Discrete bHLH transcription factors play functionally overlapping roles in pigmentation patterning in flowers of Antirrhinum majus

Nick W. Albert1*, Eugenio Butelli2*, Sarah M.A. Moss1, Paolo Piazza3, Chethi N. Waite1, Kathy E. Schwinn1, Kevin M. Davies1 and Cathie Martin2

1Plant & Food Research Food Industry Science Centre, Fitzherbert Science Centre, Batchelar Road, Palmerston North 4474, New Zealand; 2John Innes Centre, Norwich Research Park, NR4 7UH, UK; 3Oxford Genomics Centre, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, UK

Summary

- Floral pigmentation patterning is important for pollinator attraction as well as aesthetic appeal. Patterning of anthocyanin accumulation is frequently associated with variation in activity of the Myb, bHLH and WDR transcription factor complex (MBW) that regulates anthocyanin biosynthesis.
- Investigation of two classic mutants in Antirrhinum majus, mutabilis and incolorata I, showed they affect a gene encoding a bHLH protein belonging to subclade bHLH-2. The previously characterised gene, Delila, which encodes a bHLH-1 protein, has a bicoloured mutant phenotype, with residual lobe-specific pigmentation conferred by Incolorata I.
- Both Incolorata I and Delila induce expression of the anthocyanin biosynthetic gene DFR. Rosea 1 (Myb) and WDR1 proteins compete for interaction with Delila, but interact positively to promote Incolorata I activity. Delila positively regulates Incolorata I and WDR1 expression. Hierarchical regulation can explain the bicoloured patterning of delila mutants, through effects on both regulatory gene expression and the activity of promoters of biosynthetic genes like DFR that mediate MBW regulation.
- bHLH-1 and bHLH-2 proteins contribute to establishing patterns of pigment distribution in A. majus flowers in two ways: through functional redundancy in regulating anthocyanin biosynthetic gene expression, and through differences between the proteins in their ability to regulate genes encoding transcription factors.

Introduction

The diversity of coloration and patterning of animal-pollinated flowers is truly remarkable. Complex patterns serve as nectar guides, attracting pollinators and directing them towards pollen and nectar rewards. Most of our understanding of pigmentation patterning comes from the study of regulation of anthocyanin production, which provides red/purple/blue colours to plants. Patterns of anthocyanin production across petals arise primarily through transcriptional regulation of the anthocyanin biosynthetic genes (Schwinn et al., 2006), which are regulated directly by a transcriptional activation complex comprised of R2R3-Myb, bHLH and WDR proteins; the MBW complex (Baudry et al., 2004; Koes et al., 2005; Ramsey & Glover, 2005; Gonzalez et al., 2008). The R2R3-Myb proteins are particularly important for pattern formation because they are encoded by gene families with members that are expressed differentially in response to a variety of developmental and environmental cues (Schwinn et al., 2006; Hoballah et al., 2007; Albert et al., 2011; Yuan et al., 2014; Bombarely et al., 2016; Esfeld et al., 2018). The bHLH transcription factors are also encoded by small gene families and they too have the potential to contribute to pigment patterning (Dellaporta et al., 1988; Chandler et al., 1989; Ludwig et al., 1989; Goodrich et al., 1992; Hellens et al., 2010).

MBW complexes also regulate proanthocyanidin biosynthesis (Baudry et al., 2004; Bogs et al., 2007; Terrier et al., 2009; Liu et al., 2014), vacuolar hyperacidification (Quattrocchio et al., 2006; Butelli et al., 2019), and trichome and root hair development in Brassica species (Payne et al., 2000; Zhao et al., 2008) with each process controlled by a specific subgroup of Myb proteins (Zhang et al., 2003; Xu et al., 2014; Zhang & Hulskamp, 2019). Most plants have at least two bHLH transcription factors that operate within MBW complexes, belonging to two distinct clades within subgroup IIIf of plant bHLH proteins (Heim et al., 2003; Feller et al., 2011). These are described as bHLH-1 (represented by ZmR/ZmLc, AGL3, A/AGL3, A/MYC1, PhiAF13, AmDel) and bHLH-2 (represented by Zm6n, AtFT8, PhAN1, C/Noemi) (Albert et al., 2014). In many groups of plants the activity of bHLH-2 genes is essential for anthocyanin biosynthesis (Spelt et al., 2000, 2002; Park et al., 2004; Hellens et al., 2010; Butelli et al., 2019; Strazzer et al., 2019). In other plants, including Arabidopsis (Gonzalez et al., 2008; Feyissa et al., 2009)
and *Antirrhinum* (Goodrich *et al*., 1992), the bHLH-1 proteins act also in controlling anthocyanin biosynthesis directly (Zhang *et al*., 2003; Zhang & Hulskamp, 2019). bHLH-2 proteins appear to be essential for controlling proanthocyanidin biosynthesis in Arabidopsis (Nesi *et al*., 2000), petunia (Spelt *et al*., 2002), pea (Hellens *et al*., 2010), morning glory (Park *et al*., 2007), medicago (Li *et al*., 2016) and citrus (Butelli *et al*., 2019), and vacuolar acidification in petunia (Spelt *et al*., 2002) and citrus (Butelli *et al*., 2019; Strazzer *et al*., 2019). It is not yet clear whether bHLH-1 proteins that regulate anthocyanin biosynthesis also contribute to regulating these other pathways. The existence of genes encoding both clades of bHLH proteins in monocots and dicots suggests that there has been selection to maintain two types of bHLH protein which may work by distinct mechanisms or have distinct functions (Pesch *et al*., 2015; Zhang *et al*., 2019).

*Antirrhinum majus* – a classic model for understanding floral pigmentation

In bicoloured flowers, colour patterning differs between individual petals or zones within petals and is common in zygomorphic flowers such as legumes (banner, wings, keel), iris (standards, falls), violas, Aquilegia (blades, spurs) and orchids (tepals, labelum). However, suitable models with bicoloured floral patterns that are also amenable to genetic analyses have not been analysed extensively. Wild-type *Antirrhinum majus* has bilaterally symmetrical flowers consisting of self-coloured fused petals in the corolla tube which separate into two hind petals, two lateral petals and a single ventral petal comprising the corolla lobes. However, mutants of the bHLH-1 gene *Delila* have bicoloured flowers (Goodrich *et al*., 1992), providing a model genetic system for investigating bicolour patterning. Three R2R3Myb proteins, *Rosea* 1 (Ros 1), *Rosea* 2 and *Venosa*, of which Ros 1 has the strongest activity, control flower pigmentation in *A. majus* (Schwinn *et al*., 2006). Here we report the identification and functional characterisation of *Incolorata I*, a gene encoding a bHLH-2 protein in *A. majus*, and relate this to bicoloured patterning of its flowers.

