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ORIGINAL ARTICLE

Failure to launch: the self-regulating *Md-MYB10$_{R6}$* gene from apple is active in flowers but not leaves of *Petunia*

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Abstract

Key message **The *Md-MYB10$_{R6}$* gene from apple is capable of self-regulating in heterologous host species and enhancing anthocyanin pigmentation, but the activity of MYB10 is dependent on endogenous protein partners.**

Abstract Coloured foliage due to anthocyanin pigments (bronze/red/black) is an attractive trait that is often lacking in many bedding, ornamental and horticultural plants. Apples (*Malus* × *domestica*) containing an allelic variant of the anthocyanin regulator, *Md-MYB10$_{R6}$*, are highly pigmented throughout the plant, due to autoregulation by MYB10 upon its own promoter. We investigated whether *Md-MYB10$_{R6}$* from apple is capable of functioning within the heterologous host *Petunia hybrida* to generate plants with novel pigmentation patterns. The *Md-MYB10$_{R6}$* transgene (*MYB10–R6$_{pro}$:MYB10:MYB10$_{term}$*) activated anthocyanin synthesis when transiently expressed in *Antirrhinum rosea^{dorsea}* petals and petunia leaf discs. Stable transgenic petunias containing *Md-MYB10$_{R6}$* lacked foliar pigmentation but had coloured flowers, complementing the *an2* phenotype of 'Mitchell' petunia. The absence of foliar pigmentation was due to the failure of the *Md-MYB10$_{R6}$* gene to self-activate in vegetative tissues, suggesting that additional protein partners are required for Md-MYB10 to activate target genes in this heterologous system. In petunia flowers, where endogenous components including MYB-bHLH-WDR (MBW) proteins were present, expression of the *Md-MYB10$_{R6}$* promoter was initiated, allowing autoregulation to occur and activating anthocyanin production. Md-MYB10 is capable of operating within the petunia MBW gene regulation network that controls the expression of the anthocyanin biosynthesis genes, *AN1* (bHLH) and *MYBx* (R3-MYB repressor) in petals.

Keywords Anthocyanin · Flavonoid · MYB · BHLH · WDR · Petunia

Abbreviations

MBW	MYB-bHLH-WDR
bHLH	Basic-helix-loop-helix
WDR	WD-repeat

Introduction

Colouration and patterning of flowers, fruits and vegetative tissues are important consumer traits for ornamental and horticultural crop species. Anthocyanins are pigments that provide red/purple/blue colouration to plants, and are

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produced in response to a variety of developmental and environmental cues (Cheynier et al. 2013; Dixon and Paiva 1995; Martin and Gerats 1993). Breeding or engineering fruits and vegetables with enhanced anthocyanin content are of interest not only because of their aesthetic appeal, but also for their health-promoting properties (Davies and Espley 2013; Martin et al. 2011; Zhang et al. 2014). Efficient breeding (e.g. marker-assisted-selection, MAS) or engineering strategies to increase pigmentation in both ornamental and horticultural species requires an understanding of how plants normally control the production of anthocyanins.

Plants tightly regulate the production of anthocyanin pigments through coordinated transcription of the anthocyanin biosynthetic genes (Davies and Schwinn 2003). This is determined by the activity of a transcriptional activation complex, consisting of R2R3-MYB, bHLH and WD-repeat (WDR) proteins (MBW complex) (Davies et al. 2012; Koes et al. 2005; Ramsay and Glover 2005). While all three components need to be expressed simultaneously in the same cell, the R2R3-MYB genes are particularly important for establishing pigmentation patterns. This is due largely because the R2R3-MYB genes are present in families, with individual genes having diverse spatial and temporal expression domains that integrate developmental and/or environmental signals (Albert et al. 2011, 2015; Gonzalez et al. 2008; Schwinn et al. 2006; Yuan et al. 2014). Our understanding of the MBW complex activity has recently been extended to include hierarchical regulation of MBW components, such as activation of bHLH proteins (reinforcement) and R3-MYB repressors (feedback repression) (Albert et al. 2014a; Baudry et al. 2006). Furthermore, R2R3-MYB repressor proteins antagonise the activity of the MBW activation complexes, preventing inappropriate anthocyanin synthesis (e.g. in non-stressed leaves) (Albert et al. 2014a; Dubos et al. 2008). These gene regulation networks are important for providing stringent regulation of anthocyanins and may also contribute to pigmentation patterning (Albert et al. 2014b; Davies et al. 2012).

