

Title: The genetic basis of replicated bullseye pattern reduction across the *Hibiscus trionum*
Complex

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Summary

- Colorful petal patterns fulfil important functions and constitute excellent systems to illuminate the evolutionary processes that generate morphological diversity or instead support the repetitive emergence of similar forms.
- Here, we combined phylogenomic approaches, genetic manipulations, molecular techniques and bee behavioral experiments to (i) solve the species relationships across the Trionum Complex, a small *Hibiscus* clade that displays bullseye petal patterns varying in size, hue and composition, (ii) identify key genes involved in the production of bullseye pigmentation and (iii) reveal molecular events underpinning pattern variation during the evolution of the group.
- We found that epidermal cell shape, texture and pigmentation are genetically distinct and that pigmentation is the most labile feature across the group. We demonstrate that repetitive bullseye reduction events primarily occur through independent modifications of a single genetic locus encoding BERRY1, an R2R3 MYB that regulates anthocyanin pigment production in petals. We also found that buff-tailed bumblebees discriminate against flowers with smaller bullseye sizes suggesting that changing bullseye proportions impact plant-pollinator interactions.
- Our results demonstrate how repeated mutations in a single locus led to morphological variation in petal patterning, a trait shown to impact plant fitness in other species and contribute to angiosperm reproductive isolation and speciation.

Introduction

The molecular basis underlying the repeated evolution of similar morphological characteristics across the tree of life remains a central question of evolutionary biology. Replicated evolution, a term encompassing both parallel and convergent evolution, leads different species and populations to independently evolve similar features (James *et al.* 2023). While this sometimes relies on the modification of homologous genes (Lenormand *et al.* 2016), it remains unclear why some genes are more likely than others to mediate parallel or convergent changes. The evolution of color patterns in both plants and animals provides phenotypically striking traits that can be utilized to unravel the molecular mechanisms supporting the evolution of diversity (Manceau *et al.* 2010; Orteu & Jiggins, 2020; Galipot *et al.* 2021). With more than 350,000 extant species, the angiosperms are a living repository of natural diversity. The diversification of flowering plants yielded a remarkable array of morphologies and colorful patterns that decorate corollas. Remarkably, similar motifs are often found in distantly related species. Striking examples

include petal stripes in *Gazania* and *Phlox*, venation patterns in *Veronica*, *Geranium* and *Antirrhinum*, and bullseye patterns in the poached egg plant (*Limnathes douglasii*) and baby blue eyes (*Nemophila menziesii*). Such commonalities hint towards a limited but functionally important set of patterns that independently arose across distantly related angiosperms. It also provides multiple systems with which to investigate the processes that have led to the repeated emergence of similar traits.

Petal patterns play crucial roles in key biological processes including plant-pollinator communication, defense against herbivory and protection from abiotic factors and thus are likely to contribute directly to plant fitness (Fairnie *et al.* 2022). Insect pollinators can display innate or acquired preferences for given motifs influencing their choice of flower to visit (Gumbert, 2000). Importantly, petal patterns also function post-landing, acting as visual guides to lure pollinators toward pollen and/or nectar rewards and increase the chances of successful pollination. The lower petal lobes of the purple monkeyflower (*Mimulus lewisii*), for example, display prominent yellow markings surrounded by dark pink stripes with a white border. Mutants lacking these nectar guides or with reduced petal coloration exhibit significant reductions in pollinator visitation (Owen & Bradshaw, 2011). Further work in yellow monkeyflowers (*Mimulus guttatus*) reinforced the importance of pigmentation patterns for pollinator attraction as bumblebees preferred the flowers of the *red tongue* mutant with an expanded anthocyanin region over wildtype flowers with smaller anthocyanin spots (Ding *et al.* 2020). Petal patterns also play important roles to protect against abiotic stress. In wild sunflowers (*Helianthus annuus*), larger UV-absorbing bullseyes increased a flower's tolerance to desiccation (Todesco *et al.* 2022). In silverweed flowers, the size of the UV bullseye positively correlates with the amount of exposure to UV irradiation, possibly enhancing pollen protection from UV-induced DNA damage (Koski & Ashman, 2015). In both species, the UV-absorbing bullseye results from the preferential or restricted production of flavonols at the petal base which protect against environmental stress (Pollastri & Tattini, 2011). Hence, the evolution of petal patterns is shaped by both biotic and abiotic factors and investigating the processes mediating changes in petal patterning provides insights into the mechanisms facilitating the emergence of adaptations and speciation.

The patterns on the petal surface are produced by a combination of features including epidermal cells that differ in their shape, texture and pigmentation (Kay, 1981; Johnson & Midgley, 1997). Plant pigments are divided into three classes: carotenoids, betalains and flavonoids. The carotenoid and flavonoid biosynthetic pathways are widely distributed across the plant kingdom while betalains are restricted to the order Caryophyllales. Flavonoids are the predominant class of pigments in angiosperm flowers and produce a wide array of colors. Flavonoids comprise

several subgroups, including flavonols and anthocyanins, which are colorless/'cream'-like and blue/red, respectively. Flavonoid biosynthesis begins with the production of chalcone, followed by several hydroxylation steps to produce the precursors for flavonols and anthocyanins. Thereafter, the pathway branches whereby the enzymes dihydroflavonol (*DFR*) and anthocyanidin synthase (*ANS*) produce anthocyanins while flavonol synthase (*FLS*) yields flavonols, respectively (Tanaka *et al.* 2008). The accumulation of these pigments, in varying combinations and abundance, and in different regions of the petals, give rise to the spectrum of color patterns on the petal epidermis. Petal coloration can also be influenced by the physical properties of epidermal cells (Moyroud *et al.* 2017; Riglet *et al.* 2021). For instance, conical cells can focus light into the anthocyanin-containing vacuole enhancing color intensity, while semi-ordered striations of the cuticle can produce a weak iridescence and blue halo effect that increases flower salience to pollinators (Whitney *et al.* 2009; Moyroud *et al.* 2017).

The genetic components underpinning the production of petal patterns have been functionally characterized in a limited number of model species. Flavonoid biosynthesis is regulated at the transcriptional level by the MBW complex, combining two types of transcription factors (TFs), an R2R3 MYB (myeloblastosis) and a basic helix-loop-helix (bHLH) protein, and a scaffold WD-repeat protein (Ramsey & Glover, 2005). MYBs and bHLHs constitute two of the largest TF families in plants and as such, several MBW complexes exist to tightly regulate the expression of late flavonoid biosynthetic genes. In *Antirrhinum majus*, the venation pattern present on the lateral sides of the corolla tube is produced by overlapping expression patterns of the MYB TF *AmVENOSA* and its bHLH partner *AmDELILA*. Together, they combine with a WD40 co-activator to form an MBW complex that activates anthocyanin biosynthesis (Shang *et al.* 2011). *AmVENOSA* expression is restricted to a wedge of cells between the vein and adaxial epidermis while *AmDELILA* is broadly expressed in the epidermal cells of the corolla (Goodrich *et al.*, 1992; Jackson *et al.*, 1992). Therefore, the restricted expression of *AmVENOSA*, the MYB element of the MBW complex, is the main driver of the venation pattern. The mechanisms accounting for the spatial regulation of *MYB* transcription has only been explored in a few systems including *Mimulus verbenaceus* leaf stripes (LaFountain *et al.*, 2024) because many species used in the study of petal patterning cannot be genetically manipulated with ease. Hence the upstream processes accounting for the restriction of MYB expression are still poorly understood across angiosperms.

Venice mallow (*Hibiscus trionum*) produces flowers with a striking bullseye pattern. This herbaceous species has emerged as a model system to understand petal pattern evolution and development at both the macro- and microscale (Moyroud *et al.*, 2022; Riglet *et al.*, 2024). Petal epidermal cells in the two regions of the bullseye exhibit differences in color, cell shape and

texture. Cells in the proximal region (petal base) are purple, flat and elongated (tabular) and covered with a striated cuticle while cells in the distal region (petal tip) are cream-colored and conical in shape with a smooth surface (Vignolini *et al.*, 2015). Differences in pigmentation between the two regions appear between stages 1 and 2 of floral development while differences in cell shape and texture emerge later between stages 3 and 4 (Moyroud *et al.*, 2022). The proximal and distal regions are specified very early during development long before the epidermal cells acquire their characteristic features. During stage 0, a band of large elongated cells appear to mark the position of the future bullseye boundary and divide the petal epidermis into proximal and distal domains (Riglet *et al.*, 2024). *H. trionum* belongs to the *Malvaceae* family and falls into the Trionum Complex that appears to have experienced a recent radiation event (Craven *et al.*, 2011). The size of the bullseye varies extensively across the Trionum Complex providing an attractive system to investigate the processes underpinning petal pattern variation and possibly replicated evolution. In this study, we first used a phylogenomic approach to solve species relationships across the Trionum Complex and uncovered multiple instances of bullseye pigmentation reduction. Combining molecular approaches and genetic manipulations *in planta*, we found that independent occurrences of bullseye size reductions are due to recurring modifications of an R2R3 MYB-encoding locus, *BERRY1*. This locus restricts anthocyanin production to the petal base and acts as a principal driver of bullseye pattern formation. Importantly, pollinator behaviour assays revealed that bumblebees easily discriminate between flowers of varying bullseye size, suggesting that repeated mutations within a single locus are sufficient to generate morphological variations in a trait likely to impact speciation.

Materials and Methods

Plant material and growing conditions

Wildtype *H. trionum* L. CUBG seeds (voucher CGE00046422) were sourced from the Cambridge University Botanic Gardens, Cambridge, UK. *H. trionum* L. seeds from Bream Head, New Zealand (vouchers AK253689 & CGE00046417) were sourced from Dr. Brian G. Murray (University of Auckland). *H. trionum* L. Commercial seeds (voucher CGE00080883) were supplied by Doekele G. Stavenga (Netherlands). Seeds of *H. richardsonii* Sweet ex Lindl. (vouchers AK251841 & CGE00046420) were also sourced from Dr. Brian G. Murray (University of Auckland). Seeds of *H. verdcourtii* Craven, population 1 (voucher CGE00046415), population 2 (voucher CGE00046414), population 3 (voucher CGE00080882), population 4 (voucher CGE00046413), population 5 (voucher CGE00046366) and *H. tridactylites* Lindl. population 6 (voucher CGE00046364) and population 7 (voucher CGE00046363)] were provided by Stephen B.

Johnson (New South Wales, Department of Primary Industries). Seeds were imbibed in 90°C H₂O for 10 min and germinated in the dark at 30°C for 48 h. Seedlings were transferred to Levington High Nutrient M3 compost and grown under glasshouse conditions consisting of a 16 h light/8 h dark photoperiod at 25°C with a minimum radiance of 88 W.m⁻². Wildtype species and transgenic lines used in this study are summarized in Table S1 and S2, respectively.

Phenotyping

Open flowers (stage 5, Moyroud *et al.*, 2022) were collected in the morning and imaged on black velvet using a Panasonic DMC-FZ38 camera. Dissected petals were imaged with a Keyence microscope (VHX5000 and VHX7000 models) fitted with a VH-Z100R lens. Petals with the adaxial side facing upward were scanned with a black and white background using the Epson Perfection V850 Pro scanner. Total petal and purple pigmented areas were measured from scanned images using ImageJ version 1.53 (<https://imagej.net/ij/>). For each flower, 3 petals were measured and a minimum of 10 flowers were measured per genotype.

Flavonoid extraction and quantification

Sample collection and extraction: Stage 5 petals were dissected into proximal, boundary and distal regions and flash frozen along with ovary tissue in liquid nitrogen. For each sample, ~40 mg of tissue was homogenized in a tissue lyser. 1 ml of acidic methanol (70:30 MeOH:1% acetic acid) was added to the tissue, vortexed and then incubated overnight on a rotating wheel at 4°C. Petal debris was pelleted for 3 min at 13,148 x g and the supernatant was collected and stored at 4°C until the following day. The extraction was repeated with 1 ml of acidic methanol (90:10 MeOH:1% acetic acid). Supernatants from both extractions were pooled before reducing to ~250 µl by evaporation in a miVac centrifugal concentrator (Genevac). The final volume was measured before measurements were taken.

Sample quantification: Flavonols and anthocyanins were quantified using a spectrophotometer set to A₃₅₀ and A₅₃₀, respectively. Three technical replicates (three dilutions) per extraction were measured. Concentrated extracts were diluted in 1 ml MeOH to measure flavonol concentration, and in 1 ml 90:10 MeOH:1N HCl to measure anthocyanin concentration. Absorbance units (AU) were converted to concentration (mg flavonol or anthocyanin equivalent per g of fresh weight tissue) based on the following calculation:

$$\frac{\left(\frac{(\text{absorbance} \times \text{conversion factor} \times \text{volume total cuvette} \times \text{volume total extract})}{\text{volume sample loaded}} \right)}{\text{mass of tissue}}$$

202 To determine conversion factors, standard curves were generated using flavonol quercetin 3-D-
galactoside (Cayman Chemicals item 18648) and anthocyanin cyanidin 3-O-glucoside (chloride)
204 (Cayman Chemicals item 16406) as reference compounds.

206 **Crosses**

For each cross, pollen was transferred by hand to the recipient flower. To avoid self-fertilization,
208 recipient flowers without visible pollen on their stigma were selected for crossing.

H. trionum CUBG x *H. richardsonii*: five independent crosses of *H. trionum* CUBG and *H.*
210 *richardsonii* were performed. F1 hybrids were identified based on the presence of a pigmented
bullseye and microscopy imaging. F1 hybrid plants were allowed to self-pollinate and flower
212 phenotypes of the next generation (F2) individuals were characterized.