**Materials and Methods**

**Plant material**

The *del* mutant, line Jl:8, is closely related to the wild-type line, Jl:7. The *incolorata I* mutant, line Jl:569, was obtained from Professor Linnert, Frei University of Berlin in 1984 and outcrossed to Jl:7. Segregation in the F2 allowed the selection of ivory *inc I* : *del* homoyzogotes. The *mutabilis* mutant was identified from several individuals segregating in an F2 population from a cross between a stock line carrying *decipiens* and Jl:7 (Stubbe, 1966). The origins of *det*<sup>inc</sup> (Jl:602; Jl22) and *det*<sup>dec</sup> (Jl:23) have been described by Goodrich *et al*., (1992) and Martin *et al*., (1991) and lines Jl:32, Jl:33, Jl:56, Jl:520, and Jl:522 have been described by Fincham & Harrison, (1967), Coen *et al*., (1986) and Almeida *et al*., (1989).

**Construction of a cDNA library from RNA from lobes of del flowers**

RNA was extracted from red petal lobes from a *del* line (Jl:8). cDNA was synthesised from polyA<sup>+</sup> RNA. EcoRI linkers were ligated to the cDNA ends and cloned into the EcoRI site of λgt10. The resultant library contained c. 6 × 10<sup>6</sup> PFU.

**Screening of the cDNA library**

Approximately 5 × 10<sup>5</sup> PFU were screened using a 32P-labelled fragment from a cDNA clone of *Anl* (Spelt *et al*., 2000) amplified from petals of line V26. Three positive plaques were identified. A 2.4 kb *Kpn*I fragment from the largest cDNA insert was subcloned into the *Kpn*I site of pBluescript (Stratagene) and named pJAM1494.

**RNA preparation for RNA gel blots**

RNA was extracted from petals and RNA gel blots were run as described by Martin *et al*., (1985). Probes for biosynthetic genes were prepared as described in Martin *et al*., (1991) and Schwinn *et al*., (2006).

**Isolation of genomic clones**

A library of genomic DNA from Jl:522 was prepared in λEMBL4 (Martin *et al*., 1991) and screened with pJAM1494. DNA inserts from positive plaques were subcloned as EcoRI fragments into pBluescript. DNA was also isolated from *inc I*<sup>1</sup> : *del* and *inc I*<sup>2</sup> : *del* lines digested with EcoRI, size-fractionated and cloned into λNM1149. Inserts were screened with pJAM1494 and the positive EcoRI inserts were subcloned into pGEM-T (Promega) and sequenced.

**Amplification of *inc I*, *delila* and *Delila*-like alleles from genomic DNA**

was performed with iProof polymerase (Bio-Rad) and gene-specific primers (Supporting Information Table S1). Sequences have been submitted to GenBank with the following accession numbers: *det*<sup>inc</sup>, genomic DNA, MW027119: *inc I*<sup>1</sup>, genomic DNA, MW027120; *Incolorata I*, cDNA, MW027121; *WDRI*, cDNA, MW027122.

**Phylogenetic tree**

Deduced amino acid sequences were aligned using Muscle (Edgar, 2004) to generate a maximum likelihood phylogenetic tree using PhyML (Guindon *et al*., 2010) with 1000 bootstrap replicates using Geneious (10.0.9) software. Amino acid sequence alignments are shown in Dataset S1.

**Biologist transformation of *inc I* petals**

Complementation assays of *inc I*<sup>1</sup> : *del* petals were performed by biolistic transformation of petals (Albert *et al*., 2015). GFP-ER was used as an internal control for identifying transformed cells (Haselhoff *et al*., 1997).
Isolation of AmWDR1

WDR1 was isolated by 3′ Rapid Amplification of cDNA Ends (RACE) PCR (Frohman et al., 1988) from line JI:75 using degenerate oligonucleotides K112 and K113 for first and second amplification rounds, respectively. The 5′ end of the WDR1 gene was isolated using a Universal Genome Walker kit (Clontech) and gene-specific primers K127 and K128 for the first and second amplification rounds, respectively. The full-length WDR1 cDNA was amplified from RNA from petals using primers K133 and K134 and cloned into pJAM1502. For all primers see Table S1.

RNA isolation and qRT-PCR

Whole petals of wild-type (JI:522), rosea\textsuperscript{densea} and incolorata I flowers, or dissected wild-type (JI:522) and delila (JI:8) petals (tubes and lobes) were sampled. Total RNA was extracted using Plant RNeasy Isolation kits (Qiagen). For qRT-PCR analysis, cDNA was prepared from 1 μg total RNA using iScript\textsuperscript{TM} qGDNA clear cDNA synthesis kits (Bio-Rad), and diluted 20-fold for analysis. qRT-PCR was performed using SsoAdvanced\textsuperscript{TM} Universal SYBR\textsuperscript{®} Green Supermix (Bio-Rad), gene-specific primers (Table S1) were normalised to the geometric mean of Cyclophilin and EF1\textalpha (Albert et al., 2014). Means ± SEM of three biological replicates were calculated. Two-tailed Student’s \(t\)-tests between wild-type and the corresponding mutant samples were performed to determine significant differences. Data were transformed (log10) to account for unequal variance.

Full-length transcripts of Delila and Delila-like were amplified with gene-specific primers (Table S1) and 2GRobust polymerase (Kapa Biosciences), from cDNA synthesised using Superscript II\textsuperscript{TM} reverse transcriptase (Life technologies) and oligo (dT)\textsubscript{12-18}.

Transient dual luciferase assays in Nicotiana benthamiana

The ability of different transcription factors to activate the promoter of the \textit{Pallida} gene encoding dihydroflavonol-4-reductase (DFR) was assessed by combining transient expression via agroinfiltration of \textit{N. benthamiana} leaves with a quantitative dual luciferase reporter system.

Effectors plasmids encoding transcription factors were obtained by cloning coding sequences first into the pDONR207 entry clone (Thermo Fisher Scientific/Invitrogen) and subsequently transferred to the destination vectors pJAM1502 (Ros, Inc1, WDR1) or pMDC32 (Del) using Gateway cloning technology (Thermo Fisher Scientific/Invitrogen). Both vectors are Gateway-compatible binary plasmids that allow strong, constitutive expression of the genes of interest driven by double CaMV 35S promoters (Curtis & Grossniklaus, 2003; Luo et al., 2007).

The reporter constructs p2532, p2534 and p2536 were obtained by PCR amplification of the DFR promoter from different \textit{A. majus} accessions followed by cloning into the pGreen II 0800-LUC vector as \textit{KpnI}—\textit{NcoI} fragments to control the expression of the firefly-derived luciferase reporter gene. This vector also contains a \textit{Renilla} luciferase gene under the control of a CaMV 35S promoter as an internal control to normalise the values of the experimental reporter gene for variations caused by transfection efficiency (Hellens et al., 2005). The reporter constructs containing deletions in the DFR promoter (p2551, p2557, p2565 and p2571) were obtained using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) using plasmid p2532 as a template and primers listed in Table S1.

