

# Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10

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## Summary

Anthocyanin concentration is an important determinant of the colour of many fruits. In apple (*Malus × domestica*), centuries of breeding have produced numerous varieties in which levels of anthocyanin pigment vary widely and change in response to environmental and developmental stimuli. The apple fruit cortex is usually colourless, although germplasm does exist where the cortex is highly pigmented due to the accumulation of either anthocyanins or carotenoids. From studies in a diverse array of plant species, it is apparent that anthocyanin biosynthesis is controlled at the level of transcription. Here we report the transcript levels of the anthocyanin biosynthetic genes in a red-fleshed apple compared with a white-fleshed cultivar. We also describe an apple MYB transcription factor, *MdMYB10*, that is similar in sequence to known anthocyanin regulators in other species. We further show that this transcription factor can induce anthocyanin accumulation in both heterologous and homologous systems, generating pigmented patches in transient assays in tobacco leaves and highly pigmented apple plants following stable transformation with constitutively expressed *MdMYB10*. Efficient induction of anthocyanin biosynthesis in transient assays by *MdMYB10* was dependent on the co-expression of two distinct bHLH proteins from apple, *MdbHLH3* and *MdbHLH33*. The strong correlation between the expression of *MdMYB10* and apple anthocyanin levels during fruit development suggests that this transcription factor is responsible for controlling anthocyanin biosynthesis in apple fruit; in the red-fleshed cultivar and in the skin of other varieties, there is an induction of *MdMYB10* expression concurrent with colour formation during development. Characterization of *MdMYB10* has implications for the development of new varieties through classical breeding or a biotechnological approach.

**Keywords:** anthocyanin, *Malus × domestica*, *MdMYB10*, regulation, transcription factor.

## Introduction

The accumulation of anthocyanin pigments in apple fruit is an important determinant of fruit quality. Usually restricted to the skin of apples, these pigments provide essential cultivar differentiation for consumers and are implicated in the health attributes of apple fruit (Boyer and Liu, 2004). Anthocyanins belong to the diverse group of ubiquitous secondary metabolites collectively known as flavonoids. In plants, flavonoids are believed to have a variety of functions, including defence and protection against light stress, and the pigmented anthocyanin compounds play an important physiological role as attractants in plant/animal interactions (Harborne and Grayer, 1994; Koes *et al.*, 1994).

Differences in colour attributable to anthocyanins may be due to a number of factors including the number of hydroxyl groups on the B-ring, the sugars and acyl side groups (Harborne, 1967), the environment of the vacuole including its pH or the accumulation of specific metal ions (Brouillard, 1988), or cellular ultrastructure (Noda *et al.*, 1994). One of the most common anthocyanin pigments is cyanidin, which, in the form of cyanidin 3-*O*-galactoside, is the pigment primarily responsible for red colouration in apple skin (Lancaster, 1992; Tsao *et al.*, 2003). The enzymes operating in this biosynthetic pathway in apple have been well characterized (Honda *et al.*, 2002; Kim *et al.*, 2003).

In apple, pigment biosynthesis may be induced by light, particularly UV, and various stress treatments including cold (Dong *et al.*, 1998). There is also evidence that the anthocyanin biosynthetic enzymes are coordinately induced during development of apple fruit (Lister and Lancaster, 1996). This suggests that expression of the genes encoding the biosynthetic enzymes is coordinately regulated by one or a few regulatory proteins.

There are numerous reports of the regulation of genes in the anthocyanin pathway by transcription factors (TFs), and collectively these have established that the components of the regulatory complex controlling anthocyanin biosynthesis are conserved in all higher plants (Holton and Cornish, 1995). MYB TFs have been shown to play an important role in transcriptional regulation of anthocyanins (reviewed in Martin and Paz-Ares, 1997). Plant MYBs have been implicated in controlling pathways as diverse as secondary metabolism (including the anthocyanin pathway), development, signal transduction and disease resistance (Jin and Martin, 1999). They are characterized by a structurally conserved DNA-binding domain consisting of single or multiple imperfect repeats; those associated with the anthocyanin pathway are of the two-repeat (R2R3) class. Regulation also appears to be specific to discrete subsets of structural genes, acting either early or late in the anthocyanin biosynthetic pathway (Davies and Schwinn, 2003). In the leaves of perilla (*Perilla frutescens*), coordinated transcriptional regulation controls virtually all steps of anthocyanin biosynthesis from the genes encoding chalcone synthase (CHS) to the final genes encoding anthocyanin transporter proteins (Saito and Yamazaki, 2002). In contrast, in grape (*Vitis vinifera*), specific regulation by *MybA* is restricted to the very late steps in anthocyanin production, including UDP-glucose:flavonoid 3-O-glycosyltransferase (UFGT), but not the earlier biosynthetic steps (Kobayashi *et al.*, 2002).

There are 126 R2R3 MYB TFs in *Arabidopsis thaliana* L. Heynh, which can be divided on the basis of their sequence into 24 subgroups (Stracke *et al.*, 2001). The *Production of Anthocyanin Pigment 1* (*PAP1*) MYB gene of *Arabidopsis* (Borevitz *et al.*, 2000) falls into subgroup 10 (when the phylogeny of Stracke *et al.*, 2001 is used), and its protein demonstrates a high degree of amino acid conservation with other known MYB regulators of anthocyanin production. When *PAP1* was over-expressed in transgenic *Arabidopsis*, this led to up-regulation of a number of genes in the anthocyanin biosynthesis pathway from phenylalanine ammonia-lyase (PAL) to chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR) (Borevitz *et al.*, 2000; Tohge *et al.*, 2005).

The R2R3 MYB factors that regulate anthocyanin biosynthesis have been shown to interact closely with basic helix-loop-helix (bHLH) TFs (Mol *et al.*, 1996; Winkel-Shirley, 2001). Examples include the maize *ZmC1* MYB and *ZmB* bHLH, the petunia *AN2* MYB and *AN1/JAF13* bHLHs, and the *Antirrhinum* *Rosea1*, *Rosea2* and *Venosa* MYBs and the

*Mutabilis* and *Delila* bHLHs (Goff *et al.*, 1992; Goodrich *et al.*, 1992; Mol *et al.*, 1998; Schwinn *et al.*, 2006). Over-expressing lines of *PAP1* have elevated transcript levels of the *TT8* gene (Genbank accession NM\_117050) that encodes a bHLH protein involved in regulating condensed tannin and anthocyanin biosynthesis (Nesi *et al.*, 2000; Tohge *et al.*, 2005). The dependency of these specific MYB-related proteins on a bHLH co-regulator to activate transcription is linked to the presence of a small number of amino acid residues (a signature motif) in the highly conserved R2R3 binding domain of the MYB proteins, as revealed by comparison between the structure of the maize P protein (which activates in a bHLH-independent manner) and the bHLH-dependent maize C1 protein (Grotewold *et al.*, 2000). Substitution of just six amino acids from the R2R3 domain of MYB C1 into the corresponding positions in MYB P resulted in a mutant with bHLH-dependent behaviour, similar to C1. More recently, it was suggested that this might be a key mechanism that allows MYBs to discriminate between target genes (Hernandez *et al.*, 2004).

An additional level of anthocyanin regulation involves a separate class of proteins, containing WD40 domains, which form complexes with MYB and bHLH proteins (Baudry *et al.*, 2004, and reviewed in Ramsay and Glover, 2005). Examples include *an11* in petunia (de Vetten *et al.*, 1997) and *TTG1* in *Arabidopsis* (Walker *et al.*, 1999). However, a ubiquitous role for the WD repeat in the transcriptional complex regulating anthocyanin biosynthesis has yet to be demonstrated. Indeed, in maize, the WD repeat protein appears to function in regulating anthocyanin biosynthesis only in kernels. It may be that some bHLH proteins are more dependent on the participation of WD repeat proteins in the regulatory complex than others.

