Physiological and genetic control of anthocyanin pigmentation in different species

Povero, G.

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Chapter 2

Specification of anthocyanin pigmentation patterns in plants

with Francesca Quattrocchio and Ronald Koes

1.1 Anthocyanins: natural functions and possible applications

Plant secondary metabolism involves the production of a wide array of molecules that are not essential for basic growth and development, but have important roles in resistance to stress, pollination and seed dispersion, as well as in the interaction of plants with microbes, fungi, and other plants (Nascimento and Fett-Neto, 2010).

Anthocyanins represent a class of flavonoid/phenolic secondary metabolites that confer pigmentation, providing the red and blue/purple colours familiar in many flowers and fruits (Holton and Cornish, 1995). These compounds are synthesized to attract pollinators and other animals for seed dispersal, and are stored in the acidic vacuole of specialized cells (Holton and Cornish, 1995; Koes et al., 2005; Tanaka and Ohmiya, 2008). Anthocyanin accumulation has been shown to have a large repertoire of functions ranging from signalling fruit quality and ripening (Willson and Whelan, 1990), protection against various kind of biotic and abiotic stresses in vegetative tissues, providing a screen from photoinhibition caused by high levels of visible light (Smillie and Hetherington, 1999), and protection from oxidative damage (Neill and Gould, 2003).

The attractive purple tomatoes producing anthocyanins in their skin (Figure 2A; described in chapters 1, 4 and 5) are just an example of coloured fruits, as in nature many types of berries show intense anthocyanin pigmentation. The esthetical value of anthocyanins in fruits, cut flowers and decorative plants confers commercial value to these compound, and the vast knowledge available on the molecular biology of anthocyanin synthesis allows the modification of flower colour, for example in ornamental crops. In 1987, Meyer and colleagues demonstrated for the first time that genetically modified (GM) plants can contribute significantly to breeding of new colors in ornamental cultivars. Although until now only few commercial GM ornamental varieties are on the market, it is expected that more products will appear over the next years, when GM plants -in particular for non-food crops- might become more acceptable to the consumers and to the authorities. Huge opportunities for application of GM plants in the flower business are waiting to be exploited. An interesting example is the possibility of creating cultivars of chrysanthemum, gerbera, and rose harbouring blue flowers by engineering the anthocyanin pathway towards the accumulation of delphinidin-derived anthocyanins (Tanaka and Brugliera, 2007; Gould et al., 2008). Genetically modified carnation cultivars, such as "Moonshadow™" and "Moonlight™", are already available. These transgenic lines represent two interesting examples of new varieties displaying modified flower colour (http://www.gmo-compass.org/eng/gmo/db/92.docu.html). Petals of "Moonshadow™", accumulate delphinidin derivatives that contain an additional hydroxyl group at the C-5', compared to the anthocyanins accumulated by common carnation. This has been achieved by introducing a *DFR* and the *F3'5'H* genes from petunia in a white carnation variety (a *dfr* mutant; Tanaka et al., 2009). Also pink-flowered varieties of lobelia (*Lobelia erinus*) have been transformed with *F3'5'H* from lisianthus, and this has lead to the introduction of delphinidin synthesis and blue flower colour (Tanaka et al., 2005). Already in 2004, the Japanese company Suntory, produced the first biotechnology-driven "Blue Roses" by transformation of a *F3'5'H* gene, (http://www.suntory.com/news/2004/8826.html). A new GM rose variety ("Blue Rose APPLAUSE") produced recently by the same company, displays 100% blue pigments (delphinidin) in the petals (http://www.suntory.com/news/2009/10592.html)Document19.

The positive reaction of the market to these new products gives reason to think that more transgenic varieties will be developed for commercially interesting species.

Besides their attractive and protective roles in plants, a growing interest for anthocyanins comes from findings related to their health properties in human diet, and to possible applications in the preparation of cosmetics like crèmes and other products. Over the past decade we became aware of a vast array of health benefits arising from the consumption of fruits and vegetables in relation to the presence of secondary plant metabolites, including anthocyanins and other flavonoids, because of their well-documented anti-oxidant effects (Prior, 2003). Anthocyanins entered the human diet in ancient times and have also been part of the traditional herbal medicines used by North American Indians, Europeans, and Chinese. Anthocyanin-rich extracts (habitually derived from dried leaves, berries, storage roots, or seeds) have been used historically to treat diverse conditions like hypertension, pyrexia, liver disorders, dysentery and diarrhea, kidney stones and urinary tract infections, and the common cold (Konczak and Zhang, 2004).

In more recent times, anthocyanins have been shown to be active in preventing coronary heart disease and cholesterol-induced atherosclerosis and have anti-inflammatory and anticarcinogenic activities (Prior, 2003; Lila, 2004; Galvano et al., 2007). A still increasing number of scientific studies have shown that anthocyanins improve vision and blood circulation (Konczak and Zhang, 2004), decrease capillary permeability and fragility, and are involved in membrane strengthening (Lila, 2004). Although the biological effects of anthocyanins and flavonoids are ascribed to their antioxidant activity (Pietta, 2000), it is also proposed that they may affect signalling pathways in animal cells (Meiers et al., 2001; Williams and Grayer, 2004).

Whatever the real origin of their precious properties, it is certain that the presence of high levels of anthocyanins and other flavonoids is a desirable attribute for crops species. However, most crops contain sub-optimal levels of such compounds, therefore an increasing number of approaches have been adopted to stimulate the production of flavonoids in plants (Schijlen et al., 2004). Due to the effect of these compounds on human health, the production of food plant varieties with increased contents in anthocyanins, could become one of the main targets of plant biotechnology in the next years.

1.2 Anthocyanin biosynthesis and regulation

The ubiquitous and dispensable nature of pigments for plant viability has made possible to identify flavonoid/anthocyanin mutants in several species. This has facilitated the genetic and molecular dissection of the pathway, which is highly conserved among higher plants.

Anthocyanins are synthesized through a branch of the more general flavonoid biosynthetic pathway (Winkel-Shirley, 2001; Schijlen et al., 2004). The most common anthocyanins were first isolated in Germany in 1915, and included pelargonidin (from the red form present in *Pelargonium*, geranium), cyanidin (from the purple colour of *Centaurea cyanus*), and delphinidin (from the blue colour of *Delphinium*). All other anthocyanins originate from these compounds through different degrees of hydroxylation, *O*-methylation, glycosylation, and acylation (Schijlen et al., 2004).

Genes involved in this biosynthetic pathway belong to two different classes: those encoding the enzymes that catalyze the step-by-step construction of the anthocyanin molecule (structural genes), and those regulating the expression of the structural genes (regulatory genes). Regulatory genes encode transcription factors (TFs) and other regulatory proteins, which control the activity of the promoter of the structural genes, modulating in this way the temporal and spatial coordination of their transcription.

Two main classes of TFs have been shown to regulate anthocyanin production in all studied species: R2R3 MYB-type and bHLH-type TFs (Schijlen et al., 2004; Quattrocchio et al., 2006; Allan et al., 2008).

The current idea of the regulation of anthocyanin biosynthesis is based on a multitude of experimental data indicating that these two types of TFs physically interact with each other and, together with a "WD40" repeat factor (WDR), form a MYB-bHLH-WDR transcription complex directly responsible for the activation of the structural gene transcription (Koes et al., 2005; Quattrocchio et al., 2006). The physical interaction between these TFs is mediated by a specific motif in the R3 repeat of the MYB domain and the N-terminal region of the bHLH protein (Goff et al., 1992; Zimmermann et al., 2004). The WD40 regulators are proposed to work through post-translational activation of the MYB-bHLH complex. Several studies, among which the ones mentioned in this chapter, contributed to a model for the regulatory network controlling anthocyanin synthesis and accumulation that has proven to be conserved in all plant species investigated so far (Figure 1).