Effectors and reporter plasmids were transformed into \textit{Agrobacterium tumefaciens} strain GV3101. Reporter plasmids were co-transformed with the helper plasmid pSoup (Hellens et al., 2005). Liquid cultures were grown overnight with selection (kanamycin 50 mg l\textsuperscript{-1}, rifampicin 25 mg l\textsuperscript{-1}) and harvested by centrifugation. Cells were washed and resuspended in 10 mM MgCl\textsubscript{2}, 10 mM MES pH 5.6, 200 μM acetylsyringone to \(A_{600}=0.2\). \textit{Agrobacterium} suspensions were infiltrated into the abaxial surface of expanded leaves of 3-wk-old \textit{N. benthamiana} plants. For each combination, five injected areas were treated as biological replicates. Agroinfiltrated leaves were harvested after 3 d and luciferase activity was measured immediately using the Dual-Glo Luciferase Assay System kit (Promega). Leaf discs of 4 mm in diameter were collected in 1.5 ml white transparent tubes containing 100 μl of PBS. A volume of 75 μl of luciferase assay reagent was added and firefly luminescence was measured on a Glomax 20/20 single tube luminometer (Promega) after 10 min. \textit{Renilla} luminescence was measured on the same instrument 10 min after the addition to the same sample of 75 μl of Stop & Glo reagent. Results were expressed as the ratio of firefly to \textit{Renilla} luciferase activity (Luc/Ren).

Results

Classic mutants \textit{incolorata I} and \textit{mutabilis} are allelic and affect anthocyanin biosynthesis

The classic \textit{delila} mutant of \textit{Antirrhinum majus} has bicoloured flowers with red lobes, pigmented with anthocyanin, and an ivory-coloured tube (Wheldale, 1907; Baur, 1910). \textit{Del} encodes a bHLH transcription factor that regulates anthocyanin pigmentation in both tubes and lobes (Goodrich et al., 1992). However, knockout mutants of \textit{del} have fully pigmented lobes and consequently the bicolour pattern in \textit{del} mutants has been predicted to arise from the activity of a second, unknown protein, active in the petal lobes (Goodrich et al., 1992; Schwinn et al., 2006). Two mutants affect lobe pigmentation: \textit{incolorata I} (\textit{inc I}) and \textit{mutabilis} (\textit{mut}) (Stubbe, 1966; Linnert, 1972). The \textit{inc I} mutant was originally identified by its ivory flowers and complete absence of anthocyanins in vegetative tissues (Fig. 1) (Stubbe, 1966; Linnert, 1972). The \textit{inc I} mutant line was obtained from the IPK Gatersleben collection of \textit{Antirrhinum} germplasm in 1984 and outcrossed to JI:7 (a full red, self-coloured line (\textit{Inc II/Inc I: Dell Del})). Segregation in the F\textsubscript{2} showed 9/56 plants segregating for \textit{del} in addition to full red (43/56; \textit{Inc II/Inc I} and \textit{Inc II/Inc I} and ivory \textit{inc I}: \textit{del}/\textit{homoygotes} (4/56). This suggested that the original \textit{inc I} mutant line was homozygous for \textit{del}, which was confirmed by crossing a reselected \textit{inc I} line to JI:8 (\textit{del/del}). All F\textsubscript{1} plants lacked tube pigmentation (\textit{del/del}) but had full red lobes.
confirming that inc I was a recessive mutation, visible only in combination with homozygous del alleles (Fig. 1).

The mutabilis mutation was described originally by a series of alleles in the IPK Gatersleben collection, linked (2 cM) to Del (Stubbe, 1966). While these original mutants have been lost from the collection, we identified an individual with the mutabilis phenotype from an F2 population from a cross between a stock line carrying decipiens (Stubbe, 1966) and wild-type (JJ:7). The rediscovered mutabilis mutant has pale pink blushing/speckling on the petal lobes (Fig. 1), but completely lacks tube pigmentation because it is homozygous for del. The speckling of the lobes is much more evident in plants grown in the field than in those grown in the glasshouse (Fig. S1) with strong pigmentation on the abaxial epidermis of the lobes except where tissue has been shaded before unfolding, suggesting that this allele is sensitive to light. An F2 population from a cross between the mutabilis mutant (mut:del) and a full red wild-type line (JJ:7 Mut:Del), segregated for flower colour patterning. Phenotypes fell into three categories: ivory tube with pale, speckled lobes (mut:del parental phenotype; 56 plants), full red tube and lobes (wild-type parental phenotype; 200 plants) and ivory tube with red lobes (del phenotype; 8 plants). Recombinants with a red tube and pale, speckled lobes were not observed, indicating that Del activity is epistatic to Mut in regulating pigmentation. Further attempts to replace del with Del alleles in the mut mutant line by outcrossing proved unsuccessful, confirming that the mut phenotype, like that of inc I, is observed only in the presence of nonfunctional del alleles.

Crosses between inc I and mut did not complement the pigmentation phenotype, showing the mutations to be allelic (Fig. S2). The gene was named Incolorata I, and the incolorata I mutant allele was named inc I1 (Stubbe, 1966; Linnert, 1972). The mutabilis mutant carries a weak allele of inc I, designated inc I2.

The redundancy of Inc I with Del in regulating anthocyanin biosynthesis suggested that the genes encode similar proteins. Del encodes a bHLH transcription factor (Goodrich et al., 1992), belonging to the R/JAF13 bHLH-1 clade of subgroup IIII (Heim et al., 2003). In other species, a second class of bHLH proteins (TT8/AN1 bHLH-2 clade) regulates anthocyanin and proanthocyanidin biosynthesis (Nesi et al., 2000; Spelt et al., 2002). We screened cDNA libraries generated from lobe tissue with a del background with a cDNA probe of the ANI gene from petunia line V26. Multiple cDNA clones corresponding to a new bHLH sequence were identified: the longest cDNA insert was subcloned as a 2.4 kb KpnI insert in pBluescript (pJAM1494). Sequencing confirmed this contained a full-length cDNA. The wild-type Incolorata I gene was identified in a 24 kb genomic DNA insertion in λEMBL4 from line JJ:522. Fragments of the gene were also cloned from inc I2 genomic DNA and sequenced. A 4 bp duplication (CATG) was identified within exon three of the inc I2 mutant line, resulting in a frame-shift, confirming this gene to be Incolorata I. We were unable to identify any sequence variants in genomic DNA that could account for the weak phenotype of the inc I2 allele but RNA gel blots from wild-type and inc I2 mutant flowers (Fig. S3) revealed an absence of Inc I transcript in the mutant, suggesting that the inc I2 mutant phenotype results from considerably reduced levels of Inc I expression.