H. verdcourtii pop. 2 x *H. verdcourtii* pop. 3: pollen was transferred by hand from a *H. verdcourtii*
214 flower (St. George, red bullseye, “*H. ver2*”) to an open *H. verdcourtii* (St. George, pale yellow with
pink halo bullseye, “*H. ver3*”). F1 hybrid plants were allowed to self-pollinate and flower
216 phenotypes of the F2 generation were characterized.

218 **Rapid genomic DNA extraction to isolate *HrBERRY1***

Fresh leaf tissue (~1 cm²) was ground with a plastic pestle before addition of 250 µl Tris HCl-
220 EDTA extraction buffer with 10% SDS. The samples were briefly vortexed and spun for 1 min at
13,148 x g to collect genomic DNA from the supernatant. DNA was precipitated with isopropanol
222 and the pellet was washed with 70% EtOH. The pellet was dried before resuspension in 40 µl
ddH₂O.

224

Genotyping the F2 progeny from wildtype *H. trionum* and *H. richardsonii* crossing

226 A single bud per individual was collected from *H. trionum* CUBG and *H. richardsonii* parents, and
from 27 F2 individuals randomly chosen among >100 F2 individuals that had been phenotyped
228 and assigned to one of three phenotypes (*H. trionum* CUBG-like, *H. richardsonii*-like, hybrid-like).
Genomic DNA was extracted from selected individuals. 800 mg of mixed petal/bud tissue was
230 divided into 50 mg aliquots, homogenized, and incubated with 1 ml CTAB buffer (2% w/v) + 10 µl
2-mercaptoethanol (Sigma M3148) at 55°C for 30 min. Supernatant was collected from pelleted
232 samples and incubated with 10 mg.ml⁻¹ RNase A (ThermoFisher Scientific EN0531) at 32°C for
30 min. DNA was extracted using 25:24:1 phenol-chloroform-isoamyl alcohol saturated with 10
234 mM Tris, pH 8.0, 1 mM EDTA (Sigma P0269) and 24:1 chloroform-isoamyl alcohol mixture (Sigma
25666). DNA was separated from protein and other cellular debris by carefully removing the upper

aqueous layer after each extraction. The DNA was precipitated overnight at -20°C in chilled isopropanol and washed twice with 70% EtOH. DNA pellets were dried and resuspended in 30 µl low TE buffer (10 mM Tris HCl pH 8, 0.1 mM EDTA pH 8.0). Genomic DNA purity and concentration were analyzed using a NanoDrop (ThermoFisher Scientific). *HtBERRY1* or *HrBERRY1* gene copies were determined by PCR using allele-specific primers (Table S3). A 1200 bp band indicated the presence of the *HtBERRY1* allele and a 400 bp band indicated the presence of the *HrBERRY1* allele. Samples were genotyped in a blind manner. To confirm the genotyping results, a further 11 F2 individuals with a *H. richardsonii*-like phenotype were tested. *HtACTIN1* was included as a positive control for the presence of genomic DNA.

Genotyping the F2 progeny from *H. ver2* and *H. ver3* crossing

Fresh leaf tissue (~1 cm²) was collected from *H. ver2* and *H. ver3* parents, and 33 F2 individuals. The tissue was ground with a plastic pestle before addition of 250 µl Tris HCl-EDTA extraction buffer with 10% SDS. The samples were briefly vortexed and spun for 1 min at 13,148 x g to collect the genomic DNA from the supernatant. DNA was precipitated with isopropanol and the pellet was washed with 70% EtOH. The pellet was dried before resuspension in 40 µl ddH₂O. Samples were genotyped in a blind manner to check for the presence of *HvBERRY1*. The presence of *HvBERRY1* was determined by PCR using *HvBERRY1*-specific primers (Table S3). F2 samples with a pigmented red bullseye produced a 421 bp band that indicated the presence of *HvBERRY1* while those without a pigmented bullseye did not produce a PCR product. *HvACTIN1* was included as a positive control for the presence of genomic DNA.

Tissue collection, RNA extraction and Illumina sequencing to produce the mixed tissue transcriptome for phylogenomic analyses

Whole bud, flowers and fruit tissue were collected from each of the following species and populations: wildtype *H. trionum*, *H. trionum* diploid New Zealand naturalised race, *H. trionum* Commercial, *H. richardsonii*, *H. verdcourtii* pops. 1-5, *H. tridactylites* pops. 6 & 7. Upon collection, bud tissue was flash frozen in liquid nitrogen. Per species/population, three biological replicates were collected with each biological replicate corresponding to one individual plant. Frozen samples were homogenized using a pestle and mortar and total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma STRN250). ~40 mg of tissue was used for each RNA extraction. An on-column DNase I treatment (Sigma DNASE70) was performed between the first two column washes. RNA was eluted in 30 µl nuclease-free H₂O (Promega MC1191) and purity

was analyzed using a NanoDrop (ThermoFisher Scientific) and RNA ScreenTape (Agilent 5067-5576, 5067-5577, 5067-5578) on the Agilent TapeStation 2200 system.

Samples with minimum 1.7 µg total RNA (>50 ng µl⁻¹), and A260/280 value of 2.0 were shipped on dry ice to Azenta Life Sciences, Leipzig (formerly GeneWiz) for RNA-seq library preparation and sequencing. Library preparation was performed with polyA selection and sequencing using an Illumina HiSeq machine (2x 150 bp configuration, ~350 M raw paired-end reads per lane, at least 30M reads per sample). Raw reads (accession numbers SAMN47801648 to SAMN47801669) have been deposited on GenBank SRA (<https://www.ncbi.nlm.nih.gov/sra>).

Phylogenomic transcriptome analysis

Raw sequence reads for the outgroups (*Hibiscus cannabinus*, *Hibiscus syriacus* and *Hibiscus sabdariffa*), were downloaded from the NCBI Short Read Archive (SRA) accessions (SRX1084555 [Zhang *et al.* 2016], SRX2142897 [Kim *et al.* 2017], SRX3341155 [Loo *et al.* 2016]). The sequences were assembled using the same procedure as the *H. trionum* stage 1 petal transcriptome (described below).

We reduced the redundancy for each transcriptome using cd-hit-est v.4.7 (Fu *et al.* 2012), with the settings “-c 0.99 -n 10 -r 0”. Initial homology inference was conducted by performing an all-by-all blastn v.2.7.1 with the settings “--e-value 10 --max_target_seqs 1000”. Markov clustering, as implemented in the program mcl (van Dongen, 2000) with an inflation value of 1.4, was used to refine the clusters further. We then performed two rounds of phylogeny-based data cleaning. In the first round, the sequences were aligned using MAFFT v.7.407 (Katoh & Standley, 2013) with the settings “—auto —maxiterate 1000” the alignments were cleaned for 10% column occupancy using the program pxclsq with the settings “-p 0.1” from the phyx package (Brown *et al.* 2017). A tree for each cleaned alignment was inferred using maximum likelihood as implemented in FastTree v.2.1.1 (Price *et al.* 2010) with the settings “-gtr -nt”. The resulting trees had any tips with an absolute branch length of 0.4 subs/site or a relative branch length of 0.6 subs/site removed. Only the transcript with the most informative sites was retained for any instance where the same taxa were sister to one another. Any subclades containing at least 4 taxa and were on a branch with a 1.0 subs/site length were divided into separate homolog trees. During the second round of cleaning, the same process was performed, however, the maximum likelihood tree inference was conducted using IQtree v.1.6.12 (Nguyen *et al.* 2015) using the best inferred model of evolution (Kalyaanamoorthy *et al.* 2017) and 1000 ultrafast bootstrap replicated. The orthologs were extracted from the trees using the Maximum Inclusion approach with an

absolute branch length setting of 0.4 subs/site and a relative branch length setting of 0.6 subs/site. A coalescent based maximum quartet support species tree was estimated using Astral v.5.7.3.

Tissue collection, RNA extraction and Illumina sequencing for *H. trionum* stage 1 petal transcriptome

Tissue was collected from five individual *H. trionum* CUBG plants to generate five biological replicates: for each biological replicate, stage 1 petals were harvested and dissected to separate proximal and distal region. Tissue from these 10 samples were flash frozen in liquid nitrogen, ground to a fine powder, and total RNA extracted using the Spectrum Plant Total RNA kit (Sigma STRN250) with 40 mg of starting material. An on-column DNase I (Sigma DNASE70) treatment was performed between the first two column washes. RNA was eluted with 30 µl nuclease-free H₂O (Promega MC1191). RNA purity was analyzed using a NanoDrop (ThermoFisher Scientific) and RNA ScreenTape (Agilent 5067-5576, 5067-5577, 5067-5578) on the Agilent TapeStation 2200 system. Samples with minimum 1.75 µg total RNA (>50 ng.µl⁻¹), and A260/280 value of 2.0 were shipped on dry ice to Azenta Life Sciences, Leipzig (formerly GeneWiz) for RNA-seq library preparation and sequencing. Library preparation was performed with polyA selection and sequencing using an Illumina HiSeq machine (2x 150bp configuration, ~350M raw paired-end reads per lane, at least 30M reads per sample). Raw reads (accession numbers SAMN47793371 to SAMN47793390) have been deposited on GenBank SRA (<https://www.ncbi.nlm.nih.gov/sra>).

***H. trionum* CUBG stage 1 petal transcriptome assembly and differential gene expression analysis**

RNA-seq reads were used to assess gene expression and compare transcript abundance between proximal and distal regions of stage 1 (S1) petal primordia, as defined in Moyroud *et al.* 2022. First, a reference transcriptome (Supplementary Data 1) was created by combining the raw reads from S1 proximal and distal petal tissue of a single plant. To produce this reference transcriptome the raw reads were cleaned, assembled into a database with chimeric sequences removed, and their coding regions for transcripts predicted using the previously excluded chimeric sequences. Cleaning raw reads used Rcorrector v1.0.3 (Song & Florea, 2015), to remove sequencing errors, and Trimmomatic v0.38 (Bolger *et al.* 2014), to filter for quality and remove the adapters added during library preparation. The cleaned reads were then assembled into a transcriptome with Trinity v2.8.3 (Grabherr *et al.* 2011). The procedure followed Yang and Smith (2013) with a database composed of the genomes of *Gossypium raymondii*, *Arabidopsis thaliana*, and *Theobroma cacao*. Chimeric sequences were filtered by BLAST® v2.2.31 (Altschul *et al.*

1997) and isoforms and assembly artifacts removed using Corset v1.07 (Davidson & Oshlack, 2014). Finally, coding regions were predicted using the program Transdecoder v5.3.0 (Haas *et al.* 2013) and a BLAST database composed of the same taxa as the chimera database. The reference transcriptome was then used as a guide for transcriptome assembly corresponding to each biological replicate. Raw reads from each sample were first mapped onto the reference transcriptome using Kallisto v.0.44.0 (Bray *et al.* 2016). The transcript level- abundance was converted into counts using the program tximport (Soneson *et al.* 2015). Fold change and dispersion of these counts were calculated using DESeq2 (Love *et al.* 2014). Finally, the top blastn hit between the transcriptome sequences and the NCBI nr database was used for automatic gene annotation. Genes with log2-fold change <-2 or >2 and adjusted p-value < 10⁻⁵ were considered differentially expressed. Results of the differential gene expression analysis and assembled transcripts from stage 1 *H. trionum* CUBG petals are provided in Supplementary Data 1 & 2.

Phylogenetic analysis to establish the identity of selected R2R3 MYBs

The amino acid sequences of HtBERRY1 (HRI_002225000), HtBERRY2 (HRI_002225200) and HtCREAM1 (HRI_001263100) were used to conduct a blast search against the genome of *H. trionum* available on GenBank (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_030270665.1/). This yielded another six HtBERRY-like sequences (HRI_002224000, HRI_002224800, HRI_002224600, HRI_000181400, HRI_001579800, HRI_002131000) and another three HtCREAM-like sequences (HRI_003566500, HRI_00625700, HRI_001075200). Those 12 *H. trionum* sequences were combined with protein sequences of subgroup 6 MYB genes known to regulate anthocyanin production in other species: ROSEA1 (DQ275529), ROSEA2 (DQ275530) and VENOSA (DQ275531) from *Antirrhinum majus*; PELAN (KJ011144) and NEGAN (KJ011145) from *Mimulus lewisii*; MYB113-like (XM_012977147) from *Mimulus guttatus*; DEEP PURPLE (HQ116169) and PURPLE HAZE (HQ116170, HQ428100) from *Petunia hybrida* as well as amino acid sequences of all MYB genes identified in the genome of *Arabidopsis thaliana*, *Gossypium raimondii* and *Theobroma cacao* were acquired from Jiang *et al.* 2020, He *et al.* 2016 and Du *et al.* 2022, respectively. In total, 483 MYB sequences were used for phylogenetic reconstructions and those are provided in Supplementary Data 3. The 483 sequences were aligned using MAFFT v7.490 with the setting “—auto —maxiterate 1000”. The alignment was then cleaned for a minimum of 10% column occupancy using the program pxclsq from the phyx package. A maximum likelihood

tree was then inferred from the cleaned alignments using the WAG model of evolution, with gamma rate variation and 1000 ultrafast bootstrap replicates as support (Hoang *et al.* 2018).

cDNA synthesis

cDNA was synthesized from 0.5 ug total RNA using SuperScript III Reverse Transcriptase (Invitrogen). Oligo(dT)₁₅ primers were used for first strand cDNA synthesis for gene isolation and measuring expression by RT-PCR or qPCR. First-strand cDNA synthesis followed the manufacturer's guidelines, with incubation with SuperScript III RT at 50°C for 1 hr followed by an inactivation step at 70°C for 15 min.