Plants with elevated quantities of anthocyanins typically arise because of altered expression of R2R3-MYB activators. In maize, several alleles of the *pl* locus (encoding an R2R3-MYB) exist that confer enhanced light-dependent pigmentation (pl_{bol3}) or constitutive ectopic pigmentation ($Pl_{Rhoades}$) throughout the plant (Cone et al. 1993; Pilu et al. 2003). Red/purple cultivars of *Perilla fructescens* occur because of the enhanced expression of an anthocyanin MYB regulator (Gong et al. 1999), and transposon-mediated activation of R2R3-MYB genes is the mechanism responsible for generating purple cauliflower and blood oranges (Butelli et al. 2012; Chiu et al. 2010). However, in red-fleshed apples (*Malus* × *domestica*), ectopic anthocyanin regulation occurs by a distinct mechanism. The apple R2R3-MYB gene *Md-MYB10* normally regulates skin colour in fruit, but in varieties where *Md-MYB10* is expressed ectopically, plants have red foliage and fruits with red flesh (Espley et al. 2006). This ectopic expression pattern occurs because of an unusual allele of *Md-MYB10* ($MYB10_{R6}$), which has an altered promoter allowing MYB10 to regulate its own expression, resulting in enhanced anthocyanin accumulation throughout the plant (Espley et al. 2009).

Transgenic approaches have been taken to generate plants with increased anthocyanin pigmentation by expressing MYB or bHLH regulatory genes. Overexpression of R2R3-MYB factors in petunia generated plants with novel bronze and dark leaf phenotypes (Albert et al. 2011) and had lost the light requirement previously observed for petunias expressing a bHLH gene (Albert et al. 2009; Schwinn et al. 2014). Similarly, plants overexpressing R2R3-MYB genes results in plants with dark foliage in *Arabidopsis*, *Mimulus*, *Medicago, Trifolium* and tobacco (Borevitz et al. 2000; Butelli et al. 2012; Peel et al. 2009; Yuan et al. 2014). However, in each of these examples, the transgenes were expressed from a *CaMV35S* promoter. Alternative promoters to regulate high-anthocyanin traits would be desirable, because of intellectual property constraints, problems with transgene stability and potential increased regulatory burden to approve plants containing sequences derived from plant pathogens.

In this study, we investigate whether the $Md\text{-}MYB10_{R6}$ gene is capable of autoregulation in the heterologous species *Petunia hybrida* for the purpose of generating plants with enhanced foliar and floral pigmentation. We examine the requirements for the $Md\text{-}MYB10_{R6}$ gene to autoregulate and to interact with the anthocyanin regulatory factors present in petunia.

Materials and methods

Transformation

Dual luciferase promoter activation assays upon the *Md-MYB10-R6* promoter were performed using *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves as described in Espley et al. (2009).

The construct pART27-MdMYB10 g ($MYB10\text{–}R6_{pro}$:-*MYB10*:$MYB10_{term}$) is described in Espley et al. (2009) and was used for biolistic and stable transformation. Petals of *Antirrhinum rosea*^{dorsea} (line 112 W) or pre-cultured 'Mitchell' petunia leaf discs were biolistically transformed with 2 µg of pART27-MdMYB10 g with 2 µg pPN93 ($35S_{pro}$:*GFP-ER*) using 1.0 µm gold particles, as described in Albert et al. (2015). Petunia leaf discs (5 mm), cut from

surface-sterilised leaves from glasshouse-grown plants were pre-cultured for 6 days, abaxial surface down on preculture medium (solid half-MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg, et al. 1968), 20 μM cupric sulphate, 13.3 μM benzylaminopurine (BAP), 1.1 μM Indole-3-acetic acid (IAA), 200 μM acetosyringone and 87.6 mM sucrose) in Petri plates prior to bombardment.