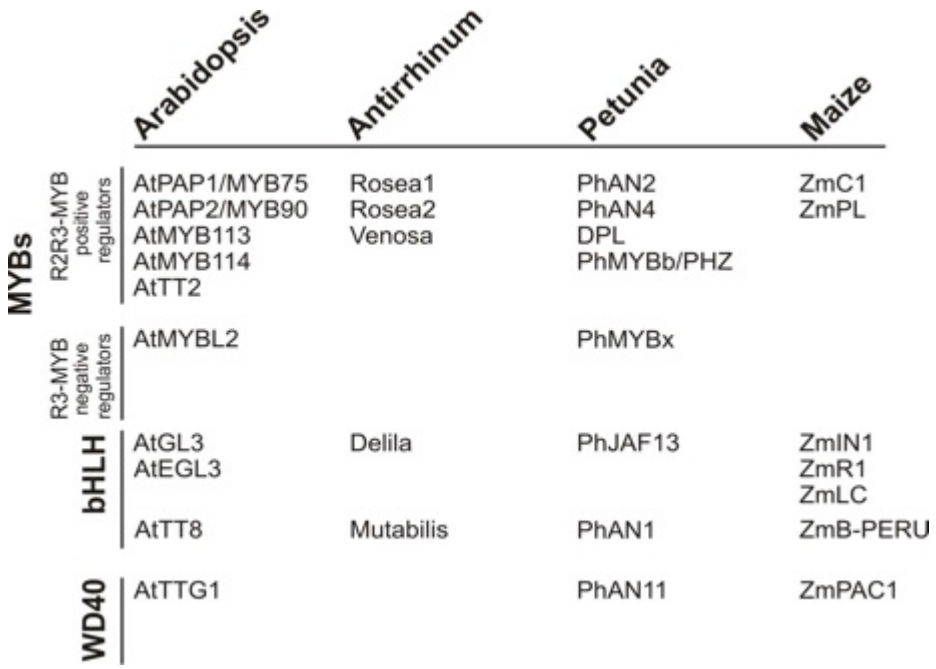

Figure 1. Transcription factors belonging to the MYB-bHLH-WD40 gene families involved in anthocyanin regulation in different model species.

1.3 Anthocyanins in flowers

Pigmentation in flowers is coupled with the choice of some plants to attract pollinators in order to efficiently produce offsprings. Most of the pink, red, orange, scarlet, purple, and blue pigments found in flowers are anthocyanins. Other flavonoids, such as aurones, chalcones, and some flavonols, carotenoids and other minor pigments, play a more limited role in flower colour (Gould et al., 2008).

Flower pigmentation is a key factor in the pollination strategy (pollination syndrome). In insect-pollinated species, single-gene mutations can lead to modifications in flower color and in this way affect the number of visits by the chosen pollinator or favour those of alternative ones. Both conditions are not favourable to reproduction as they result in a lower number of successful visits (Bradshaw and Schemske, 2003; Hoballah et al., 2007).

Specific pigmentation patterns are crucial to determine the rate of successfull pollinations by: i) influencing the number of visits and the effectiveness of pollen transfer, and ii) providing guides

for the location of pollen and nectar, or for more suitable landing regions on the flower (Shang et al., 2011).

Specific pigmentation patterns can be observed in flowers of different plant species. These patterns are highly variable in different species, but also among flowers of the same species in relation to age, and even among different areas inside the same corolla (Shang et al., 2011). The display of pigmentation patterns on petals includes the generation of spots, stripes, irregular blotches, venation, or combination of these, due to the different colouration of individual cells within the corolla (Sasaki and Takahashi, 2002; Medel et al., 2003; Heuschen et al., 2005; Lunau et al., 2006; Ushimaru et al., 2007; Shang et al., 2011).

Many studies describe how anthocyanin biosynthesis is regulated in species that present different reproductive strategies. The white-flowering model plant Arabidopsis is wind pollinated and the coloured flower species Antirrhinum and petunia are both pollinated by insects. The comparison of the regulation of anthocyanin production in species with different pigmentation patterns gives the opportunity to understand how such differences are generated.

1.3.1 Anthocyanins in Arabidopsis

In Arabidopsis, anthocyanins accumulate in vegetative tissues, whereas they are absent in the flower. In particular, anthocyanins and other flavonoid pigments can be produced in variable amounts in leaves, stems, sepals, trichomes, and seed coats, depending on light level, presence or absence of stresses, and nutrition, while they are not synthesized in roots, petals, or stamens (Figure 2B).

The major anthocyanins found in Arabidopsis are glycosylated and acylated cyanidin derivatives (Bloor and Abrahams, 2002). The MYB proteins PAP1, PAP2, MYB113, MYB114, and MYBL2 have been shown to be involved in anthocyanin biosynthesis in this species (Borevitz et al., 2000; Dubos et al., 2008; Gonzalez et al., 2008; Matsui et al., 2008). The R2R3-MYB PAP1, PAP2, MYB113, and MYB114 activate tissue-specific anthocyanin accumulation (Borevitz et al., 2000; Gonzalez et al., 2008). Another R2R3-MYB, named *TT2*, controls the proanthocyanidin production in seeds (Nesi et al., 2000), while, the R3-MYB protein MYBL2 inhibits anthocyanin biosynthesis by interfering with the formation of the MYB-bHLH-WD40 complex (Dubos et al., 2008; Matsui et al., 2008). In general, expression of any of the R2R3-MYB activators from the *35SCaMV* promoter results in induction of anthocyanin production (Borevitz et al., 2000; Gonzalez et al., 2008) while expression of any of the anthocyanin related R3-MYBs from the same promoter reduces anthocyanin pigmentation. Besides its role in specifying the fate of epidermal cells, the R3-MYB CAPRICE (CPC) represses anthocyanin production when ectopically expressed in Arabidopsis (Zhang et al., 2009) and in the heterologous hosts tobacco (Zhu et al., 2009) and petunia (Kroon, 2004).

The combination of activator- and repressors-MYBs to fine tune the pattern of anthocyanin deposition has been shown in different species. Two bHLH factors GLABROUS3 (GL3), and ENHANCER OF GLABRA3 (EGL3) control anthocyanin biosynthesis in Arabidopsis vegetative tissues (Nesi et al., 2000; Zhang et al., 2003). A third bHLH protein, TESTA GLABRA 8 (TT8) is involved in the regulation of proanthocyanin, anthocyanin, and mucilage biosynthesis (Baudry et al., 2006). *TT2* overexpression, results in ectopic expression of *TT8* e.g. in roots (Nesi et al., 2000), indicating that TT2 may regulate TT8, (similar to of the mechanisms shown in petunia for the induction of *AN1* expression by *AN2*; Spelt, 2000).

The WD40 factor TTG1 is required for anthocyanin synthesis, as well as trichome development, root hair development, seed coat pigmentation and morphology and has been shown to be functionally homologous to the petunia AN11 protein (Walker et al., 1999).

Other levels of regulation of pigment deposition are recently getting discovered. For example, the coloration of the stems of *Arabidopsis thaliana*, (with the strongest anthocyanin

pigmentation at the junction between rosette and stem) is an accumulation pattern controlled by the miR156-targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes (Gou et al., 2011) otherwise known to be involved in flower transition.

Another example are three members of the *LATERAL ORGAN BOUNDARY DOMAIN (LBD)* family, LBD37, LBD38, and LBD39 which are negative regulators of the anthocyanin pathway (Rubin et al., 2009).

1.3.2 Anthocyanin pigmentation in Antirrhinum

In the model plant *Antirrhinum majus*, yellow aurone and cyanidins are present in both inner and outer epidermal layers of the corolla lobes (Schwinn et al., 2006). To a lesser extent, cyanidins are also present in the epidermal layers of the corolla tube (Jackson et al., 1992; Schwinn et al., 2006). The combination aurones/cyanidins provides a visual target for pollinating bumblebees, attracting them to the mouth of the fused corolla (Schwinn et al., 2006).

