Painted flowers: Eluta generates pigment patterning in Antirrhinum

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Painted flowers: *Eluta* generates pigment patterning in *Antirrhinum*

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Summary

- In the early 1900s, Erwin Baur established *Antirrhinum majus* as a model system, identifying and characterising numerous flower colour variants. This included *Picturatum*/*Eluta*, which restricts the accumulation of magenta anthocyanin pigments, forming bullseye markings on the flower face.
- We identified the gene underlying the *Eluta* locus by transposon-tagging, using an *Antirrhinum* line that spontaneously lost the nonsuppressive *el* phenotype. A candidate MYB repressor gene at this locus contained a CACTA transposable element. We subsequently identified plants where this element excised, reverting to a suppressive *Eluta* phenotype. *El* alleles inhibit expression of anthocyanin biosynthetic genes, confirming it to be a regulatory locus. The modes of action of *Eluta* were investigated by generating stable transgenic tobacco lines, biolistic transformation of *Antirrhinum* petals and promoter activation/repression assays.
- Eluta competes with MYB activators for promoter *cis*-elements, and also by titrating essential cofactors (bHLH proteins) to reduce transcription of target genes. *Eluta* restricts the pigmentation established by the R2R3-MYB factors, *Rosea* and *Venosa*, with the greatest repression on those parts of the petals where *Eluta* is most highly expressed.
- Baur questioned the origin of heredity units determining flower colour variation in cultivated *A. majus*. Our findings support introgression from wild species into cultivated varieties.

Introduction

The rediscovery of Mendel's laws of inheritance and segregation (Correns, 1900; Tschermak-Seysenegg, 1900a,b; de Vries, 1900; Bateson, 1901, 1902) signalled the birth of modern genetics, sparking widespread excitement for how this could be applied to the variation between flowers found in home gardens. An early advocate for genetics, Erwin Baur became fascinated by garden snapdragon (*Antirrhinum majus*, 'Antirrhinum'), establishing it as a model genetic system to understand how heredity units contribute to observed flower colours and patterning. During the early 1900s, seed catalogues such as those from the Haage & Schmidt Nursery (Erfurt, Germany) had extensive collections of Antirrhinum with diverse colours and patterns (> 100 varieties), including magenta, yellow, bi-colours (e.g. *delila*), striped and bullseye patterns (e.g. *Picturatum*; Fig. 1a, Supporting Information Figs S1, S2). These patterns arise owing to differences in concentrations and patterning of magenta (anthocyanin) and yellow (aurone) pigments. Baur was interested in understanding the sources of this variation, and whether variation was caused by the lack of heredity units (alleles) in the cultivated varieties. However, 'wild' *A. majus* is not a uniform species (Schwarz-Sommer *et al.*, 2003), but rather there are numerous closely related types from different locations that exhibit varied colour patterns and morphologies (Baur, 1910), hampering clear identification of a '-wild-type' (WT). Baur described and analysed numerous variants, including *Picturatum* (painted flowers), which had reduced anthocyanin pigmentation resulting in a washed-out appearance of the flowers. Accessions were sourced from the nursery of Haage & Schmidt in Erfurt, Germany (Baur, 1910). He showed that *Picturatum* (factor G) segregated as a single locus dominant/semi-dominant to full red (although he found it difficult to distinguish Gg from GG; Baur, 1910). *Picturatum* later became known as *Unicolorata* and then *Eluta*, described by Baur's student Hans Stubbe (1941), and factor F came to be known as *Rosea* (Schwinn *et al.*, 2006). Despite this long history, the gene underlying *Eluta* remains to be conclusively identified and characterised. *Eluta* serves as a fascinating example to address

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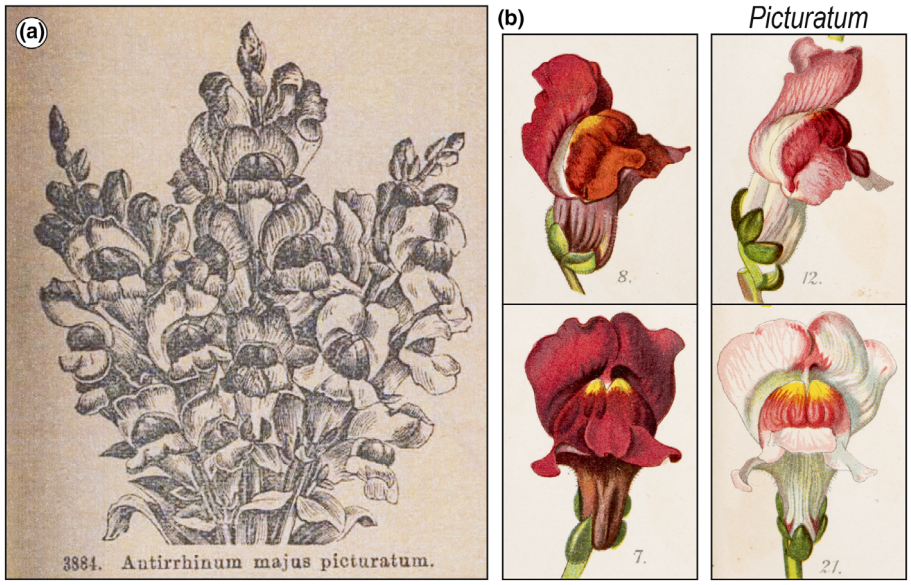

Fig. 1 Early illustrations of *Picturatum/Eluta* phenotypes in Antirrhinum. (a) Illustration of Antirrhinum *Picturatum* in the 1910 seed catalogue from Haage & Schmidt Nursery (Erfurt, Germany). (b) Illustrations of full red and *Picturatum* phenotypes from Baur's description of Antirrhinum colour variants (Baur, 1910; Supporting Information Fig. S2).

Baur's questions regarding the origin of variation conferring pigmentation patterning of flowers.

Since these early genetic discoveries, many of the genes underlying natural variants or induced mutants determining flower colour and patterning have been identified in Antirrhinum. Some encode anthocyanin biosynthetic enzymes, including *Nivea/Chalcone Synthase*, *Incolorata II/Flavanone 3-Hydroxylase*, *Pallida/Dihydroflavonol 4-Reductase* and *Candica/Anthocyanidin Synthase* (Martin *et al.*, 1985, 1991; Sommer & Saedler, 1986). Loci that regulate the timing and spatial patterning of anthocyanins have also been identified, including *Rosea*, *Venosa*, *Delila* (also listed in Haage & Schmidt, 1910) and *Incolorata I*. *Rosea* and *Venosa* encode R2R3-MYB transcription factors (Schwinn *et al.*, 2006; Shang *et al.*, 2011), while *Delila* (subgroup (SG) IIIf, bHLH-1 clade), *Del-like* (SGIIIf, bHLH-1) and *Incolorata I* (SGIIIf, bHLH-2) encode bHLH transcription factors (Goodrich *et al.*, 1992; Albert *et al.*, 2021; Martin *et al.*, 2023). These MYB and bHLH transcription factors may act together with a WD-repeat (WDR) protein, to form a complex (MBW complex) that activates transcription of the anthocyanin biosynthetic genes. Because all three components are involved in regulating anthocyanin production, complex pigmentation patterns (e.g. vein association, bicolouration) can arise from the overlapping spatial expression of the MBW components (Shang *et al.*, 2011; Davies *et al.*, 2012; Albert *et al.*, 2021). In addition to transcriptional activators, several classes of repressors have been shown to antagonise the formation or activity of the MBW complex. Subgroup 4 R2R3-MYB proteins repress transcription of flavonoid biosynthetic genes (Tamagnone *et al.*, 1998; Aharoni *et al.*, 2001; Matsui *et al.*, 2008; Albert *et al.*, 2014a). These MYB proteins can be incorporated into MBW complexes and recruited to anthocyanin biosynthetic genes, repressing transcription through C-terminal domains (EAR motif or TLLLFR). By contrast, the R3-MYB repressors (e.g. *Petunia hybrida* PhMYBx, *Arabidopsis thaliana* AtCPC and *Mimulus gattutus*/*Erythranthe guttata* MgROI) inhibit the formation of MBW complexes by titrating bHLHs through binding ('squelching'; Zhu *et al.*, 2009; Yuan *et al.*, 2013; Albert *et al.*, 2014a). The combined activity of activators and repressors has the potential to generate a diverse array of complex pigmentation patterns (Albert *et al.*, 2014b; Ding *et al.*, 2020).

Studies to date demonstrate the importance of R2R3-MYB activators in determining the pigmentation intensity and patterning in plants (Schwinn *et al.*, 2006). These genes usually exist as small gene families with distinct spatial and temporal expression patterns. In Antirrhinum, alleles of *Rosea* (consisting of three linked genes) generate patterns ranging from solid magenta to pale/white flowers (Schwinn *et al.*, 2006; Tavares *et al.*, 2018). These may be combined with the vein-associated stripes controlled by *Venosa* (Shang *et al.*, 2011), and contribute to the natural diversity of pigmentation of wild species. Similarly in petunia, *MYB* gene family members control full petal colour, vein-pigmentation (venation) and blushed patterns (Albert *et al.*, 2011; Zhang *et al.*, 2021), and in Mimulus (*Erythranthe* species) full colour or spots (Yuan *et al.*, 2014). In Antirrhinum, *Eluta* represents an additional locus that modifies anthocyanin patterns established by *Rosea* and *Venosa*, but it remains unclear how this occurs.

The genus *Antirrhinum* contains over 20 closely related species, arising from adaptive radiation and speciation in Europe following the last ice age. Extensive phenotypic variation exists, and the species occupy distinct and often challenging environments, ranging from alpine to coastal conditions. Baur's dilemma in identifying a homogenous WT *A. majus* was because, although they are distinct species, many can form interspecific hybrids. In some locations, such as the Pyrenees, hybridisation between self-incompatible species with distinct colour morphs occurs naturally, for example between yellow *Antirrhinum striatum* and magenta *Antirrhinum pseudomajus* (Whibley *et al.*, 2006). However, the resulting hybrids are not an intermediate orange

phenotype, but instead, dominant alleles from each parental species repress and restrict the localisation of the pigment produced in the other species: *Eluta* inhibits and restricts magenta anthocyanin production in parts of the flower and *Sulfurea* (*Sulf*) restricts yellow aurones (Whibley *et al.*, 2006; Bradley *et al.*, 2017). It is thought that species separation is maintained because of pollinator preferences for particular morphs, and/or foraging behaviours, such as re-visiting flowers of the same type (e.g. colour, shape and scent, known as flower constancy; Whibley *et al.*, 2006; Tastard *et al.*, 2014; Jaworski *et al.*, 2015). Thus, repressive alleles are important for maintaining parental phenotypes in hybrids. Interestingly, both *Eluta* and *Sulfurea*, each of which restricts pigmentation, are dominant alleles.

In this study, we traced the origins of the original *Eluta* variants described by Baur & Stubbe back to the early 1900s and identified the gene responsible for the *Picturatum* phenotype by transposon-tagging. We investigated the modes of action of Eluta and related the activity of *Eluta* alleles to both pattern formation and the pigmentation phenotypes in wild *Antirrhinum* species.