Stable transgenic petunias (cultivar 'Mitchell') were generated by *Agrobacterium*-mediated transformation (*A. tumefaciens* strain GV3101) of leaf discs grown for 1 day on preculture medium (above), essentially as described in Conner et al. (2009). Thirteen independent transgenic lines with three clonal copies were generated and grown in a greenhouse for evaluation and characterisation. Plants were grown in greenhouses with ambient lighting, and were heated at 15 °C and vented at 25 °C (Albert et al. 2009).

qRT-PCR

Total RNA was isolated from leaf and petal tissue by Hot Borate extraction (Hunter et al. 2002) from three independent transgenic lines containing the transgene and from two independent wild-type 'Mitchell' controls. First strand cDNA was synthesised from 1 μg of DNaseI treated total RNA using Superscript III reverse transcriptase (Life Technologies) primed with oligo dT and diluted 20-fold with sterile water. qRT-PCR was performed as described in Albert et al. (2011) normalised to the geometric mean of *ACT2* and *EF1α*. *Md-MYB10* transcripts were amplified using oligonucleotide primers described in Espley et al. (2006). Statistical significance was performed using one-way ANOVA upon log-transformed data. Log transformation was performed because of unequal variance. Treatment means were compared using Fisher's Least Significant Differences at $P = 0.05$ (5 % LSD).

Results and discussion

Previously, the *Md-MYB10*$_{R6}$ gene was shown to autoregulate itself and activate the synthesis of anthocyanins in the leaves of transformed apple plants (Espley et al. 2009). We were interested to test whether the *Md-MYB10*$_{R6}$ gene would self-regulate and drive high levels of anthocyanin synthesis in a heterologous host species.

The ability of heterologous transcription factors to activate reporter gene expression through the *Md-MYB10*$_{R6}$ promoter was examined using a dual luciferase assay upon *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. The *Md-MYB10*$_{R6}$ promoter:luciferase construct alone had very little activity in *N. benthamiana* leaves. However, co-transformation with *CaMV35S*$_{pro}$*:MYB* and *CaMV35S*$_{pro}$*:bHLH* anthocyanin regulators from apple (Md-MYB10 + Md-bHLH3) or petunia (Ph-DPL + Ph-AN1) resulted in strong activation (Fig. 1a). The responsiveness of the *Md-MYB10*$_{R6}$ promoter to Ph-DPL and Ph-AN1 suggests that the *Md-MYB10*$_{R6}$ gene may be able to self-regulate in other species.

A pART27 binary vector with the *Md-MYB10*$_{R6}$ gene under the control of its native promoter (Fig. 1b) was used for transient expression assays. This binary vector was biolistically transformed into pre-cultured petunia cv. 'Mitchell' leaf discs or into petals of *rosea*^{dorsea} snapdragon, which has pale flowers due to the loss of MYB gene activity (Schwinn et al. 2006). The *Md-MYB10*$_{R6}$ construct complemented the *myb*$^{-}$ mutant phenotype in *Antirrhinum* petals, restoring anthocyanin synthesis, which colocalised with the internal GFP control (Fig. 1c). This construct also resulted in anthocyanin accumulation in transformed callus cells on petunia leaf discs (Fig. 1d). These results suggest that the *Md-MYB10*$_{R6}$ gene is able to express and autoregulate in both leaf-callus and petal tissue in species other than apple.

Stable transgenic petunias containing the *Md-MYB10*$_{R6}$ transgene were generated by *Agrobacterium*-mediated transformation of petunia 'Mitchell' leaf discs. Thirteen independent transgenic lines were generated, with clonal copies, and were grown in a greenhouse. Regenerated petunia plants transformed with the *Md-MYB10*$_{R6}$ construct failed to accumulate anthocyanins in leaves of any independent transgenic lines (Fig. 2). Wild-type 'Mitchell' petunia plants typically produce anthocyanins in the pedicel and sepals in response to ambient light conditions, due to the activity of DPL and PHZ (R2R3-MYB), AN1 (bHLH) and AN11 (WDR) (Albert, et al. 2011, 2014a). Similarly, *Md-MYB10*$_{R6}$ petunias accumulate anthocyanin in the pedicel and sepals, but fail to show an enhanced phenotype, suggesting the *Md-MYB10*$_{R6}$ transgene is not active in these tissues. The genetic background transformed, cv. Mitchell, has white petals due to null alleles of the R2R3-MYB gene *AN2* (Quattrocchio et al. 1998). The *Md-MYB10*$_{R6}$ transgene complements *an2*, restoring anthocyanin synthesis to the petals consistently across independent transgenic lines (Fig. 2). The level of pigmentation in petals of *Md-MYB10*$_{R6}$ petunias exceeded that achieved when the petunia anthocyanin R2R3-MYB genes *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*) were expressed from a *CaMV35S* promoter in the same petunia cultivar used in this study (Albert et al. 2011). The petunia cultivar 'Mitchell' contains a dominant pigmentation intensity trait that inhibits anthocyanin synthesis in petals, but not leaves (Albert et al. 2011), although the basis for this trait is currently unknown. The petal pigmentation in *Md-MYB10*$_{R6}$ petunias demonstrates that the transgene is capable of expressing to high levels in petunia and activating anthocyanin synthesis. However, anthocyanin