Previous genetic data suggested that Mut/Inc I and Del were closely linked, 2 cM apart (Stubbe, 1966). From our crosses of inc I2:del × Inc I:1; Del a total of 264 F2 plants had 64 del/ del individuals of which 56 had speckled/ivory flowers (inc I2 homozygotes) and 8 (del/del) individuals had red lobes and so carried at least one Inc I1 allele. Assuming all the latter to be heterozygous Inc I1:inc I2, we calculated there to have been 8 recombination events among 128 gametes giving a recombination fraction (r) of 8/128 = 0.0625 (±0.0214) and the distance between Inc I and Del, (d) c. 6.7 (± 2.19) cM, meaning Inc I and Del lie about 6.7 cM apart. In the high quality genome sequence for A. majus (Li et al., 2019) Incolorata I (Am02g53780) and Delila (Am02g33340) are both located on chromosome 2, 20.7 Mb apart (Fig. 2a).

Lying 5.85 Mb beyond Del on chromosome 2 is a sequence encoding a third bHLH protein, very similar to Del, which we named Delila-like (Am02g28470) (Fig. 2a,c). The Delila-like gene encodes a bHLH transcription factor in the bHLH-1 clade, and its intron/exon structure (established by amplification of a cDNA), was characteristic of bHLH-1 genes (Fig. 2b).

Phylogenetic analysis of the predicted amino acid sequences of Incolorata I, Delila and Delila-like together with other bHLH transcription factors that regulate flavonoid biosynthesis.
confirmed that they represent the two bHLH clades within subgroup IIIf (Fig. 2c). The sequence similarity and the conserved exon/intron structure between Inc I and genes such as ANI1 (petunia) and TT8 (Arabidopsis), with the bHLH domain encoded entirely in exon 7, placed Inc I firmly within the bHLH-2 clade.

**Confirmation of functional redundancy of Del, Del-like and Inc I in controlling anthocyanin biosynthesis**

We confirmed the functional redundancy of Inc I, Del and Del-like in inducing anthocyanin biosynthesis by bombarding petal lobes of the incI del double mutant line with plasmids carrying each cDNA driven by the CaMV 35S promoter. All three genes were able to restore anthocyanin production to single cells of the petal lobes, by contrast to the CaMV 35S: GFP control (Fig. 3).

**Genetic evidence that Del acts redundantly with Inc I in inducing anthocyanin pigmentation**

Croses were performed between incolorata I (inc I), mutabilis (inc I) and an unstable delilarecurrens mutant (del rec). JI:602 del rec flowers have red petal lobes, but the petal tubes are ivory with red sectors, resulting from the excision of a transposable element within the Del gene, that restores Del activity (Goodrich et al., 1992). Del revertant sectors were able to complement both inc I and inc I del mutant backgrounds, restoring pigmentation in lobes (Fig. 4a). Revertant Del sectors were observed that extended throughout the tube and lobes, confirming Del to be active in both tissues and to mask the phenotype of inc I mutations (Figs 4, S4). Individuals were recovered in the F3 generation where the transposable element had excised from the Del locus in the germline of an inc I: del I1 parental plant. These plants had full red flowers with coloured lobes and tubes (Fig. 4b).

The functional redundancy of Inc I and Del was investigated further by a cross between the stock line JI:23 (Martin et al.,) and inc I. JI23 carries a weak allele of Delila (del 23) that results in slightly reduced anthocyanin pigmentation in the tube, particularly the upper region of the tube (Fig. S5). The F3 population from JI:23 Inc II Inc I: del 23 × inc I Inc I: del I 2 segregated for flower colour in 96 plants scored, with six distinct phenotypic classes and their frequencies confirmed that they represent the two bHLH clades within subgroup IIIf (Fig. 2c). The sequence similarity and the conserved exon/intron structure between Inc I and genes such as ANI1 (petunia) and TT8 (Arabidopsis), with the bHLH domain encoded entirely in exon 7, placed Inc I firmly within the bHLH-2 clade.

**Confirmation of functional redundancy of Del, Del-like and Inc I in controlling anthocyanin biosynthesis**

We confirmed the functional redundancy of Inc I, Del and Del-like in inducing anthocyanin biosynthesis by bombarding petal lobes of the incI del double mutant line with plasmids carrying each cDNA driven by the CaMV 35S promoter. All three genes were able to restore anthocyanin production to single cells of the petal lobes, by contrast to the CaMV 35S: GFP control (Fig. 3).

**Genetic evidence that Del acts redundantly with Inc I in inducing anthocyanin pigmentation**

Croses were performed between incolorata I (inc I), mutabilis (inc I) and an unstable delilarecurrens mutant (del rec). JI:602 del rec flowers have red petal lobes, but the petal tubes are ivory with red sectors, resulting from the excision of a transposable element within the Del gene, that restores Del activity (Goodrich et al., 1992). Del revertant sectors were able to complement both inc I and inc I del mutant backgrounds, restoring pigmentation in lobes (Fig. 4a). Revertant Del sectors were observed that extended throughout the tube and lobes, confirming Del to be active in both tissues and to mask the phenotype of inc I mutations (Figs 4, S4). Individuals were recovered in the F3 generation where the transposable element had excised from the Del locus in the germline of an inc I: del I1 parental plant. These plants had full red flowers with coloured lobes and tubes (Fig. 4b).

The functional redundancy of Inc I and Del was investigated further by a cross between the stock line JI:23 (Martin et al.,) and inc I. JI23 carries a weak allele of Delila (del 23) that results in slightly reduced anthocyanin pigmentation in the tube, particularly the upper region of the tube (Fig. S5). The F3 population from JI:23 Inc II Inc I: del 23 × inc I Inc I: del I 2 segregated for flower colour in 96 plants scored, with six distinct phenotypic classes and their frequencies confirmed that they represent the two bHLH clades within subgroup IIIf (Fig. 2c). The sequence similarity and the conserved exon/intron structure between Inc I and genes such as ANI1 (petunia) and TT8 (Arabidopsis), with the bHLH domain encoded entirely in exon 7, placed Inc I firmly within the bHLH-2 clade.
lobes (inc I:inc I: del23:del) with a flush of pigmentation on the lobes (inc I:inc I: del23:del); three ivory flowers (inc I:inc I: del:del), from a family of 15 plants. These data demonstrated that Del acts within both the tube and lobes to regulate anthocyanin pigmentation, acting redundantly with Inc I in the lobes, but predominating in the tube of the flowers.

Interestingly by comparing the flowers of Inc II:Inc I: del23:del with those of inc I:inc I: del23:del, it was apparent that inc I activity affected anthocyanin accumulation in the tubes of the flowers as well as in the lobes, although its contribution to anthocyanin accumulation is substantially greater in the lobes than in the tubes (Fig. 5). This was confirmed by comparing the colour of Inc II:Inc I: del23:del flowers to inc I:inc I: del23:del flowers (Fig. 5). By contrast, although Del operates in both the lobes and the tubes, its contribution is substantially greater in tubes than in lobes as shown by comparing the phenotypes of inc I:inc I: del23:del and inc I:inc I: del23:del3 (Fig. 5).

