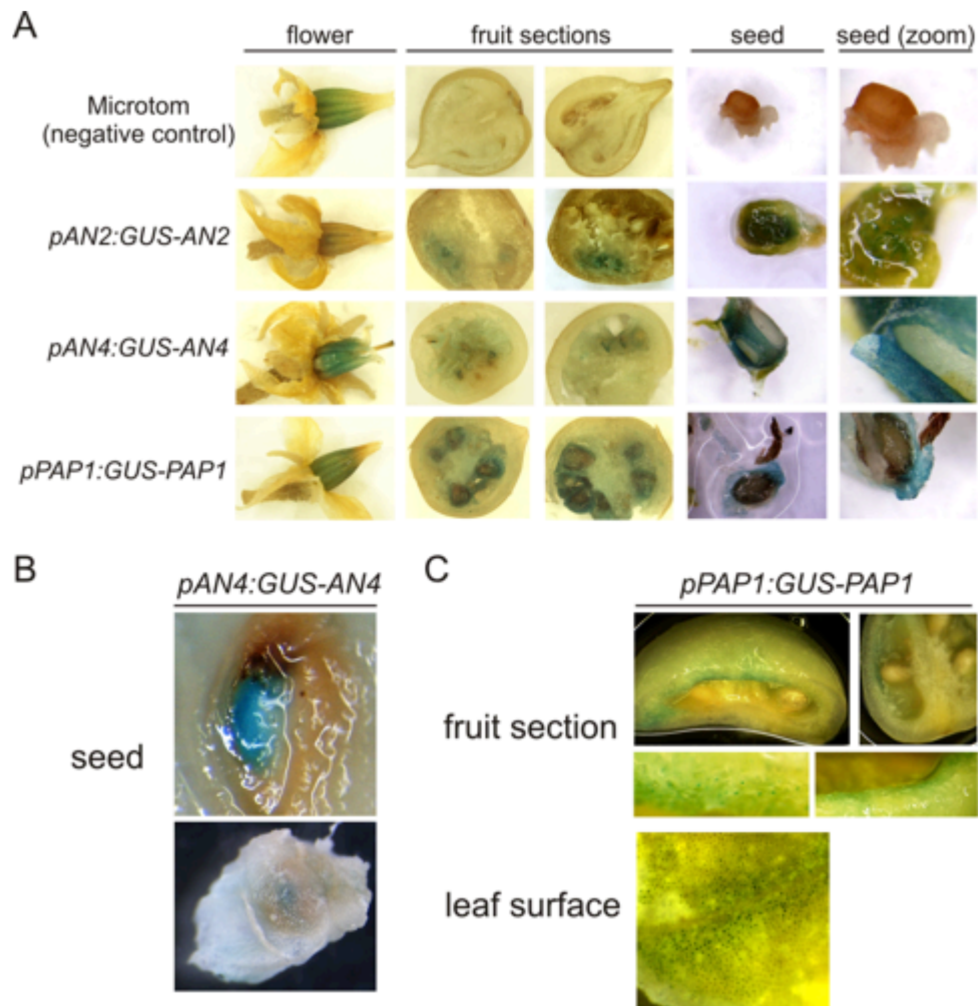

Figure 3. GUS staining showing the activity of chimeric gene constructs in tomato (MicroTom). See material and methods for the different protocols of GUS staining showed in this Figure.

(A) Visualization of GUS expression in tissues of tomato plants transformed with the genomic fragment containing *PhAN2*, *PhAN4*, and *AtPAP1*. GUS staining was mainly visible in anthers, seeds, and placenta of the fruit.

(B) Details of seed and placenta of tomato plants transformed with *pAN4:GUS-AN4*.

(C) Micrographs of fruit sections and leaf surface of plants transformed with *pPAP1:GUS-PAP1*.

In all these pictures it is clear that the GUS activity is localized in the nucleus of the cells (as expected as GUS is expressed as fusion protein to the MYB factor)

Conclusions and biological significance of the work described

The relatively young discipline Evo-Devo tries to explain how highly conserved "master genes" can have generated during evolution the enormous variety of shapes, colors and structures that we observe in nature. Very conserved groups of genes control morphological traits in animals, inflorescence architecture and deposition of anthocyanin pigments in higher plants. The mechanism of pigment deposition in plants, gives a nice experimental system to unravel how the very same protein could generate distinct pigmentation patterns and gives a key to look at how evolution have shaped variation.

An approach like the one carried out in this study can assess whether the activity of specific promoters will maintain in heterologous hosts the same pattern shown in the original species. If this is the case, it would provide a strong evidence that differences in the cis-regulatory elements in the gene are responsible for the appearance of new pigmentation patterns. If, on the other hand, the promoters assume in heterologous species the expression pattern of the endogenous gene, it would mean that changes in upstream regulators (regulators of regulators) are responsible for the differences in expression of the MYB genes and, consequently, the different pigmentation patterns.