Materials and Methods

Antirrhinum germplasm

Antirrhinum majus L. *Eluta* stock lines were generated from Sippe50, originally sourced from IPK Gatersleben (Leibniz Institute of Plant Genetics and Crop Plant Research). Sippe50 has been maintained as a pure stock since at least 1906 (Schick & Stubbe, 1932), containing the *Eluta* variant (*El/El*) and the *delila* mutant (*del/del*). *El* stocks free of *del* were generated by crossing with full red genotype JI522 (*el/el*, *Del/Del*) to F_2. Homozygous *El/El Del/Del* individuals were maintained by self-pollination. Stock JI668 (el^{ul668}) was established from a selection of a magenta individual arising from a cross between JI594 (*Pal*, *El*) and JI2 (pal^{rec}, *el*) stocks. Stock 594 was originally obtained from IPK Gatersleben Germany. *Antirrhinum majus* 'Princess' (syn. 'Lucky lips') was sourced from Kings Seeds New Zealand. The origins of *Antirrhinum latifolium* Mill. 'Pyrea' (IPK), *A. majus* L. 'Barcelona', *Antirrhinum molle* L., *Antirrhinum mollissimum* Rothm., *Antirrhinum graniticum*, *Antirrhinum australe* Rothm and, *A. majus* $rosea^{dorsea}$ (ros^{dor}) are described in Schwinn *et al.* (2006).

Phylogenetic trees

Maximum likelihood phylogenetic trees were generated from MUSCLE (Edgar, 2004) sequence alignments using PHYML (Guindon & Gascuel, 2003), within GENEIOUS PRIME (v.2022.0.1), using amino acid sequence and WAG substitution model (MYB repressor) or nucleotide sequence and GTR model (*Eluta* alleles).

Cloning and PCRs

The coding sequences for *Eluta* were amplified from cDNA from Sippe50 (*El*) and JI522 (*el*) flowers, prepared with Superscript II™ reverse transcriptase (Life Technologies) and oligo $(dT)_{12–18}$, using gene-specific primers (Table S1) and 2GRobust polymerase (Kapa Biosciences, Wilmington, MA, USA). These were cloned into pENTR/D-TOPO (Life Technologies) and recombined into a gateway vector pNWA101 to generate overexpression constructs ($35S_{pro}$*:CDS:OCS*). Plasmid constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *El* and *el* alleles were amplified for sequencing from genomic DNA with iProof polymerase (Bio-Rad) and gene-specific primers (Table S1).

Nicotiana benthamiana infiltrations

Nicotiana benthamiana Domin. ('Northern Territory') leaf infiltrations were performed as described previously (Albert *et al.*, 2021) with *A. tumefaciens* (GV3101) strains harbouring *35S:El*, *35S:Inc I*, *35S:Ros1*, *35S:GFP*, *35S:El T98M* or *35S:el M97T* constructs. Plants were photographed 6 d postinfiltration. Dual luciferase assays were performed on *Pallida* (*DFR*) promoter sequences (Albert *et al.*, 2021).

Antirrhinum bombardments

Antirrhinum biolistic experiments were performed as detailed in Schwinn *et al.* (2006), with the modifications detailed here. A maximum of 10 μg of plasmid DNA was used and mixed with gold particles as per Schwinn *et al.* (2006); 5 μl of the gold suspension was used to bombard the petals with each tub of at least three petals being bombarded twice. $CaMV35S_{pro}$*:mGFP5-ER* (Haselhoff *et al.*, 1997) was included as an internal positive control for successful transformation.

Stable *Nicotiana tabacum* transformations

Stable transgenic *N. tabacum* L. 'Samsun' plants were generated using *A. tumefaciens* (strain GV3101) containing a $CaMV35S_{pro}$*:* $El^{Sippe50}$ or $CaMV35S_{pro}$*:GFP-ER* construct (Horsch *et al.*, 1985). Independent transformation events were maintained by selecting and tracking a single shoot per explant. Clonal copies were maintained for independent transformants. Tobacco lines containing $CaMV35S_{pro}$*:Delila* have been described previously (Butelli *et al.*, 2012). Three biological replicates consisting of clonal copies of a single transgenic tobacco line were performed and each biological replicate consisted of pooled petal tissue from ≥ 4 stage four flower buds. Tissue was used for RNA isolation and quantitative reverse transcription polymerase chain reaction and flavonoid analysis.

RNA-seq differential gene expression analysis

Total RNA was extracted from whole petals of WT JI522 (*el/el*), heterozygous (*El/el*) and homozygous (*El/El*) *Eluta* genotypes (derived from an F_2 population of Sippe50 × JI522) using an RNeasy Plant mini kit (Qiagen). Stranded libraries (100-bp pair-ended) were prepared and sequenced with HiSeq 2000 platform at the Australian Genome Research Facility (Melbourne, Vic., Australia). The raw sequence reads were cleaned and trimmed

using SortMeRNA 2.1 and Trimmomatic 0.36, respectively, and then were mapped to the reference genome (snapdragon_IGDBV1) using Salmon 0.9.1. Comparative analysis on differentially expressed genes was performed using the DESeq2 R package.

qPCR

Quantitative reverse transcription polymerase chain reaction was performed as described in Albert *et al.* (2021), using RNA isolated from tobacco petals, or from the dissected petals of *Antirrhinum*, using gene-specific primers (Table S1). Tobacco data were normalised to the geometric mean of *NtEF1α* and *NtL25*. *Antirrhinum* data were normalised to the geometric mean of *AmPP2A*, *AmUCE E2*, *AmRNA RMD*, *AmEF1α* and *AmCyclophillin*.

Chemistry

Flavonoids were extracted from 25 mg freeze-dried, ground tobacco petal tissue in 1 ml methanol : water : formic acid (80 : 19 : 1). Flavonols were quantified by UHPLC as described in Albert *et al.* (2018), except that they were quantified as quercetin rutinoside equivalents. Anthocyanins were quantified using an alternative UHPLC programme. The analytical column used was a Zorbax SBC18 2.1 × 150 mm, 1.8 µm (Agilent, Santa Clara, CA, USA), maintained at 50°C. A binary solvent programme was used with Solvent A (formic acid : MQ water, 5 : 95) and Solvent B (acetonitrile) at a flow of 0.35 ml min^{-1} using a SBC18 column. The initial solvent composition was 95%A, 5%B until 0.5 min, then changed to 80%A 20%B at 10 min, and 5%A 95%B at 15 min. After a 1.5-min hold at 5% A 95%B, the composition was returned to 95%A 5%B ready for the next injection. The total UHPLC analysis time was 20 min per sample. All solvent gradients were linear. The injection volume was 2 µl. Spectral data (260–600 nm) were collected for the entire analysis. Anthocyanins quantified at 530 nm as cyanidin-3-galactoside equivalents.

Yeast two-hybrid

Yeast two-hybrid (Y2H) experiments were performed as described previously (Albert *et al.*, 2014a), with yeast strains harbouring constructs for *El* (Sippe50), *el* (JI522) or *Del*. After mating, diploid strains were selected on SD media lacking Leu and Trp, replicated on SD media lacking Leu, Trp, and His and supplemented by increasing concentrations of 3-AT (0–100 mM).

Statistical significance tests

One-way ANOVA with Fisher's least significant difference ($P < 0.05$) were performed using Genstat (v.22.1.0.532). For quantitative reverse transcription polymerase chain reaction data, log_{10} transformation was performed because of unequal variance.

Results

The *Eluta* phenotype and its genetic characterisation

The *Picturatum* variants analysed by Baur were sourced from Haage & Schmidt seed distributers (Baur, 1910). A picture from their 1910 seed catalogue shows Antirrhinum *Picturatum* flowers with a bullseye colouration pattern (Fig. 1a), consistent with illustrations in Baur (1910; Figs 1b, S2). In 1911, Baur reported the 'coupling' of factor F (subsequently identified as *Rosea*; Schwinn *et al.*, 2006) with *Picturatum* (factor G; Baur, 1911) in the second report of gene linkage, following that of Bateson in the same year (de Vilmorin & Bateson, 1911). *Picturatum* was subsequently renamed *Unicolorata* and then *Eluta* (Stubbe, 1966). German geneticists established a line called Sippe50 as comparator for all their subsequent mutant screens. Sippe50 has been maintained by self-pollination since 1906 and was reported as meeting the criteria of an ideotype, with the exception of the factors *Uni* (now *Eluta*) and *delila* (Schick & Stubbe, 1932). The suppressed *Picturatum/Eluta* phenotype is enhanced in *delila* (factor D) backgrounds, such as Sippe50 (Fig. 2a; *El/El del/del*).

Interestingly, most of the mutations affecting flower colour were identified from nursery collections such as those of Haage & Schmidt before Sippe50 was adopted as the standard line, and it may be that the restricted pigmentation, caused by the combination of *Eluta* and *delila* alleles, limited the subsequent identification of new colour pattern mutants. The identification of *Eluta* before the advent of induced mutagenesis (which started in Antirrhinum in 1923) suggests that this was a 'natural variant' already present in *A. majus*, perhaps derived from outcrossing between species which was/is frequent between different species of European *Antirrhinum*.

Eluta encodes a R2R3-MYB repressor

In *A. majus* backgrounds, *Eluta* limits anthocyanin pigmentation to a 'bullseye' in the flower face, colouring the pollinator-landing platform and reducing petal tube colouration to a spot at the base of the flower tube (Fig. 2a).

Eluta is closely linked to the *Rosea* locus (Baur, 1911; Stubbe, 1966; Schwinn *et al.*, 2006), so we searched for genes encoding repressors (e.g. *R3-* and *R2R3-MYB* repressors, *SQUAMOSA-Promoter Binding Protein-Like*) that were located on the same chromosome as *Rosea1* (chr 6), making use of a high-quality genome assembly of *A. majus* (Stock JI7; Li *et al.*, 2019). We identified an R2R3-MYB gene, Am06g36700, located *c.* 170 kb from *Rosea1* (Am06g36450; Fig. 2b). This gene was identified as a candidate for *Eluta* within the 50-kb mapping interval containing the *Eluta* locus (Tavares *et al.*, 2018). Phylogenetic analysis of the gene, herein named *Eluta*, shows it falls within a well-supported clade of R2R3-MYB repressors that regulate phenylpropanoid biosynthesis (subgroup 4; SG4; Fig. 2c; Martin *et al.*, 2023).