To confirm that del23 was indeed a weak allele of Del, cDNA and genomic DNA were amplified from flowers of JI:23 (Fig. S6). The genomic sequence of Del in JI:23 (del23) had 29 SNPs within the coding sequence (14 were nonsynonymous) compared with the reference genome (Goodrich et al., 1992; Li et al., 2019), additional SNPs (79) and small insertions into introns, including one near a splice acceptor site within intron five (Fig. S6b) that resembled an intron acceptor site. Sequences of 8/11 clones of the Del cDNA from this line were mis-spliced into the insertion, resulting in mRNA sequences that encoded an insertion of an extra amino acid within a conserved region of the predicted Del protein, one transcript spliced correctly, one retained two introns and one had an entire portion of the cDNA missing (Fig. S6b). We concluded that this insertion causes mis-splicing of the Del mRNA in a significant proportion of the transcripts in JI:23 flowers, confirming that del23 was a weak del allele.

Identification of target genes of Inc I in anthocyanin biosynthesis

To determine the target genes regulated by Inc I, transcript analyses of biosynthetic genes were conducted in dissected tubes and lobes of del or wild-type flowers (Fig. 6a) or whole petals of wild-type, rosea doresea (a mutant of Rosa 1 which encodes an R2R3Myb transcription factor) and inc I:del flowers (Fig. 6b). Transcripts of the genes encoding DFR, anthocyanidin synthase (ANS) and flavonoid 3-glycosyltransferase (3GT) were at trace amounts in inc I:del flowers and the transcript levels of flavanone 3-hydroxylase (F3H) were substantially reduced. Transcripts of the genes encoding chalcone synthase (CHS) and chalcone isomerase (CHI) were not reduced (Fig. 6a). Similarly, transcript abundance for F3H, DFR, ANS and 3GT were severely reduced in whole petals of rosea doresea, and inc I:del (Fig. 6b). These data were confirmed by RNA gel blots of the inc I:del double mutant, mutabilis (Fig. S7) establishing that mutation of inc I causes down regulation of expression of the genes encoding F3H, DFR, ANS and 3GT (Fig. 6a; Martin et al., 1991), but not CHS or CHI. These effects were very similar to those of mutations in Del (Martin et al., 1991; Jackson et al., 1992), suggesting that the two transcription factors share the same target genes in anthocyanin biosynthesis.

Model of MBW regulation of Pallida (DFR)

Some time ago, Almeida et al. (1989) proposed a model for bicoloured patterning of delila mutants based on analysis of mutations in the promoter of the Pallida (pal) gene encoding DFR (Coen et al., 1986; Almeida et al., 1989), which proposed that Del and a lobe-specific transcription factor bound to the same upstream region of the DFR promoter (Box C: Fig. S8) in tubes and lobes respectively. At the time, none of the regulatory genes had been identified but, with these regulatory genes now to
hand, we tested this model using some of the same DFR promoter mutations. We expressed Del, Inc I, Ros 1 and a cDNA encoding a WD repeat protein from An11 in petunia (WDR1) under the control of the CaMV35S promoter in Nicotiana benthamiana, and tested the different Antirrhinum DFR promoter sequences using the dual luciferase assay to measure promoter responsiveness.

The wild-type DFR promoter has two starts of transcription (TS), one 66 bp upstream of the initiating ATG (TS1) and the second (TS2), 99 bp upstream of the initiating ATG (Coen et al., 1986). TS1 is the stronger initiation site in A. majus (Coen et al., 1986; Robbins et al., 1989). TS1 is preceded by a TATA box (TATA-1: /C0 to /C93) and TS2 is preceded by TATA-2 (/C120 to /C127; Fig. 7a). There was significant induction of luciferase driven by the wild-type DFR promoter (p2532) by Ros 1 alone. This was possibly due to binding of Ros 1 to an AC-box which lies between −184 and −190 nucleotides upstream of the initiating ATG codon of the DFR gene (AC-box 1; Fig. 7a) or to a second AC-box (AC-box 2) lying between −157 and −162 bp upstream of the start of translation of the DFR gene. This transcriptional response to Ros 1 alone may be a feature specific to tobacco. AC-box 1 is likely to be a Myb binding site, because its deletion following transposon excision reduced DFR gene expression and anthocyanin production to very low levels in the tube of the flower (Almeida et al., 1989). Just eight bp gene proximal to the AC-box 1 box is G-box 1 (CACGTG: /C171 to /C176), a recognised binding site for bHLH transcription factors (Fig. 7a; equivalent to Box B in Almeida et al., 1989; Goodrich et al., 1992). Displacement of these sequences (AC-box 1 box and G-box 1) from the downstream TATA boxes/transcriptional start sites causes complete loss of DFR expression in A. majus as shown by the insertion of Tam 3 at −169 bp upstream of the initiating ATG in the DFR promoter (Fig. S8; Carpenter et al., 1987).

There was substantial induction of luciferase activity when Del was included together with Ros 1 on the wild-type DFR promoter (p2532), but no further enhancement was observed when WDR1 was also included (Fig. 7b). This suggested that Ros 1 and Del act together to induce DFR expression and the WDR1 does not enhance this interaction on the DFR promoter. In combination with Ros 1, Inc I gave similar increases in luciferase...
activity on the wild-type DFR promoter but inclusion of the WDR1 protein slightly enhanced the induction of luciferase by Ros 1 and Inc I suggesting that Inc I and WDR1 interacted positively in the MBW complex to induce transcription from the DFR promoter.

Deletion of the sequences between −93 and −116 bp upstream of the initiating ATG removed the TATA-1 box associated with TS1 (p2557). This deletion resulted in loss of induction of the DFR promoter except for the background response to Ros 1, suggesting that the MBW complex primarily regulates transcription of the DFR gene using TATA-1 and TS1. The remaining transcriptional response to Ros 1 alone, presumably is mediated by TATA-2 and TS2.

Assay of a mutant DFR promoter lacking 12 nucleotides immediately downstream of AC-box 1 that included G-box 1 known to be recognised by bHLH proteins, (p2534), abolished the induction of luciferase activity by both Del and Inc I (with or without the WDR1 protein) (Fig. 7b). These data indicated that both bHLH proteins, Del and Inc I, recognise and bind G-box 1 to activate DFR gene expression. G-box 1 is therefore likely bound by both bHLH proteins in the MBW complex and the Myb protein, Ros 1, likely binds to the AC-box 1 in the wild-type promoter especially when interacting with Del. Deletion of the DFR promoter involving loss of G-box 1, AC-box 1 and 200 nucleotides further upstream placed another G-box (G-box 2) −188 to −193 bp upstream of the initiating ATG codon of the
DFR gene. Dual luciferase assays using this promoter mutation, which in *A. majus* results in almost complete loss of DFR expression (Almeida et al., 1989), did not restore responsiveness to Del nor to Inc I plus the WDR1 protein, over and above responsiveness to Ros 1 alone. This showed that the sequence context and proximity of the AC-box and G-box motifs relative to the basal transcriptional machinery assembled around the TATA box are important in defining the response of the target gene to the MBW complex.