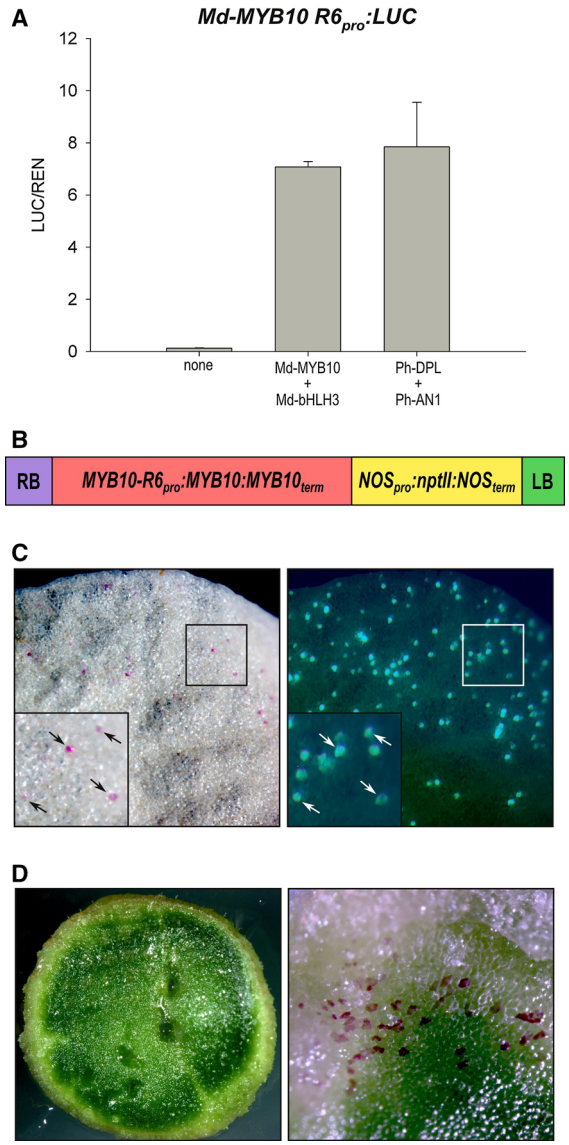

Fig. 1 Transient analysis of the *Md-MYB10*$_{R6}$ transgene. **a** Promoter activation assay upon the *Md-MYB10*$_{R6}$ promoter, using a dual luciferase assay. *Agrobacteria* containing the reporter and effector constructs were coinfiltrated into *Nicotiana benthamiana* leaves. The effector constructs express each transcription factor from a *CaMV35S* promoter: Ph-DPL (R2R3-MYB), Ph-AN1 (bHLH), Md-MYB10 (R2R3-MYB) and Md-bHLH3 (bHLH). Firefly luciferase (LUC) values are reported relative to the Renilla luciferase (REN) control; mean ± SE ($n = 4$). **b** T-DNA of the binary vector used for transformation experiments, comprising the self-regulating *Md-MYB10*$_{R6}$ gene, with native promoter and terminator sequences and the *NOS*$_{pro}$*:nptII:NOS*$_{term}$ (KanR) selectable marker. **c** Inner surface of the dorsal petal of *Antirrhinum majus rosea*^{dorsea}, 9 days after bombardment with *Md-MYB10*$_{R6}$ gene and *35*$_{pro}$*:GFP-ER*. Tissue was photographed with white light to show anthocyanin-accumulating cells and blue light to view GFP fluorescence. Arrows indicate anthocyanin producing cells. **d** Petunia leaf discs after 6 d preculture on regeneration medium (*left*) and 3 d post bombardment with the *Md-MYB10*$_{R6}$ transgene (*right*)