Distinct species of *Antirrhinum* display a lot of variation in intensity and patterning of the anthocyanin pigments in the flower. Many of these species lack anthocyanins (acyanics), or are very palely pigmented. However, also many palely

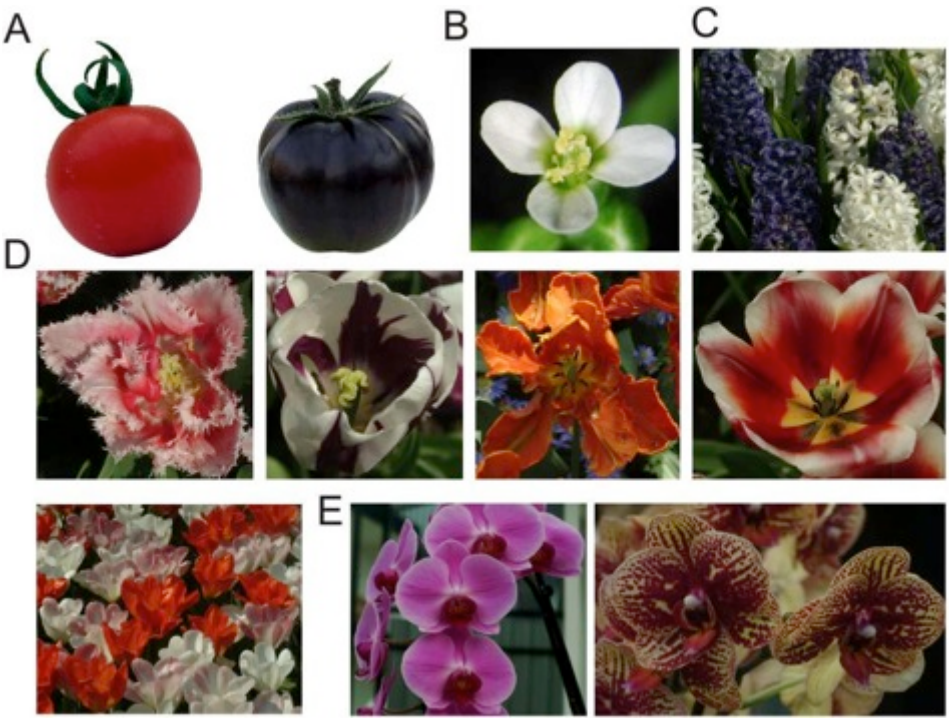

Figure 2. Pigmentation in different species.
(A) Common red tomato (*Solanum lycopersicum* L.) *vs.* anthocyanin-producing tomato.
(B) White Arabidopsis (*Arabidopsis thaliana* L.) flower. Anthocyanins are not produced in the corolla (photo taken from http://plantdev.bio.wzw.tum.de/).
(C) White "Carnegie Hyacinth" *vs*. anthocyanin pigmented "Blue Jacket Hyacinth".
(D) Distinct varieties of tulips displaying different pigmentation (and/or morphological) patterns.
(E) Varieties of Phalaenopsis displaying different pigmentation patterns.

pigmented species display pigment stripes associated with the veins (venation; Shang et al., 2011).

Different MYB and bHLH regulators of anthocyanin pigmentation have been characterized in *Antirrhinum majus* which are involved in the formation of these patterns of pigment deposition. The *Delila* gene encodes a bHLH factor required for the activation of the late anthocyanin biosynthetic genes (LBGs) in the corolla tube (Martin et al., 1991; Goodrich et al., 1992), while in the lobe, another

bHLH gene, *Mutabilis*, acts redundantly with *Delila* in activating the same target genes (Schwinn et al., 2006).

Three genes encoding related R2R3-MYB proteins, named *Rosea1*, *Rosea2*, and *Venosa*, control the intensity and pattern of anthocyanin pigmentation in the flower of *A. majus* (Schwinn et al., 2006): *Rosea1* is involved in the corolla pigmentation in both the adaxial (inner) and abaxial (outer) epidermis, while *Rosea2* gives a weak pigmentation, mainly in the adaxial epidermis of the corolla lobes (Schwinn et al., 2006; Shang et al., 2011).

Venosa modulates anthocyanin production in adaxial epidermal cells that overlie the veins of the corolla (venation). The activity of *Rosea1-2* and *Venosa* are a primary source of natural variation in patterning as shown by recent studies on distinct *Antirrhinum* species that report how pigmentation differences observed in at least six species are caused by variations in the activity of the *Rosea* and *Venosa* loci (Schwinn et al., 2006). All these observations strongly suggest that R2R3-MYB genes are major players in the definition of flower pigmentation patterns (at least in Anthirrinum).

1.3.3 Anthocyanins in petunia

Petunia is a very good model for the study of anthocyanin biosynthesis, thanks to the large collection of mutants affecting the flavonoid pathway and other aspects of pigment accumulation available in this species (Mol et al., 1999). Petunia also displays different domains of pigmentation which can independently accumulate pigment: petal limb, flower tube, anthers, and vein-associated accumulation of pigment.

In petunia, anthocyanin regulatory loci have been identified with the help of a well-established transposon tagging strategy. This approach has allowed, next to the identification of many structural genes of the pathway, the isolation of the regulators ANTHOCYANIN1 (AN1), AN2, and AN11 (deVetten et al., 1997; Quattrocchio et al., 1999; Spelt et al., 2000). Another anthocyanin regulatory gene from petunia, encoding the transcription factor JAF13, was isolated by homology with the maize anthocyanin regulatory gene *LEAF COLOUR* (*LC*), followed by functional characterization (Quattrocchio et al., 1998). All the above mentioned regulators control the anthocyanin pathway via modulation of the transcription of the biosynthetic genes.

AN1 and JAF13 belong to two separate clades of bHLH proteins (Quattrocchio et al., 1998): JAF13 being closely related to the maize regulator LC, and AN1 to the maize INTENSIFIER1 (IN1) which, on the contrary of AN1, is supposed to be an inhibitor of anthocyanin accumulation. Functional analysis of AN1 showed that it is instead an activator of gene transcription and this apparent contradiction could be explained by the presence of many *IN1* transcripts that are probably misspliced and encode truncated proteins possibly behaving as inhibitors (Burr et al., 1996).

AN2, an R2R3-MYB, activates anthocyanin biosynthesis in petunia petals (Quattrocchio et al., 1999), and can interact with AN1, JAF13, and LC in yeast two hybrids assays (Kroon, 2004). The function of AN2 appears to be redundant as pigmentation of limbs is only reduced in *an2* mutants, while pigmentation in other tissues of the flower like anthers, petal tube, pedicel, and seed coat is totally not affected (Quattrocchio et al., 1999). As described in chapter 6 of this thesis, additional genes, representing *AN2* paralogs, are involved in the pigmentation of tubes and anthers. It is important to notice that the ectopic expression of *AN2* driven by the *35S* promoter results in increased *AN1* transcription in leaves (Spelt et al., 2000), indicating the existence of a hierarchy of regulation. *35S:AN2* does however, not increase transcription of *AN1* in petals, and *AN1* expression is not abolished in petals of *an2* loss-of function mutants. Both *AN2* and its paralog *MYBb2*/*AN4* appear to control *AN1*, but most probably according to a distinct mechanism as in *an2* loss-of function mutants, pigmentation in petals is affected, but *AN1* transcripts are not (Spelt et al., 2000), while in *an4* mutants both pigmentation and *AN1* transcription are lost in anthers (chapter 6).

Another MYB, named *DEEP PURPLE* (*DPL*), controls anthocyanin production in vegetative tissues and contributes to floral pigmentation (Albert et al., 2011). In particular, *DPL* regulates anthocyanin pigmentation in the veins of the flower tube. The MYB gene *PHZ* shown to be involved in light-induced anthocyanin accumulation on exposed petal surfaces (bud-blush; Albert et al., 2011) turned out to be identical to *MYBb*.

MYBx is a single MYB repeat protein that interacts with AN1 in yeast two hybrid assays (Quattrocchio et al., 2006) and lacks a transcription activation domain (Kroon, 2004). MYBx is homologous to AtCAPRICE (CPC), it is expressed in developing flowers (Kroon, 2004) and is repressed under high light (Albert et al., 2011). The overexpression of *MYBx* inhibits anthocyanin biosynthesis and vacuolar acidification in petals (Kroon, 2004), mimicking the *an1* mutant and suggesting that MYBx sequesters AN1 into inactive complexes, in this way repressing transcription of AN1 target genes (Kroon, 2004).

The WD40 protein AN11 required for the activation of anthocyanin biosynthesis in all tissues (deVetten et al., 1997), is a cytosolic WD40 repeat protein probably involved in post-translational control of the protein complex that activates anthocyanin genes transcription, as supported by the observation that AN11 can interact in yeast two hybrid assay with, AN1, JAF13 and AN4.

