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Chapter 6

A role for MYB-type transcription factors as master-regulators of the anthocyanin pathway

with Cornelis Spelt, Arthur Kroon, Francesca Quattrocchio and Ronald Koes

(to be submitted for publication)

Abstract

Anthocyanin accumulate in widely divergent patterns in Angiosperms, and thereby acquired different functions. A wealth of genetic data showed that the pigmentation patterns are dictated by the expression patterns of conserved “toolkit” genes, encoding MYB, BHLH and WD40 transcription regulators that activate subordinate structural anthocyanin genes encoding the enzymes of the pathway. How the expression patterns of these toolkit genes, and thereby pigmentation patterns, diverged is largely unknown, as upstream regulators have not been identified. Here we show that *ANTHOCYANIN4 (AN4)* of petunia encodes a MYB protein that is required for expression of the bHLH gene *AN1* in anthers. Although AN4 and its close paralog MYBb1 are closely related to AN2, which activates pigmentation in the petal limb, the encoded proteins display some striking functional differences. When expression of all three members of this MYB subfamily is blocked, pigmentation is lost in all floral organs, and *AN1* expression is lost in anthers, but not in petals. Our data show that regulatory anthocyanin genes are activated by distinct mechanisms in different tissues, and that alterations in the expression pattern of the MYB regulator are sufficient to alter the expression pattern of its HLH partner and thereby the pigmentation pattern.

Introduction

Angiosperms display a wide variation in anthocyanin pigmentation patterns that are associated with divergent functions of these pigments. For example, anthocyanins in flowers are important to attract pollinators (Hoballah et al., 2007; Quattrocchio et al., 1999), while in seedlings they help to protect the tissue from high light intensities. The pattern of pigmentation is dictated by the tissue-specific expression of structural genes encoding the enzymes of the anthocyanin pathway, which, in turn, is controlled by a triad of transcription regulators belonging to the MYB, HLH and WD40 families respectively (for review see: Koes et al., 2005). The role of this MYB-HLH-WD40 complex in the activation of anthocyanin synthesis is widely conserved among Angiosperms, but in some specific (groups of) species it has acquired some additional functions that seem unrelated to anthocyanin synthesis. For example, in *Arabidopsis* the MYB-BHLH-WD40 complex is also involved in the specification of trichomes in aerial tissues, the specification of non-hair cells in the root epidermis, and the formation of mucilage in the seed (reviewed in Koes et al., 2005; Ramsay and Glover, 2005), while in petunia it drives the acidification of the central vacuole in petal cells (Quattrocchio et al., 2006c; Verweij et al., 2008).

To understand how the anthocyanin accumulation patterns, and thereby their functions, diverged during evolution it is essential to identify the regulatory components that limit the expression patterns of the structural and or regulatory genes. In the species analyzed, the WD40 regulator is expressed in a wide pattern, including many tissues that lack anthocyanins, indicating that the WD40 protein is not the limiting factor that restricts pigmentation to certain tissues. The expression patterns of the MYB and HLH regulators, however, closely mirror the pigmentation pattern in most species, and ectopic expression of the HLH and MYB partner suffices for ectopic expression of

structural anthocyanin genes and ectopic pigmentation. This suggests that the evolution of distinct pigmentation patterns relied primarily on alterations in the expression of these MYB and HLH genes. However, because the transcription regulation of these MYB and HLH genes has not been studied much and upstream activators have not been identified, the molecular basis for the divergence of their expression patterns remained largely unknown.

In petunia anthocyanins accumulate primarily in petals and anthers and a few cells in vegetative tissues. The WD-40 protein ANTHOCYANIN11 (AN11) and the HLH protein AN1 activate in combination with the MYB gene *AN2* structural anthocyanin genes in the limb of the petal (de Vetten et al., 1999; Quattrocchio et al., 1999; Spelt et al., 2000), and in combination with the MYB gene *PH4* a distinct pathway involved in the acidification of the vacuole in petal cells (Quattrocchio et al., 2006c; Spelt et al., 2002). *AN11* is expressed in virtually all tissues, while expression of *AN1* is restricted to all tissues that are pigmented (de Vetten et al., 1997; Spelt et al., 2002). Petunia contains an additional HLH gene, *JAF13*, which appears to be the homolog of *DELILA* from *Antirrhinum* and *R* genes from maize (Quattrocchio et al., 1998). Although gain of function experiments show that *JAF13* can activate anthocyanin genes as efficiently as *AN1*, its role in pigmentation is poorly understood, because *an1* mutations eliminate anthocyanins from all tissues (Quattrocchio et al., 1998; Spelt et al., 2000), while mutations of *JAF13* only causes a barely detectable reduction of anthocyanin synthesis in the petal limb (unpublished data).

Transient expression experiments in maize revealed that the expression of the MYB regulator *C1* and one of the *R* gene, which encode HLH factors, is both necessary and sufficient for ectopic activation of downstream structural anthocyanin genes (because the WD40 partner is already ubiquitously expressed) (Goff et al., 1990), and similar results have been obtained with a variety of dicot species, including petunia (Baudry et al., 2004; Quattrocchio et al., 1993; Quattrocchio et al., 1998). This together with the finding that the MYB and HLH anthocyanins regulators of maize, petunia and many other species can directly interact, lead to the idea that transcription activation of structural genes depends on the binding of a MYB-HLH complex (Baudry et al., 2004; Goff et al., 1992; Quattrocchio et al., 2006a; Zimmermann et al., 2004; for review see: Koes et al., 2005). Such a model would predict that it takes at least two regulatory changes to alter the expression of the MYB and of the HLH gene and activate the anthocyanin pathway in a novel pattern. Indeed in transgenic tomato plants constitutive expression of the MYB and HLH regulators *ROSEA* and *DELILA* is sufficient for ectopic activation of anthocyanin synthesis in transgenic tomato plants (Butelli et al., 2008), while constitutive expression of *DEL* alone enhances the pigmentation, but does not alter the pattern of pigmentation (Mooney et al., 1995).

Gain of function experiments, suggested that the MYB proteins might have dual role as, (i) an activator of the gene encoding its HLH partner, and (ii) together with the HLH partner (in a complex) as an activator of the structural anthocyanin genes. For example, ectopic expression of the petunia MYB protein *AN2* is sufficient for ectopic transcription of the HLH gene *AN1*, in leaves stems and other tissues, resulting in the ectopic activation of downstream structural anthocyanin genes and pigment synthesis (Quattrocchio et al., 1998; Spelt et al., 2000). Similar results were obtained with the MYB gene *TRANSPARENT TESTA2 (TT2)* and the HLH gene *TT8* in *Arabidopsis*, which regulate the synthesis of proanthocyanidins in the seed coat (Nesi et al., 2001). However, because *an2* or *tt2* loss of function mutations do not reduce the expression of *AN1* or *TT8* the significance of the gain of function data and the potential role of these MYB proteins as master regulators of the (pro) anthocyanin pathway remained unclear.

Mutation of *AN2* reduces, but does not completely abolish, pigmentation and expression of structural anthocyanin genes in the petal limb, but has no effect on pigmentation of the petal tube, anthers, pedicel or the seed coat, suggesting that the function of *AN2* is redundant with other genes.

One candidate locus to contain such a redundant gene is *AN4*, because *an4* mutations cause a phenotype complementary to *an2* and eliminates pigmentation of the anthers, but has no effect on the petal limb. Moreover, ectopic expression of *AN2* can restore the defects in *an4* anthers, suggesting that *AN4* may contain or control a *AN2*-like MYB gene (Quattrocchio et al., 1998; Spelt et al., 2000). Contrary to *an2*, mutation of *AN4* eliminates expression of *AN1* providing unequivocal evidence that *AN4* is an upstream regulator of *AN1* (Spelt et al., 2000).

To address the role of *AN4* and MYB proteins in the regulation of *AN1*, we focused on the *MYBb1* gene that we had isolated in a yeast two hybrid screen with an *AN1* bait. *MYBb1* encodes a MYB protein with high similarity to *AN2* (Kroon, 2004; Quattrocchio et al., 2006c) that is expressed at very low levels in the petal limb, the petal tube and in anthers. We found a closely paralog, *MYBb2* that is expressed in anthers and proved to be identical to *AN4*, providing direct evidence that transcription of *AN1* is controlled by its MYB partner, at least in anthers. However, in loss of function mutants for *AN2*, *AN4* and *MYBb1* transcription of *AN1* in petals was not affected, nor could it be inhibited by a dominant negative *AN4*-EAR fusion protein, indicating that *AN1* regulation by MYB partners may be specific for certain tissues only, or may be redundant with distinct pathways that do not rely on these MYB proteins.

Results

In a yeast 2-hybrid screen of a petal cDNA library with the N-terminal 238 amino acids of *AN1* (*AN1*¹⁻²³⁸), we identified 4 distinct MYB proteins that could interact with this conserved domain, namely *AN2*, *MYBa*, *MYBb1* and *MybX* (Kroon, 2004; Quattrocchio et al., 2006c). *MYBa* is encoded by *PH4* and controls a pathway involved in vacuolar acidification (Quattrocchio et al., 2006c), while *MYBx* is a small (single MYB repeat) protein similar to *TRY* and *CPC* from *Arabidopsis* that acts as an inhibitor of *AN1* activity (Kroon, 2004). Recently others renamed *MYBb1* as *DEEP PURPLE (DPL)* (Albert et al., 2011), but because it is practice that the first name given to a gene has priority, we use its original name here (*MYBb1*).

MYBb1 interacts in a yeast two-hybrid assay with *JAF13*, the apparent petunia ortholog of *DELILA* from *Antirrhinum*, as well as with *AN1* (Figure 1A). Sequencing of full size *MYBb1* cDNA and genomic fragments showed that the gene encodes an R2R3 MYB protein of 261 amino acids that is encoded by three exons (Figure 1B and 1C). Figure 1D shows that *MYBb1* is more similar to R2R3-MYB domain proteins that activate anthocyanin synthesis, such as, *AN2* from petunia, and homologs from tobacco (*NtAn2*), *Arabidopsis* (*PAP1*, *PAP2*; Borevitz et al., 2000; Gonzalez et al., 2008), tomato (*ANT1* and *SIAN2*; Mathews et al., 2003), than to MYBs involved in specification of petal cell shape in *Antirrhinum* (*MIXTA*; Noda et al., 1994) and petunia (*MYB1*; Baumann et al., 2007; van Houwelingen et al., 1998), or to MYB proteins from petunia that control vacuolar pH (*PH4*; Quattrocchio et al., 2006c) or synthesis of volatiles (*ODO1*; Verdonk et al., 2005). Although the C-terminal part of MYB proteins display in general little or no sequence conservation, conserved motifs can be found in some specific subgroups of MYBs (Jiang et al., 2004; Stracke et al., 2001). *MYBb1* contains, like *AN2*, a motif that is typical for the N9 subgroup, as defined by Jiang et al. (2004), which corresponds to subfamily 6 of Stracke et al. (2001). Besides this 6/N9 signature *MYBb1* and *AN2* share additional conserved motifs at the C- and N termini (Figure 1B). The degree and pattern of sequence identity of *MYBb1* and *AN2* suggests that they are recently duplicated paralogs and may have similar functions.