Although studies into the activation and repression of anthocyanin biosynthesis in apple fruit have shown developmental and environmental control, to date this has not been characterized at the level of transcriptional regulation. The control of anthocyanin accumulation in apple is a key question in understanding and manipulating the colour of the fruit. Identification of the factors that exert this control will provide tools for moderating the extent and distribution of anthocyanin-derived pigmentation in fruit tissue. In this study, we compared two genotypes, the white-fleshed commercial cultivar *Malus × domestica* 'Sciros' (Pacific Rose™) and the red-fleshed cultivar *Malus × domestica* 'Red Field' Open Pollinated ('Red Field'), which is highly pigmented throughout all tissues including fruit (skin and cortex) and leaves. We were able to isolate the cDNA of a subgroup 10 MYB, *MdMYB10*, from the highly pigmented red-fleshed cultivar. We compared expression of the major anthocyanin biosynthetic genes, and our candidate TF, in the different apple varieties by quantitative RT-PCR (qPCR). From our apple EST database (Newcomb *et al.*, 2006), several candidate bHLH partners were also isolated. We

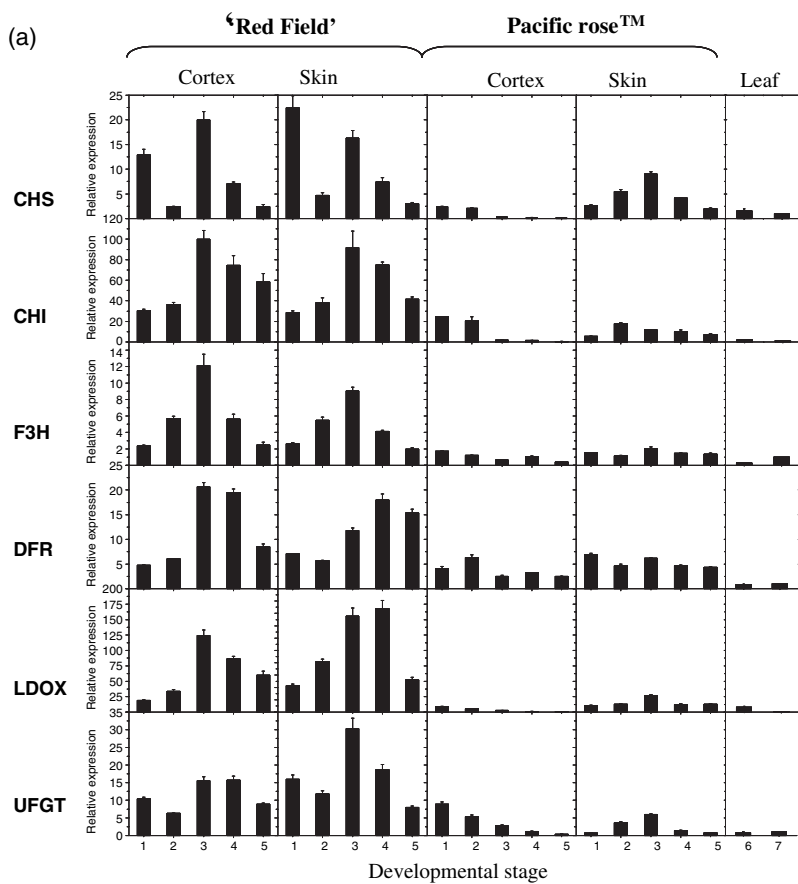
further show that this transcription factor is functionally active in driving anthocyanin accumulation in both heterologous and homologous systems, generating pigmented tobacco leaf patches using transient transformation methods and highly pigmented anthocyanin-rich apple plants using stable transformation techniques. In expression analysis studies, the behaviour of *MdMYB10* suggests that it is responsible for red apple fruit colour.

**Results**

*The RNA expression profile of anthocyanin biosynthetic enzymes suggests coordinated regulation in apple fruit*

The levels of transcripts encoding six anthocyanin biosynthetic genes were compared between white-fleshed Pacific

Rose™ and red-fleshed 'Red Field' apples by qPCR. Striking differences were observed. For all the genes assayed, transcript levels in 'Red Field' were significantly higher during all stages of fruit development than the levels found in Pacific Rose™ (Figure 1a). Transcript abundance in 'Red Field' was enhanced throughout fruit development in both skin and cortex, with the highest transcript levels at stage 3 (102 days after full bloom, DAFB). This correlated with the degree of pigmentation observed during tissue sampling, with the most intense pigmentation being observed early in development (40 DAFB) and then again in mid-summer (102 DAFB). These levels were subsequently sustained through to fruit maturation in late summer (Figure 1b). In Pacific Rose™ cortex tissue, there were comparatively low transcript levels for all the anthocyanin biosynthesis genes assayed, with a general decline in expression during fruit



**Figure 1.** Expression profiling of apple anthocyanin genes.

(a) Data from qPCR analysis of the apple anthocyanin biosynthetic genes in the cortex, skin and leaf of the apple cultivars 'Red Field' and Pacific Rose™. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3β-hydroxylase; DFR, dihydroflavonol 4-reductase (denoted as DFR1 in the text); LDOX, leucoanthocyanidin dioxygenase; UFGT, uridine diphosphate (UDP)-glucose:flavonoid 3-O-glycosyltransferase. Samples referred to on the x axis: (1) 40 days after full bloom (DAFB), (2) 67 DAFB, (3) 102 DAFB, (4) 130 DAFB, (5) 146 DAFB, (6) 'Red Field' leaf and (7) Pacific Rose™ leaf. Error bars are SE for three replicate reactions.

(b) Fruit development series and leaves of 'Red Field' and Pacific Rose™. Numbering from 1 to 7 as for (a).



development. Moderate activity was observed in the skin of Pacific Rose™, with a peak of expression midway through development (102 DAFB), concomitant with the onset of enhanced levels of pigmentation during fruit maturation.

Expression of all genes was detectable in the leaves of both cultivars but was relatively low, with little difference between 'Red Field' and Pacific Rose™. As multiple copies of these biosynthetic genes exist in apple, it is possible that different family members or alleles may be differentially expressed in such diverse tissues as fruit and leaf. To test this, two family members of DFR were identified, denoted as DFR1 (Genbank accession AF117268) (shown in Figure 1) and DFR2 (Genbank accession DT040651), and were assayed with gene-specific primers. Transcript levels of DFR1 were shown to be up-regulated in pigmented fruit tissue but not to any great extent in leaf (as shown in Figure 1). In contrast, DFR2 showed little or no activity in fruit but was highly elevated in the leaves of 'Red Field' (Figure S1).

Cloning of transcription factor candidates from pigmented apple flesh

Previous studies, in other species, have shown that subgroup 10 MYBs are key determinants of plant pigmentation. Within publicly available apple EST databases (199 900 nucleotide sequences in April 2006); there were no MYB TFs showing high homology in BLAST searches to Arabidopsis PAP1 or subgroup 10 MYBs from other species. The qPCR results for gene transcripts encoding the enzymes of the anthocyanin biosynthetic pathway were analysed to determine the most suitable tissue/time point for relevant MYB transcription factor isolation. We chose tissue from the cortex of 'Red Field' that showed the highest expression levels for the anthocyanin biosynthesis genes, and then

applied a degenerate PCR approach to gene isolation. Overlapping sequence alignments of cDNAs cloned after PCR showed that the best candidate, MdMYB10, when translated into protein, shared a high degree of homology with other MYB TFs at the R2R3 domain, and, in particular, with anthocyanin regulators from other species (Figure 2).

MdMYB10 is closely related to the Arabidopsis subgroup 10 MYBs, including PAP1, with 77% amino acid identity over the R2R3 DNA-binding domain and 58% identity to the entire protein. For Arabidopsis PAP2, these amino acid percentage identities are 75% and 57%, respectively, whilst for other species figures for overall identity are as follows: petunia AN2 60%, tomato ANT1 57%, maize C1 58% and maize P 26%. A bootstrapped circular phylogenetic tree, generated using MEGA version 3.1 (Kumar *et al.*, 2004), shows MdMYB10 in the same clade as Arabidopsis PAP1, PAP2, AtMYB113 and AtMYB114 (Figure 3a). MdMYB10 clusters with known MYBs involved in anthocyanin regulation from a diverse range of species (Figure 3b). Sequences of MdMYB10 from three cultivars, 'Red Field' (GenBank accession number DQ267896), Pacific Rose™ (DQ267897) and Granny Smith (DQ267989), were analysed. Within the coding region there were only minor nucleotide differences, which produced no changes in the predicted amino acids encoded. The only exception is a two-base change resulting in a leucine to glutamine amino acid change in helix 3 of the R2 domain (see marked residue in Figure 2). Both versions appear in the 'Red Field' and Pacific Rose™ cultivars.