To confirm the identity of the *Eluta* gene, we analysed a line derived from an *Eluta* genotype (suppressed pigmentation) that

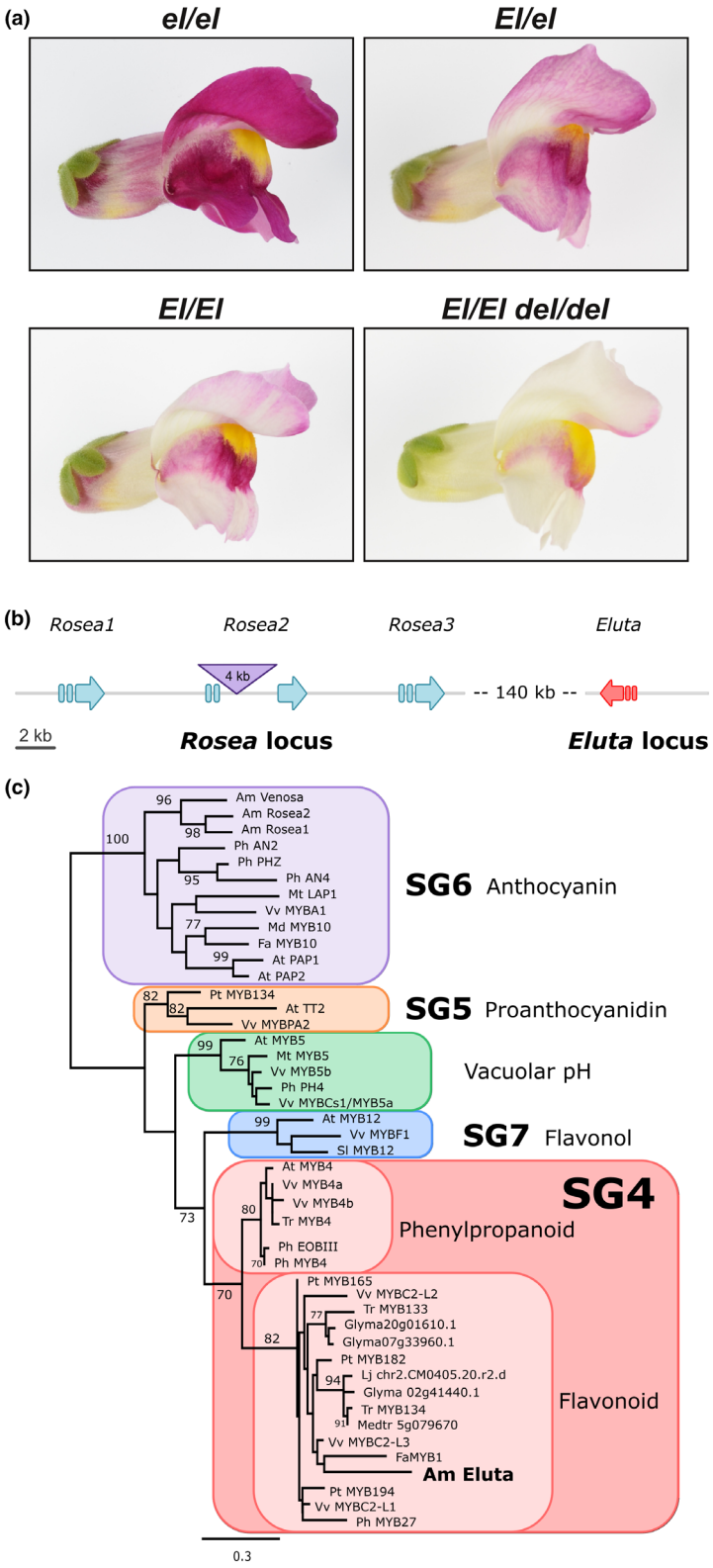

Fig. 2 *Eluta* encodes a subgroup 4 MYB repressor. (a) The presence of *Eluta* alleles inhibits anthocyanin accumulation in a dose-dependent (semi-dominant) manner in *Antirrhinum majus*. This is enhanced in *delila* backgrounds lacking an active gene encoding the bHLH protein. (b) Illustration of the linkage between the complex *Rosea* locus and *Eluta*. The purple triangle represents a transposable element fragment within *Rosea2*. (c) Maximum likelihood phylogenetic tree of the MYB domain of R2R3-MYB transcription factors belonging to activator and repressor subgroups known to regulate phenylpropanoid biosynthesis. Subgroup 4 (SG4) MYB proteins are transcriptional repressors. Support ≥ 70% shown (1000 bootstrap replicates). The sequence alignment and full species names for gene accessions are provided in Supporting Information Fig. S3.

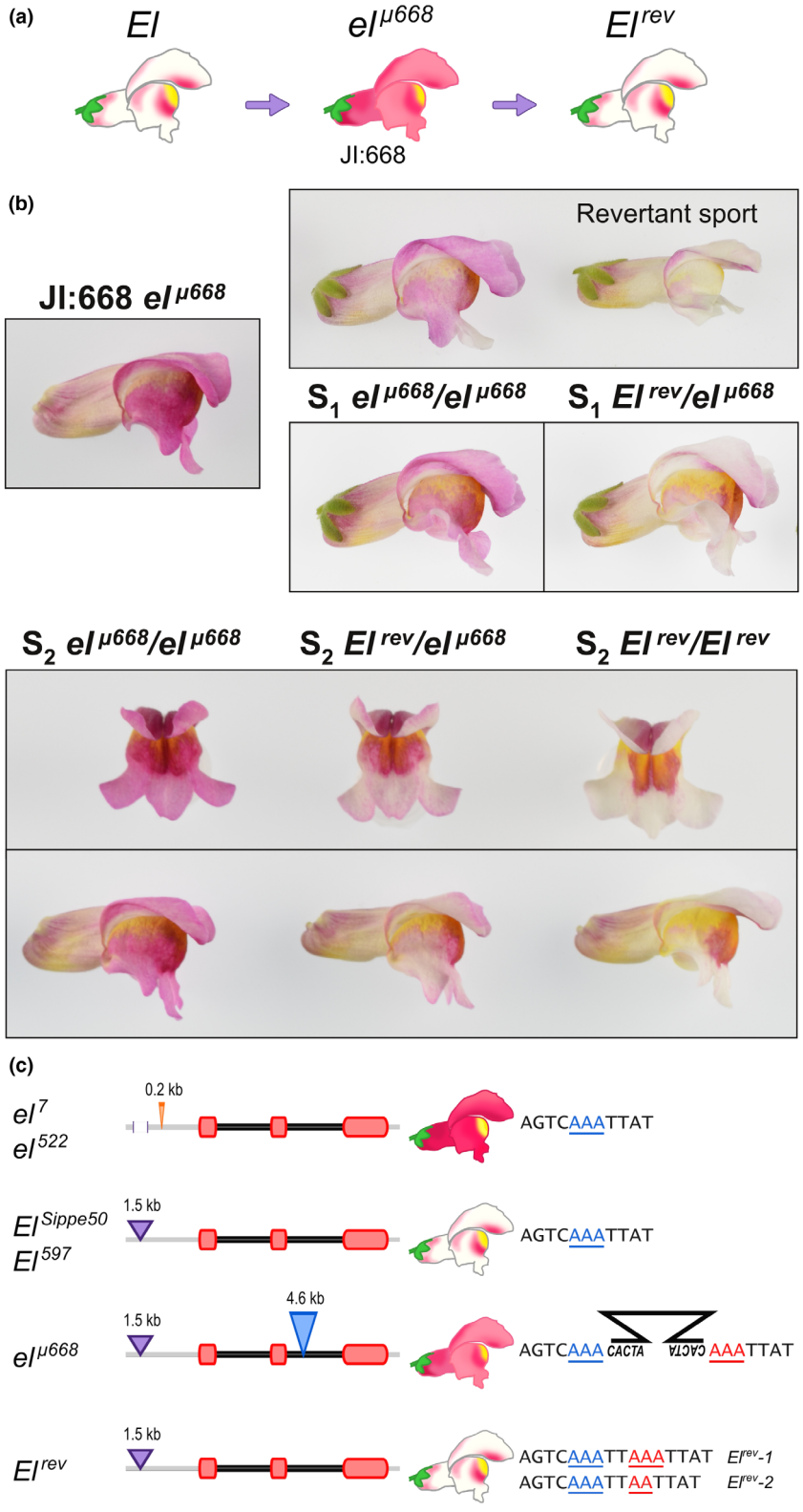

Fig. 3 Transposon-tagging *Eluta*. (a) A magenta flower colour mutant of *Antirrhinum majus* was derived from a pale patterned *Eluta* genotype, represents an unstable allele at the *Eluta* locus ($el^{\mu668}$) and has been maintained as the JI668 stock line. Plants of this line produced a bud-sport with pale flowers (El^{rev}), shown in (b). The flower colour phenotypes in the S_1 progeny had magenta ($el^{\mu668}$) and pale magenta flowers ($El^{rev}/el^{\mu668}$). The S_2 progeny produced magenta, pale magenta and near-white flower colour phenotypes, corresponding to the dosage of the revertant El^{rev} alleles. (c) Schematic representation of *Eluta* alleles in stock lines JI7, JI522, Sippe50, JI597, JI668 and the revertants of JI668. Exons are shown as red boxes, black lines as introns and promoter regions as grey lines. Sequence insertions are represented by triangles. JI668 contains a CACTA element (4.6 kb) within intron 2 of the *Eluta* locus: the target insertion site is indicated in blue. The insertion of the CACTA element resulted in a 3-bp duplication, shown in red. Two independent excision events ($El^{rev\text{-}1}$, $El^{rev\text{-}2}$) were identified based on their footprints, which conformed to the model for CACTA transposable element excision (Coen *et al*., 1986).

spontaneously produced magenta flowers ($el^{\mu668}$). This individual was identified and maintained as a stock line (JI668), because it represented a putative transposon-tagged allele of the *Eluta* locus (Fig. 3a). Plants of this line were grown and initially had the $el^{\mu668}$ magenta flower phenotype (Fig. 3b). However, during summer, one plant produced a bud-sport bearing flowers with a very pale phenotype. This reversion suggested that transposon excision may have occurred, which would allow identification of the affected gene. Seed were collected from self-pollinated flowers, and the resulting S_1 progeny produced 22 plants with

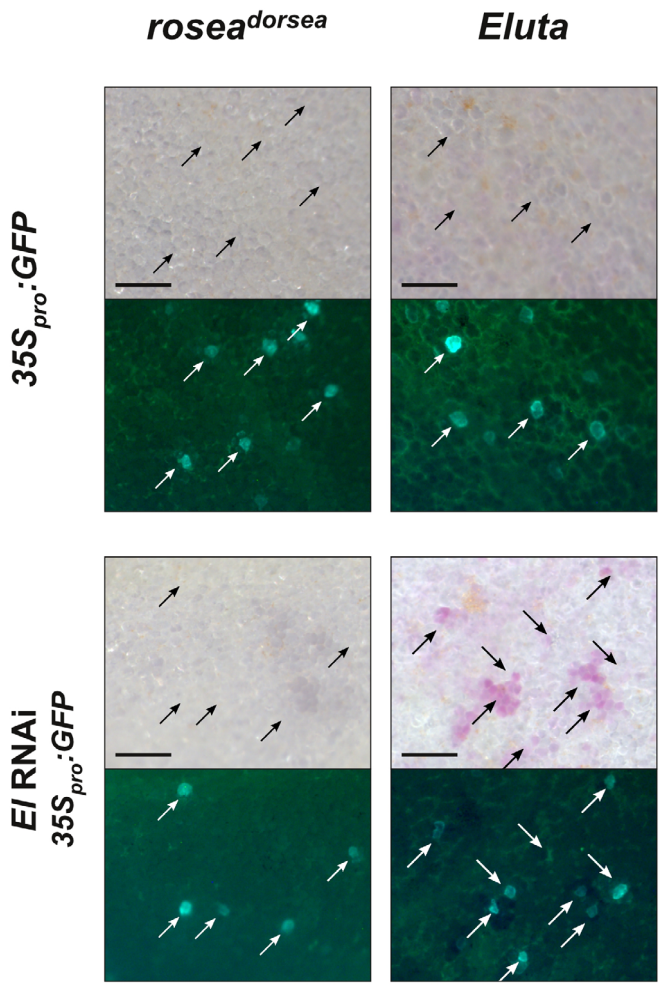

Fig. 4 Silencing *Eluta* restores anthocyanin pigmentation in Antirrhinum. Petals from *Antirrhinum majus rosea*^{dorsea} (*ros*$^{-}$, *el*/*el*) or *Eluta* (*Ros*$^{+}$, *El*/*El*) genotypes were biolistically transformed with plasmids expressing GFP alone, or with an RNA interference (RNAi) construct designed against the *Eluta* gene. Arrows indicate transformed cells, identified by GFP fluorescence under blue light. RNAi of *Eluta* restores anthocyanin pigmentation to *Ros*$^{+}$ *El*$^{+}$ petals, visible as multicellular pink foci that surround the transformed cells. Bars, 200 μm.

magenta flowers (*el*$^{\mu 668}$) and five with pale/bullseye patterned flowers (*El*^{rev}) (Fig. 3b). Self-pollinated seeds were collected from these pale flowers; the S_2 generation produced plants with magenta (*el*$^{\mu 668}$/*el*$^{\mu 668}$), pale magenta (*El*^{rev}/*el*$^{\mu 668}$) or near-white flowers with red bullseye (*El*^{rev}/*El*^{rev}), confirming the semi-dominant nature of these alleles (Fig. 3c).