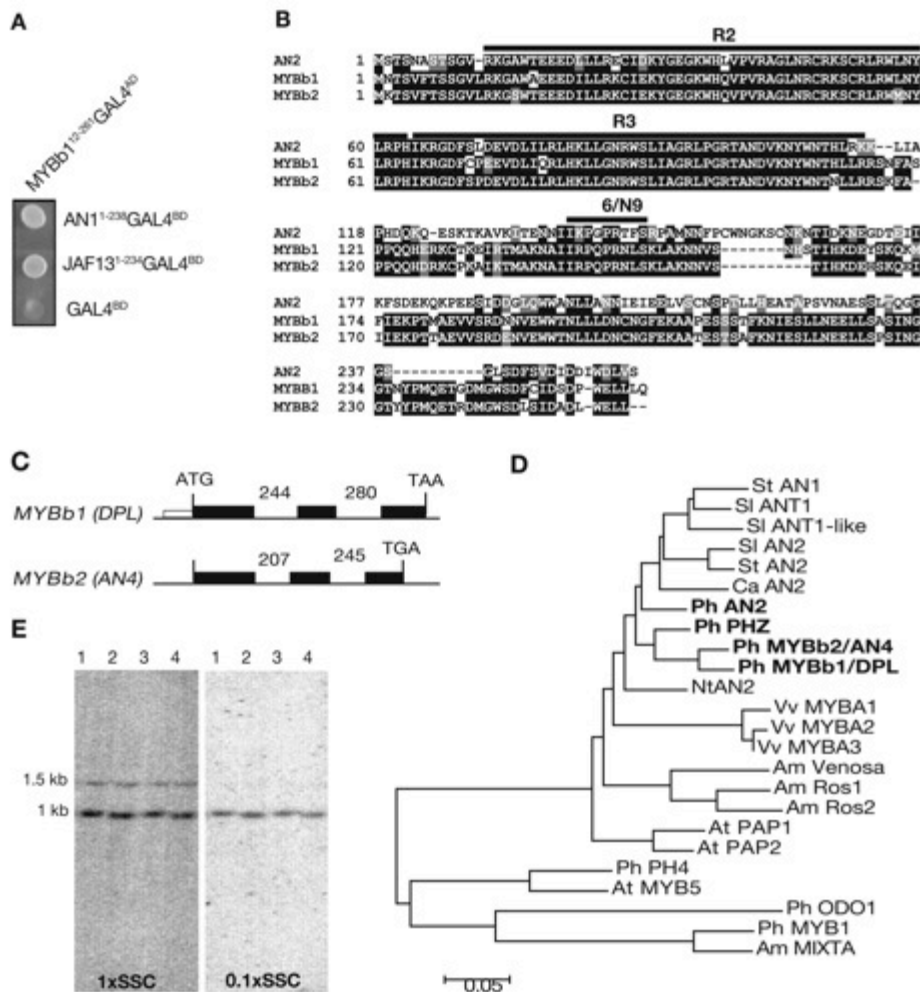


Figure 1. Molecular characterization of MYBb1 and MYBb2. **(A)** Yeast two-hybrid assay showing the interaction between a MYBb1 protein lacking the N-terminal 12 amino acids fused to the activation domain of GAL4 (MYBb1¹²⁻²⁶¹GAL4^{AD}) and the N terminal 238 amino acids of AN1 (AN1¹⁻²³⁸GAL4^{BD}) or the N terminal 234 amino acids of JAF13 (JAF13¹⁻²³⁴GAL4^{BD}). Activation of the *HIS3* and *ADE2* reporter genes is seen as growth on medium lacking both histidine and adenine. **(B)** Aligned amino acid sequences of AN2, MYBb1 and MYBb2 from the type petunia line V30. Sequence identity and similarity is indicated by the black and grey shading respectively. The R2 and R3 repeat that make up the MYB domain and the conserved signature typical of this MYB subfamily (6 or N9) are indicated on top of the sequence. **(C)** Structure of *MYBb1* and *MYBb2* genes. Exons are indicated by filled rectangles, introns are indicated by lines, with their size printed above the line. The small 5' ORF is shown as an open block of half the height. **(D)** Phylogenetic tree showing relationships between MYB proteins from various species. **(E)** Southern blot analysis of *MYBb1* and *MYBb2* genomic DNA at 1x and 0.1x SSC.

Spatiotemporal and genetic regulation of *MYBb1* expression

To investigate whether *MYBb1* might act as an activator of the anthocyanin pathway we analysed its expression by RT-PCR in tissues of the wild type petunia line V30 (Figure 2A and 2B). We used *MYBb1*-specific primers to avoid amplification of mRNA from a second closely related gene (*MYBb2/AN4*, see below). In general, the transcript level of the *MYBb1* gene appeared to be low, because a relatively high number of cycles (26) is required to detect *MYBb1* products by hybridisation. Figure 2A and 2B show that the expression pattern of *MYBb1* in line V30 is similar to that of the structural anthocyanin gene *DFR*, with one notable exception. That is, *MYBb1* is, like *DFR*, expressed in the petal tube, ovaries, sepals and petioles, but not in leaf, root or pistil. Furthermore the timing of *MYBb1* and *DFR* expression during flower development is very similar in both the anthers and the petal tube. In the petal limb, however, *MYBb1* mRNA reaches a maximum in open flowers after anthesis (stage 6), while

DFR mRNA expression peaks much earlier, in the last stages of bud expansion, before the flowers opens (stage 3-4), as in the petal tube.

To assess whether *MYBb1* expression is regulated by *AN1* or *AN2*, we examined *MYBb1* mRNA expression in mutants. Figure 2C shows that the null mutation *an1*^{W242} (Spelt et al., 2002) strongly reduces expression of *DFR* mRNA in the petal tube and the limb, but not of *MYBb1* mRNA. We observed a slight difference in *MYBb1* transcript levels between wild type and *ph4* petals, but because the effect seems only marginal this has not been studied further. Furthermore, *an2* petal limbs express equal amounts of *MYBb1* mRNA, compared to isogenic tissue in which *an2* is complemented by a *35S:AN2* transgene, suggesting that *AN2* is not necessary for transcription of *MYBb1* in petals (Figure 2D). However, the ectopic expression of *AN2* in leaf and anther tissue of *35S:AN2* plants, is sufficient for the ectopic activation of *MYBb1* mRNA up to similar levels as the petal tube (Figure 1E). The discrepancy between *AN2*-mediated ectopic activation of *MYBb* in leaf tissues and the absence of the effect in *an2* petal limbs is similar to the results obtained for *AN1* (Figure 1E; Spelt et al., 2000) and might result from redundancy of *AN2* function (see Discussion).

Figure 1 ctd.

(D) Dendrogram showing the sequence similarity between the MYB domain of proteins involved in anthocyanin and or tannin synthesis, specification of petal cell-shape, vacuolar acidification.

(E) Southern blot of *EcoRI/HindIII* digested DNA of 4 distinct *P. hybrida* W137 plants hybridized with the 3' part of the *MYBb1* cDNA. Autoradiographs after low stringency washing (1xSSC, 65 °C) and stringent washing (0.1xSSC, 65 °C) are shown.

Identification of *MYBb2*, a *AN2-MYBb1* paralog that is expressed in anthers

In anthers of *an4* mutants no anthocyanins are produced and this defect can be restored by expression of *AN2* from a *35S:AN2* transgene (Quattrocchio et al., 1998; Spelt et al., 2000). Because neither *AN2* nor *MYBb1* are strongly expressed in wild type anthers, we suspected that the petunia genome might contain one or more additional paralog(s) of *AN2* and *MYBb1* that are expressed in anthers, in an *AN4*-dependent manner.

To examine whether the petunia genome contains multiple *MYBb1* paralogs, we performed DNA gel-blot analysis. In order to avoid cross hybridisation with other *MYB* domain genes, we used the 3' half of the coding sequence of *MYBb1* as a probe, thus excluding the region encoding the conserved *MYB* domain. Figure 1E shows that this *MYBb1* probe detects a single band after stringent post-hybridization washes (0.1x SSC, 65 °C), while after washing at lower stringency (1x SSC, 65 °C) a second band is visible that apparently derives from a another gene that is distinct from *MYBb1* and from *AN2*, as the 3' part of the *AN2* gene diverged too far from the *MYBb1* probe to cause cross-hybridization.

To isolate fragments of this second *MYBb1*-like gene, we used various primers complementary to *MYBb1* in combination with a 3'-RACE primer in RT-PCR reactions on RNA from anthers (anthers contains very little *MYBb1* mRNA) of lines V30 (*AN4*) and R27 (*an4*). Several *MYBb1* primers yielded RT-PCR products on cDNA derived from anthers of line V30 (*AN4*), while none were obtained from anthers of line R27 (*an4*). Sequencing revealed that these RT-PCR products derived from a gene, temporarily named *MYBb2*, that is highly similar to *MYBb1*. We subsequently isolated cDNA and genomic fragments of *MYBb2* by a combination of PCR procedures (see Methods).

MYBb2 shares 92% nucleotide similarity with *MYBb1* throughout the entire coding sequence (Supplementary Figure S1) and encodes an R2R3-MYB protein that is highly similar to *MYBb1*, also in the C-terminal part (Figure 1C and 1D). Both *MYBb1* and *MYBb2* contain 2 introns (Figure 1B) in positions that are conserved in many plant *MYB* genes, though the introns of *MYBb* and *MYBb2* diverged substantially in sequence (70% similarity) and size (Figure 2B). This, together with the observation that we could amplify both genes from a range of petunia lines that were inbred for >40 generations, demonstrates that *MYBb1* and *MYBb2* are not allelic, but represent closely related paralogs that originated from a relatively recent gene duplication event.

To examine whether *MYBb2* might be involved in plant pigmentation, we analysed its expression in the wild type line V30 by RT-PCR. Figure 2A shows that *MYBb2* is expressed in anthers of young floral buds (stage 1-2), simultaneous with the anthocyanin biosynthetic gene *DFR*. In tubes of developing flowers, *MYBb2* mRNA expression starts in young buds and steadily increases in subsequent stages to reach a maximum in stage 5, a little later than the expression of *DFR* mRNA (Figure 2A). In petal limbs of developing

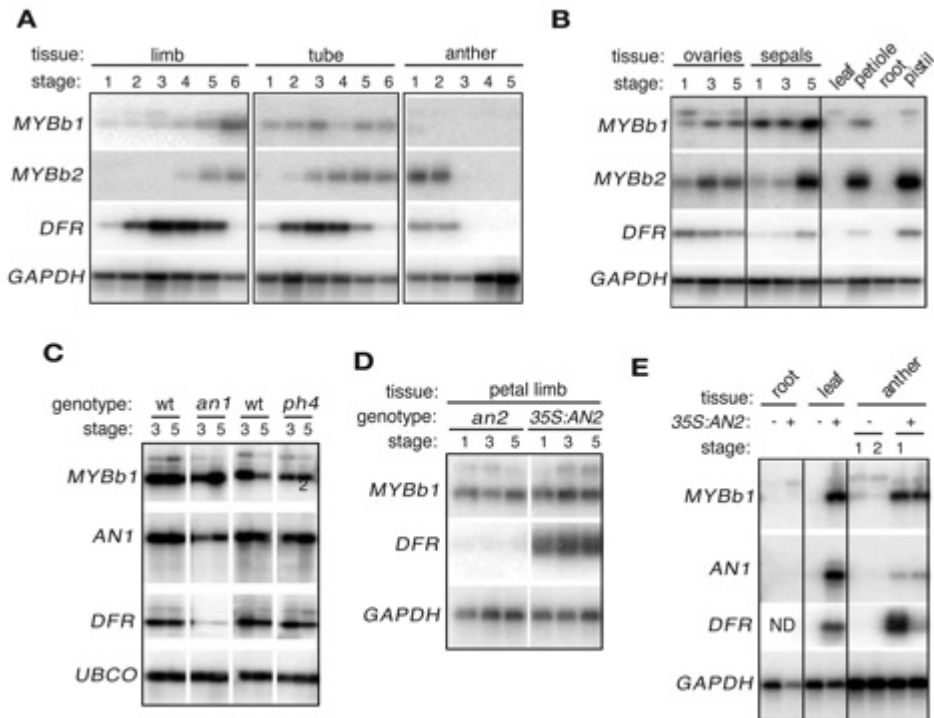


Figure 2. Spatiotemporal and genetic control of *MYBb* and *MYBb2* expression.

(A) RT-PCR analysis of mRNAs in petal limb, tube and anthers of flowers from line V30 starting from stage one (1 cm sized buds) to stage six (open flower). Transcripts of *MYBb1*, *MYBb2*, *DFR* and *GAPDH* were detected by quantitative RT-PCR, using 27 cycles for *MYBb1* and *MYBb2*. The arrow with the star points to signal originating from contaminating genomic DNA in some cDNA samples.

(B) Transcript analysis of *MYBb1*, *MYBb2*, *DFR* and *GAPDH* in the ovary, leaf, pedicel, root, pistil and sepal tissue samples for line V30. Note that for *MYBb1* and *MYBb2*, 32 cycles of amplification were used for this RT PCR.

(C) RT-PCR analysis of *MYBb1*, *AN1*, *DFR* and, as a constitutively expressed control, *UBIQUITIN CONJUGATING LIGASE (UBCO)* mRNA in wild type, *an1* and *ph4* petal limbs.