MdMYB10 has the amino acid residues that specify interaction with bHLHs (arrows on Figure 2) (Grotewold *et al.*, 2000; Zimmermann *et al.*, 2004). Candidates for bHLH co-factors were therefore selected from the HortResearch EST database (Newcomb *et al.*, 2006). In the large phylogenetic family that constitutes the bHLH type TF, there is a

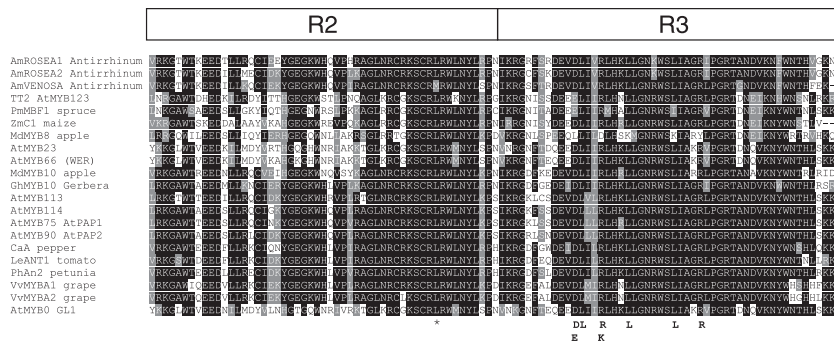
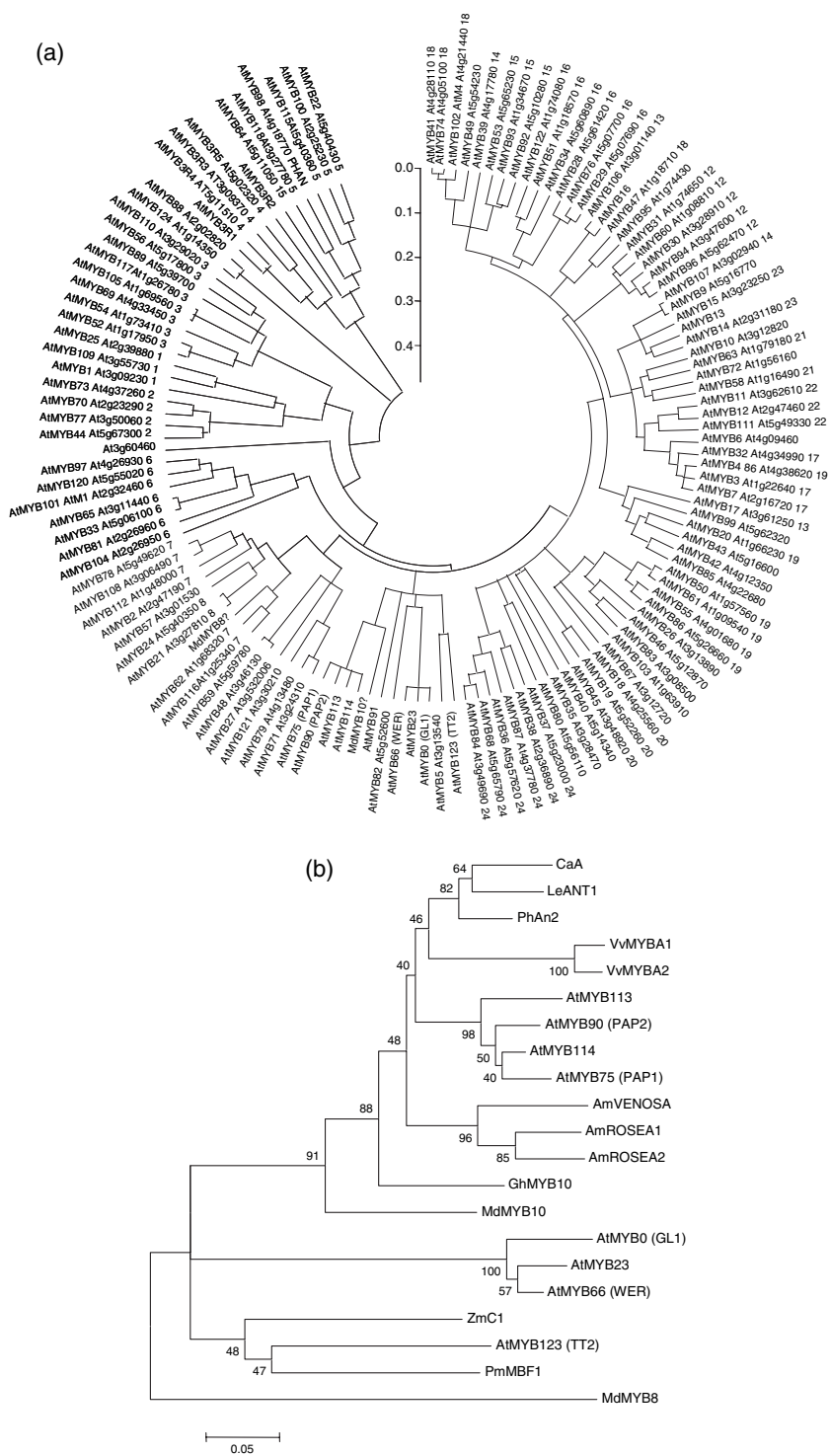


Figure 2. Protein sequence alignment of the R2R3 DNA-binding domains of MdMYB10 and other known anthocyanin MYB regulators from other species. Arrows indicate specific residues that contribute to a motif implicated in bHLH co-factor interaction in Arabidopsis (Zimmermann *et al.*, 2004). These same residues are evident in MdMYB10, suggesting a similar protein–protein interaction. The asterisk represents a two-nucleotide change that results in a leucine to a glutamine amino acid change in some alleles. Identical residues are shown in black, conserved residues in dark grey, and similar residues in light grey. MdMYB8 and AtGL1 are included as examples of MYBs that are not involved in anthocyanin regulation. The accession number of these proteins, or translated products, in the GenBank database are as follows: AmROSEA1, ABB83826; AmROSEA2, ABB83827; AmVENOSA, ABB83828; MdMYB10, DQ267896; AtPAP1, CAB09230; AtPAP2, NP176813; AtMYB113, NM105308; AtMYB114, NM105309; AtMYB66, NM121479; VvMYB1, AB242302; VvMYB2, AB097924; Ca A, CAE75745; PhAN2, AAF66727; LeANT1, AAQ55181; GhMYB10, CAD87010; PmMBF1, AAA82943; ZmC1, AAK81903; AtTT2, Q9FJA2; MdMYB8, DQ267899; AtGL1, AAC97387.



**Figure 3.** Phylogenetic relationships between Arabidopsis MYB transcription factors and anthocyanin-related MYBs of other species. (a) MdMYB10 clusters next to PAP1 (AtMYB75), within the anthocyanin MYB regulator subgroup 10. Subgroup numbers are those described by Stracke *et al.* (2001) and are shown as a suffix after most MYB descriptors. Arabidopsis genes are identified by Arabidopsis unique identifiers. (b) Phylogeny of MdMYB10 and MYB genes from other species involved in the regulation of anthocyanin biosynthesis. Full-length sequences were aligned using Clustal W (opening = 15, extension = 0.3) in Vector NTI 9.0. Conserved motifs were extracted and re-aligned as above. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) using a minimum evolution phylogeny test and 1000 bootstrap replicates. Accession numbers for genes from other species are given in Figure 2.

smaller clade termed IIIf (Heim *et al.*, 2003) that has been shown to be involved in the regulation of anthocyanin biosynthesis. Two apple ESTs encoded bHLH TFs that clustered within this clade (Figure 4a). These ESTs were sequenced and given the identifiers *MdbHLH3* (GenBank accession number CN934367), a putative homologue of the