Sequencing the *Eluta* gene (Am06g36700) in JI668 plants showed they were homozygous for an allele containing a 4.6-kb CACTA transposable element (TE) inserted within the second intron, compared with the structure of the *eluta* gene within the reference genome (Fig. 3c). Sequencing the S_1 individuals that had the revertant *Eluta* phenotype showed that the TE had excised from one allele (*El*^{rev}/*el*$^{\mu 668}$). Two independent excision events were identified in the S_1 progeny from the bud-sport, based on their footprints (Fig. 3c). Genotyping the S_2 populations confirmed the phenotypes corresponded to the dosage of the *El*^{rev} alleles. This demonstrated that stable reversions had occurred within the gene because of independent transposon excisions, conclusively determining the identity of *Eluta*.

Sequencing additional stable alleles of the *Eluta* MYB gene from WT red (*el*/*el*: JI7, 522) and *Eluta* (*El*/*El*: JI597, Sippe50) plants identified consistent polymorphisms for each allele (Fig. 3b). In particular, the *eluta* allele lacked 1.56 kb of sequence from the promoter located 1.54-kb upstream of translation start site. The 1.5-kb sequence in the promoter did not match any intact transposable elements, but a portion of it (200 bp) is present > 100 copies in the Antirrhinum reference genome. There were also several single-nucleotide polymorphisms (SNPs) and insertion/deletions (indels) between the *eluta* and *Eluta* promoter sequences.

We further tested whether this MYB gene was responsible for suppressing floral pigmentation using transient RNA interference (RNAi). A hairpin construct targeting the *Eluta* gene was transformed into Antirrhinum *Eluta* or *rosea*^{dorsea} (*el*) petal cells by particle bombardment, together with a *35S*$_{pro}$*:GFP* transformation control. Flowers from the *rosea*^{dorsea} mutant served as a negative control; they have pale flowers because of reduced expression of the MYB activator *Rosea1* (Schwinn *et al.*, 2006). Transformation with the RNAi construct restored anthocyanin pigmentation to *El* petals, forming multicellular coloured patches that surrounded the transformed cell (Fig. 4). RNAi acts noncell autonomously and can spread from the transformed cell because siRNAs are mobile. Although transformed cells were also identified by GFP fluorescence in the *rosea*^{dorsea} petals, pigmentation was never restored.

Alleles of *Eluta* from *eluta* and *Eluta* genotypes encode proteins with different amino acid sequence. Within the coding sequence there are 13 SNPs, of which nine are nonsynonymous, and three in-frame indels. This includes a nonsynonymous amino acid substitution of a threonine residue within the R3 MYB domain (*El* T98, *el* M97) that is highly conserved amongst R2R3-MYB proteins (Stracke *et al.*, 2001). We performed transient infiltration assays into *N. benthamiana* leaves to examine whether these differences resulted in altered ability to suppress anthocyanin accumulation (Fig. S4). *Nicotiana benthamiana* leaves infiltrated with *Rosea1* (*Ros1*) and *Incolorata I* (*Inc I*) accumulated anthocyanins in the infiltrated patches. However, co-infiltration with *Eluta*, *eluta*, or variants with amino acid substitutions (*El* T98M, *el* M97T) under the control of the *CaMV35S* promoter suppressed anthocyanin accumulation. These combined data confirm that the MYB repressor gene identified is responsible for the *Eluta* phenotype in Antirrhinum, and also suggest that altered expression of *Eluta* may account for the differences between *eluta* and *Eluta* genotypes, rather than differences in protein activity.

The spatial expression pattern of *Eluta* determines bullseye patterning

To characterise how *Eluta* suppresses pigmentation in Antirrhinum, we isolated and sequenced RNA (RNA-seq) from whole petals of WT (*el*/*el*), heterozygous (*El*/*el*) and homozygous (*El*/*El*) *Eluta* genotypes. Differential transcript abundance was

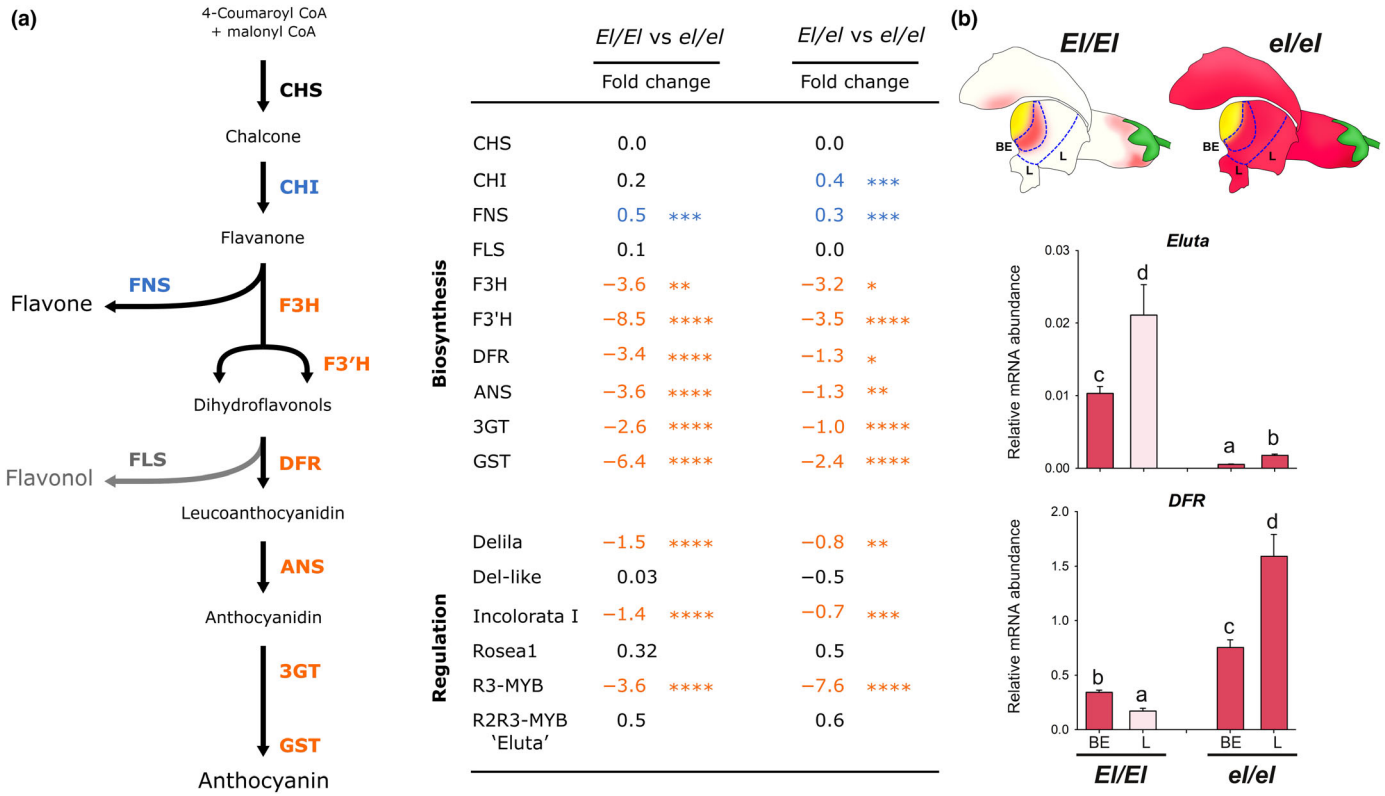

Fig. 5 *Eluta's* spatial expression pattern determines floral patterning in Antirrhinum. (a) Differential gene expression analysis (DESeq2) of RNA-seq data from whole stage three *el/el*, *El/el* or *El/El* petals. Genes that were significantly downregulated by *Eluta* are indicated in orange, those significantly up-regulated in blue. *, $P_{adj} \leq 0.05$; **, $P_{adj} \leq 0.01$; ***, $P_{adj} \leq 0.001$; ****, $P_{adj} \leq 0.0001$. (b) Petals from *El/El* or *el/el* flower buds were dissected into bullseye (BE) or lobe (L) tissues, for gene expression analyses. Transcript abundance for *Eluta* and the anthocyanin biosynthesis gene *DFR* were determined by quantitative reverse transcription polymerase chain reaction ($n = 3$ independent biological replicates, ±SEM). Letters indicate significance (one-way ANOVA with Fisher's least significant difference (LSD), $P < 0.05$). Detailed RNA-seq results displayed in this figure are summarised in Supporting Information Table S2.

analysed using DESeq2, comparing *Eluta* samples (*El/el* or *El/El*) with WT (*el/el*) (Table S2). Transcript abundance for anthocyanin biosynthesis genes *F3H/Incolorata II*, *F3′H*, *DFR/Pallida*, *ANS/Candica*, *3G*, and *GST* was reduced in *El/el* and *El/El* flowers compared with WT *el/el* (Fig. 5a). This confirmed previous analyses suggesting Eluta alters the regulation of the anthocyanin biosynthesis pathway (Martin *et al.*, 1991). The major activator of anthocyanin biosynthesis, *Rosea1* (R2R3-MYB), was highly expressed in *Eluta* genotypes, demonstrating *Eluta* does not inhibit pigmentation by reducing *Rosea1* expression. *Delila*, which encodes a bHLH protein that co-regulates anthocyanin biosynthesis with *Rosea1*, was downregulated in *Eluta* genotypes. *Incolorata I* (*Inc I*), a second bHLH that regulates anthocyanin biosynthesis in the flower lobes (Albert *et al.*, 2021), was also downregulated in *Eluta* petals. Interestingly, a gene encoding an R3-MYB repressor (Am02g17330) was expressed at higher levels in the WT (*el/el*) red petals than *Eluta*. This suggests the *bHLH* and *R3-MYB* genes may themselves be regulated by MBW activation complexes (e.g. Ros/Del/WDR), whose activity is inhibited by Eluta. Hierarchical regulation of bHLH and R3-MYB repressor genes by MBW complexes is widely conserved in flowering plants (Albert *et al.*, 2014a, 2021).