(D) RT-PCR analysis of *MYBb1* mRNA in petal limbs of an *an2* loss-of-function mutant and a transgenic line in which the *an2* mutations is complemented by *35S:AN2*. *GAPDH* is used as a constitutive expressed control.

(E) RT-PCR analysis of *MYBb1*, *AN1* and *DFR* mRNA in root, leaf and anthers of 2 developmental stages from the loss-of-function *an2* mutant (-) or a line complemented by *35S:AN2* (+). *GAPDH* is used as a constitutive expressed control.

flowers, *DFR* mRNA reaches a maximum at stage four when the bud has its maximum length but is still closed, whereas the *MYBb2* gene does not become active until stage 5, when the limb starts to unfold, and expression persists even in open flowers, when the *DFR* mRNA level has already declined (Figure 2A). This suggests that if *MYBb2* has a role in activating the anthocyanin pathway, it is unlikely to contribute much to the pigmentation of the petal limb, as its expression starts too late during petal development.

Although *MYBb1* and *MYBb2* mRNA are expressed in very similar patterns, there are some noteworthy differences. First *MYBb2* is expressed in the pistil, which is pigmented, while *MYBb1* is not. Second, *MYBb2* mRNA is much more abundant than *MYBb1* mRNA (> 30 fold), as it takes respectively 26 and 20 PCR cycles to detect these mRNAs in the petal limb and tube (Figure 2A) and in others tissues this difference is even larger (Figure 2B).

***an4* lines contain a mutation in *MYBb2* that blocks its expression**

The finding that *MYBb2* is expressed in *AN4* anthers, but not in *an4* anthers, implies that *MYBb2* expression is either regulated by *AN4*, or that *MYBb2* is identical to *AN4*. In the latter case the *mybb2^{R27}* allele from the *an4* line R27 must contain a mutation that blocks its expression, also when introduced into an *AN4* background. However, if *MYBb2* is a distinct gene that is regulated by *AN4*, than the *MYBb2^{R27}* allele should be reactivated when introduced into an *AN4* genetic background.

To introduce the *mybb2^{R27}* allele in an *an4* and *AN4* background, we crossed the lines R27 (*an4/an4*) and V30 (*AN4/AN4*), backcrossed the F1 (*AN4/an4*) to the R27 parent and selected by phenotype ten *an4/an4* and ten *AN4/an4* plants from the backcross progeny (B1). In these plants we analyzed the expression of the *mybb2^{R27}* and *MYBb2^{V30}* alleles by RT-PCR using allele-specific primers. We verified the efficiency and specificity of these allele-specific primers, by PCR amplification of genomic DNA of same plants. Figure 3A shows that *MYBb2^{V30}*-specific primers generated a 1200-bp product on genomic DNA from V30, the F1 and the *AN4/an4* backcross progeny, whereas on DNA from R27 and the *an4/an4* backcross plants only a barely detectable amount of product was generated. This indicated that the *MYBb2^{V30}* primers were indeed allele-specific, albeit not completely. Moreover, the absence of *MYBb2^{V30}* products in the 10 pooled *an4/an4* progeny indicated that *MYBb2^{V30}* is genetically linked to *AN4* (0 cross-overs in 10 chromosomes tested). The *mybb2^{R27}* primers proved to be fully allele-specific, as they produced a 1300-bp product on DNA from R27, the F1 and *an4/an4* B1 progeny, but none at all on V30 DNA.

RT-PCR experiments with the same primer pairs showed that, as expected, *MYBb2^{V30}* mRNAs were present in anthers of V30, the F1, and the *AN4/an4* backcross progeny, but not in R27 or the *an4/an4* backcross progeny. The *mybb2^{R27}* allele, however, remained inactive in the F1 (*AN4/an4*) and the *AN4/an4* backcross progeny. Therefore we conclude that the inactivity of the *mybb2^{R27}* allele in line R27 is not due to a transacting (*an4*) mutation, but due to a *cis*-acting mutation in the *mybb2^{R27}* allele itself that prevents accumulation of its mRNA. This finding, together with the co-segregation of *MYBb2^{V30}* and *AN4*, suggests that *MYBb2* and *AN4* may be identical.

MYBb2* is identical to *AN4

To obtain direct evidence that *MYBb2* is identical to *AN4* we cloned a 4.6 kb genomic fragment from the *AN4* line V30, which includes 2 kb of 5' flanking sequence upstream of the start codon and 1.4 kb of 3' flanking sequence, downstream of the stop codon, and introduced this *MYBb2^{V30}* fragment in the lines V26 (*an4*) and W115 (*an2 an4*) by *Agrobacterium*-mediated leaf disk transformation. Because of the *an4* mutation anthers of V26 and W115 lack anthocyanins, while pigmentation of the W115 petal limb is compromised by the *an2* mutation (Figure 3B). In 5 out 10 V26 transformants and 2 out of 4 W115 transformants containing the *MYBb2^{V30}* transgene pigmentation of the anthers was restored (Figure 3B-C). As expected, the pigmentation defect caused by *an2* in W115 petal limbs was

not restored (Figure 3C), meaning that the functional complementation is specific and exclusive for the *an4* mutation. Furthermore, RT-PCR analysis showed that in these transgenic plants *MYBb2*^{V30} is expressed in petals during the last stages of development of the flower and anthers of young floral buds (stage 1-2), but not in leaves (Figure 3E). Thus the genomic *MYBb2* fragment that was used contains most if not all of the cis-regulatory elements that determine its expression pattern.

Expression of the *MYBb2*^{V30} transgene resulted in the activation of *AN1* and *DFR* expression in stage 2 anthers, to similar levels as in wild type, but did not affect the expression of *CHS*, encoding the first enzyme (CHALCONE SYNTHASE) of the flavonoid pathway, and which is not affected by a mutation in *AN4* (Quattrocchio et al., 1993).

The results of these complementation experiments, together with the molecular data demonstrate that *MYBb2* is identical to *AN4*. Therefore, we refer to this gene as *AN4* from here on.

Molecular analysis of *AN4*

To investigate the cause for the absence of *mybb2*^{R27} mRNA, we compared the genomic sequences of the *an4*^{R27} and *AN4*^{V30} alleles. Several substitutions were found between the coding sequence of *mybb2*^{R27} and the *MYBb2*^{V30} allele (Figure S1), but none of these are obvious causes for the destabilisation of the *mybb2*^{R27} mRNA, like a frame shift or a non-sense mutation. For *MYBb2* eight non-silent substitutions in the coding sequence were found between V30 and R27. Four of those are at the end of the highly conserved R3 MYB

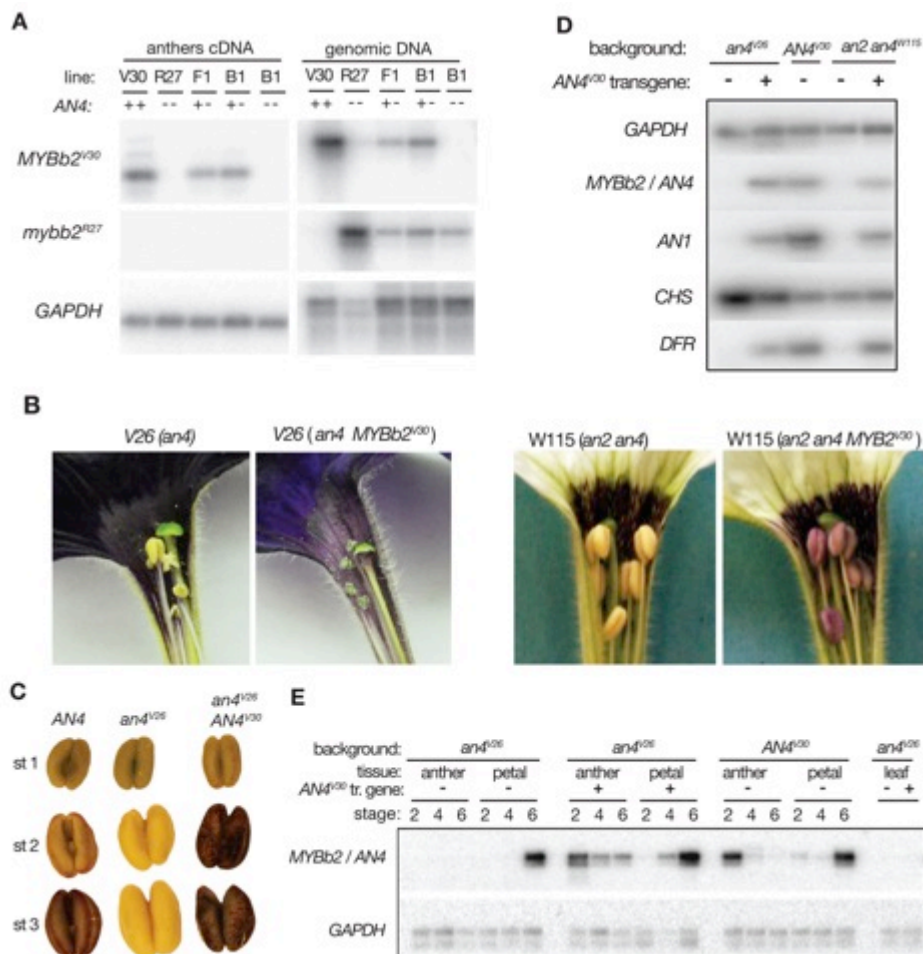


Figure 3. *MYBb2* is identical to *AN4*.

(A) *mybB2*^{R27} is an inactive allele. PCR on cDNA (left) and genomic DNA (right) from anthers of lines V30 (*AN4*), R27 (*an4*), F1 (*AN4/an4*) and pools of 10 *AN4/an4* and 10 *an4/an4* B1 progeny. The gene specific primers are indicated on the left. **(B)** Phenotypes of flowers from lines V26 (*an4*) and W115 (*an2 an4*) and complementants containing a *AN4*^{V30} transgene. V26 contains a mutation in the *POLLEN (PO)* gene, encoding Chalcone isomerase, resulting in the accumulation of (yellow) naringenin. In an *An4* and *an4* background this results in a green or yellow color. **(C)** Anthers from stage 1 to stage 3 flowers of line V30 (*AN4*), V26 (*an4*) and transgenic V26 containing *AN4*^{V30}. **(D)** RT-PCR analysis of stage 2 anthers from line V30 (*AN4*), V26 (*an4*), W115 (*an2 an4*) and transgenic siblings containing *AN4*^{V30}. **(E)** RT-PCR analysis of *AN4* expression in V30 (*AN4*), V26 (*an4*) and transgenic V26 containing *AN4*^{V30}.

repeat and, therefore, might alter the activity of the protein. However, as *an4*^{R27} does not express (stable) mRNA, these amino acid substitutions seem to be irrelevant. The *AN4*^{V30} mRNA contains in the 5' UTR region a short open reading frame (ORF) that is absent in the corresponding region of *an4*^{R27}. Such small upstream ORFs occur in many genes, including *AN2* (Quattrocchio et al., 1999), and are thought to reduce translation of the downstream ORF, but are not known to affect mRNA stability. Therefore it is unlikely that the lack of *an4*^{R27} mRNA expression is due to the absence of this upstream ORF.

PCR and sequence analysis of the 5' flanking region of *an4*^{R27} indicated that this region contained some major rearrangements (Figure 4A), because of which many primers combinations that could amplify parts of the *AN4*^{V30} promoter did not amplify products from line R27. Other primer combinations, did yield amplification products from V30, but most of these proved upon sequencing to be a mixture of two highly similar fragments, which differed for a few single nucleotide polymorphisms (SNPs) and two 8-bp insertions that resembled a transposon footprint. This indicated that some promoter sequences were duplicated in V30. When we used a 200 bp fragments, located between -1.5 and -1.7 kb (relative to the start codon) in *AN4*^{V30} as a probe on DNA gel blots, we detected a single fragment in V30 and two in R27, confirming that this region is duplicated in R27. To assess whether the duplicated promoter sequences in V30 are contiguous, we used various primers pairs that have a head to head orientations in *AN4*^{V30} in PCR reactions. None of these combinations amplified products from genomic DNA of V30, while many of them yielded products from R27, implying that the repeated *an4* promoter sequences in V30 are contiguous and organized in a direct repeat (Figure 4A) Finally, sequence analysis of various PCR fragments revealed that the *an4*^{R27} promoter contained two major rearrangements, namely: (i) an insertion of a transposon-like sequence, which has 140 bp terminal inverted repeats and is flanked by a 9-bp target site duplication, some 0.2 kb upstream of the translation start codon and (ii) a duplication of an ≥ 2800 bp region that spans the 1220 bp transposon insertion and 1440 bp of the flanking *AN4* promoter sequence (i.e. the part corresponding to -0.2 and -1.6 kb in *AN4*^{V30} promoter) (Figure 4A).