Finally, at least five other loci (*Ve1*, *Ve2*, *Ve3*, *Fine venation* and *Anthocyanin12*) affect the incidence of venation in the corolla of petunia (Martin and Gerats, 1993). Also some other type of pigmentation patterns have been studied in detail, like the colour flecks derived from transposon activity (van Houwelingen et al., 1999) and nonclonal patterns such as “Red Star” and “picotee” patterns of petunia flowers that result from endogenous short-interfering RNA instability mechanisms (Koseki et al., 2005; Saito et al., 2006).

2.1 Gene regulation and its impact on phenotypic variation

A huge amount of phenotypic variation can be observed in nature, but the molecular basis of it is largely unknown. The interest in trying to understand how such variation could originate, is the basis of a relatively new branch of biology, evolution of development (EvoDevo), which has flourished in the last 25 years.

EvoDevo asks the question of how DNA sequence changes have directed the evolution of morphological diversity (Goodman and Coughlin, 2000). Starting from the observation that gene sequences are often highly conserved among species with very different "look", the question to be asked is how highly conserved "master genes" could during evolution generate the enormous variety of shapes, colors, and structures that we observe in nature. Changes in expression pattern of these master regulators, by modifications in cis-regulatory regions (short, non-coding DNA sequences controlling the expression of a close gene), or changes in the protein-coding regions of higher hierarchy regulators, could be the origin of mutations affecting morphology.

One stream of EvoDevo, with as standard-bearer Sean Carroll, proposes that evolution of animal forms worked largely by modifications at cis-regulatory sites. Another stream, represented by Jerry A. Coyne, claims that modification in both the structure and regulation of genes have been important in adaptation (Hoekstra and Coyne, 2007).

An explicative example comes from the study of the homeobox-containing HOX transcription factors in animals. The HOX genes control body architecture (they direct the specification of segmentation patterns along the anterior-posterior body axis) and are extremely conserved from insect to mammalians and from worms to amphibians. In spite of the conservation of these master genes, these animals show impressive differences in body structure, suggesting that the main source of variation is in modifications in the expression of the conserved HOX genes.

The acquisition of new expression patterns during evolution could occur through mutations in the cis-regulatory regions of these genes. The few cases for which the origin of differences in expression pattern has been studied mostly deal with the evolution of animal form, and because of this, a general statement on the molecular basis of evolution of patterns cannot be pronounced yet (Carroll, 2008).

A "swapping" approach could be useful to investigate this subject in those systems where this is possible. A candidate gene (including cis-regulatory elements) from a species/population displaying a certain pattern could be transferred (by transformation, if possible) into a related species/population exhibiting a different pattern. If this result in the derived trait, the candidate gene is responsible for the difference between species/populations (Martin et al., 2010). Although this kind of approach is not of general application for obvious technical obstacles, it could be easily applied to several plant species. In figure 3 we report a scheme of how changes of cis elements in the promoter of genes encoding for master regulators, can result in dramatic changes in e.g. pigmentation patterns in plants.

2.2 Gene regulation for the specification of patterns in plants

It is a common idea that variation in gene expression represents an important source of phenotypic diversity. In any organism, the transcription of each gene is controlled by the combination of activators and/or repressors which bind to cis-elements in the promoter of the gene, resulting in specific gene expression patterns (Figure 3).

It could then be proposed that the differences among organisms that show different patterns, can be explained by a substantial difference in number and/or type of TFs. However, the sequence of the genomes of plants as different as Arabidopsis, poplar (Populus spp.), and maize (*Zea*

mays) showed that they contain a comparable set of genes, including TFs (Britten and Davidson, 1969; Martin et al., 2010).

Alternatively, the differences among species could originate from different expression patterns of the very same set of TFs. In a recent review Martin and colleagues (2010) pointed out that in a number of well studied cases, phenotypic variation originates from changes in expression of lower order transcription factors. The advantage in fixing mutations responsible for these variation, probably is the low number of pleiotropic effects resulting from this changes.

The same mechanisms that in animals lead to the evolution of body architecture orchestrated by HOX genes, could, in this optic, also be at the basis of the variation observed in plants. Changes in cis elements in the promoters of transcription factors controlling patterns like (e.g.) pigmentation, rather than in the proteins encoded by these or higher level regulatory genes, could then be pointed out by analyzing the same genes in species showing phenotypic differences.

However, changes that affect the functionality of cis-acting motifs are often difficult to detect also because phenotypic variation can be determined by differences in regulatory motifs localized kilobases away from the coding sequence (Salvi et al., 2007). Only the rigorous functional characterization of the promoter regions can solve each specific case. One of the most effective approaches for the solution of this problem is that of “swapping” candidate genes, as described in the previous paragraph.

Already more than a decade ago, there were sufficient convincing evidences to support the view that changes in spatiotemporal expression patterns of genes are responsible for the evolution of new morphological as well as biochemical traits (Doebley and Lukens, 1998). The idea that cis-regulatory elements of transcriptional regulators are the main site of action for evolution is now referred to as the “cisacting element model” (Martin et al., 2010) and is nowadays supported by some evidence, like the ones described in the following paragraphs.

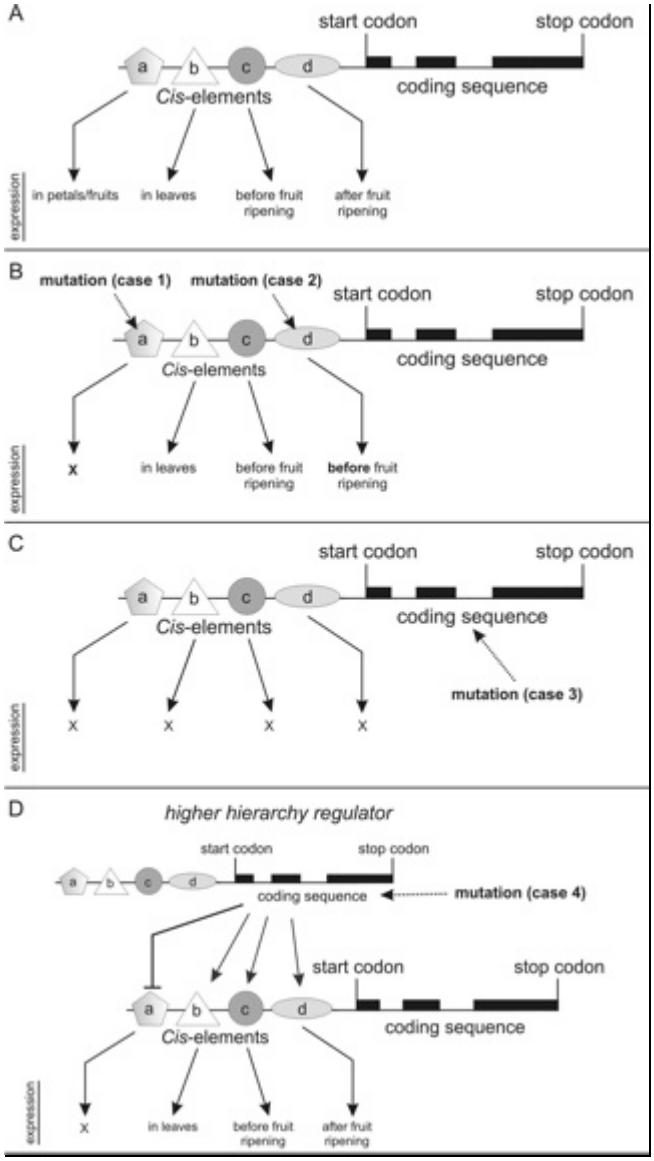

Figure 3. Schematic representation of one gene with its regulatory region in *cis*. Alternative mechanisms of mutation responsible for the variation among organisms are shown.
(A) Functional *Cis*-elements (a, b, c, and d), together with the coding sequence (from start codon to stop codon) allow a normal expression of the gene during different developmental stages and in distinct organs.
(B) Mutations in one or more *Cis*-elements (a and c in this example) can bring to loss of expression (case 1) or change of the pattern of expression (case 2).
(C) Example of a loss-of-function mutation in the coding sequence, which brings to a loss of expression of the gene and all its regulatory elements.
(D) Mutation in an higher level regulator results in modified expression of the gene (some of the cis elements are not recognized anymore, and maybe new ones are now recognised) or, in extreme cases there is no activation at all.