Construction of plant expression vectors

The coding sequences of *HtDFR1*, *HtBERRY1* and *HtCREAM1* were amplified by PCR using Q5 High-Fidelity DNA polymerase with primers listed in Table S3. Each PCR product was cloned into the EcoRV-digested pBluescript KS(-) vector. Restriction enzyme digestion was used to extract the coding sequence of each gene and insert it into a modified pGREENII (for *HtDFR1* and *HtCREAM1* overexpression) or pCAMBIA (for *HtBERRY1* overexpression) containing a 2x35S CaMV promoter driving transgene expression. The plant expression vectors also express a fluorescent eYFP under control of the promoter region of *AtUBIQUITIN10* (PromUBQ10). Final vectors were sent to Azenta (formerly Genewiz) for Sanger sequencing to validate the final construct prior to plant transformation. The final vectors used for plant transformation are summarized in Table S4.

Plant transformation

Electrocompetent *Agrobacterium tumefaciens* LBA4404 cells were transformed with plant expression vectors pVT9 (2x35S::*HtDFR1*), pAF29 (2x35S::*HtCREAM1*) or pAF52 (2x35S::*HtBERRY1*). Transformed cells were selected on LB agar plates containing 50 mg.l⁻¹ kanamycin and 25 mg.l⁻¹ streptomycin after 48 hours incubation in the dark at 30°C. Transgenic *H. trionum* lines were produced using the protocol described in Moyroud *et al.* 2022 with the following modification: 100 mg.l⁻¹ of carbenicillin was used instead of 250 mg.l⁻¹ of cefotaxime for the MS Hib plates.

Gene expression analysis using quantitative RT-PCR

To quantify gene expression levels during petal development in *H. trionum* CUBG and *H. richardsonii*, petals from stages 1 to 4 were dissected into proximal and distal regions. Stage 3

petals were dissected into proximal and distal regions to measure expression in the transgenic lines of *35S::HtDFR1* OE and KD, *35S::HtCREAM1* and *35S::HtBERRY1*. Three biological replicates were collected from individual plants for each stage. Frozen petal tissue was ground to a fine powder and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma STRN250) with ~40 mg of ground tissue used as starting material for each RNA extraction. cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's guidelines. Quantitative real-time PCR was performed using a Roche LightCycler 480 machine and the Luna Universal qPCR Master Mix (New England BioLabs). *HtACTIN1* was used as a housekeeping gene for all biological replicates and stages as in Moyroud *et al.* 2022. Data was analyzed using a modified delta Ct method, considering the real efficiency of each primer pair and one reference gene for normalization (Pfaffl, 2001; Ganger *et al.* 2017). Statistical analyses were calculated in RStudio (Version 1.1.1717). A Student's t-test, Welch's t-test or Wilcox's t-test was used depending on normality and homogeneity of variance of the samples.

Bumblebee behavior assay

Experiments with flower-naïve buff-tailed bumblebees (*Bombus terrestris* v. *audax*; Research Hive, BioBest UK) were conducted using the design published in Moyroud *et al.* 2017. Each colony was fed daily with fresh 15% sucrose solution, and twice a week with pollen grains (The Happy Health Company). Foragers were hand-marked with water-based Thorne queen marking paints in various colors to distinguish them during the experiments. For the preference tests, one *H. trionum* CUBG and one *H. richardsonii* open flower were each placed in a tube containing water equidistant from the hive entrance. Per test, one naïve bumblebee was released from the hive into the arena and the flower it chose to land on first (preference) was recorded. Forty individual bumblebees were tested for their flower preference. Statistical differences were calculated using a one sample t-test [RStudio (Version 1.1.1717)].

Results

Bullseye pattern reduction occurred several times independently across the Trionum Complex

Analyzing trait distribution across a species tree often represents a first step towards understanding how these traits vary over time and how these changes relate to the evolutionary history of a specific group of organisms. The indigenous or naturalised status of several species

in the Trionum Complex has been debated and relationships within the group have remained
438 obscure and unresolved (de Lange, 2008; Craven *et al.*, 2011). Thus, to investigate petal pattern
evolution across the Trionum Complex, we first generated transcriptomic data using RNA
440 sequencing and undertook a phylogenomic approach to clarify the relationships between the four
species within the group (Fig. 1A, Fig. S1). Whenever possible, we included several accessions
442 from each species to capture the morphological diversity existing within some species (Fig. 1B;
Craven *et al.*, 2011; Moyroud *et al.*, 2022). For instance, *H. verdcourtii* individuals from Emerald
444 and Theodore (populations 1 and 4, respectively; Fig. S1) exhibit a red bullseye, while Narrabri
individuals (population 5; Fig. S1) produce cream-colored flowers lacking a pigmented bullseye
446 (Fig. 1B). By contrast, individuals with different bullseye patterns can be found in the same region.
For example, plants with red bullseye flowers (*H. verdcourtii* population 2; Fig. S1) or cream
448 flowers with a faint pink halo (*H. verdcourtii* population 3; Fig. S1) are both present in St. George
(Craven *et al.*, 2011; Fig. 1B, Fig. S1). Intraspecific variation also exists within *H. trionum* whereby
450 a commercial accession produces a visibly smaller bullseye than our wildtype reference
accession *H. trionum* CUBG (Moyroud *et al.*, 2022; Riglet *et al.*, 2024) and wild individuals from
452 Bream Head, New Zealand (Fig. 1B, Fig. S1; Craven *et al.*, 2011; Moyroud *et al.*, 2022).

As expected, accessions assigned to the same species clustered together in our
454 phylogenomic reconstruction (Fig. 1A). Our analysis also identified *H. richardsonii*, the other
diploid species of the group (de Lange, 2008; Murray *et al.*, 2008), as sister to *H. trionum*. We
456 found that the three main features of petal epidermal cells (pigmentation, shape and texture) can
vary independently from each other. All accessions regardless of their corolla pigmentation
458 pattern produced smooth conical cells in the distal petal region (petal tip) and flat elongated
(tabular) striated cells in the proximal petal domain (petal base) except for the five *H. verdcourtii*
460 accessions that all exhibit smooth tabular cells in the proximal region (Fig. S2). Finally, our
phylogenomic reconstruction identified pigmentation to be a more labile trait than the structural
462 features (shape and texture) of epidermal petal cells as accessions lacking pigmentation at the
petal base (*H. verdcourtii* populations 3 and 5) or producing a reduced pigmented bullseye (*H.*
464 *trionum* Commercial and *H. richardsonii*) did not cluster together (Fig. 1A). The multiple instances
of bullseye pigmentation reduction across the Trionum Complex therefore provide an excellent
466 opportunity to study the genetic basis of replicated evolution.

**A single genetic locus accounts for reduction of the pigmented bullseye in *H. richardsonii*,
the sister species of *H. trionum***

We previously found that the bullseye boundary separating proximal and distal regions is specified closer to the petal base during the pre-patterning phase in *H. richardsonii* compared to *H. trionum* CUBG (Riglet *et al.*, 2024). This boundary shift is sufficient to produce a smaller structural bullseye in *H. richardsonii* as striated tabular cells cover a smaller portion of its petal. However, the pigmented area appears even smaller than the structural bullseye (Fig. 2A) suggesting that additional changes affecting the control of pigment production rather than the pre-patterning process itself must also have occurred during evolution along the lineage leading to *H. richardsonii* (Riglet *et al.*, 2024). In this study, we aim to clarify the genetic basis accounting for this reduction in pigmentation.

To precisely characterize differences in pigmentation between *H. trionum* CUBG and *H. richardsonii* bullseyes, we examined and compared the morphological and chemical features of open flowers (stage 5) from both species. We found that the pigmented bullseye covers 15% of the petal surface in *H. trionum* CUBG while the *H. richardsonii* bullseye represents only 3% of the petal surface (Fig. 2B). This five-fold reduction in pigmented bullseye size coincides with a 15-fold reduction in anthocyanin levels in the *H. richardsonii* proximal region compared to *H. trionum* CUBG (Fig. 2C). The lower amount of anthocyanin recorded in *H. richardsonii* petals could be due to reduced anthocyanin production or alternatively, increased anthocyanin degradation. We noticed that throughout bud development, *H. richardsonii* petal primordia always display a smaller pigmented area compared to *H. trionum* CUBG petals at equivalent stages (Fig. S3). This suggests differences in anthocyanin synthesis, rather than degradation, account for differences in bullseye sizes between the two species. Total flavonol content was 1.4- to 2.6-fold higher in *H. richardsonii* petal tissue compared to *H. trionum* CUBG (Fig. 2D). The reduction in anthocyanin production in *H. richardsonii* could favor precursor availability toward the flavonol branch of the flavonoid pathway, accounting for the increase in flavonols recorded in the proximal and boundary regions of *H. richardsonii* petals (Fig. 2D). Taken together, our data indicates the pigmented bullseye of *H. trionum* CUBG is generated by restricting anthocyanin synthesis to the proximal petal while flavonols are preferentially produced in the distal petal and that the smaller size of the pigmented bullseye in *H. richardsonii* is likely due to reduced anthocyanin synthesis.

To start uncovering the molecular mechanisms accounting for the difference in bullseye pigmentation between *H. trionum* CUBG and *H. richardsonii*, we crossed the two species together (Fig. 3A). Hybrid flowers displayed a pigmented bullseye similar in size to *H. trionum* CUBG (Fig. 3A and 3B). Closer inspection of the boundary region (Fig. 3C) revealed differences in the composition and pattern of cell types between the hybrid and the *H. trionum* parent. In the parental *H. trionum* CUBG, the boundary region was characterized by three cell types: (1) striated,

pigmented, tabular cells, (2) smooth, pigmented, tabular cells, and (3) smooth, non-pigmented, tabular cells (Figs. 3C, Fig. S4). In *H. richardsonii*, the reduction in bullseye pigmentation resulted in only two cell types at the boundary: (1) striated and (2) smooth, non-pigmented, tabular cells (Figs. 3C, Fig. S4). Interestingly in the hybrid, striated, non-pigmented, tabular cells replaced the smooth, pigmented, tabular cells found in *H. trionum* CUBG (Figs. 3C, Fig. S4). Although the hybrid appears identical to *H. trionum* CUBG at the macroscale, the hybrid differs in boundary composition. In the F2 selfing population, the two parental and hybrid phenotypes were recapitulated and these morphologies segregated in a Mendelian ratio as 40, 41 and 96 individuals displayed a *H. trionum* CUBG-like, *H. richardsonii*-like and hybrid-like phenotype, respectively. This indicates that a single locus likely accounts for differences in pigmented bullseye dimensions.

Expression patterns of a *DFR* and a *FLS* *Hibiscus* homolog correlate with petal pigmentation

Flavonoid biosynthesis is often targeted by evolutionary processes to generate diversity across flowering species (Kellenberger & Glover, 2023). We hypothesized that differences in petal patterning between *H. trionum* CUBG and *H. richardsonii* are also a consequence of modifications to the flavonoid pathway. This biosynthetic pathway is well-characterized (Fig. 4A) and includes the production of a common dihydroflavonol precursor before splitting into two main branches that lead to anthocyanin or flavonol synthesis. *DFR* and *FLS* are key enzymes involved in the production of anthocyanins and flavonols, respectively. From the *H. trionum* genome, we isolated three homologs of each gene. We measured their expression in the proximal and distal regions of developing *H. trionum* CUBG petals (Fig. 4B-C, Fig. S5). Among the three *DFR* homologs, *HtDFR1* was expressed at the highest levels in the proximal region: *HtDFR1* expression was eight- to 11-fold higher at the petal base compared to the distal region throughout development (Fig. 4B). *HtDFR2* and *HtDFR3* expression levels in the proximal petal were almost negligible and we concluded that they are not likely involved in anthocyanin production at the petal base (Figs. S5, S6). *HtFLS2* was highly expressed during the first three stages of petal development (Fig. 4C) before declining at stage 4, following a similar temporal dynamic yet complementary spatial expression pattern to *HtDFR1* as its expression was four- to 19-fold higher in the distal region (Fig. 4C). Neither *HtFLS1* nor *HtFLS3* were expressed at levels comparable to *HtFLS2* (Figs. S5, S6). These results demonstrate that *HtDFR1* and *HtFLS2* are likely the main players in shaping the bullseye pattern, and the spatial accumulation of anthocyanins and flavonols (Fig. 2C-D) directly reflects their expression patterns.

To determine whether *HtDFR1* was responsible for bullseye anthocyanin production, we generated 35S::*HtDFR1* transgenic lines in *H. trionum* CUBG. Four strong overexpression lines with ectopic pigmentation in the distal petal were recovered along with six transgenic lines that produced flowers devoid of a pigmented bullseye (Figs. 4D, S7). We hypothesized this was due to *HtDFR1* silencing triggered by transcript overaccumulation as seen in *Arabidopsis* T-DNA supertransformants (Schubert *et al.*, 2004). To test this hypothesis, we quantified *HtDFR1* transcription levels in transgenic lines exhibiting both phenotypes. We found the presence of distal pigmentation was accompanied by a 300-fold increase in *HtDFR1* expression in the distal region (Figs. 4E, S7). Conversely, *HtDFR1* transcript levels were very low (15-fold lower than WT expression) in the proximal petal of white flowers, confirming that *HtDFR1* expression matched the strength of the phenotype observed. We concluded the absence of pigmentation was due to *HtDFR1* silencing such that the transgenic lines producing white flowers corresponded to *HtDFR1* knockdown. Total anthocyanin and flavonol measurements in both lines confirmed visual observations. Anthocyanin levels were five-fold higher in the distal petal of the *HtDFR1* overexpression line and almost absent from petals of the *HtDFR1* knockdown line (Fig. 4F). Flavonol levels were two-fold higher throughout the petal of the *HtDFR1* knockdown line (Fig. 4G). The lack of *HtDFR1* activity in the knockdown lines likely increased precursor availability for flavonol biosynthesis, possibly accounting for increased flavonol levels.