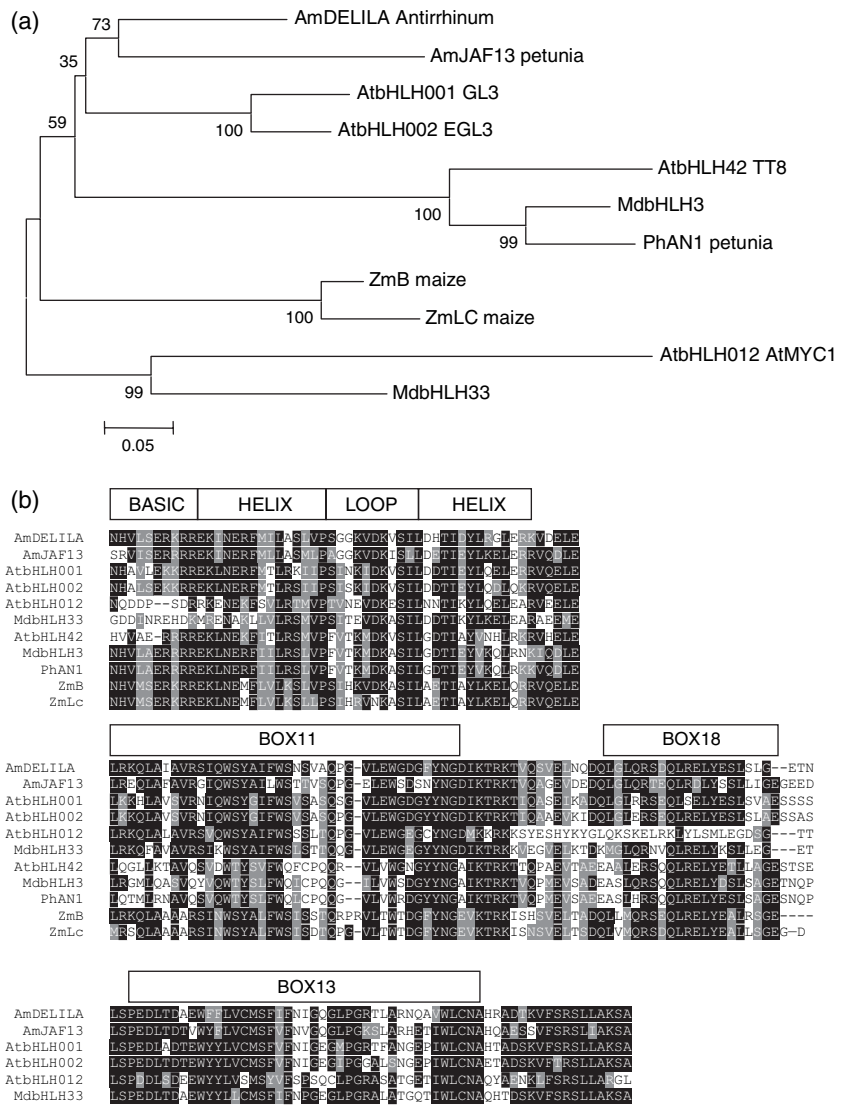
Arabidopsis *TT8* gene, and *MdbHLH33* (GenBank accession number DQ266451), a putative homologue of *Delila* (from *Antirrhinum*, Goodrich *et al.*, 1992). N-terminal domains identified as important for MYB interaction (Heim *et al.*, 2003) were present in both *MdbHLH3* and *MdbHLH33* (Figure 4c).

**Figure 4.** MdbHLH3 and MdbHLH33 share homology at the bHLH motif with anthocyanin bHLH regulators from other species.

(a) Unrooted phylogenetic tree showing that MdbHLH3 clusters in the same group as AtTT8 whilst MdbHLH33 clusters in the same group as AtMYC1. Full-length sequences were aligned using Clustal W (opening = 15, extension = 0.3) in Vector NTI 9.0. Conserved motifs were extracted and re-aligned as above. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) using a minimum evolution phylogeny test and 1000 bootstrap replicates.

(b) Protein sequence alignment, with the structure of the bHLH binding domain indicated. The accession numbers of these proteins, in the GenBank database are as follows: AmDELILA, AAA32663; ATMYC1, BAA11933; EGL1, Q9CAD0; G13, NP680372; PhJAF13, AAC39455; MdbHLH3, CN934367; MdbHLH33, DQ266451; PhAN1, AAG25928; AtTT8, CAC14865; ZmB, CAA40544; ZmLC, AAA33504.

(c) N-terminus of the same bHLH subset showing regions (boxes 11, 18 and 13) conserved within the bHLH IIIb clade (according to Heim *et al.*, 2003). Identical residues are shown in black, conserved residues in dark grey, and similar residues in light grey.



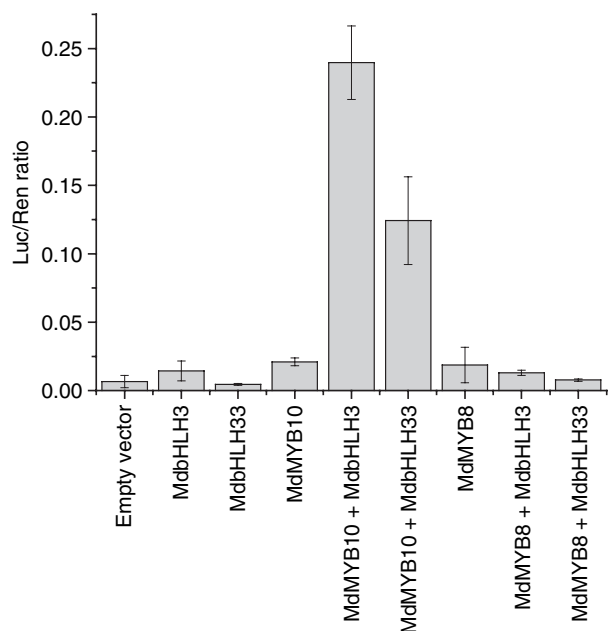
*Transient luminescent assays of apple transcription factor activity*

The dual luciferase system has been demonstrated to provide a rapid method of transient gene expression analysis (Hellens *et al.*, 2005; Matsuo *et al.*, 2001). It requires no selectable marker and results can be quantified by a simple enzymatic assay. We used *Nicotiana tabacum* to test the interaction of our candidate TFs with an Arabidopsis anthocyanin biosynthesis gene promoter AtDFR (*TT3*, GenBank accession number NM\_123645). This is known to be regulated by Arabidopsis *PAP1* and *PAP2* MYB TFs (Tohge *et al.*, 2005; Zimmermann *et al.*, 2004). To investigate the interaction between MYB and bHLH TFs, co-transformations were performed with bHLH class putative regulators from apple: *MdbHLH3* and *MdbHLH33*. The results from the promoter assay indicated a significant increase in activity when

*MdMYB10* was co-transformed with an apple bHLH (Figure 5). The highest activity was observed when *MdMYB10* was co-infiltrated with *MdbHLH3*, although *MdbHLH33* also activated *MdMYB10*. These results reflect previous work in a transient protoplast transfection system in which an Arabidopsis DFR promoter:GUS fusion was activated by *PAP1* only in the presence of a bHLH (Zimmermann *et al.*, 2004). The apple MYB *MdMYB8* (which falls in subgroup 8, Figure 3) was selected to probe the specificity of *MdMYB10* against the DFR promoter: *MdMYB8* activity was low under all conditions and showed no activation by either bHLH (Figure 5).

*Transient colour assays of apple transcription factor activity*

We have established a simple method to reveal anthocyanin pigment accumulation in *Nicotiana tabacum* via *Agrobac-*



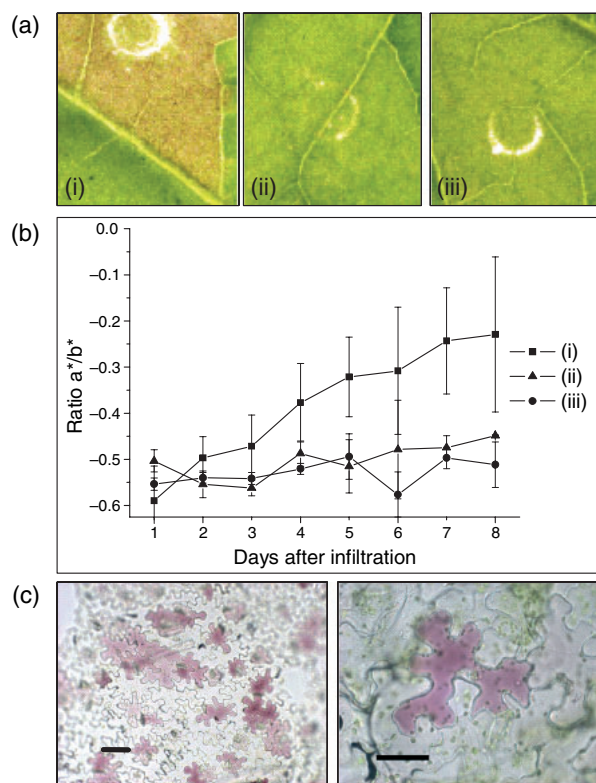
**Figure 5.** Interaction of *MdMYB10* and apple bHLH TFs in transient tobacco transformation assays affects the activity of the DFR gene promoter. The dual luciferase assay shows promoter activity expressed as a ratio of DFR promoter luciferase (LUC) to 35S Renilla (REN), where an increase in activity equates to an increase in LUC relative to REN. Error bars are the SE for six replicate reactions.