To unravel the recognition of sequence motifs by the MBW complexes further, new mutations of the DFR promoter were created (p2551; p2565 and p2571). Removal of 12 bp downstream of G-box 1 (loss of /C0 154 to /C0 165 bp), including AC-box 2 (AACACC; /C0 157 to /C0 162; p2551) gave good induction of the DFR promoter in response to Ros 1 and Del, although inclusion of WDR1 in this assay inhibited luciferase activity to the levels achieved by Ros 1 alone (Fig. 7b). Interestingly, loss of AC-box 2 eliminated responsiveness to Inc I and WDR1. We tested the effects of the MBW components on deletion of AC-box 1 (/C0 180 to /C0 192; p2571; Fig. 7b). This caused large reductions in responsiveness of the DFR promoter to Del with Ros 1 alone, and by contrast to all other versions of the DFR promoter, the presence of WDR1 enhanced expression by the Ros 1–Del complex suggesting that Ros 1–Del binding to AC-box 2 (in the absence of AC-box 1) and G-box-1 is enhanced by WDR1. Loss of AC-box 1 eliminated the response to Inc I, although there was perhaps a small induction when Inc I was combined with WDR1. To confirm these results, we tested a promoter deletion that included both AC boxes and G-box 1 (–154 to –192; p2565). This deletion completely eliminated responsiveness of the DFR promoter to the MBW complex (Fig. 7b), confirming that the MBW components work through the combined AC-box 1, G-box 1 and AC-box 2 upstream activator sequence (UAS) and implying that there are structural and consequently functional differences in the way Ros 1 interacts with the bHLH-1 and bHLH-2 proteins on the DFR promoter. A model for how this might work is proposed in Fig. 7c.

Of course, the effects of the MBW complex on anthocyanin production reflect the net responses of all anthocyanin biosynthetic genes and the relative changes in enzyme and transporter activity on anthocyanin accumulation. To investigate the net effect of the MBW components on the regulation of anthocyanin production in *N. benthamiana*, we tested different combinations of regulatory proteins for induction of anthocyanins in the leaves of *N. benthamiana* ‘Northern Territory’ which does not suffer from reported problems with induction of anthocyanins in leaves of the more commonly used laboratory strain (Bally et al., 2015; Thole et al., 2019). Anthocyanin levels were significantly induced by Ros 1 on its own (>25 fold) and these were further elevated by inclusion of a vector expressing Del in the transient assay (>45

---

**Fig. 6** Anthocyanin biosynthesis genes are downregulated in *incolorata i* petals of *A. majus*. (a) Wild-type (WT) and delila mutant flowers were separated into tube and lobes for analysis of CHS, CHI, F3H, DFR, ANS and 3GT transcript abundance by qRT-PCR in tube (T) and lobe (L) tissue of WT and delila mutant petals. (b) Petals from whole flowers of WT, ros dorsea and inc I : del were analysed for CHS, CHI, F3H, DFR, ANS and 3GT transcript levels by qRT-PCR. Bar colour indicates pigmentation status. Means ± SEM, *n* = 3 biological replicates. Means that are significantly different (Student’s t-test) from WT are indicated (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

---

©2020 The Authors
New Phytologist ©2020 New Phytologist Foundation

New Phytologist (2021) 231: 849–863
www.newphytologist.com
fold) (Fig. 8). Inclusion of a vector expressing the WDR1 protein repressed the induction of anthocyanins by Ros 1 and Del significantly, suggesting that the combination of Ros 1 and Del is negatively impacted by inclusion of the WDR1 protein on anthocyanin production. Induction of anthocyanin production by the combination of Ros 1 and Inc I was not as great as for Ros 1 and Del, but was enhanced slightly by inclusion of the WDR1 protein suggesting that the WDR protein interacts positively with Inc I to promote net anthocyanin production by the MBW complex in N. benthamiana.
We also tested the functionality of Del-like in inducing anthocyanin production in *N. benthamiana*. Although Del-like did induce a small increase in anthocyanin produced in leaves, this was not significantly greater than Ros 1 alone. Inclusion of WDR1 in the inoculum reduced this small increase below levels for Ros 1 alone (Fig. 8). We concluded that the weak enhancement of anthocyanin biosynthesis by Del-like in *N. benthamiana* suggested that it plays a negligible role in controlling anthocyanin biosynthesis directly in flowers of *A. majus*.

The regulatory network controlling expression of the components of the MBW complex

In petunia, the bHLH-1 protein (JAF13) forms an MBW complex that regulates the expression of the *An1* gene (*bHLH-2*), enabling new MBW complexes to form with specific Myb proteins that target anthocyanin, proanthocyanidin and acidification pathways (Albert et al., 2014). While features of this hierarchical regulatory network have been reported in several plants (Baudry et al., 2006; Albert et al., 2014; Albert, 2015; Liu et al., 2014; Montefiori et al., 2015; Li et al., 2020), it is not known whether hierarchical regulation is universal.

Hierarchical regulation was investigated by analysing the expression of anthocyanin regulatory genes in petals (Fig. 9). *Ros I* abundance was unaffected by the *del* mutation and transcripts were present in both tube and lobe tissues. *Inc I* transcripts were detected only in the lobes of *del* mutants but, interestingly, *Inc I* was expressed in both the tube and lobes of wild-type petals, indicating that Del promotes *Inc I* expression in tubes. *Delila-like* transcripts were detected in the lobes and tubes of both wild-type and *del* mutants albeit at relatively low levels, suggesting that *Del-like* is not regulated by the MBW complex. *WDR1* transcripts were reduced in the tube of *del* mutants compared with wild-type, suggesting that its expression is controlled, in part, by the MBW complex.

Analysis of transcript levels in *ros<sup>del</sup>* lines confirmed which regulatory genes were under MBW control. *Inc I* showed significantly lower levels of expression in *ros<sup>del</sup>* compared with wild-type flowers, confirming that it was under MBW control. Although *Del-like* showed reduced expression in *ros<sup>del</sup>*
transcript levels detected were so low as to make the significance of the reduction ambiguous. Del was not impacted in its transcript levels in *ros* compared with wild-type, confirming that Del is not regulated by the MBW complex. *Ros 1* expression in *inc 1 del* showed no significant changes in transcript levels compared with wild-type, confirming that *Ros 1* is not controlled by the MBW complex.