Although *HtDFR1* overexpression produced distal petal pigmentation, anthocyanin levels remained lower in the distal petal than in the proximal region suggesting that other factors, such as substrate competition between ectopic *HtDFR1* and endogenous *HtFLS2*, limit anthocyanin production in the distal petal. A clear bullseye pattern was still visible in the *HtDFR1* knockdown line despite the near-complete absence of anthocyanin (Fig. 4D, Fig. S7), highlighting the contribution of the structural elements (differences in cell shape and texture between the proximal and distal regions) to the visual appearance of the petal. This also confirms the gene regulatory network regulating pigment production differs from the upstream processes initiating boundary formation during the pre-patterning phase (Riglet *et al.*, 2024).

To identify the genetic changes accounting for reduced anthocyanin production in *H. richardsonii*, we isolated *HrDFR1* and *HrFLS2* in *H. richardsonii*. We did not find any differences in their amino acid sequences compared to the *H. trionum* CUBG orthologs. Next, we measured their expression throughout *H. richardsonii* petal development and found that relative to *H. trionum* CUBG, *HrDFR1* expression was significantly reduced across all petal stages in both proximal (17- to 107-fold lower) and distal (two- to nine-fold lower) tissue (Fig. 5A, Fig. S8). The expression profile of *HrFLS2* was similar to *HtFLS2* such that its expression was higher in the distal petal

compared to the proximal petal (Fig. 5B, Fig. S8). Therefore, the reduction of bullseye pigmentation in *H. richardsonii* correlates with a severe reduction in *HrDFR1* expression across the petal primordia.

Changes at the *BERRY1* locus are responsible for differences in pigmented bullseye size between *H. trionum* CUBG and *H. richardsonii*

MBW complexes, comprising a MYB, a bHLH and a WD40 component often regulate the expression of genes encoding key enzymes such as *DFR* and *FLS* in the flavonoid biosynthesis pathway (Xu *et al.*, 2014). We reasoned that some of the TFs regulating *HtDFR1* transcription must be preferentially expressed in the proximal region of the petal. To identify these regulators, we dissected stage 1 petal primordia into proximal and distal regions and performed RNA-seq to detect genes differentially expressed just before the emergence of a pigmented bullseye (Fig. S9A, Supplementary Data 1 & 2). Using a four-fold cutoff score, we identified 938 and 343 genes preferentially expressed in the proximal or distal petal regions, respectively (Fig. S9B). Among those ~1300 genes, three MYBs attracted our attention: two were almost exclusively expressed in the proximal petal, which we named *HtBERRY1* and *HtBERRY2*, and one was preferentially expressed in the distal petal that we named *HtCREAM1*. Phylogenetic analysis of the MYB family revealed that the two *HtBERRY* genes belong to subgroup 6 (Fig. S10A, Supplementary Data 3 & 4), a clade that comprises the *A. thaliana* *PAP1/2* genes and other known regulators of petal pigment production in snapdragon, petunia, monkeyflowers, cotton, *Nigella*, *Clarkia* and orchids (Schwinn *et al.*, 2006; Albert *et al.*, 2011; Yuan *et al.*, 2014; Cai *et al.*, 2023; Yuan *et al.*, 2023; Martins *et al.*, 2017; Lin & Rausher, 2021a; Hsu *et al.*, 2015). *HtCREAM1* fell within a clade comprising cocoa and cotton orthologs but lacked *Arabidopsis* genes (Fig. S10B), making it difficult to predict its role.

qRT-PCR analysis in *H. trionum* CUBG confirmed the differential expression of *HtBERRY1/2* and *HtCREAM1* along the proximo-distal axis of the petal throughout development. *HtBERRY1* transcription was always higher in proximal tissue (≥ 15 -fold), increasing until stage 3 before reducing to almost negligible levels (Fig. 6A). *HtBERRY2* expression mirrored that of *HtBERRY1*, but its overall transcription level was lower compared to its paralog. *HtCREAM1* expression complemented *HtBERRY1/2* expression patterns such that its expression was always higher in the distal tissue at early stages (≥ 5 -fold in the distal region) but dropped off by stage 3 (Fig. 6A).

To investigate the functions of these MYB genes and establish their contribution to bullseye pigmentation, we generated *HtBERRY1* and *HtCREAM1* overexpression lines (Fig. 6B).

For both genes, we obtained three independent lines of *35S::HtBERRY1* and *35S::HtCREAM1*. The *35S::HtBERRY1* lines (*HtBERRY1* OE) produced flowers with ectopic pink pigmentation in the distal petal (Fig. 6C, Fig. S7). Their corollas were entirely colored as the distal region produced 30-fold more anthocyanin than wildtype (Fig. 6C), however a distinct bullseye pattern was still visible (Fig. 6B). This phenotype was consistent with the predicted role of *HBERRY1* as a positive regulator of anthocyanin production. We also measured higher anthocyanin levels in the rest of the petal and ovary (Fig. 6C), further validating *HtBERRY1*'s ability to promote anthocyanin production. Flavonols levels decreased by 30% and 50% in the proximal and boundary regions, respectively, when compared to wildtype. This could be due to competition between anthocyanin and flavonol production with the increase in anthocyanin biosynthesis resulting in less precursor available for flavonol synthesis. Flowers from the *35S::HtCREAM1* (*HtCREAM1* OE) lines displayed a paler bullseye (Fig. 6B, Fig. S7). Flavonoid quantification indicated this was due to reduced anthocyanin production in the proximal region (60% decrease compared to wildtype; Fig 6C). Interestingly, the lack of anthocyanin was not accompanied by increased flavonol accumulation. We found that flavonol content was reduced to levels resembling that of the *HtBERRY1* OE line (Fig. 6C) suggesting constitutive *HtCREAM1* overexpression dampens the entire flavonoid pathway.

To begin dissecting the gene regulatory network controlling pigment production in the *H. trionum* CUBG petal, we measured expression of the three MYB regulators and the structural genes *HtDFR1* and *HtFLS2* in both *HtBERRY1* and *HtCREAM1* OE lines (Fig. 6D). We found increased *HtFLS2* expression in the distal region of *HtCREAM1* OE petal (1.5-fold) suggesting *HtCREAM1* promotes *HtFLS2* transcription. In the *HtBERRY1* OE line, *HtBERRY1*, *HtBERRY2* and *HtDFR1* expression in the proximal petal increased by ~15-, six- and four-fold compared to wildtype, respectively. This indicates that *HtBERRY1* promotes anthocyanin production through the activation of *HtDFR1* transcription. Interestingly, both *HtCREAM1* and *HtFLS2* expression decreased (four- and 1.7-fold, respectively) in the distal petal when *HtBERRY1* was constitutively overexpressed. *HtBERRY1* could repress *HtCREAM1* expression and perhaps *HtFLS2* directly, or more likely indirectly via *HtBERRY1* activation of a yet-to-be-identified inhibitor of *HtCREAM1* (IOC) in the distal region. Collectively, our results allow us to propose a small gene regulatory network accounting for the restriction of anthocyanin production to the proximal petal (Fig. 6E).

Having identified some of the genetic interactions regulating pigment production in the petal of *H. trionum* CUBG, we hypothesized that one of those interactions or actors of this network was impaired in *H. richardsonii*, interfering with anthocyanin biosynthesis (Fig. 2C). First, the expression of *HrCREAM1* could be enhanced in *H. richardsonii*, repressing anthocyanin

production. Alternatively, *HrBERRY* gene expression could be reduced, yielding a smaller pigmented bullseye in *H. richardsonii*. To test these hypotheses, we isolated orthologs of *BERRY1/2* and *CREAM1* in *H. richardsonii* and measured their expression in developing petals of *H. richardsonii* (Fig. 7A, Fig. S11). The expression of *HrCREAM1* was three- to 76-fold lower, not higher, than *HtCREAM1* in both the proximal and distal petal, allowing us to reject the first hypothesis (Fig. 7A, Fig. S11). *HrBERRY1* expression was scarcely detected and *HrBERRY2* transcription was reduced by at least 2.5-fold in the proximal region, likely accounting for the lowered expression of *HrDFR1* in developing petals of *H. richardsonii* (Fig. 5A).

We also examined the coding sequence of each MYB regulator. We identified a single base pair substitution (A>T, 105bp) in the first exon of *HrCREAM1* but this was a synonymous mutation encoding a glycine residue at position 35. We found the coding sequence of *HrBERRY1* contained eleven SNPs compared to its ortholog in *H. trionum* CUBG (three substitutions in exon one, four in exon two and four in exon three) and a large 2063 bp insertion in the second intron (2445 bp vs. 382 bp in the second intron of *H. trionum* CUBG). Two of the SNPs within the exons introduced premature stop codons: a T>A substitution at position 17 bp and G>A substitution at position 159 bp. Thus, the *HrBERRY1* transcript would produce short, truncated proteins that are likely non-functional (Fig. 7B). Overall, our findings suggest a scenario where the low expression of *HrDFR1* is caused by a lack of *HrBERRY1* activity. If this is the case, the functional *H. trionum* CUBG and non-functional *H. richardsonii* *BERRY1* alleles should segregate with the bullseye size phenotype. To test this hypothesis, we examined allele segregation in the F2 population described in Fig. 3A. In a blind experiment, 38 F2 individuals were genotyped using allele-specific primers highlighted in Fig. 7B. We found that in all 38 F2 individuals, the genotype at the *BERRY1* locus matched the F2 phenotype (Fig. 7C): all *H. trionum* CUBG-like F2 plants were homozygous for the *HtBERRY1* allele, all *H. richardsonii*-like F2 individuals were homozygous for the *HrBERRY1* allele, and all hybrid-like F2 plants carried a copy of each allele. This strongly indicates that the reduced pigmented bullseye of *H. richardsonii* is due to the loss-of-function mutations in *BERRY1* or due to change in a closely linked locus. We examined the genome of *H. trionum* and found that *HtBERRY2* is relatively close to *HtBERRY1* (~25kb). However, *BERRY2* is expressed at much lower levels than *BERRY1* in both *H. trionum* and *H. richardsonii* (Fig. S11). Importantly, the two *BERRY2* paralogs orthologs are 98% identical (differing in 5 out of 249 amino acids) and *HrBERRY2* appears to code for a fully functional protein, unlike *HrBERRY1*. Hence, we concluded that the reduction in bullseye pigmentation is very likely caused by a non-functional *BERRY1* gene in *H. richardsonii*.

A loss of BERRY1 activity also accounts for absence of bullseye pigmentation in some populations of *H. verdcourtii*

Based on our phylogenetic reconstruction and distribution of bullseye features across the Trionum Complex (Fig. 1), the last common ancestor of those four *Hibiscus* species likely exhibited a pigmented bullseye. Reduction in bullseye size then occurred several times during the subsequent evolution of the group, including within *H. verdcourtii*, generating intraspecific variation (Fig. 1B).

Interestingly, we amplified *HvBERRY1* in populations 1, 2 and 4 who yield individuals with red bullseye flowers, but we failed to amplify this gene in populations 3 and 5 whose flowers are devoid of red pigmentation in the petal base (Fig. 1B). To determine if *BERRY1* was involved in *H. verdcourtii* pigmentation variation, we crossed red bullseye individuals from population 2 with plants from population 3 lacking a pigmented bullseye (Fig. 8A). The F1 hybrid produced flowers resembling those from population 2 (Fig. 8A), indicating that the pigmented bullseye trait is also dominant in *H. verdcourtii*. We found that bullseye color segregated in a 3:1 ratio in the F2 population as 37 and 11 individuals displayed a red and pale bullseye phenotype, respectively, leading us to conclude that pigmentation loss in *H. verdcourtii* was due to a change affecting a single locus. To test whether *HvBERRY1* could be the causative locus, we performed blind genotyping of 34 F2 individuals (Fig. 8B). Our results demonstrate that the presence/absence of *HvBERRY1* is a perfect predictor of the presence/absence of a red bullseye (Fig. 8B), supporting the idea that the loss of *HvBERRY1* accounts for the loss of petal pigmentation in *H. verdcourtii* (Fig. 8C, Fig. S12).

Bumblebee preference assays favor larger pigmented bullseyes

The fact that sister species and distinct populations from the same species exhibit pigmented bullseyes of contrasting sizes raises the interesting possibility that a change in pattern dimension could influence pollinator selection, reduce genetic exchange between populations and thus play a role in reproductive isolation and speciation. We previously showed that buff-tailed bumblebees (*Bombus terrestris* v. *audax*) can discriminate between 3D-printed plastic discs mimicking the proportions of *H. trionum* CUBG and *H. richardsonii* bullseyes and have an innate preference for discs with the larger bullseye (Riglet *et al.*, 2024). Real flowers, however, are complex structures with differences in shape, scent, and temperature, that along with pigmentation variation may also influence pollinator responses. To test bumblebee behavior in a more realistic setting, we conducted preference test experiments using real flowers from each species. We found that *B. terrestris* chose to land on *H. trionum* CUBG flowers over *H. richardsonii*

85% of the time (n= 40; 34 bees selected *H. trionum* CUBG vs. six bees selected *H. richardsonii*, $p = 3.51 \times 10^{-7}$, one-sample t-test), indicating that the preference for the larger bullseye stands in a real-flower context.