2.3 Patterns variation by changes in cis-elements in the promoter of regulatory genes

Inventory of genes shown to be involved in natural variation in different plant species (Alonso-Blanco et al., 2009) are the material necessary to define how important variation in cis-acting regulatory motifs contributed to evolution in plants. For example, in tomato, changes in the promoter region of the gene *FW2.2*, involved in the modulation of fruit size, cause its misexpression. The effect of this mutation is a large fruit phenotype (Frary et al., 2000). In this case relatively small changes in the promoter of a gene have tremendous effect on the fruit phenotype.

Also for the *teosinte branched* (*tb1*) gene it has been shown that changes regulatory elements are at the basis of some dramatic morphological differences that distinguish maize (*Zea*

mays ssp. mays) from its wild ancestors, the teosinte (*Z. mays ssp. parviglumis* and *mexicana*). Clark and colleagues (2006) demonstrated indeed that sequences localized more than 41 kb upstream of *tb1* act in *cis* to regulate *tb1* transcription and are responsible for the phenotypic changes acquired during domestication of this species. Similarly, in rice, a single-nucleotide polymorphism (SNP) in the 5' regulatory region of the *qSH1* gene is responsible of the loss of seed shattering because of loss of formation of the abscission layer. Interestingly, the presence of this SNP is associated with seed shattering in japonica subspecies of rice, and this suggested that it was a target of artificial selection during rice domestication (Konishi et al., 2006). One other example is the evolution of a 41bp cis-regulatory motif named MEM1 in the promoter of the PEPC genes (encoding a carboxylase), which has been shown to be the basis of mesophyll specific expression in the C4 dicot *Flaveria trinervia* (Akyildiz et al., 2007). The changes required to convert an element with no obvious function into a mesophyll-specific cis-acting element have been tracked down to two nucleotides. This is a superb example of how small changes in nucleotide sequence can be sufficient to create a novel pattern of gene expression and a totally new phenotype.

Finally, several lines of evidence indicate that in the *Ranunculaceae* duplications in a flower organ identity B-function MADS box factor, followed by changes in expression, may have represented the basis of the evolution of petaloid organs (Kramer et al., 2003) in apetalous species such as *Thalictrum thalictroides*, as an adaptation to insect pollination (Di Stilio et al., 2009).

Changes in gene expression pattern can also drive the evolution of physiological traits by affecting metabolic pathways. For example, the evolution of floral scent in *Clarkia*, has been studied through the analysis of the differences between the species *Clarkia brewery*, and its probable ancestor *C.concinna*. *C.brewery* emits large amounts of specific scents by highly expressing genes like linalool-synthse (*Lis*), whereas a limited expression of this gene can be found in *C.concinna* (Dudareva et al., 1996), suggesting that changes in the regulation of this gene expression are the origin of scent variation between the two species.

Next to small sequence changes, also large rearrangements in the non-transcribed flanking regions of genes can result in the acquisition of new cis-regulatory elements, which give rise to novel patterns of gene expression and/or novel phenotypes. For example, in tomato, a chimeric gene resulting from the insertion of a duplicated copy of a metabolic enzyme into the 5' end of the homeobox-containing gene *LeT6* resulted in overexpression of the homeodomain protein and conversion of unipinnate compound leaves into three- or fourfold pinnate compound leaves (Chen et al., 1997).

Also transposable element-induced alterations in the promoter of genes can be source of new expression patterns. One example comes from an insertion in the promoter of the maize alcohol dehydrogenase gene which resulted in quantitative, organ-specific alteration of its expression (Kloeckenergruissem and Freeling, 1995).

From this long list of specific examples, we can definitely say that, changes in regulation originating from alterations in regulatory sequences has played a role in generating novel phenotypes during evolution. More importantly, the phenotype generated is not always a monstrous disruption of normal developmental patterns, but can (in some cases) become a new convenient adaptation.

The examples described are a good collection of cases, but to define a general mechanisms for the generation of new patterns, a good "model" for the study of pattern variability in the plant kingdom would be of great value. Plant pigmentation, with its huge variety among different species (or even among different ecotypes of the same species) provides a nice model to look at the specification of patterns, with the advantage that variations can just be spotted by changes in colours.

2.4 Regulation of anthocyanin pigmentation patterns in different species

The biosynthesis and specification of anthocyanin colours in different species has been influenced by mechanisms of selection orchestrated either by nature or by humans, or both. Domestication has often promoted an increase or a refinement in the pigments already present in the plant material, while in other cases a selection for agronomic traits has discarded pigmentation (Gonzali et al., 2009). This because when pigmentation happened to be linked to not desiderable traits, it was eliminated (together with the undesiderable character) by breeding, or in some cases probably because strongly coloured food is not always considered attractive. Tomato, for instance (*Solanum lycopersicum* L.), has fruits rich in carotenoids that lack anthocyanins, although some *Solanum* species taxonomically close to *S.lycopersicum* have retained the ability to produce anthocyanins in fruits (Jones et al., 2003). As all *S. lycopersicum* cultivated varieties have fruits that do not produce anthocyanins, it is probable that the loss of anthocyanin pigmentation in the fruit took place relatively early in the domestication of this species (Willits et al., 2005). This character has been introgressed -through interspecific crosses- in cultivated tomato, demonstrating that it is possible to reactivate the whole pathway in fruits. Unfortunately, the identity of the loci conferring the anthocyanin phenotype in fruits of certain genotypes is unknown, making it impossible for the moment to establish the mechanism by which fruit pigmentation was lost. This subject is further discussed in chapters 1, 4, and 5 of this thesis.

Another example of loss of anthocyanin related to domestication comes from maize. In wild type varieties of maize anthocyanins are normally synthesized in kernels and vegetative tissues (Selinger and Chandler, 1999). The varieties of maize normally diffused in the western world produce anthocyanin free kernels which seem to better meet the taste of the consumers, but in this case the section has operated on a loss of function mutation in the MYB encoding locus C1. More similar cases are known in domestication (white grape is one of those) in which a loss of function mutation of the MYB regulator is responsible for the phenotype. Examples like these show that also a mechanism of mutation in the coding sequence of the regulator plays a role in adaptative changes, although it only gives limited possibilities in the type of new phenotypes it can generate.

In several flowers, specific floral traits are associated with the choice for specific pollinators. This phenomenon is known as “pollination syndrome” (Hoballah et al., 2007). In the genus *Petunia*, species with hawk moth pollination syndromes (Wijsman et al., 1983; Ando et al., 2001) like *Petunia axillaris*, have white flowers with long and narrow corolla tubes that produce large amounts of volatiles at dusk (when nocturnal moths are active) and massive amounts of nectar (Ando et al., 1995; Ando et al., 2001; Stuurman et al., 2004; Hoballah et al., 2005; Oyama-Okubo et al., 2005; Hoballah et al., 2007). In *Petunia integrifolia* instead, flowers are violet/red, the corolla tube is short and wide and contains low quantities of nectar. These flowers emit small amounts of volatiles (Stuurman et al., 2004; Hoballah et al., 2005), and are mainly pollinated by solitary bees (Ando et al., 2001). Interestingly, the lack of anthocyanin pigments in *P.axillaris* is due to a loss of function mutation in the gene encoding of the MYB transcription factor *PhAN2*, through inactivation the coding region (Quattrocchio et al., 1999). This suggested that coloured petals, like the ones observable in *P.integrifolia*, represent the ancestral phenotype, whereas white flowers represent a “derived” phenotype (Hoballah et al., 2007). The case of speciation in petunia seems to be of the same category of those just described for pigmentation changes related to maize and grape domestication, where not changes in regulatory sequences, but rather in the coding sequence of a regulator are responsible for the acquisition of a new phenotype.

However, pigmentation patterns in petunia have been generated also by a mechanism involving paralogs of *PhAN2* which drive the accumulation of anthocyanin in different organs, such as flower tube, anthers, and vegetative tissues. Phylogenetic analysis of these *PhAN2* paralogs indicates that they most probably originated relatively recently by duplications (as supported by the very high level of homology) followed by the acquisition of new cis regulatory elements that conferred different

domains of expression. The *PhAN4* and *PhMYBb* (chapter 6) drive biosynthesis of anthocyanins in anthers and tube respectively.

In Figure 3 we summarize the different mechanisms leading to new pigmentation patterns that we have described so far.