While the degree of differential expression observed for some anthocyanin-related genes may be considered modest in the RNA-seq data (Fig. 5a), it is important to note that whole petals were sampled, combining both suppressed (pale/white) and non-suppressed (red bullseye) tissue. For this reason, a refined quantitative reverse transcription polymerase chain reaction analysis was performed using dissected petal tissue from *El/El* and *el/el* flower buds, comparing the 'bullseye' region with the lobes (Fig. 5b). Transcript abundance for *Eluta* was > 10-fold higher in *El/El* than in *el/el* flowers. The spatial pattern of *Eluta* expression was similar in *El/El* and *el/el* samples, with the highest expression present in the lobes and lower expression in the bullseye region. Conversely, transcript abundance for *DFR* displayed an inverse expression pattern to *Eluta* in *El/El* tissues, corresponding with the suppressed pigmentation phenotype. Other components of the anthocyanin regulatory network were also examined (Fig. S5), showing similar expression patterns to the DESeq analysis. Thus, the spatial expression pattern for *Eluta* determines the restricted patterns of anthocyanin accumulation.

Eluta suppresses anthocyanin biosynthesis by competing for *cis*-elements and cofactors

We examined whether Eluta could inhibit anthocyanin biosynthesis by occupying promoter *cis*-elements required by MYB activators (Rosea1; Ros1), using a series of promoter mutants from the

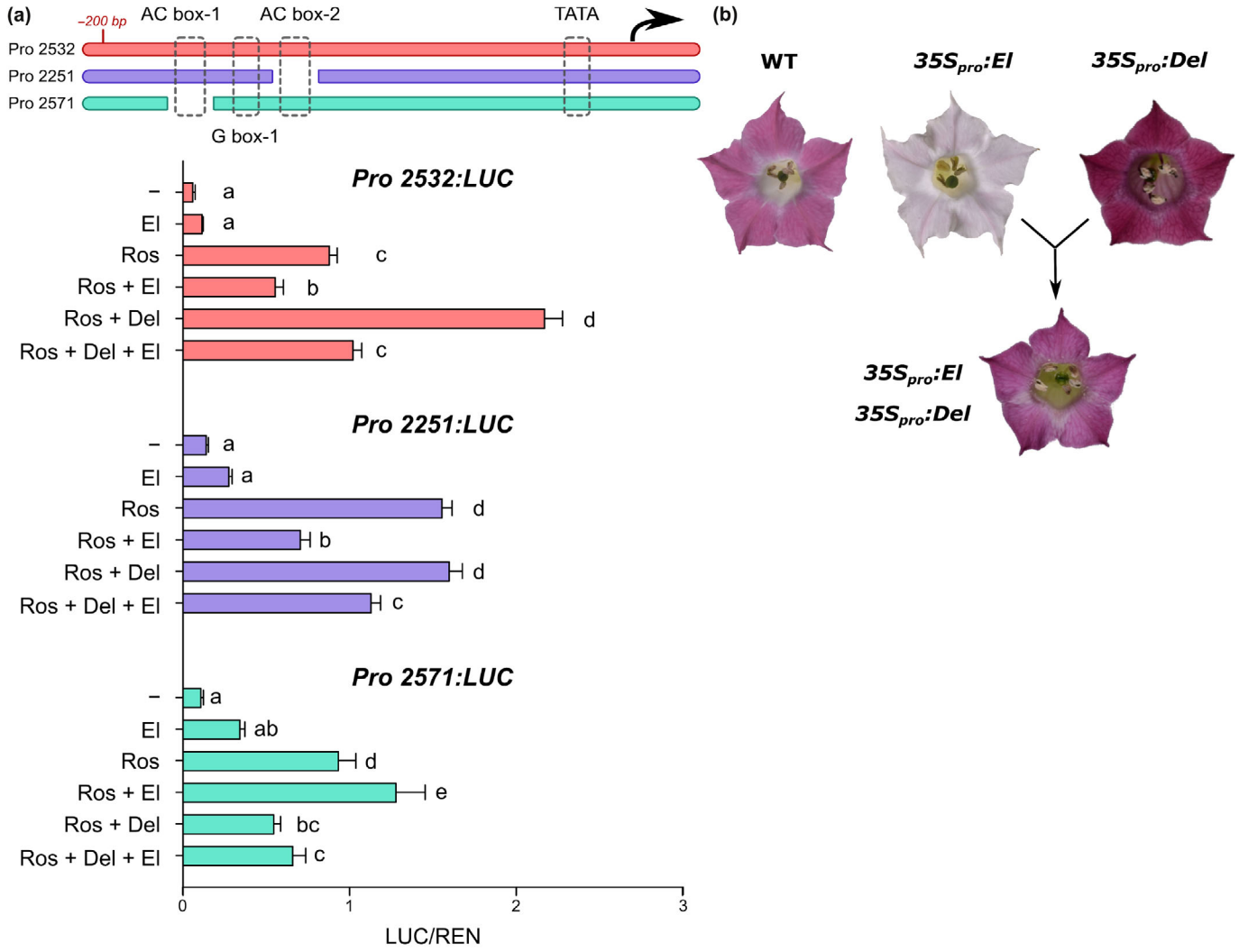

Fig. 6 Anthocyanin suppression by Eluta involves multiple mechanisms. (a) The promoter of *Pallida*, which encodes dihydroflavonol 4-reductase (DFR), contains two AC boxes recognised by MYB transcription factors and a G-box recognised by bHLH proteins within the proximal promoter region. Compared with a wild-type (WT) sequence (Pro2532), mutations of the *Pal* promoter contain deletions that remove AC box-1 (Pro2571) or AC box-2 (Pro2251) (Albert *et al*., 2021). Promoter activation assays were performed using a dual luciferase reporter assay of *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. Different combinations of effectors were co-transformed: reporter construct only (−), Rosea1 only (Ros), Rosea1 and Eluta (Ros + El), Rosea1 and Delila (Ros + Del), and Rosea1, Delila and Eluta (Ros + Del + El). Mean LUC/REN values, $n = 5 \pm$ SEM, are shown. Letters indicate significance (one-way ANOVA with Fisher's least significant difference (LSD), $P < 0.05$). (b) *Nicotiana tabacum* (tobacco) flowers from lines expressing *Eluta*, *Delila* or both, and the WT control.

key anthocyanin biosynthetic gene *Pallida* (encoding DFR). These mutant promoters lack AC boxes that have been shown to be necessary for activation by Rosea1 (Albert *et al*., 2021; Fig. 6a); *Pro2532* is the WT promoter and contains both AC boxes, *Pro2251* contains only AC box-1, and *Pro2571* contains only AC box-2.

In dual luciferase assays, Rosea1 activated the WT *Pro2532* strongly, with further enhancement when Delila (Del) was included (Fig. 6a). This activation by Rosea1 was repressed when Eluta was co-infiltrated, with or without Delila. Similar inhibition by Eluta was observed in combinations containing Rosea1, the bHLH Incolorata I and AmWDR1 (Fig. S6). Assays with *Pro2251* driving luciferase expression were similarly activated by Rosea1 and Rosea1 plus Delila, and repressed by Eluta (Fig. 6a). However, *Pro2571* responded differently. While Rosea1 was able to activate this promoter, this was not enhanced by Delila (it was reduced), and co-infiltration with Eluta had no repressive effect. These data confirmed that activation by Ros1 can use both AC boxes, but showed that Eluta requires AC box-1 for its repressive activity.

Anthocyanin suppression by Eluta is enhanced in backgrounds where the bHLH *delila* is not functional (Martin *et al*., 1991; Fig. 2a), despite the presence of a second bHLH, *Incolorata I*, in the flower lobes. This suggested bHLH concentration may be an additional component of Eluta repressive activity. MYB repressors have been proposed to inhibit gene expression by competing

with MYB activator proteins for bHLH partners (disrupting assembly of MBW complexes), or by integrating into MBW complexes and inhibiting transcription through active repression motifs (EAR, TLLLFR) (Pesch & Hülskamp, 2004; Albert *et al.*, 2014a). Eluta contains the [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R motif within the MYB domain necessary for binding bHLH partners (Zimmermann *et al.*, 2004), but it lacks identifiable repression motifs in its C terminus that are found in other subgroup 4 R2R3-MYB proteins. We confirmed that Eluta can bind the bHLH transcription factor Delila using Y2H assays (Fig. S7) but observed no difference in interaction strength between proteins encoded by *Eluta* and *eluta* alleles.

If Eluta can exert repressive activity by sequestering bHLH proteins, we reasoned that supplying additional bHLH protein should restore pigmentation to *Eluta* petals. These bHLH genes were transformed into Antirrhinum petals by biolistic transformation (Fig. S8). Overexpressing *Delila* restored anthocyanin pigmentation in *Eluta* flower petals, observed as single pink cells co-localised to GFP fluorescence. By contrast, *Incolorata I* alone was unable to restore pigmentation to transformed *Eluta* petal cells. Recently, Albert *et al.* (2021) showed that, unlike Delila, Incolorata I activity was enhanced by WDR proteins. Repeating the complementation assay with *Incolorata I* and *AmWDR1* restored pigmentation (Fig. S8). We established a transgenic tobacco system to confirm the results from transient expression in Antirrhinum. Tobacco plants (*N. tabacum*) expressing *Eluta* (*El*^{Sippe50}) from a *CaMV35* promoter produced flowers with pale flowers (Figs 6b, S9). The degree of anthocyanin suppression corresponded to the expression level of *Eluta*, and correspondingly, the suppression of the anthocyanin biosynthesis gene *DFR*. Floral pigmentation could be restored if crossed to existing tobacco lines that express *Delila* (Fig. 6b). These data suggested that titration of bHLH partners might represent an additional mechanism of inhibition by Eluta, although probably not on the *DFR* promoter, where binding of Eluta to the AC-1 box appears to be essential for its repressive activity (Fig. 6).