Discussion

Resolving the phylogenetic history of the Trionum Complex sheds light on bullseye pattern evolution

Relationships within the *Hibiscus* genus are historically difficult to resolve (Pfeil & Crisp, 2005; Hanes *et al.*, 2024). Here, we demonstrate how phylogenomic approaches can help illuminate the evolutionary history of previously recalcitrant groups like the Trionum Complex. Our phylogenetic reconstruction divided the Trionum Complex into four distinct clades and positioned *H. richardsonii* as sister species to *H. trionum*. This finding corroborates previous morphological and ecological studies where the status of *H. richardsonii* as a separate species or a variant of *H. trionum* was debated (de Lange, 2008; Craven *et al.*, 2011).

Resolving the relationships within the Trionum Complex also enabled us to illuminate the evolution of petal patterning. We found that cell shape distribution was fixed across the complex, as conical cells were exclusively located in the distal region while tabular cells were only present in the proximal petal. Unlike the other members of the complex, *H. verdcourtii* lacked striations in the proximal petal region regardless of the presence of a pigmented bullseye (Fig. S2). This indicates the genetic mechanisms controlling cell shape, cuticle texture and pigmentation are distinct and the ability to produce a striated cuticle was gained after the divergence of *H. verdcourtii* or lost along the lineage leading to *H. verdcourtii*. Our analysis established pigmentation as the most labile feature of epidermal petal cells, as accessions with a reduced pigmented bullseye did not cluster together in the species tree. Such versatility in petal pigmentation suggests it is an important feature undergoing selection and contributes to bullseye evolution.

HtBERRY1 plays an important role in the production of a pigmented bullseye in *Hibiscus*

In *Dianthus caryophyllus*, *Rosa multiflora* and *Camellia japonica* flowers, expression of *DFR* and *FLS* homologs correlates with red and white flowers, respectively (Luo *et al.*, 2016). In *Clarkia gracilis*, *DFR* expression patterns coincide with petal spot formation during development (Martins *et al.*, 2013). Here, we also demonstrate compartment-specific expression of *DFR* and *FLS* prior to the emergence of pigmentation patterns at very early stages of development (stage 1). Our results are consistent with recently published transcriptome data for later stages of petal

development (stage 3-4) in *H. trionum* (Koshimizu *et al.*, 2023). Our findings indicate strong preferential expression of *HtDFR1* in the proximal petal region is central to restricting anthocyanin production to the petal base. Other flavonoid pathway genes (chalcone synthases, flavonoid 3'-hydroxylases, flavonoid 3'5'-hydroxylases and anthocyanidin synthases) are either uniformly present across the petal or slightly higher in the distal domain (Fig. S13). Our results also argue for functional divergence between the three *DFR* genes and between the three *FLS* paralogs. Functional divergence due to changes in enzymatic capabilities was reported for *FLS* paralogs in rapeseed (*Brassica napus*) and *Arabidopsis* (Owens *et al.*, 2008; Schilbert *et al.*, 2021) and for *DFR* homologs in morning glory (*Ipomoea* sp.; Des Marais & Rausher, 2008). In *Hibiscus trionum*, such diversification involved divergence of expression patterns between paralogs but whether this was also accompanied by changes in coding sequences affecting the biochemical properties of the enzymes they code for remains to be elucidated.

The restriction of *HtDFR1* activity to the petal base is due in part to the localized expression of one of its activators, *HtBERRY1*, to the proximal petal region as constitutive overexpression of *HtBERRY1* is sufficient to trigger ectopic expression of *HtDFR1* and yield anthocyanin production in the distal region. We found that the close paralog of *HtBERRY1*, *HtBERRY2*, is also preferentially expressed in the proximal petal region, although at much lower levels than *HtBERRY1*. Both corresponding proteins are highly similar (82.9% identity, 94.8% similarity) suggesting that *HtBERRY2* could also participate in *HtDFR1* regulation and pigment production. It will be interesting to test this hypothesis in the future and determine whether the residual expression of *HrDFR1* in absence of a functional *HrBERRY1* is due to the activity of *HrBERRY2*. Interestingly, the coloration obtained in the distal domain in *HtBERRY1* OE line was not as intense as in the proximal domain and anthocyanin levels remained lower (Fig. 4D-F). This could be due to several factors. First, the lack of or restricted availability of a *HtBERRY1* partner could limit its ability to activate *HtDFR1* expression in the distal region. Alternatively, other TFs contributing to *HtDFR1* expression could be absent in the distal region. Secondly, differences in the chromatin landscape at the *HtDFR1* promoter could impair effective binding of *HtBERRY1* to *cis*-regulatory elements. However, our expression data (Fig. 6D, Fig. S14) suggests that *HtDFR1* is expressed at similar levels in both proximal and distal regions in the *HtBERRY1* OE line and rules out both hypotheses. Instead, limited expression of enzymes acting downstream of *HtDFR1* in the same region could account for dampened anthocyanin production. We found that apart from *ANS*, other anthocyanin biosynthesis genes were uniformly present throughout the developing petal primordia (Fig. S13). *HtANSa* is expressed at high levels in both petal domains but slightly higher in the proximal domain (less than 2-fold difference). Therefore, it is possible

that increasing *HtANSa* expression in the distal petal domain could boost color intensity, but it is unlikely a limiting factor for anthocyanin production when *HtBERRY1* is overexpressed. Finally, competition with *HtFLS2* for precursor availability could prevent *HtDFR1* from synthesizing anthocyanin effectively in the distal petal region. The fact that *HtCREAM1* and *HtFLS2* are both downregulated in the distal region of *HtBERRY1* OE makes it unlikely that competition between *HtDFR1* and *HtFLS2* is the main cause of the paler hue of the distal domain of *HtBERRY1* OE line. Taken together, our results suggest that *HtBERRY1* promotes pigment production by at least two different routes. First, by inducing the expression of *HtDFR1*, and second, by reducing the transcription of *HtCREAM1* and *HtFLS2*, limiting possible competition between *HtDFR1* and *HtFLS2* in the proximal region and thus favoring anthocyanin synthesis over flavonol production in the center of the bullseye.

Whether *HtBERRY1* directly represses *HtCREAM1* and/or *HtFLS2* transcription remains to be tested. If this is the case, *HtBERRY1* must be able to act as both an activator and a repressor by forming different MBW complexes involving different bHLH partners. The R2R3 MYB subfamily, to which *HtBERRY1* belongs, is the largest of the four MYB families and can be characterized by two adjacent imperfect MYB repeats (Wu *et al.*, 2022). Aside from other functions, over half of R2R3 MYBs are involved in the regulation of specialized metabolism such as phenylpropanoid biosynthesis which results in end products such as flavonoids. The importance of MYB regulation in the flavonoid pathway has been highlighted across several species including *Petunia hybrida* and *Antirrhinum majus*. PhDPL, PhPHZ, AmROSEA1/2 and AmVENOSA all induce expression of *DFR* orthologs (Schwinn *et al.*, 2006; Albert *et al.*, 2011b; Shang *et al.*, 2011). However, a dual role for a single R2R3 MYB as a transcriptional activator and repressor has not been previously reported. Alternatively, *HtBERRY1* could act indirectly, by activating the expression of a repressor that downregulates *HtCREAM1* and *HtFLS2*. Overall, our results highlight that *HtBERRY1* plays an important role in controlling anthocyanin production across the petal surface during bullseye formation.

Studies in a range of species have shown that the spatial control of *MYB* genes is central to the emergence of pigmentation patterns in many angiosperms (Ding *et al.*, 2023). Unfortunately, little is known regarding the upstream regulators of R2R3 MYBs during flower development. In snapdragon, anthocyanin production is controlled by the R2R3 MYB *VENOSA* and its bHLH partner *DELILA*. *DELILA* is expressed throughout the petal epidermis while *VENOSA* is only transcribed around the vasculature such that pigment production is limited to the epidermal cells directly above the vasculature where both *VENOSA* and *DELILA* are present (Shang *et al.*, 2011). A signal emerging from differentiating vascular cells could induce local

VENOSA expression or alternatively, the same upstream signal that specifies vasculature formation (e.g. auxin signaling) could in parallel promote VENOSA transcription. In the Trionum Complex, the bullseye pattern exhibits strong polarity along the petal proximo-distal axis, reflecting the differential expression of *HtBERRY1*. Hence, cues that specify and pattern the proximo-distal axis of lateral organs are likely to act as upstream regulators of *HtBERRY1* expression. Interestingly, the venation patterning in snapdragon is prominent in the distal corolla but largely lacking in the proximal region (Shang *et al.*, 2011). This suggests that the ability of an epidermal cell to activate anthocyanin production is dependent on its position both relative to the vasculature and along the petal length. Therefore, these positional signals are not mutually exclusive and can be combined to enable cells to perceive and integrate spatial information from multiple inputs. The processes that drive proximo-distal polarity establishment in petal primordia remain elusive (Salvi *et al.*, 2025) but future studies aiming at uncovering the mechanisms that spatially restrict *HtBERRY1* transcription during petal development represent a promising avenue to shed light on these obscure processes.

Independent modifications of a single locus lead to repeated reductions in bullseye dimensions

The occurrence of forms closely resembling each other across the tree of life supports the notion that similar features repeatedly arose independently through evolution. However, the molecular basis supporting phenotypical convergence is often unclear. Here, we used reduction in petal pattern dimensions across a small group of *Hibiscus*, the Trionum Complex, to unravel the molecular underpinnings of reduction in bullseye pigmentation. Our results uncovered a case of replicated evolution, where the size of the petal pigmented area shrunk several times independently across the Trionum Complex. In at least three cases, this bullseye reduction was due to distinct changes affecting the same MYB-encoding locus, *BERRY1*, triggering either reduction in expression and premature stop codons of *HrBERRY1* in *H. richardsonii*, or complete gene loss of *HvBERRY1* from the genome of two populations of *H. verdcourtii*. Occurrences of replicated evolution driving the pigmentation changes have been documented in both plant and animal species. Geographically isolated populations of agouti-colored beach mice share an independently selected allele contributing to the cryptic coloration that assists in protection against predators. (Wooldridge *et al.*, 2022). The transition to red flower color in *Ipomea* and the shift from blue to red flowers of Andean *Ipomoea* have been observed multiple times (Streisfeld & Rausher, 2009; Smith & Rausher, 2011). In both cases, the change in flower color was accompanied by

mutations affecting flavonoid hydroxylases that redirect the anthocyanin biosynthesis pathway toward the production of pelargonidin derivatives (red pigments).

Findings by Wheeler *et al.*, 2022 suggest regulators of the flavonoid pathway evolve faster than structural genes. In this study, we demonstrate clear instances of replicated evolution that impact petal patterning through independent modifications to the BERRY1 transcription factor, rather than through changes affecting *DFR* or *FLS* loci. To date, all studies examining floral pigment pattern production highlight that restriction of MBW complex activity to specific petal regions is central to the production of a pigmented motif on the petal surface, and this is often achieved by precisely controlling MYB expression (Albert *et al.*, 2014). Consistently, changes in petal patterning during evolution often rely on genetic variation of MYB TFs. In *M. lewisii*, a two-component activator-inhibitor system involving two MYB TFs regulates pigmentation spots on *Mimulus* corollas. Genetic variation in *RTO* encoding for the MYB repressor component accounts for the presence of both spotted and red-tongue petal pattern phenotypes in natural populations (Ding *et al.*, 2020). R2R3 MYBs, rather than their bHLH partners, are the main drivers of localized anthocyanin synthesis in *Antirrhinum*, petunia and maize (Piazza *et al.*, 2002; Schwinn *et al.*, 2006; Albert *et al.*, 2011). Furthermore, variation affecting the expression or functionality of subgroup 6 MYBs in *Clarkia* is sufficient to explain petal pattern variation across the genus (Lin & Rausher, 2021a; Lin & Rausher, 2021b). Why MYBs rather than bHLHs tend to limit the production of pigment to subregions of the epidermis is unclear. It could reflect fundamental differences in the transcriptional regulation of both families, or instead indicate that the MYB components are less likely to have pleiotropic effects than their bHLH partner. Together, these findings underscore that evolution often recycles molecular mechanisms to generate petal diversity, revealing that the investigation of regulatory networks provides critical insights into how repeated evolutionary outcomes are achieved.

Our findings also suggest that changes in pattern dimensions may have contributed to the diversification of the Trionum Complex by impacting plant-pollinator interactions as bumblebees exhibit a strong innate preference for the larger bullseye of *H. trionum* CUBG over the reduced one of its sister species *H. richardsonii*. The geographical distributions of *H. trionum* and *H. richardsonii* overlap in the North Island of New Zealand (Craven *et al.* 2011) and we have shown the two species are cross-fertile when hand-pollinated in greenhouse conditions (Fig. 3A). In the future, it will be important to determine whether hybridization mediated by pollinating insects also occurs in the wild or whether the difference in bullseye size, combined with other factors, favors reproductive isolation.