*terium* infiltration. Subgroup 10 MYBs and anthocyanin-related bHLHs were syringe-infiltrated into the underside of expanding tobacco leaves. Pigmentation was evident at infiltration points as early as 4 days post-infiltration for *MdMYB10* when co-infiltrated with an apple bHLH (Figure 6a). The degree of pigmentation increased over the experimental period of up to 10 days.

Pigmentation was also observed with infiltration of *MdMYB10* alone (Figure 6a), but not when either apple bHLH or *MdMYB8* alone were infiltrated (data not shown). The results demonstrate the efficacy of this assay as a useful visual reporter system to study the regulation of the pigmentation processes.

Colour intensity and hue were quantified by measurement with a Minolta chromometer, using the  $L^*a^*b^*$  system. This confirmed the visible transition from green to red. The data are shown as a ratio of  $a^*/b^*$  (Figure 6b), where the change from negative towards positive indicates a shift from green to red. It should be noted that there was variability in pigmentation between replicates of a given treatment, particularly between leaves of different ages (Figure 6b).

To verify cellular build-up of anthocyanin compounds, microscopic images were obtained from epidermal peels, removed from the abaxial surface of the leaf, 1 week after co-inoculation with *MdMYB10* and *MdbHLH3* (Figure 6c). This illustrated the transformation of individual cells with the candidate genes and the accumulation of anthocyanin pigments within the vacuoles of epidermal cells.



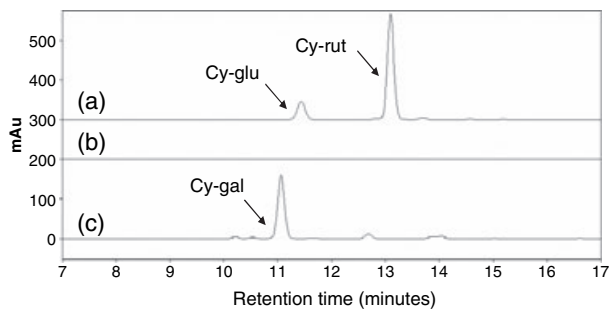
**Figure 6.** Development of colour due to transient transformation of *Nicotiana tabacum* leaves.

(a) Digital images of infiltration sites 8 days after transformation with: (i) *MdMYB10* + *MdbHLH3*, (ii) *MdMYB10* alone, and (iii) empty vector control. (b) Colour measurement by Minolta chromameter, shown as  $a^*/b^*$  ratio. A shift towards positive indicates a colour change from green towards red; (ii) to (iii) as above. Error bars are the SE for six replicate reactions.

(c) Microscope images showing anthocyanin accumulation in tobacco epidermal cells infiltrated with *MdMYB10* + *MdbHLH3* at magnifications of 20 $\times$  (left) and 40 $\times$  (right). Scale bars = 50  $\mu$ m.

#### HPLC analysis of anthocyanins in tobacco and apple

To confirm the identity of the anthocyanins synthesized after transformation of tobacco with selected TFs, samples of leaf tissue were removed and extracted, and the soluble anthocyanins analysed by HPLC. When *MdMYB10* and *MdbHLH3* were co-transformed in tobacco leaves, two major peaks were observed (Figure 7a), representing cyanidin-3-glucoside (LC-MS molecular ion [M<sup>+</sup>] with a mass of 449 Da; major fragment of 287 Da corresponding to the M<sup>+</sup> of cyanidin with a 'neutral loss' of 162 Da corresponding to a hexose sugar residue) and cyanidin-3-O-rutinoside (LC-MS M<sup>+</sup> at 595 Da, with major fragments of 449 Da [cyanidin hexoside], 287 Da [cyanidin] and neutral losses of 146 Da [deoxyhexose sugar; probably rhamnose residue] and a 162 Da hexose sugar residue). No observable anthocyanin peaks were found in the extracts of tobacco leaf transformed with *Agrobacterium* carrying empty vector control alone.



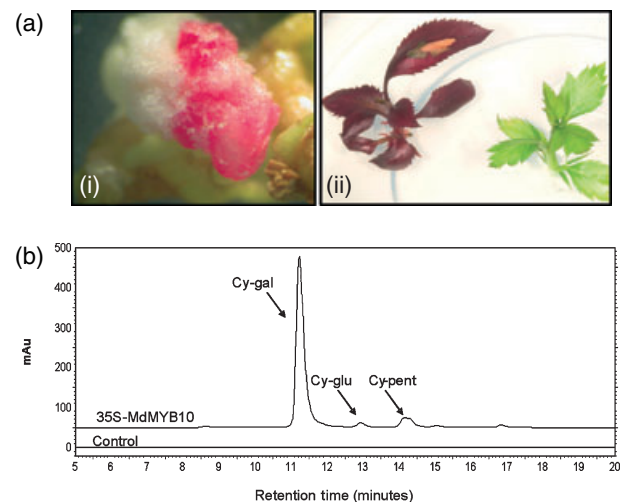
**Figure 7.** Anthocyanin profiles of *N. tabacum* and apple. HPLC traces at 520 nm of (a) *MdMYB10* + *MdbHLH3*, (b) tobacco leaf infiltrated with empty vector control, and (c) Pacific Rose<sup>TM</sup> apple fruit skin. Peaks identified are as follows: cy-glu, cyanidin-3-glucoside; cy-rut, cyanidin-3-O-rutinoside; cy-gal, cyanidin-3-galactoside.

(Figure 7b). To compare with compounds naturally occurring in apple, anthocyanins from the skin of apple (Pacific Rose<sup>TM</sup>, mature fruit) were also extracted. HPLC confirmed the predominance of cyanidin-3-galactoside (LC-MS as for glucoside above) in apple skin, with traces of cyanidin glucoside and three pentosides (Figure 7c) as previously described (Tsao *et al.*, 2003).

An HPLC comparison (not shown) of apple flesh phenolic extracts showed that the phenolic profile at 280 nm of 'Red Field' was almost identical to that of Pacific Rose<sup>TM</sup>, with the exception that anthocyanins were present only in the flesh of the former. No significant change in levels of phenolic acids, flavonoids, chalcones or procyanidins was observed.

#### Generation of 35S-MdMYB10 transgenic apple plants

To test the function of *MdMYB10* in a homologous system, apple explants (cultivar 'Royal Gala') were transformed with 35S-*MdMYB10* and a kanamycin selectable marker. Regenerated callus of kanamycin-positive transformants was highly pigmented (Figure 8a). Shoots regenerated from this callus maintained colour, whilst control plants transformed with an empty vector consistently showed no pigmentation at either the callus or regenerated shoot stages. qPCR analysis confirmed a massive elevation of *MdMYB10* transcript level but no apparent elevation in either *MdbHLH3* or *MdbHLH33* transcript levels (Figure S2). The suggestion that different gene family members of biosynthetic enzymes are leaf- or fruit-specific was confirmed in these transgenic plants. *MdDFR1* showed a slight increase in transcript levels in the 35S-*MdMYB10* transgenic lines compared to the control plants, and *MdDFR2* showed even higher levels (Figure S1). HPLC analysis of *MdMYB10* transgenic apple leaves showed a predominant peak of cyanidin-3-galactoside (Figure 8b) and confirmed the presence of smaller amounts of cyanidin glucoside and three derivatives of cyanidin pentoside.



**Figure 8.** Over-expression of *MdMYB10* in apple elevates anthocyanin production.

(a) Pigmented callus (i) and apple plants (ii) transformed with 35S-*MdMYB10* (left) and empty vector control plant (right).