Origin of El^{Sippe50} allele

Pale, patterned flowers are distributed throughout all three sections of the *Antirrhinum* genus (Streptosepalum, Kickxiella and Antirrhinum), often manifesting as a spot of vein-associated anthocyanin pigmentation on the dorsal petals (dorsal petal sinus; Whibley *et al.*, 2006; Wilson & Hudson, 2011; Vargas *et al.*, 2017), consistent with the activity of *Eluta* alleles restricting patterns established by *Venosa* (MYB) (Fig. 7a). The patterns in Sippe50 and related stock or commercial varieties differ, because they contain both *Rosea*$^{+}$ activity and restriction/suppression by *Eluta*. Possible sources of *Eluta* alleles available to horticulturalists at the end of the 19th century were accessions of *A. latifolium* and *Antirrhinum meonanthum*, which were kept as accessions in the germplasm collection of IPK Gatersleben by Baur's successor, Hans Stubbe. In particular, one accession of *A. latifolium* ('Pyrea') originating from the Pyrenees has restricted pigmentation and venation patterning in the mouth of the flower, and crucially, had previously been shown to contain suppressive *Eluta* alleles (Schwinn *et al.*, 2006). We amplified and sequenced *Eluta* alleles from accessions of *Antirrhinum* species and a commercial *A. majus* cultivar with bullseye patterning ('Princess'). None of the alleles sequenced contained mutations or variants that would be likely to result in nonfunctional proteins. Phylogenetic analysis of the nucleotide sequence of these alleles (Figs 7, S10) showed that sequence from *A. latifolium* 'Pyrea' formed a clade with sequences from *A. majus* Sippe50, JI668 and a commercial variety, *A. majus* 'Princess', sharing 100% nucleotide identity. This likely derivation of the *Eluta* allele from *A. latifolium* 'Pyrea' is shown in the context of a broader tree (Figs S11, S12). Previous genetic analysis found that *A. latifolium* 'Pyrea' contains a weak allele of the *rosea* locus that generates blushed phenotypes similar to *A. majus rosea*^{dorsea}; a reduced expression allele that contains numerous promoter alterations (Schwinn *et al.*, 2006). The analysis of the promoter of *Rosea1* showed that *A. latifolium* 'Pyrea' contains an identical sequence to the *A. majus rosea*^{dorsea} (Fig. S13). Thus, sequencing data and genetic crosses confirmed that *A. latifolium* 'Pyrea' contains linked alleles of *rosea*^{dorsea} and *Eluta* (*ros*^{dor}*.El*). Sequencing the promoter of *Rosea1* from Sippe50 showed that it contains an identical allele to the *Ros*+ allele present in *A. majus* JI7, supporting our conclusion that the bullseye patterning of Sippe50 was derived by recombination between *Ros1.el* and *ros*^{dor}*.El* alleles from an *A. majus* × *A. latifolium* cross.

Discussion

The diversity of flower colour and patterning is remarkable, often derived from overlapping layers of colour patterns that are under separate genetic control. Antirrhinum has provided a powerful model for investigating the genetic basis for how such patterns are formed, since the beginning of the 20th century. Antirrhinum flowers with the '*Picturatum*' phenotypes of washed-out or painted patterns were already ubiquitous in the first decade of the 20th century, available from commercial seed distributers when Erwin Baur was sourcing plant materials for his genetic studies. Yet the gene responsible remained uncharacterised, despite early identification of the *Picturatum/Eluta* phenotypes. The identification of a transposon-tagged *Eluta* genotype and characterisation of the subsequent excision events provides unequivocal evidence that an R2R3-MYB gene is responsible for generating suppressed and restricted anthocyanin patterns in Antirrhinum.

Identification of Eluta

The existence of mechanisms modifying pigmentation patterns and intensity has long been apparent. This genetic variation is particularly evident in Antirrhinum, with > 100 varieties described in Haage & Schmidt's (1910) seed catalogue. We can now assign genes to phenotypes illustrated by Baur (1910; Fig. S2): alleles for the MYB activators *Rosea* (magenta, e.g. *purpurea*) and *Venosa* (venation patterning, *rubro-striatum*, *rubro venosum*), the bHLH *Delila* (bicolouration, *delila*), *Sulfurea* (diffuse yellow, *sulphureum*, *luteum*; restricted yellow, *hellgelbem auge*) and *Eluta* (restricted anthocyanin, *Picturatum*). Baur (1919)

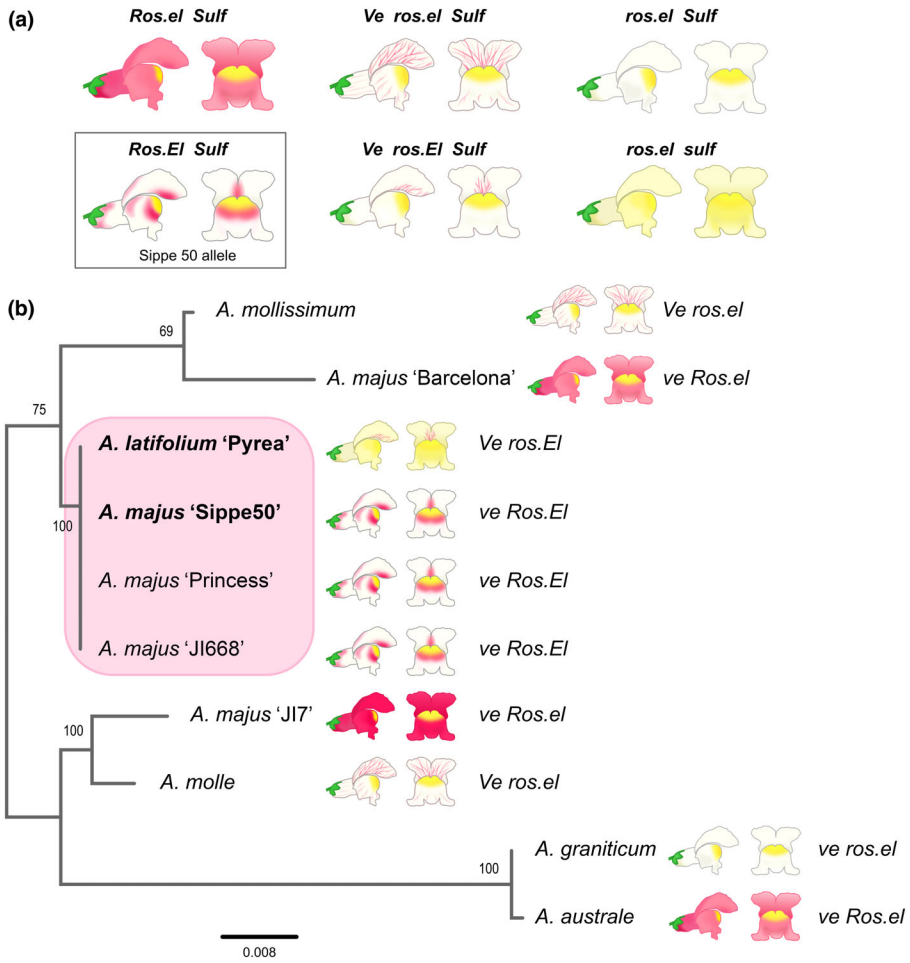

Fig. 7 Phylogenetic analysis of *Eluta* alleles from *Antirrhinum* accessions. (a) Diverse colour morphs can be formed through the activities of the anthocyanin activators *Rosea* and *Venosa*, and their restricted patterns that form with suppressive *Eluta* alleles. Yellow aurone pigments are restricted to the flower mouth by *Sulfurea*; *sulf/sulf* individuals have diffuse yellow pigmentation. (b) A maximum likelihood tree of *Eluta* nucleotide sequences from selected European Antirrhinum species. Bootstrap support > 65% (1000 replicates) is shown. The genotype of each sample is shown for the *Venosa* (*Ve*), *Rosea* (*Ros*) and *Eluta* (*El*) loci. All taxa are *Sulfurea*$^{+}$ (*Sulf*) except *Antirrhinum latifolium* (*sulf/sulf*; pale yellow).

was able to recreate many of the colour combinations available in Haage & Schmidt's catalogue in the F_2 progeny from a yellow-flowering genotype and a magenta-flowering genotype (Fig. S1; The parental genotypes correspond to *sulf*, *del*, *ros.El* and *Sulf*, *Ros.el*, *Del*). The *Picturatum* variants correspond to patterns established between *Eluta* alleles (factor G) and those involved in activating anthocyanin biosynthesis (*Rosea*, *Delila*, *Incolorata I*). We have been able to show that the dosage of *Eluta* (Fig. 2) reproduces the weaker, washed-out *picturatum* phenotypes (*El/el*) (Fig. 1b top), or stronger bullseye patterning (*El/El*) (Fig. 1b lower) described by Baur (1910). Thus, *Eluta* corresponds to the gene underlying the *picturatum* phenotypes and contributes to the phenotypic variation observed in cultivated Antirrhinum.

Basis of restricted pigmentation patterns

Eluta forms colour patterns by restricting the colour patterning established by the anthocyanin activators. The expression of anthocyanin biosynthesis genes is reduced (Fig. 5), corresponding to the dosage of suppressive *Eluta* alleles (Fig. 5a), and the dosage of activators Rosea1 and Delila (Figs 2a, 3b). In *A. majus* backgrounds with active *Rosea1* alleles (*Ros.El*), *Eluta* restricts anthocyanins to a bullseye pattern highlighting the pollinator-landing platform. Dissected petal tissue showed the spatial pattern of *El* expression is central to pattern formation (Fig. 5b), in contrast to *Rosea1* and *Delila*, which are expressed throughout the entire petal (Fig. S5; Albert *et al.*, 2021). *Eluta* transcript abundance was highest in the petal lobe regions with the most suppressed pigmentation. Conversely, the central bullseye region that accumulated anthocyanin had decreased *Eluta* expression. These patterns vary in different genetic backgrounds, depending on where the anthocyanin biosynthesis activators are expressed: If the genetic background lacks diffuse/full anthocyanin pigmentation (*rosea1*), venation patterning on dorsal petals, controlled by another MYB family member, *Venosa*, may be visible (Shang *et al.*, 2011). The restricted patterns generated by *Ve/Del/El* result in a spot of tightly restricted venation pigmentation at the dorsal petals' sinus (Tavares *et al.*, 2018) providing a nectar guide selected by pollinating bees (Shang *et al.*, 2011). Thus, patterning arises from the overlapping domains of anthocyanin activation (*Ros/Ve*, *Del/Inc I*) and inhibition (*El*).

Suppressive *Eluta* alleles are genetic variants with quantitative differences in transcript abundance. Both *Eluta* and *eluta* alleles encode highly similar proteins that bind Delila equally well in Y2H assays (Fig. S7) and inhibit anthocyanin accumulation in *N. benthamiana* leaves (Fig. S2). Transcript abundance in *El/El* petals was substantially higher than in *el/el* petals, suggesting that these alleles differ in their ability to inhibit pigmentation because

of quantitative differences in *Eluta* expression. There are numerous sequence variations between the promoters of *Eluta* and *eluta* alleles, perhaps including gain/loss of enhancers that alter the rate of transcription. Differences in transcript abundance of the *Eluta* transgene in tobacco also corresponded to differences in pigmentation suppression (Fig. S9), highlighting that Eluta abundance directly corresponds to pigmentation outcomes.