In case of the MYB genes that we studied here, it appears that the major cause for the divergence of their expression patterns are alterations in the upstream regulatory network. In agreement with this, for example, GUS activity for the construct containing *AtPAP1* was detected in anthers and petals of transformed petunia, and in tomato leaves, likely following the pattern of the endogenous genes. However, the current results need to be confirmed and extended with additional data, such as, expression analyses of transgenes and endogenous genes in different organs for example. Such analyses are currently underway, as well as new transformations using Arabidopsis as a host. This experimental direction will be crucial to establish to what extent the evolution of distinct pigmentation patterns relied on mutations in cis-regulatory elements in these master regulators of the anthocyanin pathway as opposed to alterations in the upstream trans regulatory network. However, it cannot distinguish whether such differences in the upstream regulatory network involve alterations in proteins coding sequences or in cis-regulatory elements of upstream regulatory genes, as long as the molecular identity of these genes is unknown.

Matherial and methods

Plant material and growth conditions

The petunia (*Petunia hybrida*) line used for the transformation was W115 (*an2 an4* mutant). Tomato (*Solanum lycopersicum* L.) cv. MicroTom was used as control for the experiments illustrated in this work. Seeds were sown in pots and plants were cultivated under normal conditions in a glasshouse. Plants were grown under normal greenhouse conditions (16 hours light, 8 hours dark, maximum temperature 30 °C, minimum 20°C). Transgenic tomato plants for different constructs were grown side by side.

Preparation of the constructs

For the preparation of *35S:GFP:PhAN4* and *PhAN4transgene* constructs we followed the procedure described in chapter 6.

To study the expression patterns driven by the different MYB genes, we prepared constructs containing the whole gene region (~2 Kb promoter, coding sequence, with introns and exons, and a fragment of 500 to 1000 bp at the 3' end). We generated chimeric genes where each promoter drives expression of a protein fusion of the GUS coding sequence and the MYB sequence (Figure 4). For this

purpose we used the 'MultiSite Gateway® Pro' kit (Invitrogen, Carlsbad, CA), which subdivides the procedure in two main steps: i) BP recombination reaction between each attB-flanked DNA fragments (our inserts: MYB promoter-GUS gene-MYB gene + trail; see Figure 4) and the appropriate attP-containing donor vector, to generate an entry clone and ii) LR recombination reaction between the multiple entry clones (containing our inserts separately) and a Gateway® Destination vector using LR Clonase™ II Plus to generate an expression clone (see Figure 4).

Genomic DNA from tomato tissues was isolated as described previously (deVetten et al., 1997). PCR reactions were performed using the "Phusion® High-Fidelity DNA Polymerase" (Finnzymes, Ratastie 2, 01620 Vantaa, Finland). The amplification of the single fragments for the preparation of the constructs was performed using the following couples of primers (attb-sites are underlined):

GUS gene:
Fw: GGGGACAACTTTTCTATACAAAGTTGGCATGTTACGTCCTGTAGAAACCC
Rv: GGGGACAACTTTATTATACAAAGTTGTTTGTTTGCCTCCCTGCTGCGGTT

pPAP1:
Fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTGATAGGTCCCATAAGCAA
Rv: GGGGACAACTTTGTATAGAAAAGTTGGGTCCATGGAACAAAGATAGATAC

PAP1 (gene + trail):
Fw: GGGGACAACTTTGTATAATAAAGTTGCCATGGAGGGTTCGTCCAAAGG
Rv: GGGGACCACTTTGTACAAGAAAGCTGGGTAGCCCATATGAGAATCTGCAC

pAN2:
Fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTAACTCTTCCACCAACACATCA
Rv: GGGGACAACTTTGTATAGAAAAGTTGGGTGCATGATGTATAGTTCTCACT

AN2 (gene + trail):
Fw: GGGGACAACTTTGTATAATAAAGTTGTTATGAGTACTTCTAATGCATCAA
Rv: GGGGACCACTTTGTACAAGAAAGCTGGGTGAGGAGAGTTGCGGTAGGCT

pAN4:
Fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGTGGATAGGAAAAATGGGATTGG
Rv: GGGGACAACTTTGTATAGAAAAGTTGGGTTCATGATTGTGATGAATAAGT

AN4 (gene + trail):
Fw: GGGGACAACTTTGTATAATAAAGTTGTCATGAAAACTTCTGTTTTTACGT
Rv: GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTGCATTCAATGAGCATGAAATCCA

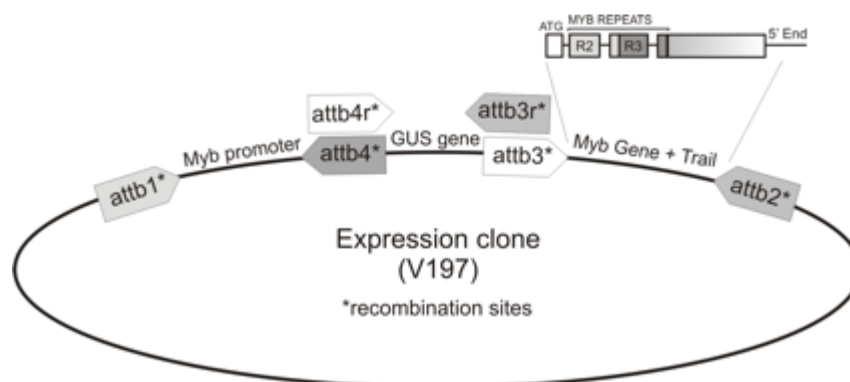

Figure 4. Schematic representation of the expression vector used for the "swap" study.