Fig. 2 Pigmentation phenotypes of *Md-MYB10*$_{R6}$ petunias. Representative pigmentation phenotypes of 'Mitchell' (WT) and *Md-MYB10*$_{R6}$ petunias. 'Mitchell' petunia flowers have white petals due to a loss of MYB gene activity (*an2*). The *Md-MYB10*$_{R6}$ restores anthocyanin synthesis to petals. Leaves lack visible anthocyanin pigmentation in WT and *Md-MYB10*$_{R6}$ petunias, and accumulate anthocyanin in the stems, pedicels and sepals

synthesis was not activated in vegetative tissues, contrasting with results in apple, where the *Md-MYB10*$_{R6}$ transgene conferred dark red foliage (Espley et al. 2009). This suggested that the *Md-MYB10*$_{R6}$ transgene failed to express in leaves, or that protein partners required by Md-MYB10 to activate anthocyanin synthesis were not present in these tissues, or both.

In order to investigate the basis for the pigmentation patterns observed in the *Md-MYB10*$_{R6}$ petunias, qRT-PCR was performed on flowers and leaves (Fig. 3). *Md-MYB10* transcripts were barely detectable in leaves, but were abundant in petal tissue. Transcript levels for an anthocyanin biosynthetic gene, *DFR*, correlated strongly with the expression of *Md-MYB10*. Thus, the lack of anthocyanin accumulation in leaves is a consequence of the lack of *Md-MYB10* expression in this tissue. The *Md-MYB10*$_{R6}$ promoter is activated by MBW activation complexes [(Espley et al. 2009); Fig. 1a], and therefore the failure of the transgene to express could be due to a lack of essential protein partners for Md-MYB10, such as MBW components (e.g. bHLH partners). Alternatively, R2R3-MYB

repressors have recently been shown to bind to MBW complexes and assert repressive activity (Albert et al. 2014a; Matsui et al. 2008), which could also account for the failure of the *Md-MYB10*$_{R6}$ transgene to express in vegetative tissues.

To further understand the activity of the *Md-MYB10*$_{R6}$ transgene, we examined the expression of endogenous MBW components in petunia. Transcript abundance for the bHLH factor *AN1* strongly correlated with the expression pattern observed for *Md-MYB10*, *DFR*, and with anthocyanin accumulation. *AN1* expression was very low in leaves (Fig. 3), but in petals *AN1* was highly expressed, and was significantly higher in *Md-MYB10*$_{R6}$ petunias compared to wild-type. Similarly, the competitive R3-MYB repressor *MYBx* (Albert et al. 2011, 2014a; Koes et al. 2005; Kroon 2004; Genbank accession KF985022) was poorly expressed in leaves, but in petals was expressed at significantly higher levels in *Md-MYB10*$_{R6}$ petunias compared to wild-type. Transcripts for *MYB27,* an active R2R3-MYB repressor, were abundant in leaves of both *Md-MYB10*$_{R6}$ and wild-type plants. In petals, *MYB27* transcript levels were greatly reduced compared to leaves, but were present in slightly higher levels than in wild-type. The enhanced transcript level for *AN1* and *MYBx* within *Md-MYB10*$_{R6}$ petunia flowers demonstrates that Md-MYB10 is capable of functioning within the MBW gene network of petunia. We have previously shown that the MBW complex regulates the expression of bHLH and R3-MYB genes, in addition to the anthocyanin biosynthesis genes (Albert et al. 2014a). Thus, the failure for the *Md-MYB10*$_{R6}$ promoter to activate in leaves does not appear to be caused by incompatibility between Md-MYB10 and endogenous bHLH and WDR proteins.