Repressive mechanisms alter floral pigmentation intensity and patterning in diverse flowering plants. While the basis of these repressive mechanisms can differ, they share common features, including epistasis with loci that activate pigmentation and act in a dominant (or semi-dominant) manner. *Eluta* in *Antirrhinum* is a particularly interesting repressor of flower colour because it alters both pigmentation intensity and patterning, compared with other characterised repressors, such as *IlMYBL1* (SG4-derived) from *Iochroma*, and *ROSE INTENSITY1* (R3-MYB) from *Erythranthe* (previously *Mimulus*), which primarily alter intensity (Yuan *et al.*, 2013; Gates *et al.*, 2018). A second notable repressor of pigmentation in *Antirrhinum* is *Sulfurea*, which restricts the biosynthesis and accumulation of yellow aurone pigments. The *Sulfurea* locus generates small interfering RNAs (siRNAs) targeting the transcript of the aurone biosynthetic gene *Chalcone 4′-O-Glucosyltransferase*, restricting aurones to a spot on the flower face (Bradley *et al.*, 2017). This mechanism resembles those for 'picotee' or 'star' patterns that form in various species because of siRNA silencing of the flavonoid biosynthesis gene *Chalcone Synthase* (Davies *et al.*, 2012). The generation of new pigmentation patterns probably reflects the spatial expression patterns of these repressor genes, as with *Eluta*.

Eluta encodes an atypical subgroup 4 R2R3-MYB repressor

Eluta encodes a SG4 R2R3-MYB repressor (Fig. 2) within the sub-clade of proteins that regulate anthocyanins (e.g. strawberry FaMYB1, petunia PhMYB27; Aharoni *et al.*, 2001; Albert *et al.*, 2011, 2014a). Conserved features of these include residues within the R2R3 region that allow binding to bHLH transcription factors and C-terminal repression domains (Albert & Allan, 2021; LaFountain & Yuan, 2021). Other well-characterised SG4 repressors, like PhMYB27, require their C-terminal domains (containing an EAR motif) to exert full repressive activity, with the residual inhibitory activity attributed to bHLH binding and titration (Jin *et al.*, 2000; Albert *et al.*, 2014a). Eluta is atypical, lacking repressive motifs, yet is a potent inhibitor of anthocyanin biosynthesis. Eluta requires an AC box within the *Pallida* (*DFR*) promoter to exert its repressive activity (Fig. 6a), suggesting it competes with Rosea1 for promoter binding sites, at least for this target gene. Such inhibition is similar to that for C1-I, a truncated variant of the maize anthocyanin activator MYB that lacks the activation domain and acts as a dominant inhibitor, possibly by competing with functional C1 proteins for *cis*-elements (Paz-Ares *et al.*, 1990; Goff *et al.*, 1991). However, competition by Eluta may not be limited to promoter binding sites, but may extend to competing with Rosea1 for bHLH proteins necessary for assembly of MB/MBW activation complexes. Eluta contains the conserved amino acid motif associated with binding SGIIIf bHLH proteins, and Eluta binds Delila in Y2H assays (Fig. S7). Supplying additional bHLH protein Delila restored pigmentation to flowers with suppressed pigmentation from Eluta activity, in both tobacco and *Antirrhinum* (Figs 6b, S6). Interestingly, supplying *Incolorata I* to Eluta-suppressed *Antirrhinum* petals failed to restore colour unless *AmWDR1* was co-transformed, suggesting that Eluta can also limit available WDR concentrations, which may themselves be bound to bHLH proteins (Baudry *et al.*, 2004). Titration or 'squelching' of essential transcription factors is a well-established inhibitory mechanism (Ptashne, 1988; Feldbrügge *et al.*, 1994). The R3-MYB repressors, such as those that inhibit the MBW complexes controlling trichome density and patterning (AtCPC, AtTRY and AtETC), or anthocyanins (PhMYBx, MgROI, AtCPC), do so by binding bHLH proteins via the motif in the R3 domain (Koes *et al.*, 2005; Albert *et al.*, 2022). Promoter-mediated and squelching repressive activities are not mutually exclusive, particularly when considering the dynamics of MB/MBW complex assembly and DNA binding, which can vary between target genes. For example, in maize, binding of C1/R to the *A1 (DFR)* promoter is mediated by C1 (MYB), yet for *Bz1 (Flavonoid 3-O-Glucosyltransferase)* binding is mediated by R (bHLH), despite requiring C1 for activating transcription (Kong *et al.*, 2012). Competitive inhibition for promoter elements is a well-established mechanism for regulating phenylpropanoid production, and the first MYB repressor described in plants, AmMYB308, operates this way, displacing stronger transcriptional activators from binding target promoters (Tamagnone *et al.*, 1998). Thus, despite Eluta lacking a typical repression domain, it is well-equipped to repress anthocyanin biosynthesis by competing with MYB activators for promoter binding sites and partners.

Phylogenetic relationship of *Eluta* alleles and the importance of patterning

Baur proposed that the origin of pigmentation diversity in commercial *Antirrhinum* cultivars might be the presence or absence of hereditary units present in wild *A. majus* (Baur, 1910). However, *A. majus* represents cultivated/garden snapdragons and lacks a wild ideotype. Baur's question can be re-framed: were the alleles of *Rosea* and *Eluta* that produced the *Picturatum/Eluta* phenotypes introgressed from wild *Antirrhinum* species that form fertile hybrids with *A. majus*? Our findings support the introgression of *Eluta* alleles (ros^{dor}.*El*) from an *A. latifolium* genotype into cultivated snapdragons. It is clear Erwin Baur also worked with *ros.El* genotypes (in addition to *Ros.El* combinations), because these are described as an example of genetic linkage between *Rosea* (Factor F) and *Eluta/Picturatum* (Factor G; Baur, 1919; Fig. S1). However, the linked *Ros.El* allele present in Sippe50 must have arisen through a recombination event between an *Eluta* allele from *A. latifolium* and a Ros^{+} allele from *A. majus*. Such a combination was possibly selected for by horticulturalists for their stronger and higher contrast bullseye patterns that would appeal to home gardeners. These alleles could then be fixed in a homozygous state, which would enable cultivars and stock lines

to be developed that would breed true (e.g. Sippe50). We identified the same *Eluta* allele in a modern *A. majus* cultivar with strong bullseye patterning ('Princess'), demonstrating that the linked *Rosea/Eluta* alleles continue to generate colour variants for home gardeners.

Eluta alleles form restricted venation patterns in wild *Antirrhinum* European species. This includes species known to contain suppressive *Eluta* alleles, such as *A. latifolium*, *A. meonanthum* and *A. striatum* (Schwinn *et al.*, 2006; Whibley *et al.*, 2006), and others, such as species within Kickxiella (e.g. *A. valentinum* and *A. sempervirens*) with very prominent restricted anthocyanin venation (Vargas *et al.*, 2017). Notably, a rare recombination event between *Rosea* and *Eluta* in *A. striatum* (*sulf*$^{-}$, *ros.El*)/*A. pseudomajus* (*Sulf*$^{+}$, *Ros.el*) hybrids allowed fine-mapping of the *Eluta* locus to a 50-kb interval (Tavares *et al.*, 2018). This interval contains the MYB repressor we identified by transposon tagging, and the *A. striatum* allele MYB gene was differentially expressed compared with *A. pseudomajus* (Tavares *et al.*, 2018).

Floral pigmentation is an important cue to attract animal pollinators for reproduction, and usually involves patterning and contrasting colours. Diffuse (nonrestricted) pigmentation (including UV-B absorption) is important for long-range target recognition, while high-contrast patterns, called 'nectar-guides', act as landing lights that direct pollinators towards the nectar or pollen reward (Owen & Bradshaw, 2011; Davies *et al.*, 2012; Richter *et al.*, 2023). Within the *Antirrhinum* genus, the patterns formed by *Eluta* (anthocyanin) and *Sulfurea* (aurone) combine to form several common colour morphs: restricted venation with restricted aurone (*Ve*, *ros.El*, *Sulf*), diffuse aurone with restricted venation (*Ve*, *ros.El*, *sulf*) and self-coloured anthocyanins with restricted aurone patterning (*Ros.el*, *Sulf*) (Fig. 7). Despite being popular with horticulturalists, orange flowers with diffuse anthocyanin and aurone pigmentation (*Ros.el*, *sulf*) were never a species ideotype, and hybrid phenotypes are shifted towards restricted patterns because *Eluta* and *Sulfurea* act dominantly. This is thought to help maintain species separation in hybrid zones (e.g. *A. striatum*/*A. pseudomajus* in the Pyrenees), as a result of pollinator behaviours such as flower constancy (Tastard *et al.*, 2014).

Conclusions

Previous studies have highlighted the importance of the R2R3-MYB activators *Rosea* and *Venosa* for generating pigmentation diversity. We show that the epistatic interactions between *Rosea/Venosa* and *Eluta* enable the formation of restricted nectar guide patterns, contributing to pigmentation diversity and species separation between and within European species in the genus, *Antirrhinum*.

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Competing interests

None declared.

Author contributions

NWA, AH, CM conceived of the research project. LC and AH provided the materials. SMAM, YZ, EB, CNW, S-MY, SBC, NNH and NWA undertook the experiments. SMAM, NNH, AH, CM, KES, KMD and NWA contributed to the experimental design and interpretation of data. SMAM, CM and NWA drafted the manuscript before all authors contributed to its improvement and agreed on its final content.

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Data availability

Raw RNA sequencing data from all samples are deposited into the SRA database (BioProject ID: PRJNA1054305). DNA sequences are available at NCBI under accession nos. OR997831–39.

References

Aharoni A, De Vos CHR, Wein M, Sun ZK, Greco R, Kroon A, Mol JNM, O'Connell AP. 2001. The strawberry *FaMYB1* transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *The Plant Journal* **28**: 319–332.

Albert NW, Allan AC. 2021. MYB genes involved in domestication and crop improvement. *Annual Plant Reviews Online* **4**: 199–242.

Albert NW, Butelli E, Moss SMA, Piazza P, Waite CN, Schwinn KE, Davies KM, Martin C. 2021. Discrete bHLH transcription factors play functionally overlapping roles in pigmentation patterning in flowers of *Antirrhinum majus*. *New Phytologist* **231**: 849–863.

Albert NW, Davies KM, Lewis DH, Zhang H, Montefiori M, Brendolise C, Boase MR, Ngo H, Jameson PE, Schwinn KE. 2014a. A conserved network of

transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *Plant Cell* **26**: 962–980.

Albert NW, Davies KM, Schwinn KE. 2014b. Gene regulation networks generate diverse pigmentation patterns in plants. *Plant Signaling & Behavior* **9**: e29526.

Albert NW, Lafferty DJ, Moss SMA, Davies KM. 2022. Flavonoids – flowers, fruit, forage and the future. *Journal of the Royal Society of New Zealand* **53**: 1–28.

Albert NW, Lewis DH, Zhang H, Schwinn KE, Jameson PE, Davies KM. 2011. Members of an R2R3-MYB transcription factor family in *Petunia* are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. *The Plant Journal* **65**: 771–784.

Albert NW, Thrimawithana AH, McGhie TK, Clayton WA, Deroles SC, Schwinn KE, Bowman JL, Jordan BR, Davies KM. 2018. Genetic analysis of the liverwort *Marchantia polymorpha* reveals that R2R3MYB activation of flavonoid production in response to abiotic stress is an ancient character in land plants. *New Phytologist* **218**: 554–566.

Bateson W. 1901. Experiments undertaken by W. Bateson and Miss E. R. Saunders. *Evolution Committee of the Royal Society, Report I*. London, UK: Harrison & Sons, 126.

Bateson W. 1902. *Mendel's principles of heredity – a defense*. Cambridge, UK: Cambridge University Press, 26–29.