The *an4* allele of line V26 has a different defect than *an4*^{R27} that abolishes its expression in anthers, but not in the petal (Figure 3E). Surprisingly, we could not detect any differences in the sequences of the *AN4*^{V30} and *an4*^{V26} alleles between position -2.5 kb upstream of the start codon and 1.4 kb downstream from the stop codon. Because we obtained the same result with multiple independent samples of V26 DNA, we can rule out that this results is caused by DNA contaminations. Moreover, DNA gel blot analysis showed that the restriction patterns of 5' flanking region of *an4*^{V26} were identical to those of *AN4*^{V30} (Figure 4B) indicating that the similarity between both alleles extends even further into the 5' flanking region, beyond position -2.5 kb. Furthermore the DNA gel blot

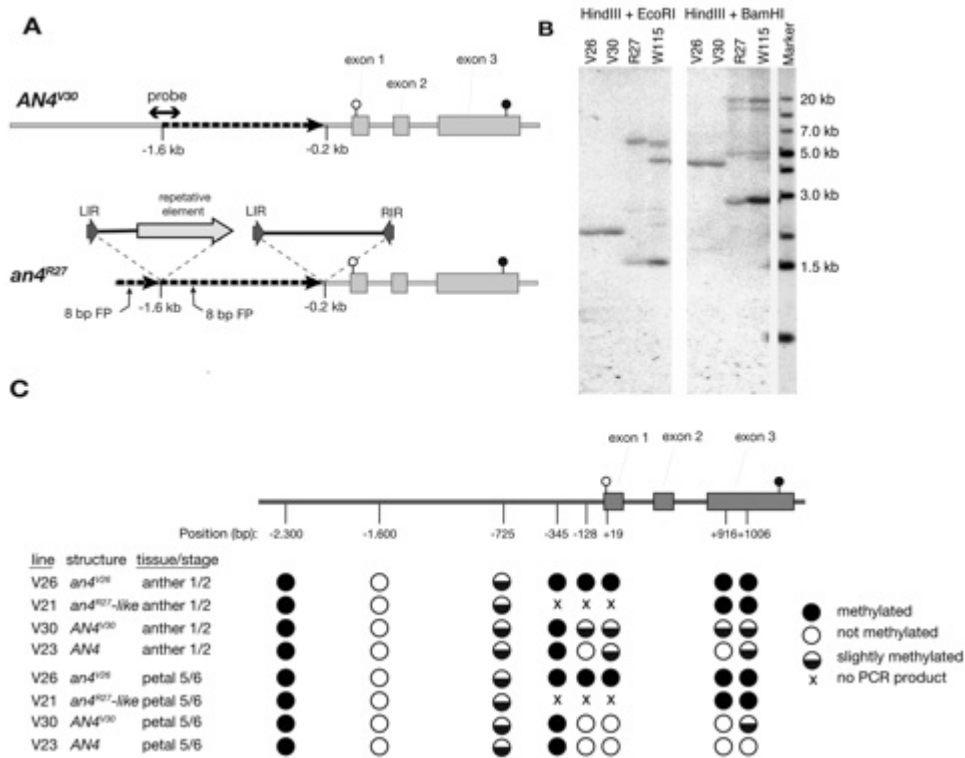


Figure 4. Analysis of mutant *an4* alleles.

(A) Maps of *AN4^{V30}* and *an4^{R27}* alleles. Exons are indicated by grey rectangles, and translation start and stop codons with open and closed circles respectively. Triangles denote transposons, and the dark grey arrows, the left and right terminal inverted repeats (LIR, RIR). The dotted arrow in *AN4^{V30}* denotes a region that is duplicated in *an4^{R27}* and 8-bp FP the position of 8-bp insertions resembling a transposon footprint.

(B) DNA-gel blot analysis of *AN4^{V30}*, *an4^{V26}*, *an4^{R27}*, *an4^{W115}* alleles. Genomic DNA was digested with the restriction enzymes indicated, size-separated and hybridized with a promoter fragment spanning -1.5 to -1.7 kb in the *AN4^{V30}*, as indicated in (A).

(C) Methylation status of *HpyCH IV* restriction sites in *AN4* and *an4* alleles. *HpyCH IV* sites in *AN4* are indicated in the diagram above: their methylation status in genomic DNA from distinct lines and tissue is indicated with open and closed circles below each site.

data rule out the possibility that *an4^{V26}* contains a large rearrangement, like a duplication, that might escape detection by PCR. Given that the 4.6 kb genomic *AN4^{V30}* fragment contains all sequences required for correct expression and complementation of *an4* (Figure 3E), and that the *AN4^{V26}* allele has the same sequence we reasoned that *an4^{V26}* is either an epiallele or contains a rearrangement in the far up- or downstream region by which it came under the (long distance) control of a cis-element that represses transcription specifically in anthers. To examine whether the inactivity of *an4^{V26}* is associated with DNA methylation, we digested genomic DNA isolated from anthers and petals of lines V26 (*an4^{V26}*), V23 (*AN4*), V30 (*AN4*) and V21 (containing an *an4* allele with a similar rearrangement as *an4^{R27}*) with the *HpyCH IV* enzyme, which is sensitive to methylation of the C in ACGT, and amplified the DNA with primers flanking individual *HpyCH IV* sites to determine whether this had been cut. Figure 4C shows that the *HpyCH IV* sites in the coding sequence and immediate upstream region (-128 and -345) were methylated in V26 anthers and petals, but not, or much less in the *AN4* alleles of V30 and V21. The coding sequence of the *an4^{R27}* allele was also methylated, but methylation in the proximal promoter region could not be determined as this region is difficult to amplify due to the rearrangements. The simplest explanation for these results is that *an4^{V26}* contains an epigenetic defect that specifically abolishes its expression in anthers, but not in the petal limb.

Functional comparison of AN4 and MYBb1 with AN2

Although AN4 (and MYBb1) are closely related to AN2, both proteins differ in that AN4 is required for the expression of *AN1*, whereas AN2 is not. This regulatory difference might stem from a functional difference in the AN4 and AN2 proteins, or from differences in the regulatory networks that control *AN1* expression in petals and anthers. To examine to what extent AN4 and AN2 proteins are similar, we used different functional assays.

First we examined whether both AN4 and AN2 co-localize to the same compartment and whether constitutive expression of AN4 can ectopically activate *AN1* expression and pigment synthesis, like AN2. Therefore, we expressed chimeric proteins in which GFP was fused to the C-termini of AN2, AN4 and MYBb1 from a transgene driven by the constitutive 35S promoter in the *an2 an4* line W115. Most of the *35S:AN2-GFP* and *35S:AN4-GFP* transformants displayed ectopic pigmentation of leaves, stem, bracts, and sepals, enhanced pigmentation of the petal tube and restored pigmentation of the petal limb (Figure 5A). The latter indicated that both *35S:AN2-GFP* and *35S:AN4-GFP* complemented the limb pigmentation defect caused by *an2* equally well. That this results in a rather pale flower color is due to other factors in the W115 background, such as leaky

allele at the *HYDROXYLATION AT FIVE1 (HF1)* locus and a dominant *FLAVANOL (FL)* allele, which forces most precursors into the flavonol pathway instead of the anthocyanin pathway (Holton et al., 1993; Wiering, 1974). However, none of the *35S:MYBb1-GFP* transformants displayed ectopic or enhanced pigmentation. Analysis of these transgenic plants by real time PCR analysis showed that *35S:MYBb1* was expressed in most of the plants analyzed but the abundance of *MYBb1-GFP* mRNAs was several orders of magnitude lower than that of *AN2-GFP* and *AN4-GFP*. Although the AN2-GFP and AN4-

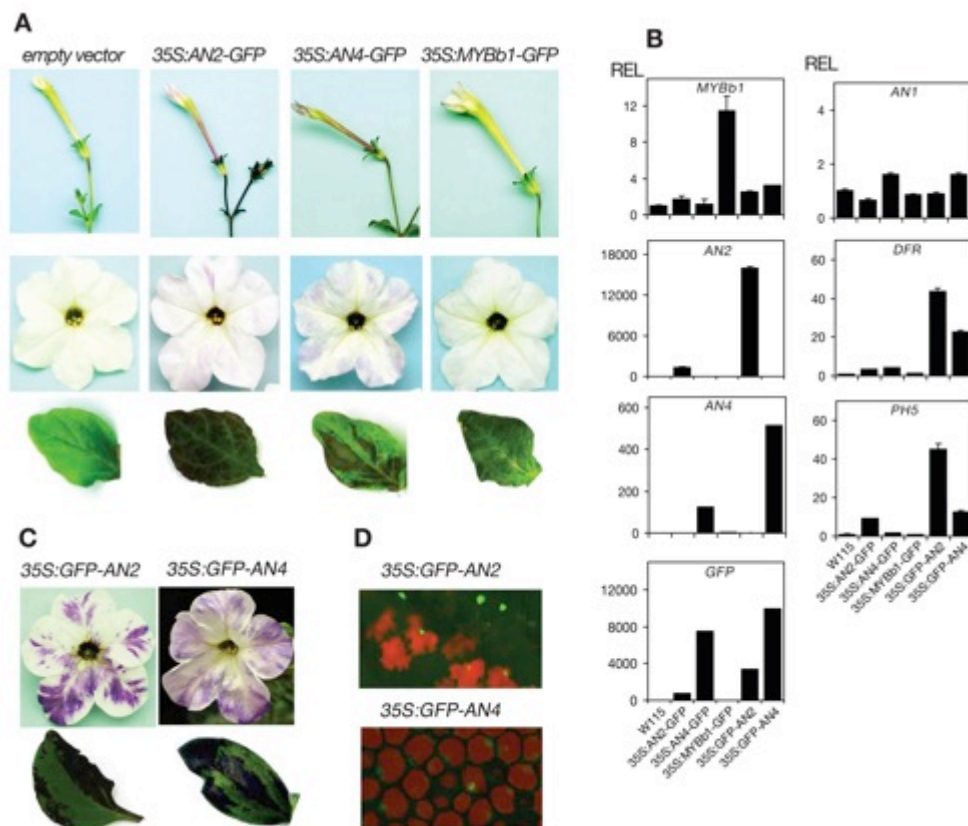


Figure 5. Constitutive expression of GFP fusions to AN2, AN4 and MYBb1.

(A) Phenotypes of inflorescence, flower and leaf of transgenic plants expressing C-terminal fusions of GFP to AN2, AN4 and MYBb1.

- (B) Phenotypes of flowers and leaves expressing N-terminal fusions of AN2 and AN4.
(C) Real Time PCR analysis of mRNAs expressed in petal limbs of the indicated genotypes.

GFP fusions complemented *an2*, they seemed treacherous as reporters of intracellular localization, because the major proteins detected in these plants by anti-GFP on immuno-blots, had a size similar to free GFP (~ 30kD), while no proteins with a size predicted for AN2-GFP or AN4-GFP (~55 kD) could be seen. This suggested that the AN2-GFP and AN4-GFP proteins were cleaved *in vivo*. Consistent with that idea, we observed that GFP fluorescence was present in both the nucleus and cytoplasm, in a similar pattern as in cells expressing free GFP (not shown).

Interestingly, transgenic plants that expressed proteins consisting of GFP fused to the N-termini of AN2 and AN4 (*35S:GFP-AN2* and *35S:GFP-AN4*), showed a very strong pigmentation of the petals and green organs, such as sepals, bracts leaves and stem (Figure 5C). Consistent with that we observed that these transgenics expressed *DFR* at very high levels, much higher than in *35S:AN2-GFP* or *35S:AN4-GFP* plants, and (re)activated also the expression of *PH5*, a gene that is involved in vacuolar acidification pathway, but not anthocyanin biosynthesis (Verweij et al., 2008). *AN1* is in the *an2* W115 petals expressed at the same levels as in wild type (*AN2*), and expression in transgenic petals was not clearly different from untransformed controls, apart for a slight increase in transformants that expressed *AN4-GFP* or *GFP-AN4* mRNA at very high levels (Figure 5B).