The low expression of *AN1* (bHLH) and high expression of *MYB27* (R2R3-MYB repressor) in leaves could account for the failure of the *Md-MYB10*$_{R6}$ transgene to express in this tissue. AN1 is an essential component of the MBW complex for anthocyanin synthesis in petunia (Albert et al. 2011, 2014a; Spelt et al. 2000) and the lack of an appropriate bHLH for Md-MYB10 would prevent activation of the *Md-MYB10*$_{R6}$ promoter. The active repressor *MYB27* antagonises the activation activity of MBW complexes, and thus could prevent the activation of the *Md-MYB10*$_{R6}$ promoter. We examined these possibilities by growing *Md-*

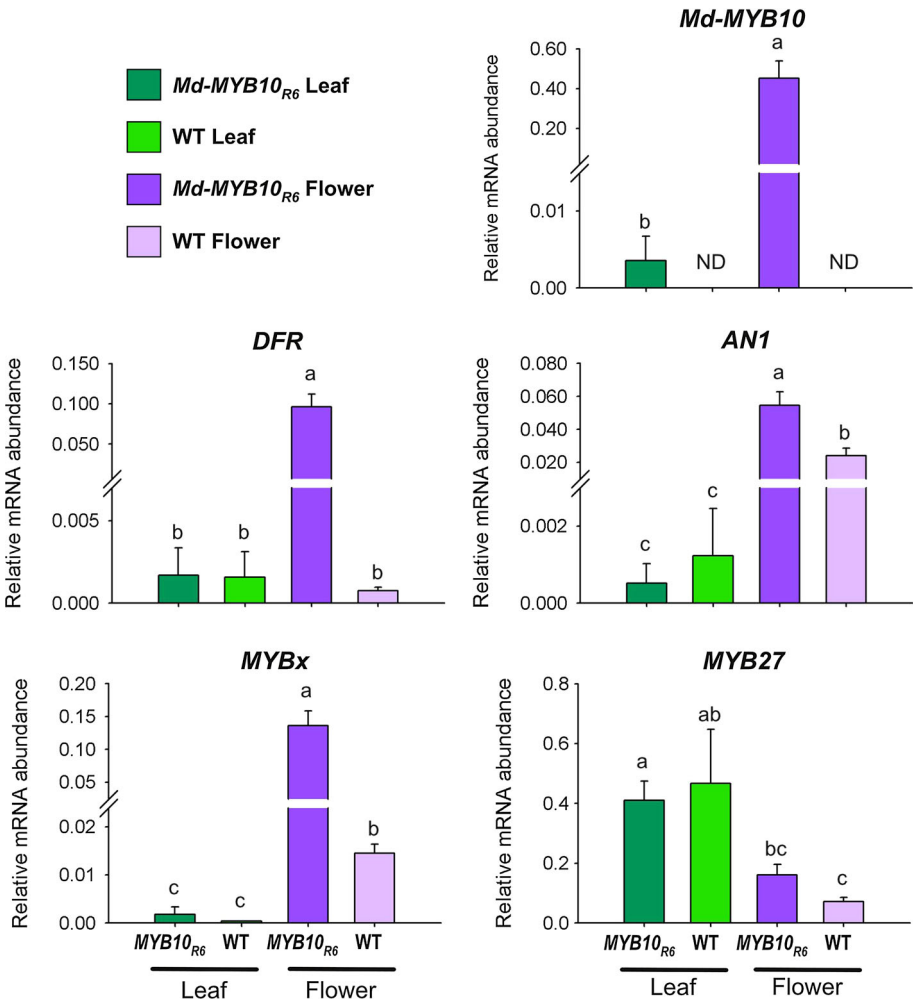

Fig. 3 Transcript abundance for anthocyanin biosynthetic and regulatory genes. Transcript abundance of *Md-MYB10*, *DFR* (anthocyanin biosynthesis gene), *AN1* (bHLH), *MYBx* (R3-MYB) and MYB27 (R2R3-MYB) in leaves and flowers of wild-type and *Md-MYB10*$_{R6}$ petunias. Transcript abundance was determined by qRT-PCR relative to the geometric mean of *Actin* and *EF1a*. Mean ± SEM $n = 2$ (WT) or $n = 3$ (*Md-MYB10*$_{R6}$) from biological replicates, consisting of independent lines, are shown. Significantly different means ($p = 0.05$) are indicated (*a*, *b*, *c*). *ND* not detected