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L. 2004. TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *The Plant Journal* **39**: 366–380.

Baur E. 1910. Vererbungs- und Bastardierungsversuche mit *Antirrhinum*. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre* **3**: 34–98.

Baur E. 1911. Vererbungs- und Bastardierungsversuche mit *Antirrhinum*. II. Faktorenkoppelung. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre* **6**: 248–251.

Baur E. 1919. *Einführung in die experimentelle verebungslehre*. Berlin, Germany: Gebrüder Borntraeger.

Bradley D, Xu P, Mohorianu I-I, Whibley A, Field D, Tavares H, Couchman M, Copsey L, Carpenter R, Li M *et al.* 2017. Evolution of flower color pattern through selection on regulatory small RNAs. *Science* **358**: 925–928.

Butelli E, Licciardello C, Zhang Y, Liu J, Mackay S, Bailey P, Reforgiato-Recupero G, Martin C. 2012. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. *Plant Cell* **24**: 1242–1255.

Coen ES, Carpenter R, Martin C. 1986. Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. *Cell* **47**: 285–296.

Correns C. 1900. *Mendels regel über das verhalten der nachkommenschaft der rasenbastrade*. Berlin, Germany: Gebrüder Borntræger.

Davies KM, Albert NW, Schwinn KE. 2012. From landing lights to mimicry: the molecular regulation of flower colouration and mechanisms for pigmentation patterning. *Functional Plant Biology* **39**: 619–638.

Ding B, Patterson EL, Holalu SV, Li J, Johnson GA, Stanley LE, Greenlee AB, Peng F, Bradshaw H, Blinov ML. 2020. Two MYB proteins in a self-organizing activator-inhibitor system produce spotted pigmentation patterns. *Current Biology* **30**: 802–814.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Feldbrügge M, Sprenger M, Dinkelbach M, Yazaki K, Harter K, Weisshaar B. 1994. Functional-analysis of a light-responsive plant bZIP transcriptional regulator. *Plant Cell* **6**: 1607–1621.

Gates DJ, Olson B, Clemente TE, Smith SD. 2018. A novel R3 MYB transcriptional repressor associated with the loss of floral pigmentation in Iochroma. *New Phytologist* **217**: 1346–1356.

Goff SA, Cone KC, Fromm ME. 1991. Identification of functional domains in the maize transcriptional activator C1: comparison of wild-type and dominant inhibitor proteins. *Genes and Development* **5**: 298–309.

Goodrich J, Carpenter R, Coen ES. 1992. A common gene regulates pigmentation pattern in diverse plant species. *Cell* **68**: 955–964.

Guindon S, Gascuel O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696–704.

Haage & Schmidt. 1910. *Haupt-Verzeichnis über Samen und Pflazen*. Erfurt, Germany: Haage & Schmidt.

Haselhoff J, Siemering KR, Prasher DC, Hodge S. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proceedings of the National Academy of Sciences, USA* **94**: 2122–2127.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. 1985. A simple and general method for transferring genes into plants. *Science* **227**: 1229–1231.

Jaworski CC, Andalo C, Raynaud C, Simon V, Thébaud C, Chave J. 2015. The influence of prior learning experience on pollinator choice: an experiment using bumblebees on two wild floral types of *Antirrhinum majus*. *PLoS ONE* **10**: e0130225.

Jin HL, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C. 2000. Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *EMBO Journal* **19**: 6150–6161.

Koes R, Verweij W, Quattrocchio F. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends in Plant Science* **10**: 236–242.

Kong Q, Pattanaik S, Feller A, Werkman JR, Chai C, Wang Y, Grotewold E, Yuan L. 2012. Regulatory switch enforced by basic helix-loop-helix and ACT-domain mediated dimerizations of the maize transcription factor R. *Proceedings of the National Academy of Sciences, USA* **109**: E2091–E2097.

LaFountain AM, Yuan Y-W. 2021. Repressors of anthocyanin biosynthesis. *New Phytologist* **231**: 933–949.

Li M, Zhang D, Gao Q, Luo Y, Zhang H, Ma B, Chen C, Whibley A, Zhang Y, Cao Y *et al.* 2019. Genome structure and evolution of *Antirrhinum majus* L. *Nature Plants* **5**: 174–183.

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES. 1985. Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the *Pallida* locus by transposon tagging. *EMBO Journal* **4**: 1625–1630.

Martin C, Li J, Albert NW. 2023. Chapter 10: A long and winding road. In: Salminen J-P, Wähälä K, Freitas V, Quideau S, eds. *Recent advances in polyphenol research, vol. 8*. Hoboken, NJ, USA: John Wiley & Sons, Ltd, 301–324.

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.

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

Owen CR, Bradshaw HD. 2011. Induced mutations affecting pollinator choice in *Mimulus lewisii* (Phrymaceae). *Arthropod–Plant Interactions* **5**: 235–244.

Paz-Ares J, Ghosal D, Saedler H. 1990. Molecular analysis of the *C1-I* allele from *Zea mays*: a dominant mutant of the regulatory *C1* locus. *EMBO Journal* **9**: 315–321.

Pesch M, Hülskamp M. 2004. Creating a two-dimensional pattern *de novo* during Arabidopsis trichome and root hair initiation. *Current Opinion in Genetics and Development* **14**: 422–427.

Ptashne M. 1988. How eukaryotic transcriptional activators work. *Nature* **335**: 683–689.

Richter R, Dietz A, Foster J, Spaethe J, Stöckl A. 2023. Flower patterns improve foraging efficiency in bumblebees by guiding approach flight and landing. *Functional Ecology* **37**: 763–777.

Sampson D, Hunter A. 1959. Inheritance of shades of bronze and pink flowers of *Antirrhinum majus*. *Canadian Journal of Plant Science* **39**: 329–341.

Schick R, Stubbe H. 1932. Die Gene von *Antirrhinum majus*. II. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre* **62**: 249–290.

Schwarz-Sommer Z, Davies B, Hudson A. 2003. An everlasting pioneer: the story of *Antirrhinum* research. *Nature Reviews Genetics* **4**: 655–664.

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*. *Plant Cell* **18**: 831–851.

Shang Y, Venail J, Mackay S, Bailey PC, Schwinn KE, Jameson PE, Martin C, Davies KM. 2011. The molecular basis for venation patterning of pigmentation and its effects on pollinator attraction in flowers of *Antirrhinum*. *New Phytologist* **189**: 602–615.

Sommer H, Saedler H. 1986. Structure of the chalcone synthase gene of *Antirrhinum majus*. *Molecular and General Genetics* **202**: 429–434.
Stracke R, Werber M, Weisshaar B. 2001. The *R2R3-MYB* gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**: 447–456.
Stubbe H. 1941. Die gene von *Antirrhinum majus* IV: Zur Angleichung der *Antirrhinum*-Nomenklatur an die Vorschläge der Nomenklatur-Kommission des VII. Internationalen Genetiker-Kongresses, Edinburgh 1939. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre* **79**: 401–443.
Stubbe H. 1966. Untersuchungen uber mutable und labile Gene. In: *Genetik und Zytologie von Antirrhinum L., sect. Antirrhinum*. Jena, Germany: VEB Gustav Fischer Verlag, 97–114.
Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C. 1998. The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell* **10**: 135–154.
Tastard E, Andalo C, Burrus M, Gigord L, Thébaud C. 2014. Effects of floral diversity and pollinator behaviour on the persistence of hybrid zones between plants sharing pollinators. *Plant Ecology & Diversity* **7**: 391–400.
Tavares H, Whibley A, Field DL, Bradley D, Couchman M, Copsey L, Elleouet J, Burrus M, Andalo C, Li M *et al.* 2018. Selection and gene flow shape genomic islands that control floral guides. *Proceedings of the National Academy of Sciences, USA* **115**: 11006–11011.
Tschermak-Seysenegg EV. 1900a. Über künstliche Kreuzung bei *Pisum sativum*. *Berichte der Deutschen Botanischen Gesellschaft* **18**: 232–239.
Tschermak-Seysenegg EV. 1900b. Über künstliche Kreuzung bei *Pisum sativum*. *Zeitschrift für das Landwirthschaftliche Versuchswesen in Österreich* **3**: 465–555.
Vargas P, Liberal I, Ornosa C, Gómez JM. 2017. Flower specialisation: the occluded corolla of snapdragons (*Antirrhinum*) exhibits two pollinator niches of large long-tongued bees. *Plant Biology* **19**: 787–797.
de Vilmorin P, Bateson W. 1911. A case of gametic coupling in *Pisum*. *Proceedings of the Royal Society of London. Series B: Containing Papers of a Biological Character* **84**: 9–11.
de Vries H. 1900. Sur la loi do disjonction des hybrides. *Comptes Rendus de l'Académie des Sciences* **130**: 845–847.
Whibley AC, Langlade NB, Andalo C, Hanna AI, Bangham A, Thébaud C, Coen E. 2006. Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* **313**: 963–966.
Wilson Y, Hudson A. 2011. The evolutionary history of *Antirrhinum* suggests that ancestral phenotype combinations survived repeated hybridizations. *The Plant Journal* **66**: 1032–1043.
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.
Yuan Y-W, Sagawa JM, Young RC, Christensen BJ, Bradshaw HD. 2013. Genetic dissection of a major QTL contributing to pollinator-mediated reproductive isolation between sister species of *Mimulus*. *Genetics* **194**: 255–263.
Zhang B, Xu X, Huang R, Yang S, Li M, Guo Y. 2021. CRISPR/Cas9-mediated targeted mutation reveals a role for AN4 rather than DPL in regulating venation formation in the corolla tube of *Petunia hybrida*. *Horticulture Research* **8**: 116.
Zhu H-F, Fitzsimmons K, Khandelwal A, Kranz RG. 2009. CPC, a single-repeat R3 MYB, is a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. *Molecular Plant* **2**: 790–802.
Zimmermann IM, Heim MA, Weisshaar B, Uhrig 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.

Fig. S1 *Rosea* and *Eluta* are linked.

Fig. S2 Deduced genotypes of Antirrhinums illustrated in fig. 1 of Baur (1910).

Fig. S3 Amino acid alignment of R2R3 MYB domain.

Fig. S4 *El* and *el* proteins suppress anthocyanin accumulation.

Fig. S5 qRT-PCR of other genes in dissected Antirrhinum petals.

Fig. S6 Eluta inhibits activation of the *Pallida/DFR* promoter by Rosea1 and Incolorata I.

Fig. S7 Eluta binds Delila.

Fig. S8 Expression of bHLH and WDR cofactors, but not *Rosea1*, partially restored pigmentation in Antirrhinum *El* petals.

Fig. S9 *Eluta* suppresses anthocyanin pigmentation in tobacco flowers.

Fig. S10 Nucleotide alignment of *Eluta* alleles.

Fig. S11 Expanded phylogenetic tree of *Eluta* alleles.

Fig. S12 Expanded nucleotide alignment of *Eluta* alleles.

Fig. S13 Phylogenetic tree of Rosea1 promoter sequences.

Table S1 Oligonucleotide primers used in this study.

Table S2 Summary of all the results of comparative RNA-seq analysis results conducted in this study.

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