Con-focal microscopy revealed that both GFP-AN2 and GFP-AN4 reside in the nucleus, consistent with the notion that these proteins act as transcription activators (Figure 5D).

Taken together these data indicate that AN2 and AN4 have a similar capacity to induce genes in the anthocyanin and the vacuolar acidification pathway, and are even interchangeable as ectopic expression of *AN4* can complement *an2*.

AN4-EAR can suppress anthocyanin synthesis in anthers, but not in petals.

To assess the potential interactions of AN4, MYBb1 and AN2 with proteins encoded by other regulators of anthocyanin synthesis or vacuolar acidification we performed systematic yeast two hybrid assays. We found that *AN4*, *MYBb1* and *AN2* can interact with the HLH protein AN1 (Figure 6A), giving a strong response that could be detected with all three reporter genes (*HIS*, *ADE* and *LacZ*). Furthermore AN4 and MYBb1, but not AN2, could interact with the WD40 protein AN11, albeit with a weaker response that could only be detected with the *HIS3* reporter, and not with the less sensitive *ADE2* and *LacZ* reporters (Figure 6A).

Next we examined whether we could eliminate the activity of AN4, MYBb1 and AN2 in plants, by constitutively expressing a fusion of AN4 and the EAR transcription repression domain. For these experiments we used the F1 hybrid M1xV30 as a host, because this hybrid is transformable and dominant for all known anthocyanin genes. We found that expression of *AN4-EAR*, phenocopied *an4* and completely abolished pigmentation and the expression of *AN1* and *DFR* in anthers, but not in the petal limb (Figure 6B-C). Thus, AN4-EAR is epistatic to transcription factors, like *AN4*, that activate *AN1* in anthers, but not to those that activate *AN1* in the petal limb.

Inactivation of AN2, AN4 and MYBb1 blocks the pigmentation of the petal limb, but not AN1 expression.

To further assess the potential role of MYBb1 in activating anthocyanin synthesis and/or *AN1* expression, we inhibited its expression via RNA interference (RNAi) by expressing an inverted repeat of a *MYBb1* cDNA fragment from the 35S promoter (*35S:AN4^{RNAi}*) in the

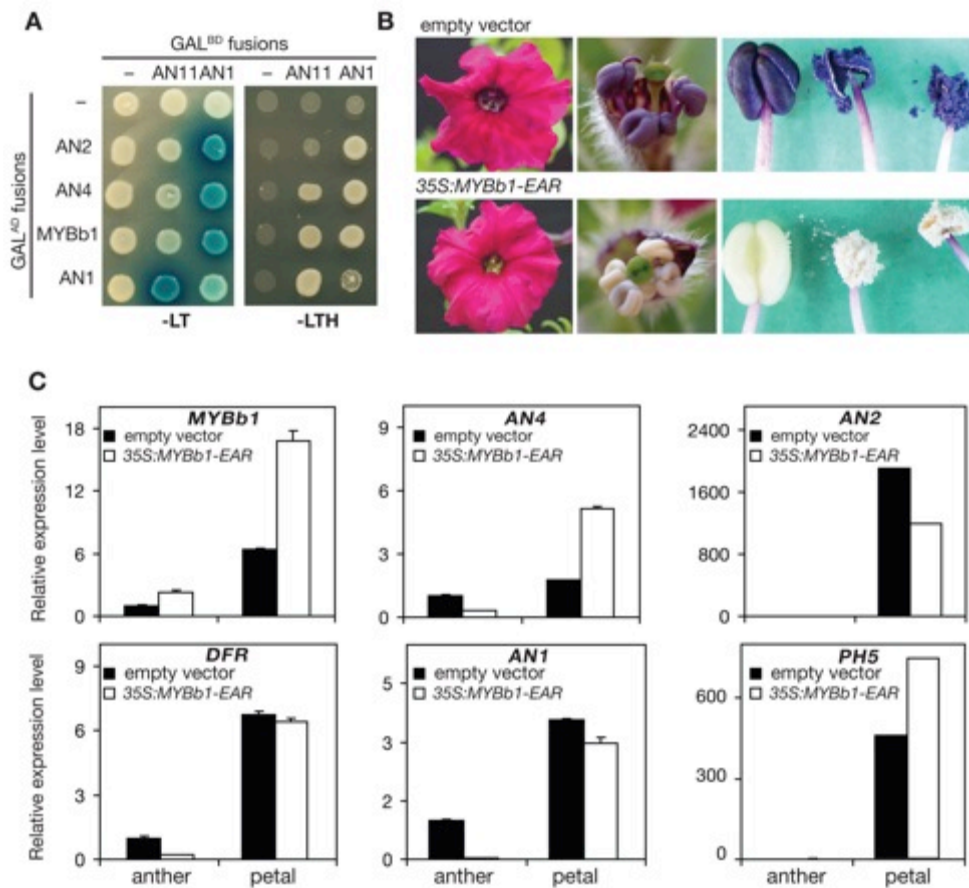


Figure 6. Comparison of functional properties of AN2, AN4 and MYBb1.

(A) Yeast two hybrid assays. Yeast strains expressing GAL^{AD} fusions of the proteins indicated on the left and GAL^{BD} fusions of the proteins indicated on top, were assayed for expression of the lacZ reporter, (left; activity is seen as blue staining) and HIS3 (right, activity seen as grow on plates lacking leucine, trauphophae and histidine (-LTH)).

(B) Phenotype of flowers and anthers of M1xV30 flowers and a transgenic sibling expressing MYBb1-EAR.

(C) Real time PCR analysis of mRNAs expressed in petals and anthers of the M1xV30 and siblings expressing MYB1-EAR.

wild type M1x V30 hybrid. In 3 out of 6 transformants pigmentation of anthers was completely abolished, while in petals pigmentation was only slightly reduced, in particular in the petal tube and the veins of the petal limb (Figure 7A). In these RNAi lines the expression of both *MYBb1* and *AN4* was reduced, which was not unexpected because of th

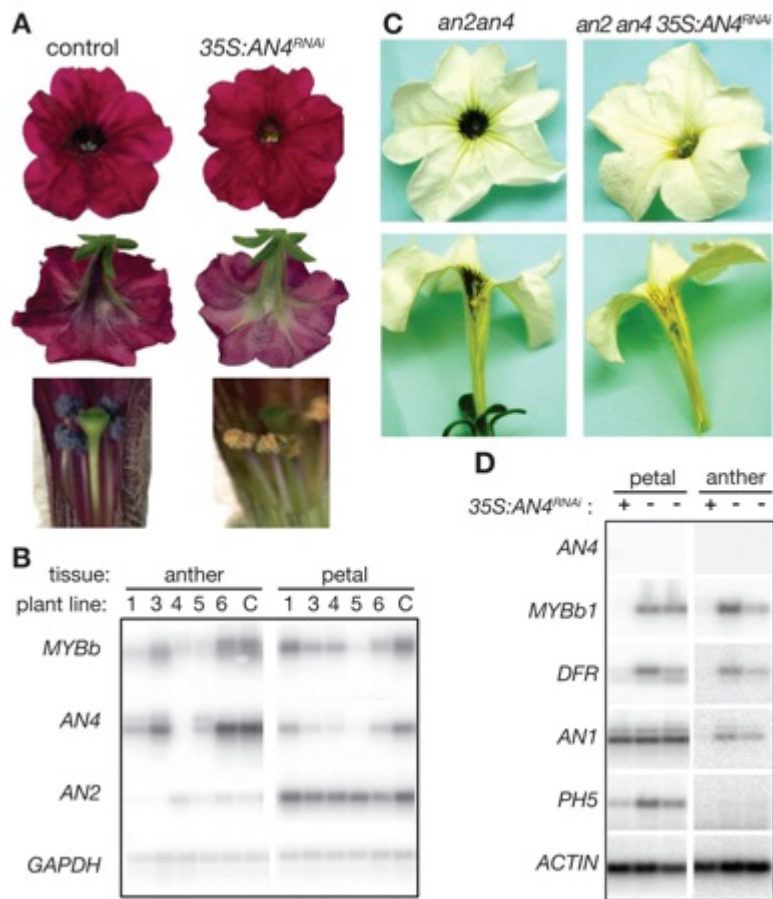


Figure 7. Phenotypes of mutants lacking expression of *AN2*, *AN4* and *MYBb1*.
(A) Phenotype of M1 x V30 flowers and a transgenic sibling expressing *AN4-IR*.
(B) RT-PCR analysis of mRNAs of M1 x V30 flowers (control, c) and 5 *35S:AN4-IR* lines.
(C) Phenotype of W115 flowers and a transgenic sibling expressing *AN4-IR*.
(D) RT-PCR analysis of mRNAs expressed in the petal limb and anthers of W115 expressing *35S:AN4-IR* (+) and control plants lacking the transgene (-).

e high sequence similarity. However, *AN2* mRNA in the petal limbs was not affected (Figure 7B). As the petal phenotype of these RNAi lines was stronger than that of *an4* mutants, this suggested a (partially redundant) role for *MYBb1* in pigmentation of (some) petal cells.

To better assess the role of *MYBb1* in the pigmentation of petals, we introduced the same *35S:AN4^{RNAi}* construct in the line W115 (*an2 an4*). W115 flowers accumulate anthocyanins in a variegated pattern in the petal tube, but pigmentation of the petal limb and anthers is abolished by *an2* and *an4*. In this background RNAi silencing of *MYBb1* severely reduced the pigmentation of the petal tube (Figure 7C). Null mutations in *AN2* strongly reduce *DFR* expression and pigment synthesis in the petal limb but do not completely abolish it, suggesting that *AN2* function in the petal limb is partially redundant. The residual expression of *DFR* in *an2* petals limbs is not due to *AN4*, as the petals limbs of W115 (*an2 an4*), also express this residual amount of *DFR* mRNA (Figure 7D). However, in W115 plants expressing *35S:AN4^{RNAi}*, the (residual) expression of *DFR* is abolished indicating that in the petal limb *MYBb1* is partially redundant with *AN2*. Also the anthers express a little residual *DFR* mRNA and this is also eliminated completely in W115 *MYBb1^{RNAi}* lines. Strikingly the *35S:AN4^{RNAi}* transgene also abolished the low amount of residual *AN1* mRNA that is expressed in *an4* anthers, but has no effect on *AN1* mRNA expression in the petal limb (Figure 7D). This indicates again

that *AN1* expression in anthers requires the AN2-like MYB proteins AN4 and MYBb1, whereas MYBb1 and AN2 are not essential for *AN1* expression in the petal limb.

Discussion

Here we present a detailed characterization of the role of the AN2/AN4 family of MYB proteins in the regulation of the anthocyanin pathway. We show that *AN4* acts in anthers as a “master” activator that acts in a feed forward loop and activates the transcription of the *AN1*, which encodes the HLH partner that is needed for activation of structural anthocyanin genes. These findings bear upon the genetic mechanisms underlying the evolution of distinct pigmentation patterns and the biological functions of anthocyanins.

It has been shown that divergence of pigmentation patterns has occurred in some cases by loss-of-function mutations in specific anthocyanin genes resulting the loss of anthocyanins from few or all tissues, depending on the functional redundancy and expression pattern of the mutated gene(s). For example the divergent pigmentation of petals in *Petunia axillaris*, which has moth-pollinated flowers with white petals, and *Petunia integrifolia*, which has bee pollinated flowers with full colored petals, arose by multiple independent mutations in *AN2* (Hoballah et al., 2007; Quattrocchio et al., 1999). *P. integrifolia* has colored anthers, while *P. axillaris* has yellow anthers and harbors an inactive *an4* with the same structure as *an4*^{R27}, indicating that also in this case divergent patterns arose by a loss-of-function mutation in an ancestral species with colored anthers, and that this mutation was later introgressed into *P. hybrida* cultivars. A similar scenario leads to the divergence of Ipomea species with either blue or red flowers (Zufall and Rausher, 2004).

It is, however, much less clear how novelty can arise during evolution and how pigmentation can be gained in tissues that were not pigmented in the ancestor. It is well established that in a wide range of species the ectopic expression of a MYB and HLH activator of anthocyanin genes is necessary and sufficient to trigger ectopic expression of structural anthocyanins gene in transient assays. This suggests that a change in the expression pattern of one of the MYB genes and one of the HLH genes may cause and create novelty in pigmentation patterns during evolution (or breeding). However, since little is known about how the transcription of the these MYB and HLH is regulated it is hard to foresee how difficult it is and how many genetic alterations are required to have both genes expressed at novel sites.