2.5 Master genes for the specification of anthocyanin patterns

Several steps of the anthocyanin pathway have been described and characterized by studying natural mutations in biosynthetic genes (Espley et al., 2009). For example, the insertion of transposons into structural genes such as chalcone synthase (Habu et al., 1998), dihydroflavonol 4-reductase (Inagaki et al., 1994), anthocyanin synthase (Hisatomi et al., 1997), cytochrome b5 (de Vetten et al., 1999) can cause flower colour polymorphism.

The study of the generation of pigmentation patterns polymorphisms is more complex as most mutations simply result in loss of function of a gene and therefore do not give the right material to dissect this problem. Instead, the study of the diversification and variation of anthocyanin "master genes" during evolution can be approached by the comparison of hortologues of such genes in different species with divergent pigmentation patterns.

The MYB genes activating anthocyanin structural genes look like the best choice for this approach, considering the following aspects:

- 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);
- some studies showed that the only expression of MYB genes is enough to trigger pigmentation in virtually any tissue of different species (see also chapters 5 and 6 of this thesis; Xie et al., 2006; Zuluaga et al., 2008);
- MYB proteins are functionally 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 factors controlling anthocyanin biosynthesis would be a less good choice as, although they have been shown to directly activate transcription of structural anthocyanin genes, they operate downstream of the MYB genes (Spelt et al., 2000) and therefore they surely are not the "master genes" we are looking for.

2.6 Examples of MYB gene modification and their effect on the phenotype

As last we report some examples of how changes in genes encoding MYB anthocyanin regulators affect pigmentation, to support the choice of these genes as "master regulators" of anthocyanin production.

Up-regulation of the expression of MYB transcription factors has been shown to induce anthocyanin production in several species (Borevitz et al., 2000; Mathews et al., 2003). Disruption of the same MYB genes by mutations (either in the promoter or in the coding sequence) results instead in reduction or even total loss of pigmentation. A well described example of the first phenomenon is a retrotransposon insertion in the promoter of Vv*MYBA1* that, together with other mutations in the adjacent Vv*MYBA2* inactivate their expression and convert red-skinned grape into white-skinned one (Walker et al., 2007). On the other hand, the purple colour of particular cauliflower varieties is due to a mutation in the MYB encoding *Pr* locus, which confers strong anthocyanin production in curds and seeds (Chiu et al., 2010). The coding regions of the wild type allele *Pr* and of the one present in

strongly pigmented varieties*, Pr-D,* share 99.2% sequence identity and both of them encode functional proteins. The comparison of the promoter sequences reveals a Harbinger DNA transposon insertion in the upstream regulatory region of the *Pr-D* allele, which introduces additional E-box *cis*-acting elements. Such alteration is proposed to be the reason of increased *Pr* gene transcription in the curds.

Constitutive expression of *AtPAP1* (by a introduction of a *35S:PAP1* construct), *SlANT1* (by activation tagging) and *MdMYB10* (by a multiple repeats minisatellite-like structure in the promoter resulting in an autoregulatory motif) has been shown to be the cause of anthocyanin accumulation in normally uncoloured plant parts respectively in *pap1-D* Arabidopsis (Borevitz et al., 2000), *antI* tomato (Mathews et al., 2003), and red-fleshed apple (Espley et al., 2007).
All these example support a role of master regulators of anthocyanin biosynthesis for the MYB transcription factors involved in the activation of the pathway.

Conclusions

One of the main goals in the study of plant evolution, will in the immediate future, be the identification of sequence changes that have generated new patterns. The study of pigmentation and in particular of anthocyanin regulation in plants represents an easy model to identify genetic changes generating new patterns. In particular, the comparison of anthocyanin master regulators (like the MYB factors at the top of the regulatory hierarchy) from different plant species (or just from different varieties or ecotypes within the same species) will help to assess the contribution of the variation in cis-regulatory elements, and/or changes in their regulators (one level up in the regulatory cascade) in the appearance of new pigmentation patterns.

Aim and outline of the thesis

In this thesis we analyzed physiological and molecular mechanisms by which the biosynthesis of anthocyanins is "orchestrated" in different species. We also studied what kind of genetic variation could have played a major role in the specification of anthocyanin pigmentation patterns during evolution.

Chapter 1 is an introduction on the characteristics of anthocyanin molecules and a short description of the biosynthetic pathway, focusing on tomato species (*Solanum lycopersicum* L.). We selected tomato as a model because of its importance as crop plant, but also because anthocyanins are produced only in vegetative tissues, while, several tomato accessions display anthocyanin accumulation in different organs. This makes it an interesting case of variation of anthocyanin accumulation within the same species. We listed and described all these lines in this chapter. Among them, some were selected for further investigations that we report in **chapters 4** and **5**.

In **chapter 2**, we reported an overview of the numerous natural functions and biotechnological applications of anthocyanins. In this chapter is also a description of the state of the art, in the study of anthocyanin biosynthesis and regulation in different model species displaying distinct pigmentation patterns. The second part of this chapter deals with the molecular basis of the variation of anthocyanin accumulation patterns during evolution. Finally, we propose a new approach to assess the contribution of different type of genetic changes to the generation of new patterns. This is based on the molecular changes occurred in "master regulators" and it aims to identify the sequences that have generated the multitude of patterns observed in plants. Experimental examples following this approach are described in **chapter 7**.

In **chapter 3** we investigated the interplay between sugars and plant hormones in modulating the expression of anthocyanin genes and the accumulation of anthocyanins in Arabidopsis.
The expression pattern of many genes involved in the anthocyanin biosynthetic pathway, including two transcription factors (*AtPAP1* and *AtPAP2*), was analyzed in Arabidopsis seedlings treated with sucrose and plant hormones. We showed that the metabolic status, signalled by the level of sugar in the plant, triggers anthocyanin production through hormones. We thus suggest the existence of a cross-talk between the sucrose and hormone signalling pathways in the regulation of anthocyanin biosynthesis.

In **chapter 4** we reported the transcriptomic characterization of selected tomato lines containing the *Anthocyanin fruit* (*Aft*) or/and the *atroviolacea* (*atv*) alleles (both mentioned also in **chapter 1**) which are responsible for anthocyanin pigmentation of the skin of the fruit. By transcript-profiling we compared red/wild type, *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* tomatoes. This analysis pointed out the existence of synergistic effects between the *Aft* and *atv* locus. Moreover, this analyses revealed that the activation of anthocyanin synthesis in tomato fruit is accompanied by a complex remodulation of gene expression. However, as the identity of the *Aft* and *atv* loci, is still unknown, the next goal became the identification and characterization of the *Aft* allele, and this is reported in **chapter 5**. We used a candidate-gene approach, and we analysed two MYB genes, *SlANT1* and *SlAN2*, that, by homology with MYBs from other species are expected to be involved in anthocyanin biosynthesis, and have been pointed as probable candidates for the *Aft* locus. The results of sequence comparison, expression analyses, and ectopic expression studies of these genes, strongly support that *SlAN2* is the *Aft* locus.

In **chapter 6** we reported the study and functional characterisation of two -very similar- petunia genes, named *PhMYBb1* and *PhAN4*. These genes are homologs of the MYB gene *PhAN2*, known to be involved in petal colouration, although they display divergent expression pattern in comparison to the latter. *PhMYBb1* and *PhAN4* regulate anthocyanin production in the tube and the anther of the flower respectively. In this chapter we also discuss the evolutionary role of gene duplication and differentiation in the specification of pigmentation patterns in distinct organs of the plant. In **chapter 7**, we extended this discussion to a more general understanding of the evolution of pigmentation patterns among species. The approach described in this last chapter was based on “gene swapping” among Arabidopsis, tomato, and petunia. New hypotheses arose from this study, which suggested new models for the evolution of pigmentation patterns, and proposed this experimental approach for the study of the genetic traits that have generated the multitude of morphological and physiological patterns during evolution.

References

Akyildiz, M., Gowik, U., Engelmann, S., Koczor, M., Streubel, M., and Westhoff, P. (2007). Evolution and function of a cis-regulatory module for mesophyll-specific gene expression in the C-4 dicot *Flaveria trinervia*. Plant Cell **19,** 3391-3402.

Albert, N.W., Lewis, D.H., Zhang, H., Schwinn, K.E., Jameson, P.E., and Davies, K.M. (2011). Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. Plant Journal **65,** 771-784.