Studies in silverweed and sunflowers suggest that floral bullseye patterns can also provide protection against UV damage and desiccation (Koski *et al.*, 2015; Todesco *et al.*, 2022). Whether the evolutionary history of the *Hibiscus* bullseye was shaped by environmental factors is not yet known but adaptations to abiotic cues could further explain the variation in pigmented bullseyes in natural populations. Future investigations within the natural habitat of these *Hibiscus* species will provide valuable information such as the identification of species-specific pollinators, if any, and the frequency of self-fertilization events associated with possible low pollinator availability.

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Author Contributions

E.M. conceptualized and designed the project. M.T.S.Y., A.L.M.F., V.T., S.Z. and L.R. performed experiments and data analysis. M.T.S.Y. validated experiments and performed statistical analysis. J.F.W. analyzed the transcriptome data and performed the phylogenomic analysis. M.T.S.Y. and E.M. prepared figures and wrote the manuscript, with input from all authors.

Competing interests

None declared.

Data, Code and Material Availability

The coding and genomic sequences of all *H. trionum* genes mentioned in this study can be accessed via the genome of *H. trionum* available on GenBank

(https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_030270665.1/) using the HRI_ reference numbers provided in the Material & Methods sections. The coding sequences of *HrBERRY1*, *HrDFR1*, *HrFLS2* and *HvBERRY1* have been deposited in GenBank under the accession number PQ456077, PQ450317, PQ450318 and PQ450319, respectively. Transcriptomic data associated with the phylogenomic analysis of the Trionum complex (accession numbers SAMN47801648 to SAMN47801669) and the comparative analysis of gene expression between proximal and distal petal regions of *H. trionum* stage 1 petal (accession numbers SAMN47793371 to SAMN47793390) have been deposited on GenBank SRA (<https://www.ncbi.nlm.nih.gov/sra>). The amino acid sequences used for assessing the placement of HtBERRY1, HtBERRY2 and HtCREAM1 in the MYB family phylogeny are provided in Supplementary Data 3. The algorithms and codes used for phylogenomic analyses, RNA-seq data analysis and differential gene expression analysis have been published elsewhere, as indicated in the references provided in the Material & Methods section.

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1210

1212 **Supporting Information**

1214 **Fig. S1.** Geographical location of *Hibiscus* species/populations used in this study.

1216 **Fig. S2.** Cell shape and texture across the adaxial petal epidermis of the 11 different accessions from the Trionum Complex used in this study.

1218 **Fig. S3.** Comparison of pigmentation throughout petal development in (A) *H. trionum* CUBG and (B) *H. richardsonii*.

Fig. S4. Light microscopy images of *H. trionum* CUBG and *H. richardsonii* boundary cell types.

1220 **Fig. S5.** Expression of *HtDFR2*, *HtDFR3*, *HtFLS1* and *HtFLS2* throughout flower development in proximal and distal petal tissue of *H. trionum* CUBG.

1222 **Fig. S6.** Normalized read counts from RNAseq data for *HtDFR* homologs and *HtFLS* homologs in proximal and distal petal tissue of *H. trionum* CUBG at stage 1 and stage 2.

1224 **Fig. S7.** Transgene expression level in various *HtDFR1*, *HtBERRY1* and *HtCREAM1* *H. trionum* CUBG lines.

1226 **Fig. S8.** Expression of *HrDFR1* and *HrFLS2* throughout flower development in proximal and distal petal tissue of *H. richardsonii*.

1228 **Fig. S9.** Identification of differentially expressed genes between the proximal and distal regions of stage 1 petal primordia in *H. trionum* CUBG.

1230 **Fig. S10.** Placement of *HtBERRY1*, *HtBERRY2* and *HtCREAM1* within the MYB family phylogenetic tree.

1232 **Fig. S11.** Expression of *HrBERRY1*, *HrBERRY2* and *HrCREAM1* throughout development in proximal and distal petal tissue of *H. richardsonii*.

1234 **Fig. S12.** Predicted gene structure of *HvBERRY1* in *H. verdcourtii* with additional allele-specific primers.

1236 **Fig. S13.** Normalized read counts for flavonoid-related structural genes, subgroup IIIf bHLH and *TTG1/LWD40* homologs in *H. trionum* CUBG Stage 1 and 2 petal tissue.

1238 **Fig. S14.** Expression of flavonoid-related structural and MYB transcription factors in distal or proximal regions of wildtype and *HtBERRY1* OE or *HtCREAM1* OE *H. trionum* CUBG lines.

1240

Table S1. *Hibiscus* species used in this study.

1242 **Table S2.** *H. trionum* CUBG transgenic lines generated in this study.

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1244 **Table S4.** Plant expression vectors generated for this study.

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The following four Supplementary Data files are provided as separate files:

1250

Supplementary Data 1. Assembled transcripts from *H. trionum*, stage 1 petals. Data related to Fig S9.

1252

Supplementary Data 2. Differential gene expression analysis between proximal and distal regions of stage 1 *H. trionum* petal primordia. Data related to Fig S9.

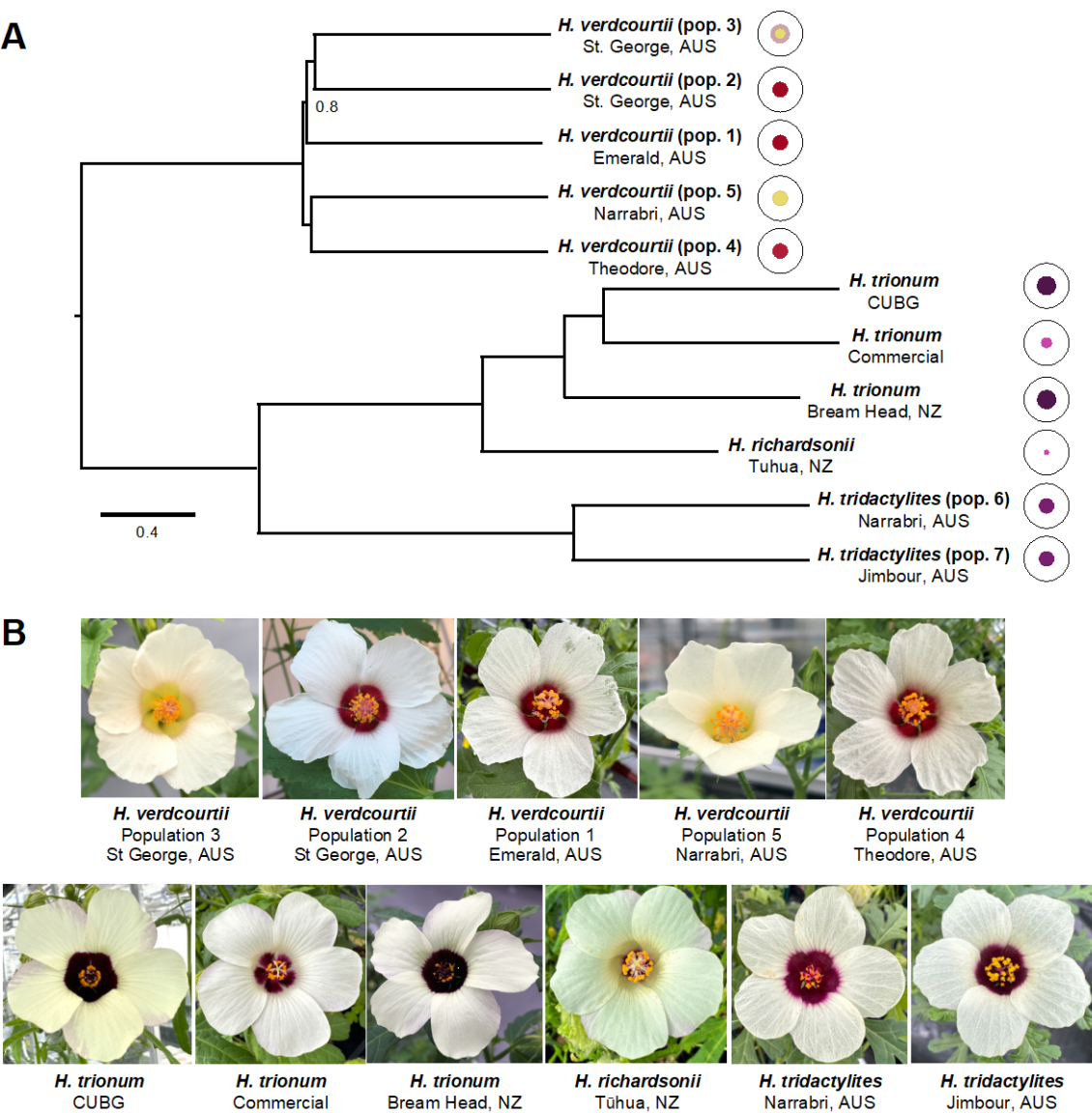
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Supplementary Data 3. Amino acid sequences of the 483 MYB sequences used to assess the phylogenetic placement of HtBERRY1, HtBERRY2 and HtCREAM1. Data related to Fig S10.

1256

Supplementary Data 4. Complete MYB family tree generated using the 483 sequences from Supplementary Data 3. Data related to Fig S10.

1258



1262

1264 **Fig. 1.** Phylogenetic relationships between Trionum Complex species. **(A)** Phylogenetic tree highlighting

1266 relationships between species and populations. Unless stated, all nodes are perfectly supported. Branch

1268 lengths are shown in coalescent units. Each species is represented by a cartoon depicting bullseye size

and color. **(B)** Representative flowers of each species/populations emphasizing variation in bullseye size

and color.

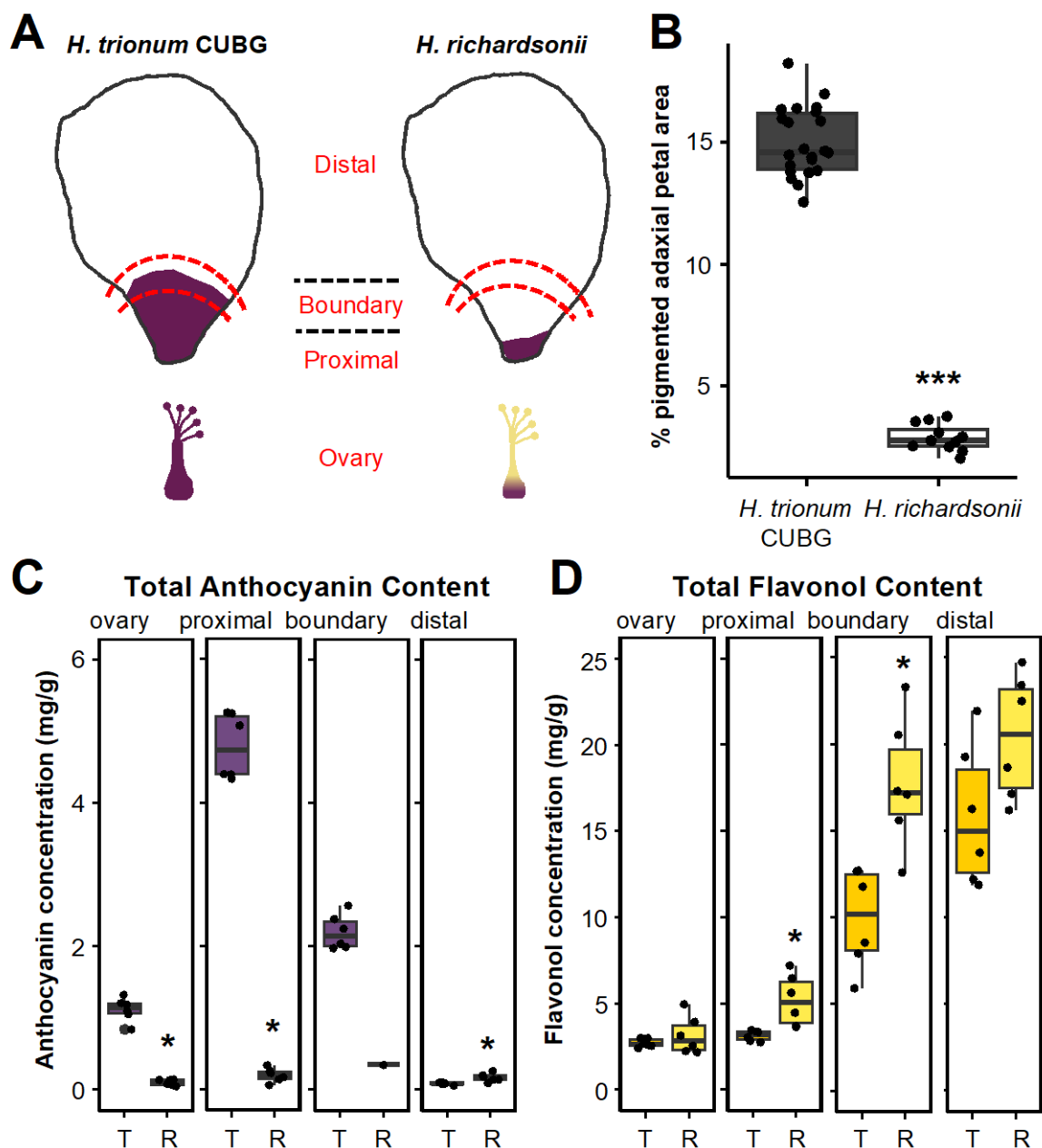


Fig. 2. Morphological differences in petal patterning between *H. trionum* CUBG and *H. richardsonii*. **(A)** Schematic depicting petal regions and ovary of a stage 5 flower. For pigment extraction assays, petals were dissected into ovary, proximal (tabular striated cells), boundary (tabular smooth cells) and distal (conical smooth cells) regions, demarcated by the red dashed lines. **(B)** The size of the pigmented bullseye was measured as a percentage of the pigmented region over total petal area. N = 22 and n = 12 flowers for *H. trionum* CUBG and *H. richardsonii*, respectively. 3 petals were measured per flower. *** p<0.001 (Student's t-test). **(C)** Anthocyanin and **(D)** flavonol quantification of *H. trionum* CUBG (T) and *H. richardsonii* (R) flowers. Stage 5 flowers were dissected into 4 regions as highlighted in (A). 6 flowers were analyzed per

species. Each data point represents the average of three technical replicates. * denotes $p < 0.05$ (Welch's or Student's t-test) of pairwise comparisons made between species in the same region.