In most species the MYB and HLH regulators are expressed in distinct but overlapping patterns. In petunia, for example, the WDR protein AN11 is expressed in virtually all tissues, while AN1 is expressed in all pigmented tissues (de Vetten et al., 1997; Spelt et al., 2000). Hence *an1* and *an11* mutants lose anthocyanins in all tissues. The MYB gene *AN2* however, is expressed in a much smaller domain and is essential for pigmentation of the petal limb only (Quattrocchio et al., 1999), whereas pigmentation of other tissues is activated by the homologs *AN4* and *MYBb1* (this paper) and a fourth gene *PURPLE HAZE* (Albert et al., 2011). A similar situation is found in *Antirrhinum* where the MYB genes *ROSEA1* (*ROS1*), *ROS2* and *VENOSA*, are expressed in different patterns than the HLH partners *DELILA* and *MIRABILIS* (Goodrich et al., 1992; Schwinn et al., 2006). This suggests that the activation of MYB and HLH genes occurs by distinct mechanisms, consistent with the finding that loss-of-function mutations in any of MYB genes does not affect the expression of their HLH partners, or vice versa. If so, it would require alterations in two distinct regulatory networks for the ectopic expression of a MYB and HLH genes and gain of pigmentation in new tissues.

Our data suggest, however, that there is more transcriptional cross-regulation of the MYB and HLH genes than previously thought. The finding that *AN4* encodes a MYB protein that is required for the expression of *AN1* mRNA in anthers provides unequivocal evidence that MYB proteins are involved in the transcriptional regulation of their HLH partners. This indicates that the ectopic

activation of *AN1* in transgenic *35S:AN2* plants (Quattrocchio et al., 1998; Spelt et al., 2000) is not caused by the unphysiologically high level of AN2 or AN4 or other "artefacts", but reflects a regulatory interaction that operates in at least some tissues (anthers) in wild type plants. Moreover, it shows that *AN2* and *AN4* are sufficient for ectopic activation of *AN1* and, in concert with the "constitutively" expressed *AN11* gene, for ectopic pigment synthesis.

The role of AN2/AN4 as activators of *AN1* does not rule out that both MYBs are directly involved in the activation of anthocyanins synthesis. In fact the available evidence suggests that these MYBs have both functions and act, in combination with unknown partners, as activators of *AN1* and subsequently as the partners of AN1, and possibly AN11, in a MYB-HLH-WD40 protein complex. Such a feed forward loop implies that the activation of *AN2* or *AN4* triggers the expression of structural anthocyanin genes with a certain delay, because first sufficient AN1 mRNA and protein needs to be synthesized before efficient activation of structural anthocyanin genes can take place. The biological relevance of this delay, if any, is difficult to envisage, because it is expected to be relatively short (< 24 hrs) compared to the timescale of the development of a flower (~1 week) and because constitutive expression of AN1 from a *35S:AN1* transgene complements *an1* efficiently, but causes no obvious alterations in pigmentation of the flower (Spelt et al., 2000). However, the delay caused by such a feed forward loop does explain why constitutive AN2/AN4 expression is sufficient for ectopic activation of structural anthocyanin genes in leaves of transgenic plants, whereas in transient expression assays, (e.g. via particle bombardment) which usually are performed in 24 hrs or less, constitutive expression of AN2 is not sufficient and a HLH partner, either AN1 or JAF13 needs to be co-expressed from a constitutive promoter as well (Quattrocchio et al., 1998; Spelt et al., 2000). Furthermore, the finding that AN1 activates *DFR* directly and that AN2 and AN4 bind to AN1 also suggest a role for AN2/AN4 in the direct activation of structural anthocyanin genes.

Given the strong evidence that AN2 and AN4 can act as activators of AN1 transcription, it remains puzzling why *AN1* expression is not affected in loss of function mutants. Even though *an2*^{W115} (Quattrocchio et al., 1999) and *an4*^{W115} are null alleles, the expression of *DFR* in the petal limb is reduced, as compared to wild type, but not abolished, while *AN1* expression is not affected at all. This indicates that the role of AN2 in the activation of *DFR* is partially redundant with other genes or pathways that are responsible for its residual expression in W115 (*an2an4*) petal limbs and might be sufficient for full expression of *AN1*. Because the silencing of *MYBb1* in W115 petals by *35S:AN4*^{RNAi} completely abolishes (residual) *DFR* expression in the petal limb, the major factor driving the residual *DFR* expression in petals appears to be MYBb1, although we cannot fully exclude that the related PURPLE HAZE protein (Albert et al., 2011) also contributes and is co-silenced in *35S:AN4*^{RNAi} lines. MYBb1 is also partially redundant with AN4 in the anthers, as the small amount of residual *DFR* and *AN1* mRNAs expressed in W115 (*an2an4*) anthers, are completely abolished by silencing of MYBb1 with a *35S:AN4*^{RNAi} transgene. However, even when in *an2an4* *35S:AN4*^{RNAi} petals *DFR* expression is completely down regulated, expression of *AN1* mRNA in this tissue is not affected, remaining as high as in wild type petals. This suggests that, in the petal limb, *AN1* is activated by a distinct pathway, and that AN2 and MYBb1, although sufficiently abundant in petal limbs, contribute little or nothing to the expression of AN1 in this tissue. This may be linked to the finding that the dominant negative MYBb1-EAR protein can shut-off the AN4-dependent activation of *AN1* in anthers, but not the AN2/MYBb1 independent activation of AN1 in the petal tube. It is very unlikely that the 35S promoter, used to drive expression of MYBb1-EAR, has insufficient activity in epidermal petal cells, as we successfully used this promoter to drive various anthocyanin genes and complement corresponding pigmentation mutants (see e.g. Figure 5; Quattrocchio et al., 1998; Spelt et al., 2000; Verweij et al., 2008) and because a PH4-EAR protein expressed from the same promoter efficiently phenocopied *ph4* mutants (unpublished data). Hence it is more probable that AN4-EAR does not affect petal pigmentation

because it fails to bind to one or more target promoters in the petals, because the corresponding *cis*-element is in this tissue not accessible due to DNA methylation or chromatin structure, or because AN4-binding sites are occupied by other proteins that bind with higher affinity.

Since the ectopic expression of MYB proteins like AN4 causes the ectopic activation of their HLH partners and, consequently, also the structural anthocyanin genes, the evolution of novel pigmentation patterns may take as little as a few changes in the regulatory region of the MYB gene to create a *cis*-element that responds to transcription factors expressed in other tissues. Analysis of AN2/AN4 homologs in tomato indicated that such a scenario contributed to the appearance of new varieties that accumulate anthocyanins in various vegetative tissues and in the fruit (chapters 4 and 5). In the same way ancestral *Petunia* species may have acquired pigmented anthers. However, it is important to note in this respect that in most species the MYB-HLH-WDR complex activates only “late” structural gene that are involved in late biosynthetic steps that are specific for anthocyanins, whereas “early” genes such as *CHS*a and *CHI*, encoding enzymes that generate the precursors of many different flavonoids, including anthocyanins, are regulated independently (Martin et al., 1991; Quattrocchio et al., 1993; Quattrocchio et al., 1998). Consequently a shift in the MYB expression pattern can only pigment novel tissues, in which the “early” steps of the pathway are already active.

Material and methods

Petunia lines and relevant genotypes

The *petunia* (*Petunia hybrida*) lines used were: R27 (AN1, AN2, *an4*, PH4), V30 (AN1, AN2, AN4, PH4); W242 (*an1*, AN2, *an4*, PH4); R149 (AN1, AN2, *an4*, *ph4*); W115 (AN1, *an2*, *an4*, PH4) V26 (AN1, AN2 *an4*). Further details on these lines and mutants alleles can be found in elsewhere (de Vetten et al., 1997; Quattrocchio et al., 2006c; Quattrocchio et al., 1993; Spelt et al., 2000; Spelt et al., 2002). The 35S:AN2 transgenic plants were created in a W115 genetic background as described (Quattrocchio et al., 1998).

To introduce the *mybb2*^{R27} into an AN4⁺ background we crossed lines R27 (*an4*) and V30 (AN4) and backcrossed the F1 (AN4*an4*) to R27. Because this backcross segregates for *HF1* (encoding Flavonone 3'5' hydroxylase) and AN4, which are both needed for anther pigmentation, we selected the sought AN4*an4* (colored anthers) and *an4an4* (yellow anthers).

Analysis of DNA and protein sequence

DNA sequences were determined as described elsewhere (Spelt et al., 2002). DNA and protein sequences were analysed with the program Vector NTI (Invitrogen, Carlsbad CA, USA). Multiple sequence alignments were produced with ClustalW algorithm in the MEGA 4.1 software package (Tamura et al., 2007) or a web-based version (<http://www.ebi.ac.uk/clustalw>). Sequence alignments were shaded using Boxshade (<http://www.ch.embnet.org/software>). The phylogenetic tree was constructed with the Minimum Evolution Tree method of Mega 4.1 using only the conserved MYB domains of the following genes and Genbank accession numbers: StAN1, AAX53089.1 (Jung et al., 2009); SIANT1 AAQ55181.1 (Borovsky et al., 2004); SIANT1-like (chapter 5); SIAN2 (Chapter 5); StAN2, AAX53091.1 (Jung et al., 2009); CaAN2, CAE75745.1 (Borovsky et al., 2004); PhAN2, ABO21074.1 (Quattrocchio et al., 1999); PhPHZ, ADQ00391.1 (Albert et al., 2011); PhMYBb2/AN4, ADQ00392.1 (this study). PhMYBb1/DPL (this study); NtAN2, ACO52470.1 (Pattanaik et al., 2010); VvMYBA1, XP_002265406.1 (predicted by automated computational analysis). VvMYBA2, BAD18978.1 (Kobayashi et al., 2004). VvMYBA3, BAD18979.1 (Kobayashi et al., 2004). AmVEN, ABB83828.1 (Schwinn et al., 2006); AmROS1, ABB83826.1 Schwinn et al., 2006; AmROS2, ABB83827.1 (Schwinn et

al., 2006); AtPAP1, AAG42001.1 (Borevitz et al., 2000); AtPAP2, AAG42002.1 (Borevitz et al., 2000); PhPH4, AAY51377.1 (Quattrocchio et al., 2006c); AtMYB5, AEE75369.1 (Stracke et al., 2001); PhODO1, AAV98200.1 (Verdonk et al., 2005); PhMYB1, CAA78386.1 (Avila et al., 1993); AmMIXTA, CAA55725.1 (Noda et al., 1994).

Yeast two hybrid (Y2H) experiments

Yeast strain PJ69a (James et al., 1996) harbouring a plasmid expressing AN1¹⁻²³⁸GAL4^{BD} was transformed with a yeast two-hybrid cDNA library prepared from petunia R27 petal mRNA (Kroon, 2004) using the Lithium acetate method (Gietz et al., 1992). Primary yeast transformants were grown on selective plates lacking leucine, tryptophane and histidine. After about 1 week of growth the colonies were replica plated using velvet cloth onto selective plates lacking leucine, adenine, histidine and adenine. Colonies that appeared were picked and grown for plasmid recovery. Plasmids from transformants selected with the second selection were purified from *E.coli* and tested in a retransformation experiment to confirm the interaction.. Yeast two-hybrid assays on strains co-expressing two defined proteins were done as described previously (Quattrocchio et al., 2006c).

To construct MYBb1-GAL4^{AD}, we amplified the *MYBb1* coding sequence from R27 petal cDNA in pAD-GAL4 (Stratagene) using primers ATGAATTCATGAATACTTCTGTTTTACGTC and GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT, and ligated it as an EcoRI-XhoI fragment in the EcoRI and SAlI site of pAD-GAL4 (Stratagene).

To generate the AN4-GAL4^{AD} construct we amplified the *AN4* coding sequence from V30 petal cDNA using primers ATGAATTCATGAAAATTCTGTTTTACGTC and GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT and ligated it as an EcoRI-XhoI fragment in the EcoRI and SAlI site of pAD-GAL4 (Stratagene).