MYB10$_{R6}$ petunias under high light conditions that have previously been shown to activate the expression of *AN1* (bHLH), *DPL* and *PHZ* (R2R3-MYB activators) while drastically reducing the transcript level of *MYB27* (R2R3-MYB repressor) (Albert et al. 2011, 2014a). Under these conditions, MBW activation complexes form that result in modest anthocyanin accumulation in 'Mitchell' petunia (Albert et al. 2011). We hypothesised that under these conditions the MBW complexes involving the endogenous petunia regulators should activate the *Md-MYB10*$_{R6}$ promoter, allowing the resulting Md-MYB10 protein to form additional MBW complexes to self-regulate its own promoter and that of the anthocyanin biosynthesis genes. Surprisingly, these treatments were not effective at inducing enhanced foliar anthocyanin synthesis (data not shown). Similarly, the observed accumulation of anthocyanin in pedicels and sepals of both wild-type and *Md-MYB10*$_{R6}$ petunias grown under ambient greenhouse lighting (Fig. 2) demonstrates that MBW complexes are forming (e.g. PHZ + AN1 + AN11) (Albert et al. 2011, 2014a). Therefore, the lack of enhancement in the *Md-MYB10*$_{R6}$ petunias suggests that factors other than the MBW components are required for the *Md-MYB10*$_{R6}$ promoter to autoregulate. This could occur because Md-MYB10 protein requires an additional protein partner to activate target genes that exist within a chromatin environment, including its own promoter, thus failing to autoregulate.

The behaviour of the *Md-MYB10*$_{R6}$ transgene in stable transgenic petunias suggests that additional proteins that are present in flowers but absent in vegetative tissues are required for Md-MYB10 to activate target genes in petunia, including the anthocyanin biosynthetic genes and the *Md-MYB10*$_{R6}$ promoter. The results from stable transgenic petunias appear to conflict with the results from the promoter activation assays (Fig. 1a) and biolistically transformed petunia leaf discs (Fig. 1d). However, in both of these transient experiments the *Md-MYB10*$_{R6}$ promoter would not exist within a normal chromatin environment. The consistent pigmentation phenotype amongst independent transgenic lines suggests that generic features of chromatin are altering the behaviour of the transgene (e.g. nucleosome occupancy), rather than random positional effects that can occur when transgenes integrate into specific chromatin environments. In maize, an additional protein partner, RIF1, has been identified that is required by the MBW complex to activate the promoters of endogenous anthocyanin biosynthetic genes but not transiently expressed genes (Hernandez et al. 2007). RIF1 likely participates in chromatic remodelling and/or altering nucleosome occupancy, and also allows different conformations of the MBW complex to exist that can bind different promoter *cis*-elements (Kong et al. 2012). Similar mechanisms may also be important for Md-MYB10, which may explain the tissue-specific activity observed in stable transgenic petunias.

We investigated the behaviour of the *Md-MYB10*$_{R6}$ gene in the heterologous host petunia to generate plants with enhanced pigmentation. While the *Md-MYB10*$_{R6}$ transgene is active in petunia, and Md-MYB10 is capable of functioning within the endogenous MBW gene regulation network, this was limited to petals. This effect is surprising, given that *Md-MYB10*$_{R6}$ transgene is capable of autoregulation in leaves of apple (Espley et al. 2009). Our findings suggest this tissue-specific expression of the *Md-MYB10*$_{R6}$ gene is primarily caused by the dependence of the Md-MYB10 protein for additional regulatory components that are present in petunia flowers but not leaves. This conclusion indicates that the use of the *Md-MYB10*$_{R6}$ gene for modifying anthocyanin content in plants is unpredictable, which may limit the use of this transgene. We are currently examining whether the *Md-MYB10*$_{R6}$ promoter can be used to autoregulate R2R3-MYB genes other than *Md-MYB10*, which may overcome the problems encountered with Md-MYB10 activity observed in petunia.

Author contribution statement MRB, NWA, RVE, RPH, KES and KMD designed experiments and interpreted data; MRB made transgenic petunia plants and transient bombardment assays; CB generated vectors and conducted luciferase assays; HN and LW conducted general molecular analysis including RNA extraction and qRT-PCR. NWA wrote the manuscript with assistance and approval from all authors.

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Compliance with ethical standards

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Conflict of interest CB, RPH and RVE are inventors on a patent family (based on WO2009139649) held by The New Zealand Institute for Plant & Food Research Ltd., on the use of the MYB10 gene and promoter for commercial activities. The authors declare that they have no other conflicts of interest.

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