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.

Alonso-Blanco, C., Aarts, M.G.M., Bentsink, L., Keurentjes, J.J.B., Reymond, M., Vreugdenhil, D., and Koornneef, M. (2009). What has natural variation taught us about plant development, physiology, and adaptation? Plant Cell **21,** 1877-1896.

Ando, T., Iida, S., Kokubun, H., Ueda, Y., and Marchesi, E. (1995). Distribution of petunia-axillaris sensu-lato in uruguay as revealed by discriminant-analysis of the live plants. Journal of the Japanese Society for Horticultural Science **64,** 381-391.

Ando, T., Nomura, M., Tsukahara, J., Watanabe, H., Kokubun, H., Tsukamoto, T., Hashimoto, G., Marchesi, E., and Kitching, I.J. (2001). Reproductive isolation in a native population of Petunia sensu Jussieu (*Solanaceae*). Annals of Botany **88,** 403-413.

Baudry, A., Caboche, M., and Lepiniec, L. (2006). TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in *Arabidopsis thaliana*. Plant Journal **46,** 768-779.

Bloor, S.J., and Abrahams, S. (2002). The structure of the major anthocyanin in *Arabidopsis thaliana*. Phytochemistry **59,** 343-346.

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.

Bradshaw, H.D., and Schemske, D.W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature **426,** 176-178.

Britten, R.J., and Davidson, E.H. (1969). Gene regulation for higher cells: a theory. Science (New York, N.Y.) **165,** 349-357.

Burr, F.A., Burr, B., Scheffler, B.E., Blewitt, M., Wienand, U., and Matz, E.C. (1996). The maize repressor-like gene *intensifier1* shares homology with the *r1/b1* multigene family of transcription factors and exhibits missplicing. Plant Cell **8,** 1249-1259.

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

Chen, J.J., Janssen, B.J., Williams, A., and Sinha, N. (1997). A gene fusion at a homeobox locus: Alterations in leaf shape and implications for morphological evolution. Plant Cell **9,** 1289-1304.

Chiu, L.-W., Zhou, X., Burke, S., Wu, X., Prior, R.L., and Li, L. (2010). The purple cauliflower arises from activation of a MYB transcription factor. Plant Physiology **154,** 1470-1480.

de Vetten, N., ter Horst, J., van Schaik, H.P., de Boer, A., Mol, J., and Koes, R. (1999). A cytochrome b(5) is required for full activity of flavonoid 3 ',5 '-hydroxylase, a cytochrome P450 involved in the formation of blue flower colors. Proceedings of the National Academy of Sciences of the United States of America **96,** 778-783.

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.

Di Stilio, V.S., Martin, C., Schulfer, A.F., and Connelly, C.F. (2009). An ortholog of *MIXTA-like2* controls epidermal cell shape in flowers of Thalictrum. New Phytologist **183,** 718-728.

Doebley, J., and Lukens, L. (1998). Transcriptional regulators and the evolution of plant form. Plant Cell **10,** 1075-1082.

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I., Routaboul, J.-M., Alboresi, A., Weisshaar, B., and Lepiniec, L. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant Journal **55,** 940-953.

Dudareva, N., Cseke, L., Blanc, V.M., and Pichersky, E. (1996). Evolution of floral scent in Clarkia: Novel patterns of S-linalool synthase gene expression in the *C-breweri* flower. Plant Cell **8,** 1137-1148.

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., and Allan, A.C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant Journal **49,** 414-427.

Espley, R.V., Brendolise, C., Chagne, D., Kutty-Amma, S., Green, S., Volz, R., Putterill, J., Schouten, H.J., Gardiner, S.E., Hellens, R.P., and Allan, A.C. (2009). Multiple repeats of a promoter segment causes transcription factor autoregulation in red apples. Plant Cell **21,** 168-183.

Frary, A., Nesbitt, T.C., Grandillo, S., van der Knaap, E., Cong, B., Liu, J.P., Meller, J., Elber, R., Alpert, K.B., and Tanksley, S.D. (2000). *fw2.2*: a quantitative trait locus key to the evolution of tomato fruit size. Science **289,** 85-88.

Galvano, F., La Fauci, L., Vitaglione, P., Fogliano, V., Vanella, L., and Felgines, C. (2007). Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides. Annali dell'Istituto superiore di sanità **43,** 382-393.

Goff, S.A., Cone, K.C., and Chandler, V.L. (1992). Functional-analysis of the transcriptional activator encoded by the maize-b gene - evidence for a direct functional interaction between 2 classes of regulatory proteins. Genes & Development **6,** 864-875.

Gonzalez, A., Zhao, M., Leavitt, J.M., and Lloyd, A.M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant Journal **53,** 814-827.

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

Goodman, C.S., and Coughlin, B.C. (2000). The evolution of evo-devo biology. Proceedings of the National Academy of Sciences of the United States of America **97,** 4424-4425.

Goodrich, J., Carpenter, R., and Coen, E.S. (1992). A common gene regulates pigmentation pattern in diverse plant-species. Cell **68,** 955-964.

Gou, J.-Y., Felippes, F.F., Liu, C.-J., Weigel, D., and Wang, J.-W. (2011). Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-Targeted SPL Transcription Factor. Plant Cell **23,** 1512-1522.

Gould K, Davies K, Winefield C. (2008). Anthocyanins: Biosynthesis, Functions, and Applications. Springer Science and Business Media, LLC, New York.

Habu, Y., Hisatomi, Y., and Lida, S. (1998). Molecular characterization of the mutable flaked allele for flower variegation in the common morning glory. Plant Journal **16,** 371-376.

Heuschen, B., Gumbert, A., and Lunau, K. (2005). A generalised mimicry system involving angiosperm flower colour, pollen and bumblebees' innate colour preferences. Plant Systematics and Evolution **252,** 121-137.

Hisatomi, Y., Hanada, K., and Iida, S. (1997). The retrotransposon RTip1 is integrated into a novel type of minisatellite, MiniSip1, in the genome of the common morning glory and carries another new type of minisatellite, MiniSip2. Theoretical and Applied Genetics **95,** 1049-1056.

Hoballah, M.E., Stuurman, J., Turlings, T.C.J., Guerin, P.M., Connetable, S., and Kuhlemeier, C. (2005). The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. Planta **222,** 141-150.

Hoballah, M.E., Gubitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., Dell'Olivo, A., Arnold, M., and Kuhlemeier, C. (2007). Single gene-mediated shift in pollinator attraction in Petunia. Plant Cell **19,** 779-790.

Hoekstra, H.E., and Coyne, J.A. (2007). The locus of evolution: Evo devo and the genetics of adaptation. Evolution **61,** 995-1016.

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

Inagaki, Y., Hisatomi, Y., Suzuki, T., Kasahara, K., and Iida, S. (1994). Isolation of a suppressor-mutator enhancer-like transposable element, tpn1, from japanese morning glory bearing variegated flowers. Plant Cell **6,** 375-383.

Jackson, D., Roberts, K., and Martin, C. (1992). Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of *Antirrhinum majus*. Plant Journal **2,** 425-434.

Jones, C.M., Mes, P., and Myers, J.R. (2003). Characterization and inheritance of the *Anthocyanin fruit* (*Aft*) tomato. Journal of Heredity **94,** 449-456.

Kloeckenergruissem, B., and Freeling, M. (1995). Transposon-induced promoter scrambling - a mechanism for the evolution of new alleles. Proceedings of the National Academy of Sciences of the United States of America **92,** 1836-1840.

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.

Konczak, I., and Zhang, W. (2004). Anthocyanins - More than nature's colours. Journal of Biomedicine and Biotechnology**,** 239-240.

Konishi, S., Izawa, T., Lin, S.Y., Ebana, K., Fukuta, Y., Sasaki, T., and Yano, M. (2006). An SNP caused loss of seed shattering during rice domestication. Science **312,** 1392-1396.

Koseki, M., Goto, K., Masuta, C., and Kanazawa, A. (2005). The star-type color pattern in *Petunia hybrida* 'Red star' flowers is induced by sequence-specific degradation of chalcone synthase RNA. Plant and Cell Physiology **46,** 1879-1883.