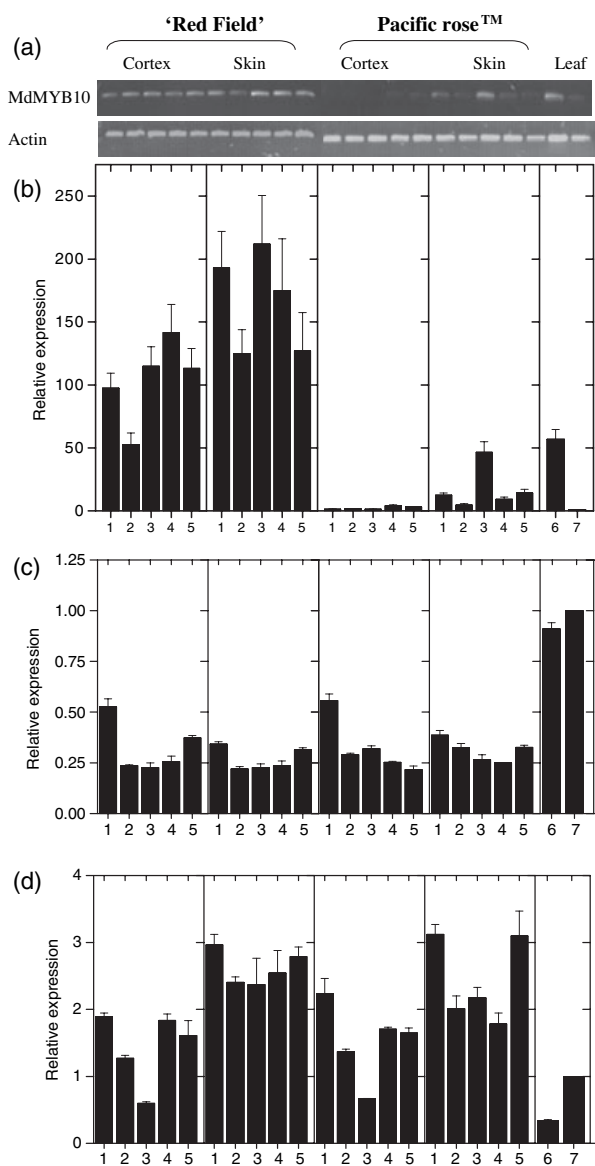
(b) Anthocyanin profiles of extracts of 35S-*MdMYB10* apple leaf (top line) and empty vector control (bottom line). Peaks identified from HPLC traces at 520 nm: cy-gal, cyanidin-3-galactoside; with minor traces of cy-glu, cyanidin-3-glucoside and cy-pent, cyanidin-3-pentoside.

#### Quantitative PCR expression analysis of *MdMYB10* in apple fruit

qPCR analysis of the expression of TF gene transcripts in the developmental series from both red- and white-fleshed varieties of apple revealed massive increases in the relative transcript levels of *MdMYB10* in the fruit tissues of 'Red Field' compared with Pacific Rose<sup>TM</sup> (Figure 9). In Pacific Rose<sup>TM</sup> cortex, transcript levels were barely detectable, whilst in Pacific Rose<sup>TM</sup> skin, transcript was detectable only by mid-season (Figure 9), and the level of transcript correlated with changes in the transcript levels of the biosynthetic genes, particularly at the 102 DAFB time point. The link between *MdMYB10* expression and UFGT expression was particularly strong (Figure 1a). Expression levels of *MdMYB10* in 'Red Field' generally followed the transcript patterns of the assayed genes encoding enzymes, with highly elevated levels throughout fruit tissues, particularly at 40 DAFB and then again at 102, 130 and 146 DAFB (Figure 9) and in leaf tissue. To confirm specificity, qPCR amplicons were sequenced and found to encode *MdMYB10*.

Transcript levels of *MdbHLH3* and *MdbHLH33* were also assayed (Figure 9c,d) but did not appear to follow the pattern displayed for the biosynthetic genes, or for *MdMYB10*, with more consistent levels of expression throughout the developmental series and in both cultivars. Again, amplicons were sequenced to verify authenticity. Transcript levels of *MdMYB8* (GenBank accession number DQ267899), an apple MYB unrelated to the anthocyanin subgroup 10 (Figure 3), were also analysed and no correla-





**Figure 9.** Expression analysis of the transcripts of *MdMYB10*, *MdbHLH3* and *MdbHLH33* during apple fruit development and in leaves.

(a) RT-PCR analysis of *MdMYB10* (220 bp) and actin (131 bp) in 'Red Field' (cortex, skin and leaf) and Pacific Rose™ (cortex, skin and leaf), and corresponding qPCR data for *MdMYB10* (b), *MdbHLH3* (c) and *MdbHLH33* (d). Gel lane and x axis numbering as follows: (1) 40 DAFB, (2) 67 DAFB, (3) 102 DAFB, (4) 130 DAFB, (5) 146 DAFB, (6) 'Red Field' leaf and (7) Pacific Rose™ leaf. Error bars shown in qPCR data are of the SE for three replicate reactions.

tive patterns with the transcript levels of the anthocyanin biosynthetic genes were observed (data not shown).

## Discussion

### *Apple anthocyanin biosynthesis is coordinately regulated*

Modern domesticated cultivars of apple show differential anthocyanin accumulation during development. Specific

anthocyanin pigments accumulate in floral tissues and during fruit maturation in apple skin. Environmental factors also cause increases in the expression of genes encoding anthocyanin biosynthetic enzymes, as demonstrated by their responsiveness to light (Kim *et al.*, 2003). It is possible that white-fleshed apples have lost the ability to produce pigmentation throughout the fruit due to a mutation in one or more biosynthetic gene(s), or in the regulatory genes as has been observed for white grapes (Boss *et al.*, 1996). In terms of the genes encoding the biosynthetic enzymes, we detected some transcript in both cortex and skin of white-fleshed Pacific Rose™, but at low levels (Figure 1a). A previous study in apple showed that all the late anthocyanin biosynthetic genes are present in the genome of a green cultivar but that transcripts were barely detectable by RNA gel-blot analysis (Kim *et al.*, 2003). Our data differ from these results in that we were able to detect expression of at least six genes, including the late biosynthetic genes, in non-pigmented apple cortex. This is probably due to the enhanced sensitivity of qPCR over RNA gel-blot analysis. In grape, it has been shown that the only difference in expression of the biosynthetic genes in red grape skin versus white grape skin was the loss of expression of UFGT in the latter (Boss *et al.*, 1996).

The anthocyanin genes in 'Red Field' followed a pattern whereby the expression levels of the biosynthetic gene transcripts peaked at 102 and 130 DAFB. There were large increases in all expressed genes in the red-fleshed compared to the white-fleshed cultivar. Only CHS differed significantly from this general pattern, with the highest expression seen early in the development of 'Red Field' fruit skin (40 DAFB), although expression did rise again at 102 DAFB. It is likely that at least some of the anthocyanin biosynthetic genes are differentially expressed according to tissue type. This is demonstrated by the transcript levels of the two versions of DFR measured: *MdDFR1* showed a large variation in differently pigmented fruit tissues but not in differently pigmented leaf tissue, whilst *MdDFR2* demonstrated the reverse.

### *Transcription factors controlling apple colour*

Sequence analysis of the *MdMYB10*-encoded protein showed that it shares homology with anthocyanin-regulating MYB TFs from other species (Figure 2). Its probable orthology to *PAP1* is supported by its position in the same clade of the MYB TF family (Figure 3a). Further analysis of *MdMYB10* cDNAs from two other apple cultivars showed a high degree of sequence conservation between such strikingly different varieties as 'Granny Smith' (white cortex and predominantly green skin) and the highly pigmented 'Red Field'. In grape, a retrotransposon-induced insertion into the gene encoding the grape MYB-related *VvmybA1* TF has resulted in a lack of anthocyanin production, generating

white grape cultivars (Kobayashi *et al.*, 2004). The similarity between the sequences of *MdMYB10* in these three apple cultivars suggests that the dramatic difference in anthocyanin accumulation in apple is not due to alterations in the coding sequence of this regulator. However, the difference in anthocyanin accumulation appears to be due to differences in the expression levels and pattern of *MdMYB10* in the two varieties.