**Discussion**

We have identified *Incolorata I* encoding a bHLH-2 protein involved in regulating anthocyanin biosynthesis in flowers of *A. majus*. Inc I has partially overlapping functions with Del, a bHLH-1 protein. Both regulate anthocyanin biosynthesis directly and their differential activity in flowers determines bicoloured patterning of petals in *del* mutants. This differential activity of the two transcription factors can also explain the weak, patterned pigmentation of mutations of the *Pallida* gene encoding DFR, an enzyme essential for anthocyanin biosynthesis (Coen et al., 1986; Almeida et al., 1989). However, Inc I and Del are not completely functionally redundant; Del positively regulates Inc I (and *WDR1*) expression in flower tubes, and it is the Del-independent expression of Inc I that gives bicoloured patterning of flowers. It is possible that Del-independent, lobe-specific expression of Inc I is dependent on Del-like, which is expressed more highly in lobes than in tubes. It is unlikely that Del-like regulates anthocyanin biosynthesis directly because *inc 1 del* homozygotes completely lack anthocyanins in their flowers, or in any other parts of the plant. In addition, the Myb transcription factor Ros 1 showed no significant promotion of anthocyanin production in combination with Del-like (Fig. 8) in *N. benthamiana*. Although Del-like does not appear to activate the expression of anthocyanin biosynthetic genes substantially it might activate *Inc I* expression specifically in lobes.

Activity of Ros 1 and Del in inducing anthocyanin biosynthesis is not enhanced by the presence of the WDR1 protein and may, in fact, be inhibited by it. Activity of Ros 1 with Inc I is enhanced by the presence of WDR1, meaning that Inc I may act in an MBW complex, but that the most effective complex of Ros1 and Del may lack the WDR1 protein. Competition between Myb and WDR proteins in forming complexes with bHLH-1 proteins has been reported by Pesch et al. (2015) in trichome formation in Arabidopsis, with the conclusion that this competition results in either an MB complex or a BW complex that regulate expression of different genes. In an extensive analysis of different subgroup III bHLH proteins, Zhang et al. (2019) demonstrated that only bHLH-1 proteins show competitive complex formation with Myb and WDRs whereas bHLH-2 proteins do not.
proteins always show enhanced complex formation with Myb and WDR proteins. This same difference between the bHLH proteins was shown by net anthocyanin biosynthesis in *N. benthamiana*, reflecting the integrated output of the MBW complex in leaves and implying that this difference also impacted expression of other genes involved in anthocyanin accumulation (Fig. 8).

Del and Inc I bind to G-Box 1 in the *DFR* promoter of *A. majus*. Both positioning and sequence context of this G-box are likely to be important for the binding and activity of Inc I and Del, as introduction of a G-box displaced upstream in a different sequence context did not rescue the induction of gene expression driven by Del or Inc I from the *DFR* promoter lacking G-box 1. The residual tube pigmentation observed in the equivalent mutants of *A. majus* (*pal-32 and pal-33; Almeida et al., 1989*) is likely to be driven by Del association with Ros 1 even where G-box 1 has been deleted, so preventing DNA binding by bHLH transcription factors. However, binding by the bHLH protein to the target gene DNA may not be essential for inducing low level anthocyanin production in *A. majus* (Fig. S8a). This ability of Del to interact with Ros 1 and promote anthocyanin production without DNA binding has been reported in tobacco (Applehagen et al., 2018), and for the B bHLH protein in regulation of the *b31* promoter in maize (Goff et al., 1992). Interestingly, the residual pigmentation observed in *pal-32 and pal-33* is restricted predominantly to the flower tube (Fig. S8a; Coen et al., 1986; Almeida et al., 1989) suggesting that, although DNA binding may not be necessary for Del participation in the MBW complex on the *DFR* promoter, DNA binding is probably essential for Inc I-WDR1 association with the MBW complex and *DFR* expression in the lobes in *A. majus* (Fig. S8b). This residual contribution of Del to induction of *DFR* gene expression is considerably lower than activation mediated by its binding to G-box 1, and might vary from target gene to target gene as several of these promoter sequences in *A. majus* lack G-box or even E-box motifs (for example *F3H*: Martin et al., 1991) that are bound by bHLH proteins.

Bicolour patterning in flowers of *A. majus* results from *Inc I* being controlled by Del in tubes, reinforced by Del controlling *WDR1* expression, which contributes positively to the activity of the Inc I-containing MBW complex. In lobes there is Del-independent expression of *Inc I* but this expression is probably dependent on an MB complex, possibly involving Del-like (which is more highly expressed in lobes than tubes) interacting with Ros 1. Del-like might have hierarchical activity on anthocyanin production through regulating *Inc I*, similar to the role of JAF13 in petunia (Albert et al., 2014) and tobacco (Montefiori et al., 2015). These findings reinforce the view that bHLH-1 and bHLH-2 proteins differ in their activity, even when their target pathways overlap, and that pigmentation patterning is established through hierarchical activities of transcription factors. The identification of transcription factors and mechanisms responsible for anthocyanin bicolouration in *Antirrhinum* provides a model for future studies to elucidate how the regulators of tissue and organ identity are linked to domain-specific pigmentation patterns.

Acknowledgements

CM, EB, PP and KES were supported by the Core Strategic Grant from the UK Biotechnology and Biological Sciences Research Council to the John Innes Centre, through the project ‘Understanding the control of flower development in *Antirrhinum majus*’ and the Institute Strategic Programmes ‘Understanding and Exploiting Plant and Microbial Secondary Metabolism’ (BB/J004596/1) and ‘Molecules from Nature’ (BB/P012523/1) from the UK Biotechnology and Biological Sciences Research Council. The Marsden Fund of New Zealand/Te Pūtea Rangahau A Marsden supported NWA, SMAM, CNW (contract PAF1501) and KES, KMD (contract CRO101). We are grateful to Prof. Peter Waterhouse (Queensland University of Technology, Brisbane, Australia) for providing the seeds of *N. benthamiana* cv Northern Territory. We thank Julie Ryan and Ian King at PFR and Peter Walker at JIC for care and maintenance of plants and Steve Mackay for laboratory support. We thank Andrew Davis and Peter Scott at JIC for photography.

Author contributions

CM, PP, NWA, SMAM and KES cloned and characterised the *Incolorata I* gene and its mutant alleles, SMAM characterised the *del* mutant allele. CM and KES undertook RNA analysis using RNA gel blots and NWA analysed gene expression by qRT-PCR. CNW performed bioptic complementation assays and KES cloned *WDRI*. EB performed dual luciferase assays in *N. benthamiana*, made all mutations of the *DFR* promoter for these assays and prepared Figs 7–9c. NWA, KMD and CM drafted the manuscript before all authors contributed to its improvement and agreed on its final content. NWA and EB contributed equally to this work.