Kramer, E.M., Di Stilio, V.S., and Schluter, P.M. (2003). Complex patterns of gene duplication in the APETALA3 and PISTILLATA lineages of the Ranunculaceae. International Journal of Plant Sciences **164,** 1-11.

Kroon, A.R. (2004). Transcription regulation of the anthocyanin pathway in *Petunia hybrida* (Amsterdam: Vrije Universiteit).

Lila, M.A. (2004). Anthocyanins and human health: An in vitro investigative approach. Journal of Biomedicine and Biotechnology**,** 306-313.

Lunau, K., Fieselmann, G., Heuschen, B., and van de Loo, A. (2006). Visual targeting of components of floral colour patterns in flower-naive bumblebees (*Bombus terrestris*; Apidae). Naturwissenschaften **93,** 325-328.

Martin, C., and Gerats, T. (1993). Control of pigment biosynthesis genes during petal development. Plant Cell **5,** 1253-1264.

Martin, C., Ellis, N., and Rook, F. (2010). Do transcription factors play special roles in adaptive variation? Plant Physiology **154,** 506-511.

Martin, C., Prescott, A., Mackay, S., Bartlett, J., and Vrijlandt, E. (1991). Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant Journal **1,** 37-49.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J., and Wagner, D.R. (2003). Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell **15,** 1689-1703.

Matsui, K., Umemura, Y., and Ohme-Takagi, M. (2008). AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant Journal **55,** 954-967.

Medel, R., Botto-Mahan, C., and Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. Ecology **84,** 1721-1732.

Meiers, S., Kemeny, M., Weyand, U., Gastpar, R., von Angerer, E., and Marko, D. (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. Journal of Agricultural and Food Chemistry **49,** 958-962.

Meyer, P., Heidmann, I., Forkmann, G., and Saedler, H. (1987). A new petunia flower color generated by transformation of a mutant with a maize gene. Nature **330,** 677-678.

Mol, J., Cornish, E., Mason, J., and Koes, R. (1999). Novel coloured flowers. Current Opinion in Biotechnology **10,** 198-201.

Nascimento, N.C.d., and Fett-Neto, A.G. (2010). Plant secondary metabolism and challenges in modifying its operation: an overview. Methods in molecular biology (Clifton, N.J.) **643,** 1-13.

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

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M., and Lepiniec, L. (2000). The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in Arabidopsis siliques. Plant Cell **12,** 1863-1878.

Oyama-Okubo, N., Ando, T., Watanabe, N., Marchesi, E., Uchida, K., and Nakayama, M. (2005). Emission mechanism of floral scent in *Petunia axillaris*. Bioscience Biotechnology and Biochemistry **69,** 773-777.

Pietta, P.G. (2000). Flavonoids as antioxidants. Journal of Natural Products **63,** 1035-1042.

Prior, R.L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. American Journal of Clinical Nutrition **78,** 570S-578S.

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.

Rubin, G., Tohge, T., Matsuda, F., Saito, K., and Scheible, W.-R. (2009). Members of the LBD Family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. Plant Cell **21,** 3567-3584.

Saito, R., Fukuta, N., Ohmiya, A., Itoh, Y., Ozeki, Y., Kuchitsu, K., and Nakayama, M. (2006). Regulation of anthocyanin biosynthesis involved in the formation of marginal picotee petals in Petunia. Plant Science **170,** 828-834.

Salvi, S., Sponza, G., Morgante, M., Tomes, D., Niu, X., Fengler, K.A., Meeley, R., Ananiev, E.V., Svitashev, S., Bruggemann, E., Li, B., Hainey, C.F., Radovic, S., Zaina, G., Rafalski, J.A., Tingey, S.V., Miao, G.-H., Phillips, R.L., and Tuberosa, R. (2007). Conserved noncoding genomic sequences associated with a flowering-time quantitative trait locus in maize. Proceedings of the National Academy of Sciences of the United States of America **104,** 11376-11381.

Sasaki, K., and Takahashi, T. (2002). A flavonoid from *Brassica rapa* flower as the UV-absorbing nectar guide. Phytochemistry **61,** 339-343.

Schijlen, E.G.W., de Vos, C.H.R., van Tunen, A.J., and Bovy, A.G. (2004). Modification of flavonoid biosynthesis in crop plants. Phytochemistry **65,** 2631-2648.

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.

Selinger, D.A., and Chandler, V.L. (1999). A mutation in the *pale aleurone color1* gene identifies a novel regulator of the maize anthocyanin pathway. Plant Cell **11,** 5-14.

Shang, Y., Venail, J., Mackay, S., Bailey, P.C., Schwinn, K.E., Jameson, P.E., Martin, C.R., and Davies, K.M. (2011). The molecular basis for venation patterning of pigmentation and its effect on pollinator attraction in flowers of *Antirrhinum*. New Phytologist **189,** 602-615.

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.

Stuurman, J., Hoballah, M.E., Broger, L., Moore, J., Basten, C., and Kuhlemeier, C. (2004). Dissection of floral pollination syndromes in petunia. Genetics **168,** 1585-1599.

Tanaka, Y., and Ohmiya, A. (2008). Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. Current Opinion in Biotechnology **19,** 190-197.

Tanaka, Y., Brugliera, F., and Chandler, S. (2009). Recent progress of flower colour modification by biotechnology. International Journal of Molecular Sciences **10,** 5350-5369.

Tanaka, Y., Katsumoto, Y., Brugliera, F., and Mason, J. (2005). Genetic engineering in floriculture. Plant Cell Tissue and Organ Culture **80,** 1-24.

Ushimaru, A., Watanabe, T., and Nakata, K. (2007). Colored floral organs influence pollinator behavior and pollen transfer in *Commelina communis* (*Commelinaceae*). American Journal of Botany **94,** 249-258.

van Houwelingen, A., Souer, E., Mol, J., and Koes, R. (1999). Epigenetic interactions among three dTph1 transposons in two homologous chromosomes activate a new excision-repair mechanism in petunia. Plant Cell **11,** 1319-1336.

Walker, A.R., Lee, E., Bogs, J., McDavid, D.A.J., Thomas, M.R., and Robinson, S.P. (2007). White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant Journal **49,** 772-785.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D., and Gray, J.C. (1999). The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. Plant Cell **11,** 1337-1349.

Wijsman, H.J.W., Dejong, J.H., and Pedersen, T.M. (1983). On the interrelationships of certain species of petunia .3. The position of petunia-linearis and *Petunia calycina*. Acta Botanica Neerlandica **32,** 323-332.

Williams, C.A., and Grayer, R.J. (2004). Anthocyanins and other flavonoids. Natural Product Reports **21,** 539-573.

Willits, M.G., Kramer, C.M., Prata, R.T.N., De Luca, V., Potter, B.G., Steffens, J.C., and Graser, G. (2005). Utilization of the genetic resources of wild species to create a nontransgenic high flavonoid tomato. Journal of Agricultural and Food Chemistry **53,** 1231-1236.

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

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology **126,** 485-493.

Xie, D.Y., Sharma, S.B., Wright, E., Wang, Z.Y., and Dixon, R.A. (2006). Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant Journal **45,** 895-907.

Zhang, F., Gonzalez, A., Zhao, M.Z., Payne, C.T., and Lloyd, A. (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development **130,** 4859-4869.

Zhang, W., Ning, G., Lv, H., Liao, L., and Bao, M. (2009). Single MYB-type transcription factor AtCAPRICE: A new efficient tool to engineer the production of anthocyanin in tobacco. Biochemical and Biophysical Research Communications **388,** 742-747.

Zhu, H.-F., Fitzsimmons, K., Khandelwal, A., and Kranz, R.G. (2009). CPC, a single-repeat R3 MYB, is a negative regulator of anthocyanin biosynthesis in Arabidopsis. Molecular Plant **2,** 790-802.

Zimmermann, I.M., Heim, M.A., Weisshaar, B., and Uhrig, J.F. (2004). Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. Plant Journal **40,** 22-34.

Zuluaga, D.L., Gonzali, S., Loreti, E., Pucciariello, C., Degl'Innocenti, E., Guidi, L., Alpi, A., and Perata, P. (2008). *Arabidopsis thaliana MYB75/PAP1* transcription factor induces anthocyanin production in transgenic tomato plants. Functional Plant Biology **35,** 606-618.