Expression analysis of *MdMYB10* showed a pattern very similar to that observed for the genes encoding the biosynthetic enzymes in 'Red Field' apples. A large increase in expression of *MdMYB10* in the fruit of this variety suggests that it is likely to be the regulator of the apple anthocyanin pathway. This association is further strengthened by expression analysis of *MdMYB10* in Pacific Rose™; it was virtually undetectable in leaf and cortex but was expressed in the pigmented skin, particularly at 102 DAFB, when colour was developing. We did not detect evidence for increased transcript levels for the two bHLH genes assayed, suggesting that, in apple, neither of these genes is positively regulated by *MdMYB10*. This is in contrast to the results for over-expressing *PAP1* in Arabidopsis (Tohge *et al.*, 2005), where *PAP1* appears to up-regulate its bHLH interactor. The proposed model for *TTG1*-dependent (WD40-containing) transcriptional networks (Zhang *et al.*, 2003) predicts increasing specificity moving down the hierarchy from WD40 through the bHLHs and down to the MYBs. Mutations in *TTG1* (encoding a WD40-containing protein) affect both anthocyanin levels and epidermal cell fate (Walker *et al.*, 1999). The MYB component of this WD40–bHLH–MYB complex appears to provide the most anthocyanin-specific effect. Our data suggest that, in apple, the MYB component, *MdMYB10*, is the limiting feature for pigment accumulation.

#### Evidence for MYB:bHLH interaction

A signature motif for the interaction between MYB and bHLH proteins, based on specific residues within the R2R3 DNA-binding domain of the MYB partner, has been proposed for maize and Arabidopsis and confirmed experimentally (Grotewold *et al.*, 2000; Zimmermann *et al.*, 2004). *MdMYB10* shares a similar residue pattern to the bHLH-dependent maize C1 protein (unlike the bHLH-independent maize MYB-related P protein). We identified the interaction signature motif in *MdMYB10*, and confirmed, using transient assays, that the protein interacts with bHLH proteins to activate transcription of DFR.

The results from transient assays in tobacco showed interaction between *MdMYB10* and two selected apple bHLH candidates (Figures 5 and 6). Whilst *MdbHLH3*, when co-infiltrated with *MdMYB10*, does appear to have a greater effect than *MdbHLH33* on the activity of the DFR promoter (Figure 5), further work is required to confirm whether either of these associations is significant in apple tissue. One

possible reason for a background level of anthocyanin accumulation with *MdMYB10* alone may be because there is an endogenous tobacco bHLH protein working within this transient assay. Using this assay, transient expression demonstrated that *MdMYB10* is effective at driving anthocyanin accumulation (Figure 6). In the presence of an apple co-activator, this accumulation was intensified and the colour appeared several days earlier, demonstrating partial dependence of *MdMYB10* activity on the presence of an apple bHLH protein in this heterologous system.

#### Tobacco foliar anthocyanin accumulation

We have used transient transformation of tobacco leaves as an assay for the anthocyanin pathway. HPLC data and microscopy show significant accumulation of anthocyanin compounds, driven by *MdMYB10* and a bHLH, confirming the functional presence of anthocyanin biosynthetic enzymes in tobacco leaves and the cellular machinery required to transport anthocyanins to the vacuole. Transient expression of *MdMYB10* in tobacco results in major anthocyanin peaks representing cyanidin-3-galactoside and cyanidin-3-O-rutinoside. Accumulation of an alternative compound to that normally observed in apple is a probable consequence of differences in the genes encoding the biosynthetic enzymes available in the two species. However, this apple TF-driven enhancement ably demonstrates the proposed conservation of MYB regulators between the species.

#### Anthocyanin accumulation in 35S-*MdMYB10* apple and the 'Red Field' red-fleshed cultivar

Over-expression of *MdMYB10* in apple generated a strong phenotype, with highly pigmented plants (Figure 8) due to enhanced levels of anthocyanin. No anthocyanin was detected in control plants by HPLC analysis in contrast to the considerable level of anthocyanins in the *MdMYB10* plants. The effect of *MdMYB10* over-expression on the pigmentation of fruit cortex will be apparent when these plantlets have fruited. In naturally occurring red-fleshed apples, high levels of *MdMYB10* expression appear to be the cause of this startling phenotype (Figure 9). Further, genetic mapping of an *MdMYB10*-derived molecular marker indicates that this locus co-segregates with the red-fleshed apple phenotype (D. Chagné, HortResearch, Auckland, New Zealand, personal communication).

#### Conclusion

The combination of bioinformatic, expression and functional data suggests that *MdMYB10* is the apple orthologue of known anthocyanin regulators from other species, such as Arabidopsis *PAP1*. Over-expression of *MdMYB10* in apple is reminiscent of *PAP1* transgenic Arabidopsis plants

(Borevitz *et al.*, 2000; Xie *et al.*, 2006). Isolation of this gene provides a crucial step in understanding the molecular regulation of anthocyanin gene transcription in apple fruit. The high levels of expression of *MdMYB10* in the cortex of the red apple variety 'Red Field' suggest that it is differences in the activity of this gene that determine the colour differences between the cortex tissues in these varieties. These differences in activity appear to result from differences in expression of the *MdMYB10* gene, as the coding sequences of this gene are virtually identical in the two varieties. Once again, a MYB transcription factor appears to be the primary determinant of differences in colour between cultivated plant varieties, as has been reported for potato, tomato, pepper (De Jong *et al.*, 2004) and grape (Kobayashi *et al.*, 2004), and in various species of *Antirrhinum* (Schwinn *et al.*, 2006). These results confirm the previous findings that it is the MYB component from the proposed regulatory complex of MYB-bHLH-WD40 that is primarily (and specifically) responsible for the level of anthocyanin accumulation. Our elucidation of the primary molecular control of colour in apple provides important information for the breeding of apples with coloured flesh.

## Experimental procedures

### Real-time qPCR expression analysis

Apple fruit were collected at six time points during the season from spring through to late summer (2003–2004): stage 1, 7 days after full bloom (DAFB); stage 2, 40 DAFB; stage 3, 67 DAFB; stage 4, 102 DAFB; stage 5, 130 DAFB; stage 6, 146 DAFB, from trees at the HortResearch orchard (Nelson, New Zealand). RNA was isolated (by a method adapted from that described by Chang *et al.*, 1993) from the fruit (six fruit from the same tree, skin and cortex separately) and the leaves of two genotypes: the white-fleshed commercial cultivar *Malus × domestica* 'Sciros' (Pacific Rose™, derived from a cross between 'Gala' and 'Splendour'), and an open-pollinated red-fleshed seedling of *Malus × domestica* 'Red Field' (a cross between 'Wolf River' and *Malus × domestica* niedzwetzkyana; Brooks and Olmo, 1972). For the first developmental fruit time point (7 DAFB), successful excision of skin from cortex was not possible, so data from this sample have been excluded. Following DNase treatment, first-strand cDNA synthesis was carried out in triplicate for each sample (to minimize variation in RNA template levels) using oligo(dT) according to the manufacturer's instructions (Transcriptor; Roche Diagnostics, Mannheim, Germany). Samples were then pooled.

Genes encoding apple anthocyanin pathway enzymes and regulators were identified by homology in the HortResearch EST database, and, where putative gene family members existed, candidates were selected when they appeared in fruit library tissues. Gene-specific primers corresponding to these genes were designed using Vector NTI version 9.0.0 (<http://www.invitrogen.com>) to a stringent set of criteria, enabling application of universal reaction conditions. To check reaction specificity, RT-PCR reactions were carried out according to the manufacturer's instructions (Platinum Taq, Invitrogen, Carlsbad, CA, USA), with a thermal profile as follows: pre-incubation at 95°C for 5 min followed by 35 cycles of 95°C (30 sec), 60°C (30 sec) and 72°C (30 sec), with a final

extension at 72°C for 5 min. The sequences of each primer pair and the relevant accession numbers are shown in Table S1.

qPCR DNA amplification and analysis was carried out using the LightCycler System (Roche LightCycler 1.5; Roche Diagnostics). All reactions were performed using the LightCycler FastStart SYBR Green Master Mix (Roche Diagnostics) according to the procedure described by the manufacturer. Reactions were performed in triplicate using 2 µl 5 × Master Mix, 0.5 µM each primer, 1 µl diluted cDNA and nuclease-free water (Roche Diagnostics) to a final volume of 10 µl. A negative water control was included in each run. Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 65–95°C melt. The raw data were analysed with the LightCycler software, version 4, and expression was normalized to *Malus × domestica* actin (*MdActin*, accession number CN938023) to minimize variation in cDNA template levels, with the Pacific Rose™ leaf sample acting as calibrator with a nominal value of 1. *MdActin* was selected for normalization due to its consistent transcript level throughout fruit tissues and leaf, with crossing threshold (Ct) values changing by < 2. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.839 and 1.945) were imported into relative expression data analysis. To ensure that the transcripts of single genes had been amplified, qPCR amplicons were sequenced and confirmed as the expected plant DNA sequences. Error bars shown in qPCR data are technical replicates, representing the means ± SE of three replicate qPCR reactions.