To generate AN11-GAL4^{BD} we amplified the *AN11* coding sequence from V30 petal cDNA in using primers TCGAATTCATGGAAAATTCAAGTCAAGAATC and TCCTCGAGCTTAT ACTTTAAGCAATTGCA and ligated it as an EcoRI-XhoI fragment in the EcoRI and SAlI site of pBD-GAL4. For PCR amplification a proofreading polymerase was used, and constructs were resequenced to ensure that not(PCR) errors had occurred. Y2H plasmids expressing AN1¹⁻²³⁸GAL4^{BD}, JAF13¹⁻²³⁴GAL4^{BD}, AN2-GAL4, AN1-GALBD and AN1-GAL4^{AD} are described elsewhere(Quattrocchio et al., 2006c).

DNA and RNA methodology

Plant DNA and RNA isolations, reverse transcription, PCR reactions and DNA- gel-blot analyses, were performed as described previously (de Vetten et al., 1997, Spelt 2000, 2002, Quattrocchio et al, 2006).

Primers used for RT-PCR analysis are listed in in Table 1. For *DFR*, *GAPDH* and *AN1*, *MYBb1* and *MYBb2/AN4* respectively 18, 16, 26, 26 and 20 cycles of amplification were used in the RT-PCRs, except where indicated differently. RT-PCR products were size-separated and detected by hybridization using a mixed ³²P-labelled probe. Radioactive hybridisation products were detected using a Phosphor imager (Molecular dynamics, Sunnyvale, CA). Amplification of genomic DNA fragments for sequencing was performed using the primers displayed in table 1.

Quantitative RT-PCR amplification (qPCR) was performed using an Opticon 1 real-time PCR machine (MJ Research) using Opticon Monitor software. *PhEF1α* was used as an endogenous control. Specific primers for each gene were used (Table 2). qPCR reactions were carried out using a Sensimix Sybr No-Rox kit (Bioline), 10 ng of cDNA template, and 4.5 pmol gene-specific primers in a final reaction volume of 15 µl. The relative quantitation of each individual gene expression was performed using the geometric averaging method (geNorm) (Vandesompele et al., 2002).

Cloning procedure, plasmid construction, and tomato transformation

All recombinations in the next part were carried out with BP or LR clonase in Gateway or Gateway compatible vectors (Invitrogen or RU Ghent). All PCR amplifications on cDNA or genomic DNA were performed with Phusion[®] High-Fidelity DNA Polymerase[™] (Finnzymes, Ratastie 2, 01620 Vantaa, Finland). As templates were used genomic DNA and cDNA from petals of line V30.

The AN4^{V30} fragment used for the complementation was amplified with primers containing attB-1 and -2 recombination sites (highlighted below by underlining):

AN4_FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCGAAAAAATGGGTA

AN4_RV: GGGGACCACTTGTACAAGAAAGCTGGGTTCACTGTCAGTTGCTTCAACAA

Entry clones were obtained by BP recombination of the PCR product and vector pDONR221 P1-P2. The resulting pEntry construct was then recombined with pKGW,0 (RU Ghent; www.psb.ugent.be/gateway/) using LR clonase. The resulting construct was transformed in *A.tumefaciens* and used for transformation of plants.

The 35S:GFP-AN2 construct was obtained by amplification of the AN2 CDS with Primer set #9 (GFP)AN2, followed by digestion with NcoI and XbaI, and ligated in pENTR4(Invitrogen) using the same restriction enzymes. This entry clone was recombined in destination vector pK7WGF2(RU Ghent) using LR Clonase, resulting in the 35S:GFP-AN2 construct.

To generate the 35S:AN2-GFP construct we amplified the AN2 coding sequence from a cDNA clone with Primer set #10 AN2(GFP), inserted it in pENTR/D by TOPO cloning and recombined this entry with the destination vector pK7FWG2 (RU Ghent) using LR Clonase, resulting in 35S:AN2-GFP.

To generate the 35S:GFP-AN4 we amplified the AN4 coding sequence from a cDNA clone with with Primer set #11 (GFP)AN4, inserted it in pENTR/D by TOPO cloning and recombined this entry with the destination vector pK7FWG2 (RU Ghent) using LR Clonase, resulting in, resulting in the 35S:GFP-AN4 construct.

To generate the 35S:AN4-GFP we amplified the AN4 coding sequence from a cDNA clone with Primer set #12 AN4(GFP), inserted it in pENTR/D by TOPO cloning and recombined this entry with the destination vector pK7FWG2 (RU Ghent) using LR Clonase, resulting in the 35S:AN4-GFP construct.

To generate the 35S:AN4^{RNAi} we amplified the AN4 coding sequence from a cDNA clone with Primer set #13 AN4(IR), and ligated it as an ECORI-XhoI fragment in pENTR4(Invitrogen) which had been cut with the same This entry clone was recombined in destination vector pK7GWIWG2(I) (RU Ghent) using LR Clonase, resulting in the 35S:AN4^{RNAi} construct.

To generate the 35S:MYBb1-EAR we amplified the MYBb1 coding sequence from a cDNA clone with Primer set #14 MYBB1(EAR), ligated as an NcoI and BamHI fragment in pENTR4 already containing the NtEAR(NtERF3) fragment. This entry clone was recombined in destination vector pK2GW7,0 (RU Ghent) using LR Clonase, resulting in the 35S:MYBB1-EAR construct.

Analysis of the methylation level of different AN4 alleles

1µg genomic DNA extracted from petal (opening flower) or anther (from 2-5mm flower buds) tissue of *petunia* lines V26(*an4*), V30(*AN4*), M1xV30(*AN4*) and V23(*AN4*) was digested with 20units HpyCH4IV (A/CGT) overnight in 100µl restriction buffer L+BSA(0.1µg/ µl). After precipitation 50ng digested DNA were used per PCR of 25µl.

In order to cover all HpyCH4IV sites 8 sets of primers (#15-22) were used.

PCR procedure was standard: 30 cycles with: 30sec 96°C; 30sec 55°C; 60sec 72°C.

PCR products were run on 1% agarose gels and analyzed.

Petunia transformation

Transgenic plants of petunia were obtained by *Agrobacterium tumefaciens*-mediated leaf disc transformation (Quattrocchio et al., 2006b) using the inbred *an2 an4* line W115 (also known as Mitchell or Mitchel diploid) or the wild type F1 hybrid M1 x V30 as a host.

Table 1. Primers used for RT-PCR analysis.

Name	Position	Sequence
<i>MYBb</i> R27	1F seq	GCTTCTCCTCCCAGC
<i>MYBb</i> R27	2R seq	CCGTTGCAGTTATCC
<i>MYBb</i> R27	3F	GGAAAGGAGCATGGGCTGA
<i>MYBb</i> R27	4R	TCATATATCTGAGTAATTCATTGG
<i>MYBb</i> R27	race 1	TGTTGAAGAGCTTTCAGGTGCTGC
<i>MYBb</i> R27	rev	CTCTAACAGGAACCTTGATGC
<i>MYBb</i> R27	R27 intron1 rev	AGTCAAGGTGCTTATACGATACTAGA
<i>MYBb</i> R27	prom. rev. R27	GCCAACACAAAGATAACTTGAG
<i>MYBb</i> R27	exon 1F	GGCTGAAGAAGAAGATATTCTCTTA
<i>MYBb</i> R27	stopR2	TATCTGAGGAATTCATTGGAGTAGC
<i>MYBb</i> R27	leader	TAGAATGATGTGTTCCACAGCTACT
<i>AN4 /MYBb</i>	Dual ATG+ EcoR1	ATGAATTCATGAAWACTTCTGTTTTTACGTC
<i>an4</i>	an4 Act.dom.	ATGAATTCGCTCCTCCTCAGCAACACGAT
<i>an4</i>	an4 V30 BDRV	TCTCTCGAGTTGCTGAGGAGGAGC
<i>an4</i>	an4 Stop	TTTCTCGAGTAATTCATTATAGTAATCCCAGA
<i>an4</i>	an4 V30 intron 1FW	GCTAAACCTCTGTAAGGAAT
<i>an4</i>	an4 R27 intron1 FW	CTAACCTCTATAAGGAACC
<i>an4</i>	an4 R27 intron1 rev	TCCTTGCTCAGTGCGCACTTTCTT
<i>an4</i>	an4 R27 tail rev	GATTACGTCAACAATTACAAGTTT
<i>an4</i>	an4 V30 tail rev	GATTACGTCAACAATTACAAGTTC
<i>an4</i>	an4 V30+R27tail rev	CTCGAGATTACGTCAACAATTACAAGT
<i>an4</i>	an4 exon 2f	TAAAGGAGGTGACTTCTCTCCA
<i>an4</i>	an4 intron 2f	CGCTCATAAAAATGAAATTAGTTG
<i>an4</i>	an4 prom.R27F	GCTTTGAACAAGTTTTGTTCAAG
<i>an4</i>	an4 prom.R27R	TCGCTGAGAAATAGTCACCTC
<i>an4</i>	an4 prom.-120bp Rev	CTAACGTGGACAACATTAAGTGC
<i>an4 V30</i>	an4 V30 leader	GAAGATAGAATATTCACAGCTACC
<i>an4 R27</i>	an4 R27 leader	AAGATAGAATGTCCACAGCTACG
<i>AN1</i>		TAGGATCCAGCCTTATCTGAGCACT
<i>AN1</i>		GGGAATTCTATGGTGTCCCAAG
<i>AN4 (MYBb2-V30)</i>		TGATCTAGAATGAATACTTCTGTTTTTACGTCG
<i>AN4 (MYBb2-V30)</i>		GATTACGTCAACAATTACAAGTTC
<i>AN4 (MYBb2-R27)</i>		TGATCTAGAATGAATACTTCTGTTTTTACGTCG
<i>AN4 (MYBb2-R27)</i>		GATTACGTCAACAATTACAAGTTT
<i>DFR</i>		ACAATGTTCCAGCTACTGTTC
<i>DFR</i>		GTAGGAACATAGTACTCTGG
<i>UBI-LIG (UBCO)</i>		GCTCTAGAATGCAGATCTTTGTCAAGACC
<i>UBI-LIG (UBCO)</i>		GCTCTAGAGGCCACCACGGAGACGGAGGAC
<i>GAPDH</i>		GGTCGTTTGGTTGCAAGAGT
<i>GAPDH</i>		CTGGTTATTCCATTACAACACTAC
<i>MYBb</i>		TGATCTAGAATGAATACTTCTGTTTTTACGTCG
<i>MYBb</i>		TGAGGTACCCATTGGAGTAGCAATCCCAG

Table 2. Primers sets used for qPCR, generation of constructs and methylation analysis.