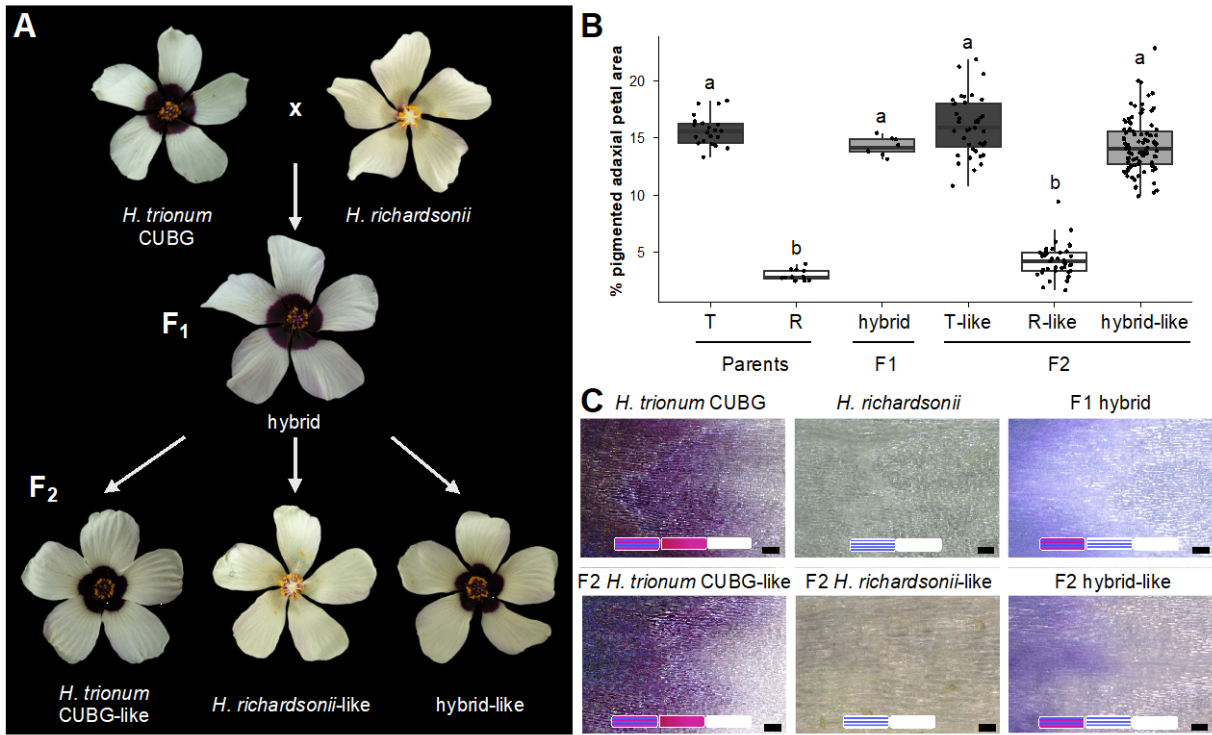


Fig. 3. Characterization of *H. trionum* CUBG, *H. richardsonii*, F1 and F2 phenotypes. (A) F1 and F2 flower phenotypes of *H. trionum* CUBG crossed with *H. richardsonii*. The F1 hybrid produced flowers with a pigmented bullseye. The F2 selfing population yielded three flower phenotypes that recapitulate the two parental and F1 hybrid flower morphologies. (B) Measurement of the pigmented bullseye size reflects F2 phenotypes that match parental (*H. trionum* CUBG = T, *H. richardsonii* = R) and F1 hybrid phenotypes. Different letters indicate statistically significant differences (Tukey's HSD, $p < 0.0004$). (C) Imaging of the boundary region of petals from the six flower morphologies from (A). The bullseye boundary region is characterized by the presence of smooth, pigmented, tabular cells in *H. trionum* CUBG and F2 *H. trionum* CUBG-like. Such cells are lacking in the bullseye boundary region of *H. richardsonii* and F2 *H. richardsonii*-like and striated, unpigmented, tabular cells are present instead. The bullseye boundary region of both F1 and F2 hybrid-like flowers also lack smooth, pigmented, tabular cells but contain both pigmented and non-pigmented striated cells. Cell types present are summarized by symbols at the bottom of each microscopy image: striated, pigmented, tabular cells = striped purple rectangle; smooth, pigmented, tabular cells = solid purple rectangle; striated, non-pigmented, tabular cells = striped white rectangle; smooth, non-pigmented, tabular cells = solid white rectangle. Scale bar = 100 μ m. Magnified images illustrating each cell type are presented in Fig. S4.

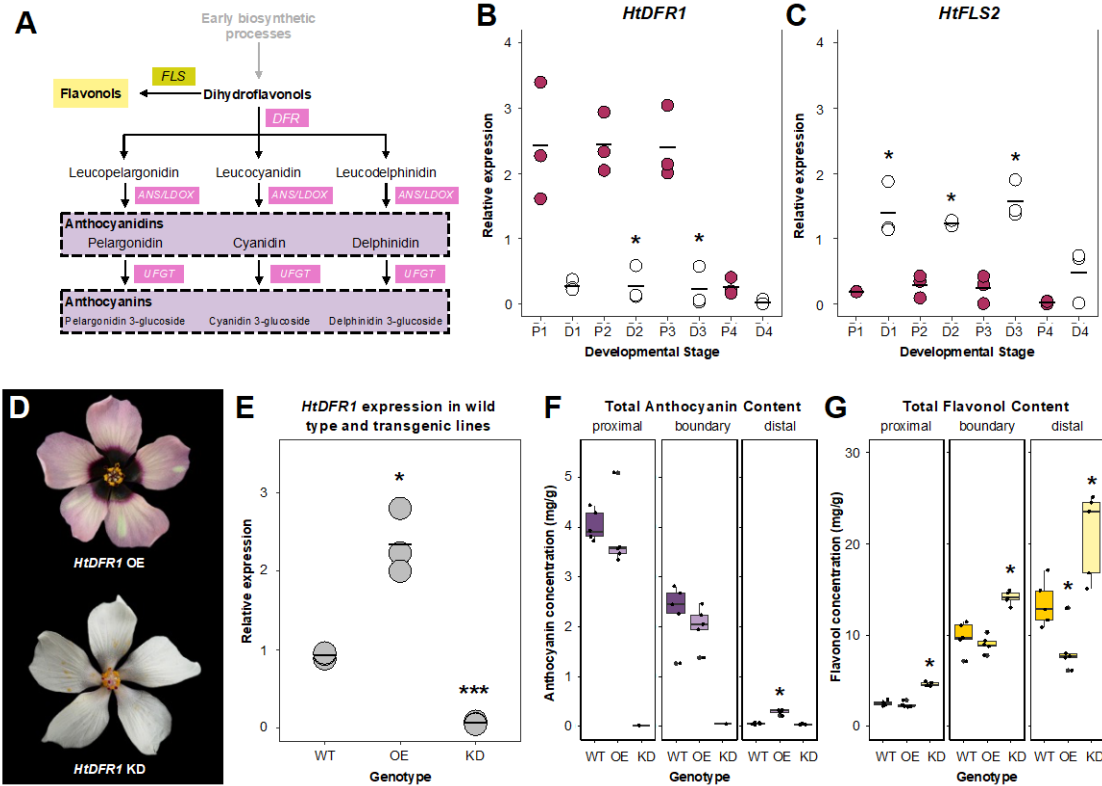


Fig. 4. Bullseye pigmentation reflects patterning of *DFR* and *FLS* expression across the petal primordia in *H. trionum* CUBG. **(A)** Simplified flavonoid biosynthesis pathway highlighting dihydroflavonols as a shared common precursor for flavonol and anthocyanin production via *FLS* and *DFR* enzyme activity, respectively. Expression of **(B)** *HtDFR1* and **(C)** *HtFLS2* throughout development in proximal (P1 to P4) and distal (D1 to D4) petal tissue of *H. trionum* CUBG. * denotes p<0.05 (Student's or Welch's t-test) of pairwise comparisons made between proximal and distal tissue of the same stage. **(D)** Phenotypes of two *35S::HtDFR1* lines. Representative images of other independent transgenic lines are provided in Fig. S7. **(E)** Quantification of *HtDFR1* expression in distal tissue of *35S::HtDFR1* OE (overexpression) and proximal tissue of KD (knockdown) lines. * denotes p<0.05 and *** denotes p<0.01 (Student's or Welch's t-test) for comparisons of *HtDFR1* against equivalent petal region in WT. The expression of *HtBERRY1* in the proximal tissue of WT is given on the left for reference. The expression of *HtBERRY1* in the distal tissue of WT is extremely low (~0.009 average relative expression compared to *HtACTIN1* and thus is not represented on this plot). **(F)** Anthocyanin and **(G)** flavonol quantification in both types of *35S::HtDFR1* lines. Stage 5 petals were dissected into three regions as highlighted in Fig. 2A. 5 flowers were analyzed per line. Each data point represents the average of three technical replicates. * denotes p<0.05 (Student's, Wilcoxon's or Welch's t-test) of pairwise comparisons made between *HtDFR1* OE vs. WT and *HtDFR1* KD vs. WT tissue of the same region. Comparisons were not made in regions that had fewer than three data points as some replicates yielded non-detectable amounts of anthocyanin. For expression data in **(B)**, **(C)** and **(E)**, three biological replicates were extracted per stage and each data point indicates an average of

three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative to *HtACTIN1* expression levels.

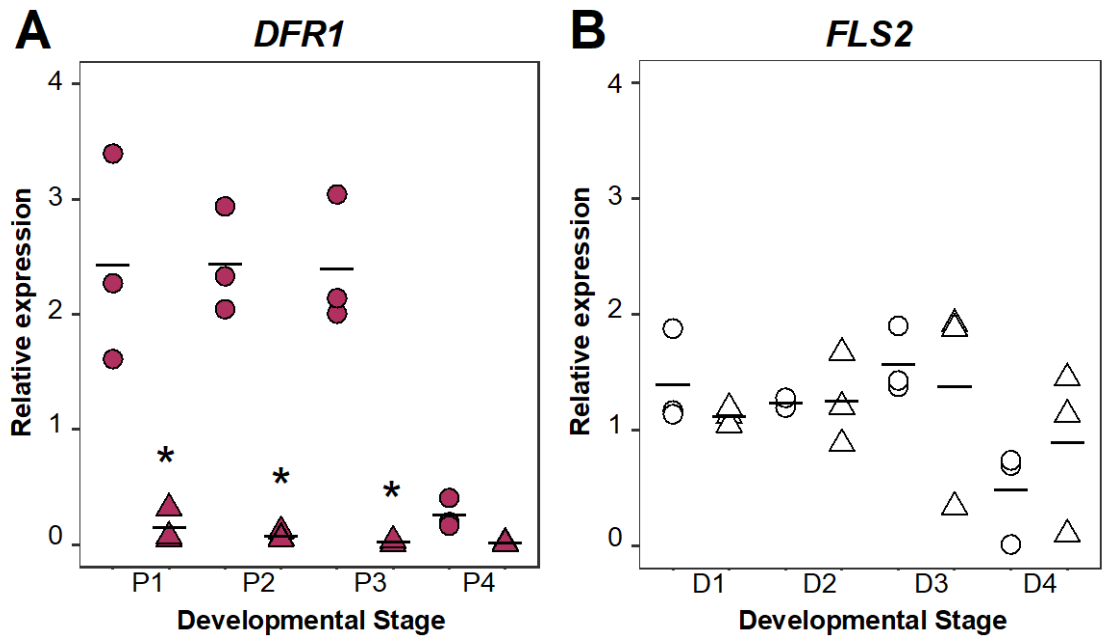


Fig. 5. Comparison of expression between *H. trionum* CUBG (circles) and *H. richardsonii* (triangles) of (A) *DFR1* orthologs in proximal petal tissue (P1 to P4) and (B) *FLS2* orthologs in distal petal tissue (D1 to D4). Three biological replicates were extracted per stage. Each data point indicates an average of three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative to *HtACTIN1* (*H. trionum* CUBG) or *HrACTIN1* (*H. richardsonii*) expression levels. * denotes p < 0.05 (Student's t-test) of pairwise comparisons made between the same region of *H. trionum* CUBG and *H. richardsonii*.