### Isolation of MdMYB10

PCR was performed using cDNA from the cortex sample of 'Red Field' (102 DAFB) using degenerate primers (with a 32-fold degeneracy) designed to the consensus DNA sequence of the R2R3 DNA-binding domain based on the sequence of anthocyanin regulators in diverse species. Numerous cDNAs encoding R2R3 MYB domains were obtained. Results from sequencing data revealed one cDNA with high identity to anthocyanin regulators, and the full-length sequence was obtained using 5' RACE (GeneRacer, Invitrogen). The complete sequence for the *MdMYB10* cDNA was compiled from overlapping fragments (Genbank accession number DQ267896). To compare the transcript from 'Red Field', full-length cDNAs were subsequently isolated from *Malus × domestica* Pacific Rose™ (DQ267897) and 'Granny Smith' (DQ267898). cDNA of *MdMYB8* (DQ267899), an apple MYB bearing little sequence homology to known anthocyanin regulators, was isolated from the HortResearch EST collection to act as a negative control.

### Dual luciferase assay of transiently transformed tobacco leaves

The promoter sequence for Arabidopsis DFR was inserted into the cloning site of pGreen 0800-LUC (Figure S3) (Hellens *et al.*, 2005) and modified to introduce an *Nco*I site at the 3' end of the sequence, allowing the promoter to be cloned as a transcriptional fusion with the firefly luciferase gene (LUC). Thus, TFs that bind the promoter and increase the rate of transcription could be identified as an increase in luminescence activity. Arabidopsis DFR (TT3, AT5g42800) was isolated from genomic Arabidopsis DNA. In the same construct, a luciferase gene from *Renilla* (REN), under the control of a 35S promoter, provided an estimate of the extent of transient expression. Activity is expressed as a ratio of LUC to REN activity such that where interaction between a TF (with or without bHLH) and the

promoter occurred, a significant increase in LUC activity relative to REN would be observed.

The promoter–LUC fusion in pGreenII 0800-LUC was used in transient transformation by mixing 100 µl of *Agrobacterium* strain GV3101 (MP90) transformed with the reporter cassette with 450 µl each of two other *Agrobacterium* cultures transformed with cassettes containing a MYB TF gene fused to the 35S promoter and a bHLH TF gene in either pART27 (Gleave, 1992) or pGreenII 62-SK binary vectors (Figure S4) (Hellens *et al.*, 2000).

*Nicotiana tabacum* 'Samsun' plants were grown under glasshouse conditions in full potting mix, using natural light with daylight extension to 16 h, until at least six leaves were available for infiltration with *Agrobacterium*. Plants were maintained in the glasshouse for the duration of the experiment. *Agrobacterium* was cultured on Lennox agar (Invitrogen) supplemented with selection antibiotics and incubated at 28°C. A 10 µl loop of confluent bacterium was re-suspended in 10 ml of infiltration buffer (10 mM MgCl<sub>2</sub>, 0.5 µM acetosyringone) to an OD<sub>600</sub> of 0.2, and incubated at room temperature without shaking for 2 h before infiltration. Infiltrations were performed according to the methods described by Voinnet *et al.* (2003). Approximately 150 µl of this *Agrobacterium* mixture was infiltrated at six points into a young leaf of *N. tabacum*, and transient expression was assayed 3 days after inoculation.

Firefly luciferase and renilla luciferase were assayed using the dual luciferase assay reagents (Promega, Madison, WI, USA). Three days after inoculation, 2 cm leaf discs (six technical replicates from each plant) were removed and ground in 500 µl of passive lysis buffer (PLB), then 10 µl of a 1/100 dilution of this crude extract was assayed in 40 µl of luciferase assay buffer, and the chemiluminescence measured. Stop and Glow™ buffer (40 µl; Promega) was then added and a second chemiluminescence measurement taken. Absolute relative luminescence units (RLU) were measured in a Turner 20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA), with a 5 sec delay and 15 sec integrated measurement.

#### Induction of anthocyanins by transient transformation of tobacco

*Nicotiana tabacum* plants were grown as described above and maintained in the glasshouse for the duration of the experiment. *Agrobacterium* cultures were incubated as for the dual luciferase assay, and separate strains containing the MYB TF gene and the bHLH TF gene fused to the 35S promoter in the pART27 binary vector were mixed (500 µl each) and infiltrated into the abaxial leaf surface as for the luciferase assay. Six separate infiltrations were performed into *N. tabacum* leaves (two plants per treatment) and changes in colour were measured daily using a Minolta CR-300 chromometer (calibrated to D65 light) (Konica Minolta, Mahwah, NJ, USA) by the L\*a\*b\* system (CIE, 1986). Infiltrations comprising *MdMYB10* together with an apple bHLH resulted in visible pigmentation after 4 days. The level of pigmentation increased throughout the experimental period; digital photographs and microscope images were taken 8 days after infiltration. To control for leaf-to-leaf variability, at least two leaves were infiltrated, and each leaf included positive controls (*Agrobacterium* cultures carrying *MdMYB10* + *MdbHLH3*) and negative controls (*Agrobacterium* with empty vector).

#### Phylogeny

Apple EST sequences were trimmed of vector, adapter and low-quality sequence regions and uploaded to Vector NTI version 9.0.0 (<http://www.invitrogen.com>). Full-length sequences were aligned

using Vector NTI Clustal W (opening = 15, extension = 0.3). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) using a minimum evolution phylogeny test and 1000 bootstrap replicates. Full MSF format files are available (Figure S6).

#### Identification and quantification of anthocyanins

Tobacco or apple samples were freeze-dried and coarsely ground before resuspension in 5 ml methanol and 0.1% HCl, extraction at room temperature for 2 h, and centrifugation at 3000 g. Aliquots of 1 ml were dried down to completion in a Labconco Centrivap Concentrator (Labconco, Kansas City, MO, USA). Samples were resuspended in 20% methanol (250 µl). Anthocyanins were identified by LC-MS analysis as described previously (Montefiori *et al.*, 2005). Identification was based on both masses (M+) of molecular ions and characteristic fragments, and comparison of retention times and fragmentation with authentic standards of cyanidin-3-O-glucoside and cyanidin-3-O-galactoside. Anthocyanins and other phenolic compounds were quantified by HPLC as described previously (Stevenson *et al.*, 2006). Quantification was achieved by reference to standards of anthocyanins and other phenolic compounds, using LC-MS data to confirm identification of peaks.

#### Transformation of apple

The binary vector pSAK277-*MdMYB10* (Figure S5) containing the *MdMYB10* cDNA driven by the 35S promoter was transferred into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method. Transgenic *Malus domestica* 'Royal Gala' plants were generated by *Agrobacterium*-mediated transformation of leaf pieces, using a method previously reported (Yao *et al.*, 1995).

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[Note added in proof: a similar gene, *MdMYB1* (Dq886414-DQ886416), has been isolated from apple and shown to regulate anthocyanin production in apple skin (Takos, A.M., Jaffe, F.W., Jacob, S.R., Bogs, J., Robinson, S.P. and Walker, A.R. (2006) Light induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiol.* **142**, 1216–1232.]

#### Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Expression data for DFR family members.

**Figure S2.** TF expression data in transgenic apple.

**Figure S3.** pGreen II 0800-LUC vector map.

**Figure S4.** pGreen II 62 SK binary vector map.

**Figure S5.** pSak 277 MDMYBIO binary vector map.

**Figure S6.** Protein sequence alignments.

**Table S1** Primers for qPCR.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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