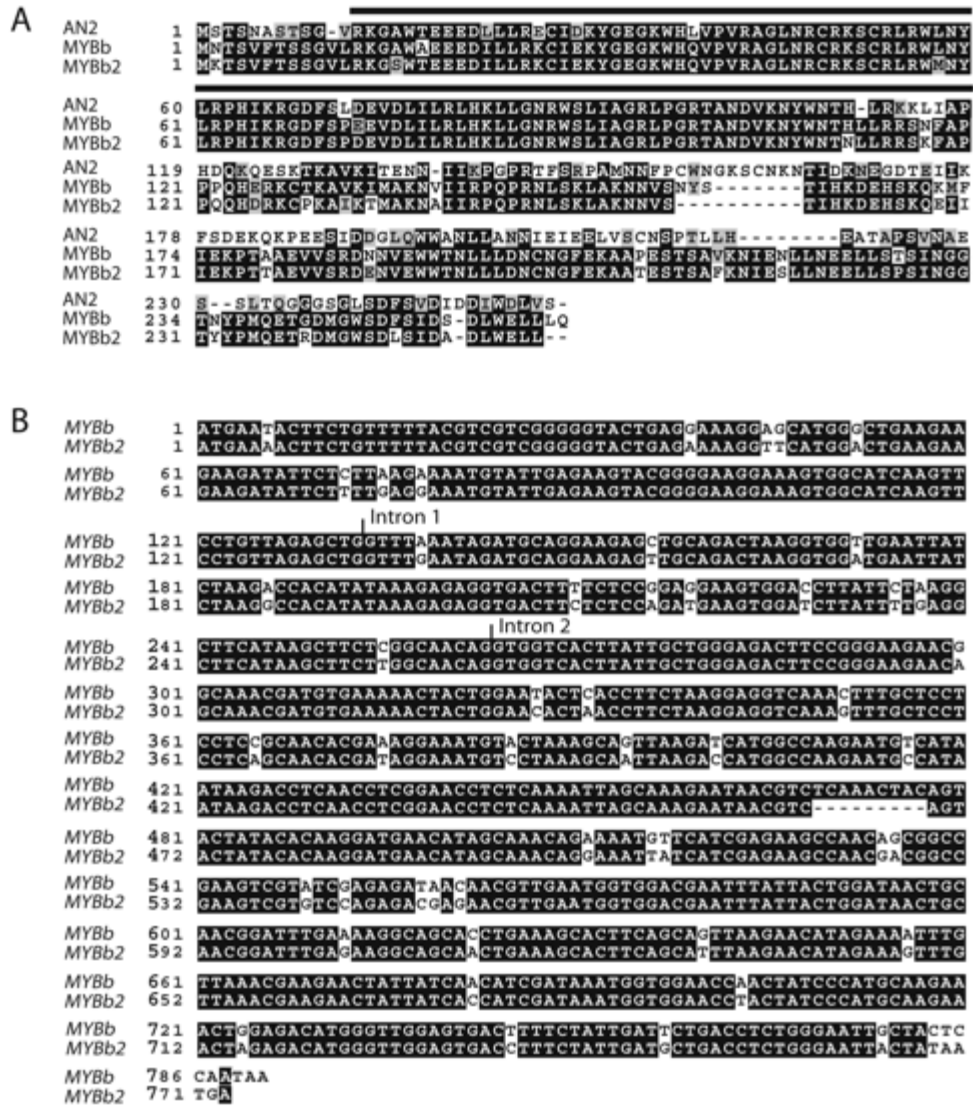
Set	Gene	Forward primer.	Reverse primer
1	EF1A	CCTGGTCAAATTGGAAACGG	CAGATCGCCTGTCAATCTTGG
2	GFP	GTACAACACAACAGCCACAACG	GATCTTGAAGTTCACCTTGATGC
3	DFR	TCATTTGCTCGTCCCACCATGC	TGGCCATTTCTCTCGGACCATC
4	AN2	GCATGAGGAAACAGCACCATCG	AAGCCACTTCCTCCTCCTTGAG
5	AN1	TAGAGCCAATCAGACGGAGGCTAC	CCCTTCTTGCAACACCTTCAC
6	MYBB1	GTATTGAGAAGTACGGGAAGGA	GCAGCTTCTCCTGCATCTATTTA
7	AN4	AAAGTGGCATCAAGTTCCTGTTA	AATTCATCCACCTTAGTCTGCAA
8	PH5	TAGCAATCCTAAATGATGGCACT	CAACTATCAGGTCTTGGAGATGG
9	(GFP)AN2	AAACCATGGTCATGAGTACTTCTA ATGCAA	TAATCTAGATCTCTCAATGGTCC CAATTA
10	AN2(GFP)	CACCATGAGTACTTCTAATGCATCA AC	ACTAACTAAATCCCATATGTCATC AA
11	(GFP)AN4	caccATGAAAACCTTCTGTTTTACGT CGTC	TAGTAATCCCAGAGGTCAGCAT C
12	AN4(GFP)	caccATGAAAACCTTCTGTTTTACGT CGTC	GATTACGTCACAATTACAAGTTC
13	AN4(IR)	ATGAATTCGCTCCTCCTCAGCAAC ACGAT	CTCGAGATTACGTCACAATTAC AAGT
14	MYBB1(EAR)	CGTCCATGGTCATGAATACTTCTG TTT	AGTGGATCCTTGGAGTAGCAAT TCCCAGAG
15	AN4-2.3kb	TCCTCTCCATTACCTAGTGTCT	CAACCAAATTTTCATCCCTCCTCCT
16	AN4-1.6kb	GGTATTCAGTGACATATTTAACTT	GGGGAAGAGAATAAGTCATAT ATATTAAGACTAG
17	AN4-745bp	AACTTCAAACCTTCTATTGGATCTT	AGCACTTCAAGTTATCTGGAGA G
18	AN4-345bp	GTATAAATTGACAAGTGCTGCAAG	ATGGAATAGTCTCTCTAGA CTG
19	AN4-128bp	CTGTAGGACAATTGAAAGATAACA	GATCTCAGGTAGCTGTGAATA TTCTATCTTC
20	AN4+19bp	GAAGATAGAATATTCACAGCTACC	TCCTTGCTCAGTGCGCACTTT CTT
21	AN4+916bp	ATGAATTCGCTCCTCCTCAGCAAC ACGAT	CTTGTGTATAGTACTGACGTT ATTC
22	AN4+1.0kb	ATGAATTCGCTCCTCCTCAGCAA CACGAT	TAGTAATCCCAGAGGTCAGCA TC

References

- 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 J* **65**, 771-84.
- Avila, A., Nieto, C., Cañas, L., Benito, J. and Paz-Ares, J. (1993). *Petunia hybrida* genes related to the maize regulatory *C1* gene and to the animal *myb* proto-oncogenes. *Plant J*. **3**, 553-562.
- Baudry, A., Heim, M. A., Dubreucq, B., Caboche, M., Weisshaar, B. and Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant J* **39**, 366-80.
- Baumann, K., Perez-Rodriguez, M., Bradley, D., Venail, J., Bailey, P., Jin, H., Koes, R., Roberts, K. and Martin, C. (2007). Control of cell and petal morphogenesis by R2R3 MYB transcription factors. *Development* **134**, 1691-701.
- Borevitz, J. O., Xia, Y., Blount, J., Dixon, R. A. and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* **12**, 2383-2394.
- Borovsky, Y., Oren-Shamir, M., Ovadia, R., De Jong, W. and Paran, I. (2004). The A locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to Anthocyanin2 of *Petunia*. *Theor Appl Genet* **109**, 23-9.
- Butelli, E., Titta, L., Giorgio, M., Mock, H. P., Matros, A., Peterek, S., Schijlen, E. G., Hall, R. D., Bovy, A. G., Luo, J. et al. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat Biotechnol* **26**, 1301-8.
- de Vetten, 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 Dev.* **11**, 1422-1434.
- de Vetten, N., ter Horst, J., van Schaik, H.-P., den Boer, B., Mol, J. and Koes, R. (1999). A cytochrome b5 is required for full activity of flavonoid 3'5'-hydroxylase, a cytochrome P450 involved in the formation of blue flower colors. *Proc. Natl. Acad. Sci. USA.* **96**, 778-783.
- Gietz, D., St Jean, A., Woods, R. A. and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.
- Goff, S. A., Cone, K. C. and Chandler, V. L. (1992). Functional analysis of the transcription activator encoded by the maize B-gene: evidence for a direct functional interaction between two classes of regulatory proteins. *Genes Dev.* **6**, 864-875.
- Goff, S. A., Klein, T. M., Roth, B. A., Fromm, M. E., Cone, K. C., Radicella, J. P. and Chandler, V. L. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of B-regulatory genes into maize tissues. *EMBO J.* **9**, 2517-2522.
- 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 J* **53**, 814-27.
- Goodrich, J., Carpenter, R. and Coen, E. S. (1992). A common gene regulates pigmentation pattern in diverse plant species. *Cell* **68**, 955-64.
- Hoballah, M. E., Gubitza, 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**, 779-90.
- Holton, T. A., Brugliera, F. and Tanaka, Y. (1993). Cloning and expression of flavonol synthase from *Petunia hybrida*. *Plant J.* **4**, 1003-1010.
- James, P., Halladay, J. and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. *Genetics* **144**, 1425-36.
- Jiang, C., Gu, X. and Peterson, T. (2004). Identification of conserved gene structures and carboxy-terminal motifs in the Myb gene family of *Arabidopsis* and *Oryza sativa* L. ssp. indica. *Genome Biol* **5**, R46.
- Jung, C. S., Griffiths, H. M., De Jong, D. M., Cheng, S., Bodis, M., Kim, T. S. and De Jong, W. S. (2009). The potato developer (D) locus encodes an R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin. *Theor Appl Genet* **120**, 45-57.
- Kobayashi, S., Goto-Yamamoto, N. and Hirochika, H. (2004). Retrotransposon-induced mutations in grape skin color. *Science* **304**, 982.
- Koes, R., Verweij, C. W. and Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* **5**, 236-242.

- Kroon, A. R.** (2004). Transcription regulation of the anthocyanin pathway in *Petunia hybrida*, (ed. Amsterdam: Vrije Universiteit).
- Martin, C., Prescott, A., Mackay, S., Bartlett, J. and Vrijlandt, E.** (1991). Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. *Plant J.* **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. et al.** (2003). Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. *Plant Cell* **15**, 1689-703.
- Mooney, M., Desnos, T., Harrison, K., Jones, J., Carpenter, R. and Coen, E.** (1995). Altered regulation of tomato and tobacco pigmentation genes caused by the *delila* gene of *Antirrhinum*. *Plant J.* **7**, 333-339.
- Nesi, N., Jond, C., Debeaujon, I., Caboche, M. and Lepiniec, L.** (2001). The Arabidopsis *TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell* **13**, 2099-114.
- Noda, K., Glover, B. J., Linstead, P. and Martin, C.** (1994). Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. *Nature* **369**, 661-664.
- Pattanaik, S., Kong, Q., Zaitlin, D., Werkman, J. R., Xie, C. H., Patra, B. and Yuan, L.** (2010). Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. *Planta* **231**, 1061-76.
- Quattrocchio, F., Baudry, A., Lepiniec, L. and Grotewold, E.** (2006a). The regulation of flavonoid biosynthesis. In *The Science of Flavonoids*, (ed. E. Grotewold), pp. 97-122. New York: Springer.
- Quattrocchio, F., Verweij, W., Kroon, A., Spelt, C., Mol, J. and Koes, R.** (2006b). 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., Verweij, W., Kroon, A., Spelt, C., Mol, J. and Koes, R.** (2006c). 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.
- Quattrocchio, F., Wing, J. F., Leppen, H. T. C., Mol, J. N. M. and Koes, R. E.** (1993). Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. *Plant Cell* **5**, 1497-1512.
- 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 J.* **13**, 475-488.
- Ramsay, N. A. and Glover, B. J.** (2005). MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends Plant Sci* **10**, 63-70.
- Schwinn, K., Venail, J., Shang, Y., 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**, 831-51.
- Spelt, C., Quattrocchio, F., Mol, J. and Koes, R.** (2000). *anthocyanin1* of petunia encodes a basic-Helix Loop Helix protein that directly activates structural anthocyanin genes. *Plant Cell* **12**, 1619-1631.
- Spelt, C., Quattrocchio, F., Mol, J. and Koes, R.** (2002). *ANTHOCYANIN1* of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *Plant Cell* **14**, 2121-35.
- Stracke, R., Werber, M. and Weisshaar, B.** (2001). The R2R3-MYB gene family in Arabidopsis thaliana. *Curr Opin Plant Biol* **4**, 447-56.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S.** (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596-9.
- van Houwelingen, A., Souer, E., Spelt, C., Kloos, D., Mol, J. and Koes, R.** (1998). Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida*. *Plant J.* **13**, 39-50.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F.** (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, 0034.1-0034.12.

- Verdonk, J. C., Haring, M. A., van Tunen, A. J. and Schuurink, R. C.** (2005). ODORANT1 regulates fragrance biosynthesis in petunia flowers. *Plant Cell* **17**, 1612-24.
- Verweij, W., Spelt, C., Di Sansebastiano, G. P., Vermeer, J., Reale, L., Ferranti, F., Koes, R. and Quattrocchio, F.** (2008). An H⁺ P-ATPase on the tonoplast determines vacuolar pH and flower colour. *Nat Cell Biol* **10**, 1456-62.
- Wiering, H.** (1974). Genetics of flower colour in *Petunia hybrida* Hort. *Genen Phaenen* **17**, 117-134.
- 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 J* **40**, 22-34.
- Zufall, R. A. and Rausher, M. D.** (2004). Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* **428**, 847-50.



Supplementary Figure S1. Sequence comparison of the coding sequences of MYBb1/DPL, MYBb2/ AN4. The nucleotide sequences, determined for the V30 alleles, were aligned using the clustalW program. The conserved nucleotides are boxed in black. Note the 9 bp insertion in *mybB* relative to *mybB2*. The position of the introns is marked above the sequences.