Petunia and tomato transformation

Transgenic plants of petunia expressing the selected MYB genes were obtained by *Agrobacterium tumefaciens*–mediated leaf disc transformation (Quattrocchio et al., 2006) of the inbred *an2 an4* line W115 (also known as Mitchell or Mitchel diploid). For tomato plant transformations, we followed the protocol described by Zuluaga et al. (2008) transforming seedlings of the MicroTom control line.

GUS staining

Protocol A. For the results showed in figures 2A-B and 3A, we performed the GUS staining incubating overnight the collected samples in a solution containing 1mM X-gluc in 50 mM Na_2HPO_4, at pH 8.0. After the incubation, ethanol 70% was used for washing and samples were visualized under the microscope.

Protocol B. For the results showed in figures 2C and 3B-C, the following procedure was carried out: samples were collected, incubated for 20 minutes at room temperature, and then washed (on ice) in the staining buffer (0.5 M sodium phosphate buffer pH 7.2, 10% Triton X-100, 100 mM potassium ferrocyanide, 100 mM potassium ferricyanide), without X-Gluc. Afterwards, the staining solution was substituted with a new staining solution containing also X-Gluc 2mM. Samples were first incubated for 20 minutes under vacuum (on ice), and then they were incubated overnight at room temperature. Afterwards, the staining buffer was removed, and samples were washed with ethanol at room temperature. Then the samples were treated with a FAA fixative (50% ethanol, 10% glacial acetic acid, 5% formaldehyde) for 30 minute at room temperature. Finally, the fixative was removed, and a solution 70% ethanol was added. GUS activity was visualized under the microscope.

References

Allan, A.C., Hellens, R.P., and Laing, W.A. (2008). MYB transcription factors that colour our fruit. Trends in Plant Science **13,** 99-102.

Borevitz, J.O., Xia, Y.J., Blount, J., Dixon, R.A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12,** 2383-2393.

Butelli, E., Titta, L., Giorgio, M., Mock, H.P., Matros, A., Peterek, S., Schijlen, E.G., Hall, R.D., Bovy, A.G., Luo, J., and Martin, C. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol **26,** 1301-1308.

Carroll, S.B. (2008). Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. Cell **134,** 25-36.

deVetten, N., Quattrocchio, F., Mol, J., and Koes, R. (1997). The an11 locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. Genes & Development **11,** 1422-1434.

Gompel, N., Prud'homme, B., Wittkopp, P.J., Kassner, V.A., and Carroll, S.B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. Nature **433,** 481-487.

Gonzali, S., Mazzucato, A., and Perata, P. (2009). Purple as a tomato: towards high anthocyanin tomatoes. Trends in Plant Science **14,** 237-241.

Holton, T.A., and Cornish, E.C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7,** 1071-1083.

Koes, R., Verweij, W., and Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends in Plant Science **10,** 236-242.

Martin, C., Ellis, N., and Rook, F. (2010). Do Transcription Factors Play Special Roles in Adaptive Variation? Plant Physiology **154,** 506-511.

Neill, S.O., and Gould, K.S. (2003). Anthocyanins in leaves: light attenuators or antioxidants? Functional Plant Biology **30,** 865-873.

Povero, G., Gonzali, S., Bassolino, L., Mazzucato, A., and Perata, P. (2011). Transcriptional analysis in high-anthocyanin tomatoes reveals synergistic effect of *Aft* and *atv* genes. Journal of Plant Physiology **168,** 270-279.

Quattrocchio, F., Wing, J.F., van der Woude, K., Mol, J.N.M., and Koes, R. (1998). Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant Journal **13,** 475-488.

Quattrocchio, F., Verweij, W., Kroon, A., Spelt, C., Mol, J., and Koes, R. (2006). PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell **18,** 1274-1291.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., and Koes, R. (1999). Molecular analysis of the *anthocyanin2* gene of *Petunia* and its role in the evolution of flower color. Plant Cell **11,** 1433-1444.

Schwinn, K., Venail, J., Shang, Y.J., Mackay, S., Alm, V., Butelli, E., Oyama, R., Bailey, P., Davies, K., and Martin, C. (2006). A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus Antirrhinum. Plant Cell **18,** 831-851.

Smillie, R.M., and Hetherington, S.E. (1999). Photoabatement by anthocyanin shields photosynthetic systems from light stress. Photosynthetica **36,** 451-463.

Spelt, C., Quattrocchio, F., Mol, J.N.M., and Koes, R. (2000). *anthocyanin1* of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell **12,** 1619-1631.

Willson, M.F., and Whelan, C.J. (1990). The evolution of fruit color in fleshy-fruited plants. American Naturalist **136,** 790-809.