ORCID

Nick W. Albert  https://orcid.org/0000-0002-8579-529X
Eugenio Butelli  https://orcid.org/0000-0001-6397-277X
Kevin M. Davies  https://orcid.org/0000-0001-5652-5015
Cathie Martin  https://orcid.org/0000-0002-3640-5080
Sarah M.A. Moss  https://orcid.org/0000-0002-0060-5486
Paolo Piazza  https://orcid.org/0000-0002-3736-1841
Kathy E. Schwinn  https://orcid.org/0000-0002-6337-3627

References

Albert NW. 2015. Subspecialization of R2R3-MYB repressors for anthocyanin and proanthocyanidin regulation in forage legumes. *Frontiers in Plant Science* 6: 1165.

Albert NW, Davies KM, Lewis DH, Zhang H, Montefiori M, Brendolise C, Boase MR, Ngo H, Jameson PE, Schwinn KE. 2014. A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *The Plant Cell* 26: 962–980.

Albert NW, Griffiths AG, Cousins GR, Verry IM, Williams WM. 2015. Anthocyanin leaf markings are regulated by a family of R2R3-MYB genes in the genus *Trifolium*. *New Phytologist* 205: 882–893.
Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E. 1991. Control of anthocyanin biosynthesis in flowers of Antirrhinum majus. The Plant Journal 1: 37–49.

Montefiori M, Brendolise C, Dare AP, Lin-Wang K, Davies KM, Hellens RP, Allan AC. 2015. In the Solanaceae, a hierarchy of bHLHs confer distinct target specificity to the anthocyanin regulatory complex. Journal of Experimental Botany 66: 1427–1436.

Nesi N, Debeaujon I, Jond C, Pellertier G, Cacho Me, Lepiniec L. 2000. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. The Plant Cell 12: 1863–1878.

Park KI, Choi JD, Hoshino A, Morita Y, Iida S. 2004. An intragenic tandem duplication in a transcriptional regulatory gene for anthocyanin biosynthesis confers pale-colored flowers and seeds with fine spots in Ipomoea tricolor. The Plant Journal 38: 840–849.

Park KI, Ishikawa N, Morita Y, Choi JD, Hoshino A, Iida S. 2007. A bHLH regulatory gene in the common morning glory, Ipomoea purpurea, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. The Plant Journal 49: 641–654.

Payne CT, Zhang F, Lloyd A. 2000. GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. Genetics 156: 1349–1362.

Pesch M, Schultheiss I, Kloppfelssch K, Ubrig JF, Koegl M, Clemen CS, Simon R, Weidkamp-Peters S, Huls Kemp M. 2015. TRANSPARENT TESTA GLABRA1 and GLABRA3 compete for binding to GLABRA3 in Arabidopsis. Plant Physiology 168: 584–597.

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

Ramsay NA, Glover BJ. 2005. MYB–bHLH–WD40 protein complex and the evolution of cellular diversity. Trends in Plant Science 10: 63–70.

Robbins TP, Carpenter R, Coen ES. 1989. A chromosome rearrangement suggests that donor and recipient sites are associated during Tam3 transposition in Antirrhinum majus. EMBO Journal 8: 5–13.

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

Spelt C, Quattrocchio F, Mol J, Koes R. 2002. ANTHOCYANIN1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. The Plant Cell 14: 2121–2135.

Spelt C, Quattrocchio F, Mol JNM, Koes R. 2000. Anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. The Plant Cell 12: 1619–1631.

Strazzer P, Spelt CE, Li S, Biek M, Federici CT, Roose ML, Koes R, Quattrocchio FM. 2019. Hyperacidification of Citrus fruits by a vacuolar proton-pumping P-ATPase complex. Nature Communications 10: 744.

Stubble H. 1966. In: Genetik und Züchtung von Antirrhinum l. sect. Antirrhinum. Jena, Germany: VEB Gustav Fischer Verlag.

Terrier N, Torregrosa L, Augeorges A, Vialot S, Verri C, Cheynier V, Romieu C. 2009. Ectopic expression of VvMybPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. Plant Physiology 149: 1028–1041.

Tholue V, Bassard JE, Ramirez-Gonzalez R, Trick M, Afshar BG, Breitel D, Hill L, Foito A, Shepherd L, Freitag 5 et al. 2019. RNA-seq de novo transcriptome assembly and flavonoid gene analysis in wild and cultivated berry fruit species with high content of phenolics. BMC Genomics 20: 23.

Whiddale M. 1907. The inheritance of flower colour in Antirrhinum majus. Proceedings of the Royal Society of London, Series B: Biological Sciences 79: 288–305.

Xu W, Grain D, Bobet S, Le Gourrieres J, Thevenin J, Kelemen Z, Lepiniec L, Dubos C. 2014. Complexity and robustness of the flavonoid transcriptional regulatory network revealed by comprehensive analyses of MYB-bHLH-WDR complexes and their targets in Arabidopsis seed. New Phytologist 202: 132–144.

Yuan YW, Sagawa JM, Frost L, Vela JP, Bradshaw HD Jr. 2014. Transcriptional control of floral anthocyanin pigmentation in monkeyflowers (Mimulus). New Phytologist 204: 1013–1027.

Zhang BP, Chopra D, Schrader A, Huls Kemp M. 2019. Evolutionary comparison of competitive protein-complex formation of MYB, bHLH, and WDR proteins in plants. Journal of Experimental Botany 70: 3197–3209.

Zhang BP, Huls Kemp M. 2019. Evolutionary Analysis of MBF Function by Phenotypic Rescue in Arabidopsis thaliana. Frontiers in Plant Science 10: 375.

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A. 2003. A network of redundant bHLH MYB proteins functions in all TTG1-dependent pathways of Arabidopsis. Development 130: 4859–4869.

Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A. 2008. The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. Development 135: 1991–1999.

Zimmermann IM, Heim MA, Weisshaar B, Ubrig JF. 2004. Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like bHLH proteins. The Plant Journal 40: 22–34.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Amino acid alignment of bHLH proteins.

Fig. S1 Differences in mutabilis phenotypes between glasshouse and field-grown plants of A. majus.

Fig. S2 Lack of complementation between inc I1 and inc I2 of A. majus.

Fig. S3 RNA gel blot showing lack of Inc I transcript in flower lobes of the mutabilis mutant of A. majus.

Fig. S4 Sectors caused by excision of a transposable element (Tam 2) from the Del gene in inc I2: delrec plants of A. majus.

Fig. S5 Phenotype of del23 allele of A. majus.

Fig. S6 Molecular analysis of del23 of A. majus.

Fig. S7 Impaired expression of anthocyanin biosynthetic genes in flowers of the inc I2 mutant of A. majus.

Fig. S8 Phenotypes of Pallida mutants of A. majus with deletions in their UAS controlling DFR expression caused by imprecise transposon excision and described by Almeida et al (1989).

Table S1 Primers used in this study.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.