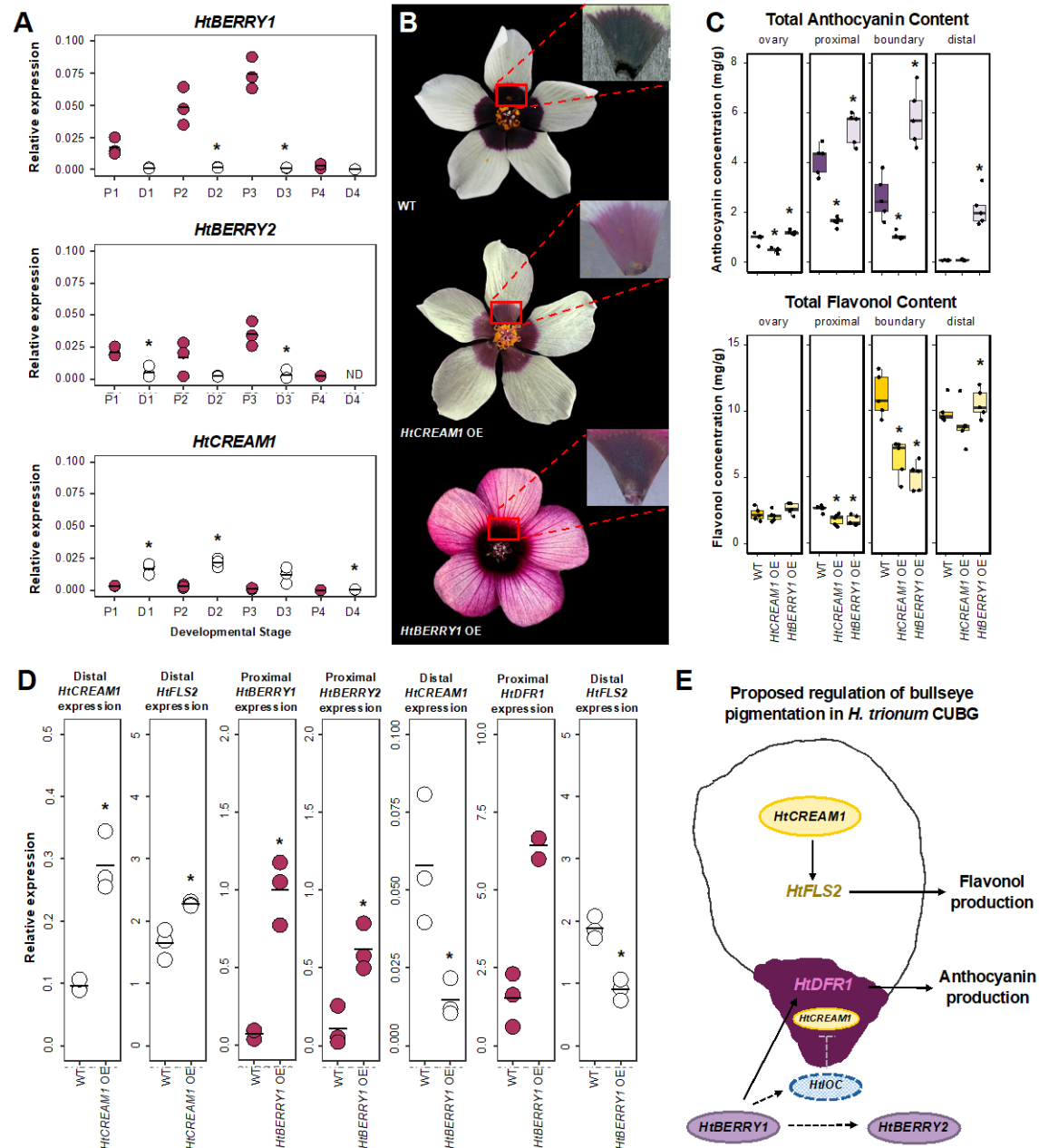


Fig. 6. Bullseye pigmentation reflects patterning of *HtBERRY1/2* and *HtCREAM1* expression across the petal primordia in *H. trionum* CUBG. **(A)** Expression of *HtBERRY1*, *HtBERRY2* and *HtCREAM1* MYB TFs throughout development in proximal (P1 to P4) and distal (D1 to D4) petal tissue of *H. trionum* CUBG. ND = non-detectable expression. * denotes $p < 0.05$ (Student's or Welch's t-test) pairwise comparisons made between proximal and distal tissue of the same stage. **(B)** Overexpression phenotypes of 35S::*HtBERRY1* and 35S::*HtCREAM1*. Representative images of other independent transgenic lines are provided in Fig. S7. **(C)** Anthocyanin and flavonol quantification in 35S::*HtBERRY1* and 35S::*HtCREAM1* lines. Stage 5 flowers were dissected into 4 regions as highlighted in Fig. 2A. 5 flowers were analyzed per line. Each data point represents the average of three technical replicates. * denotes $p < 0.05$ (Student's, Wilcoxon's or Welch's

t-test) of pairwise comparisons made between *HtCREAM1* OE vs. WT and *HtBERRY1* OE vs. WT tissue of the same region. (D) Expression of flavonoid-related structural genes and MYB transcription factors in distal or proximal regions of the overexpression lines. * denotes $p < 0.05$ (Student's or Welch's t-test) of pairwise comparisons made between the same region against WT and the transgenic line in (C) and (D). (E) Proposed regulation of bullseye pigmentation in *H. trionum* CUBG. IOC = Inhibitor of CREAM1. For expression data in (A) and (D), three biological replicates were extracted per stage and each data point indicates an average of three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative to *HtACTIN1* expression levels.

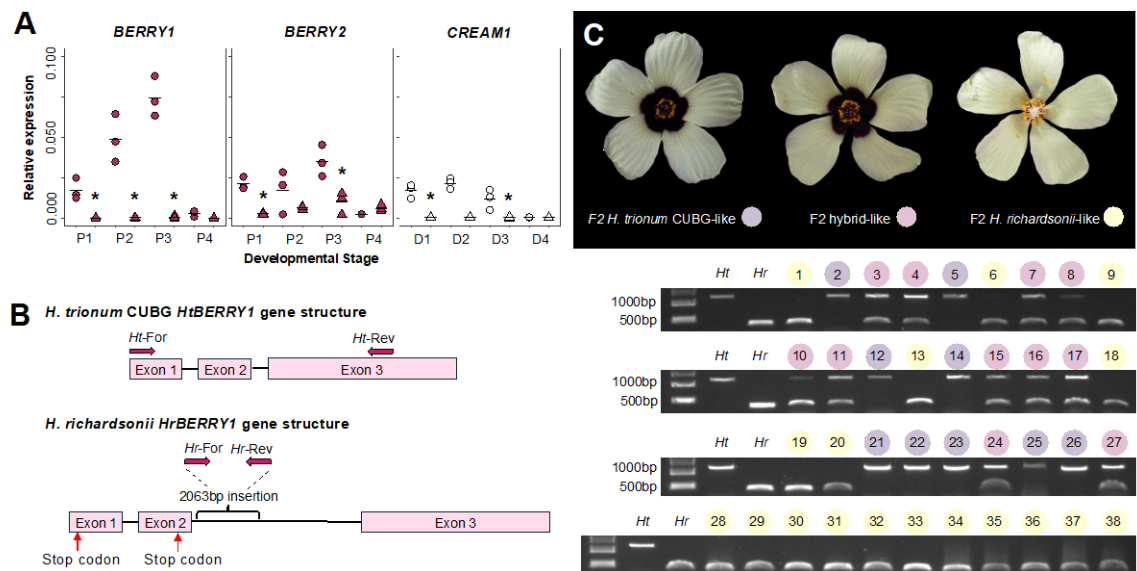


Fig. 7. Changes at the *HrBERRY1* locus are responsible for bullseye reduction in *H. richardsonii*. Comparison of expression between *H. trionum* CUBG (circles) and *H. richardsonii* (triangles) of *BERRY1* and *BERRY2* in proximal petal tissue (P1 to P4) and *CREAM1* in distal petal tissue (D1 to D4). Each data point indicates an average of three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative to *HtACTIN1* (*H. trionum* CUBG) or *HrACTIN1* (*H. richardsonii*) expression levels. * denotes $p < 0.05$ (Student's t-test) of pairwise comparisons made between the same region of *H. trionum* CUBG and *H. richardsonii*. (B) Sequencing of *HrBERRY1* revealed two premature stop codons in exons one and two and a 2063bp insertion in intron two. Allele-specific primers used in Fig. 7C are indicated above each gene structure. (C) Genotyping of the *H. trionum* CUBG (*Ht*) x *H. richardsonii* (*Hr*) F2 population. Blind genotyping of 27 F2 individuals using allele-specific primers revealed that the non-functional *HrBERRY1* allele segregates with the *H. richardsonii*-like F2 phenotype. An additional 11 F2 individuals with the *H. richardsonii*-like phenotype were included to further test the segregation hypothesis.

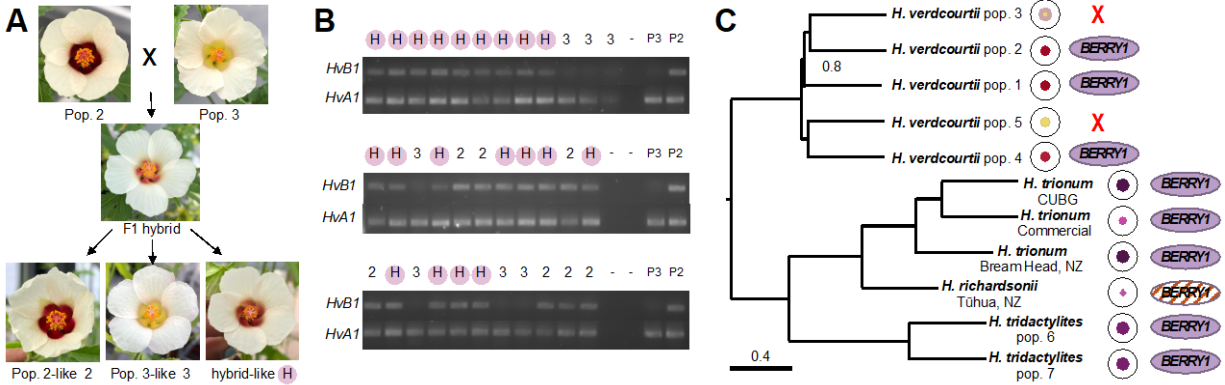


Fig. 8. Presence of *HvBERRY1* correlates with pigmented bullseyes in *H. verdcourtii*. (A) F1 and F2 flower phenotypes of *H. verdcourtii* pop. 2 crossed with *H. verdcourtii* pop. 3. The F1 hybrid produced a flower with a pale pigmented bullseye. The F2 selfing population yielded three flower phenotypes that recapitulated the two parental and hybrid flower morphologies. (B) Genotyping of the *H. verdcourtii* pop. 2 x *H. verdcourtii* pop. 3 F2 population. Blind genotyping of 34 F2 individuals using *HvBERRY1*-specific primers revealed that the presence of *HvBERRY1* segregates with a pigmented bullseye. Letters and number above gel lane provide the flower phenotype corresponding to each genotyped F2 individual: H = hybrid-like flower, 2 = *H. verdcourtii* pop. 2-like flower and 3 = *H. verdcourtii* pop. 3-like flower. The parental lines (P2 = *H. verdcourtii* pop. 2 and P3 = *H. verdcourtii* pop. 3) and a no template control (-) were included as controls. PCR results using different primer pairs are given in Fig. S12 (C) Multiple independent losses of the pigmented bullseye in the *Hibiscus trionum* Complex are a result of modifications to the *BERRY1* locus. In the *H. verdcourtii* clade, loss of *HvBERRY1* is correlated with a flower phenotype lacking a pigmented bullseye. In *H. richardsonii*, *HrBERRY1* transcripts are detected at very low level and several mutations within the coding region likely render the corresponding protein nonfunctional. Numbers next to nodes indicate support values. Branch lengths are shown in coalescent units.

1384 **New *Phytologist* Supporting Information**

1386 Article title: The genetic basis of replicated bullseye pattern reduction across the Trionum
Complex

1388

Authors: May T. S. Yeo^{1,2}, Alice L. M. Fairnie^{1,3}, Valentina Travaglia^{1,4}, Joseph F. Walker^{1,5}, Lucie
1390 Riglet¹, Selin Zeyrek^{1,2}, and Edwige Moyroud^{1,2,*}

1392 Article acceptance date: 2 April 2025

1394

This following Supporting Information is available for this article:

1396

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(B) *H. richardsonii*.

1402 **Fig. S4** Light microscopy images of *H. trionum* CUBG and *H. richardsonii* boundary cell types

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Fig. S6 Normalized read counts from RNAseq data for *HtDFR* homologs and *HtFLS* homologs in
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Fig. S7 Transgene expression level in various *HtDFR1*, *HtBERRY1* and *HtCREAM1* *H. trionum*
1408 CUBG lines.

Fig. S8 Expression of *HrDFR1* and *HrFLS2* throughout flower development in proximal and distal
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Fig. S9 Identification of differentially expressed genes between the proximal and distal regions of
1412 stage 1 petal primordia in *H. trionum* CUBG.

Fig. S10 Placement of *HtBERRY1*, *HtBERRY2* and *HtCREAM1* within the MYB family
1414 phylogenetic tree.

Fig. S11 Expression of *HrBERRY1*, *HrBERRY2* and *HrCREAM1* throughout development in proximal and distal petal tissue of *H. richardsonii*.

Fig. S12 Predicted gene structure of *HvBERRY1* in *H. verdcourtii* with additional allele-specific primers.

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The following four Supplementary Data files are provided as separate files:

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Supplementary Data 3. Amino acid sequences of the 483 MYB sequences used to assess the phylogenetic placement of *HtBERRY1*, *HtBERRY2* and *HtCREAM1*. Data related to Fig S10.

Supplementary Data 4. Complete MYB family tree generated using the 483 sequences from Supplementary Data 3. Data related to Fig S10.

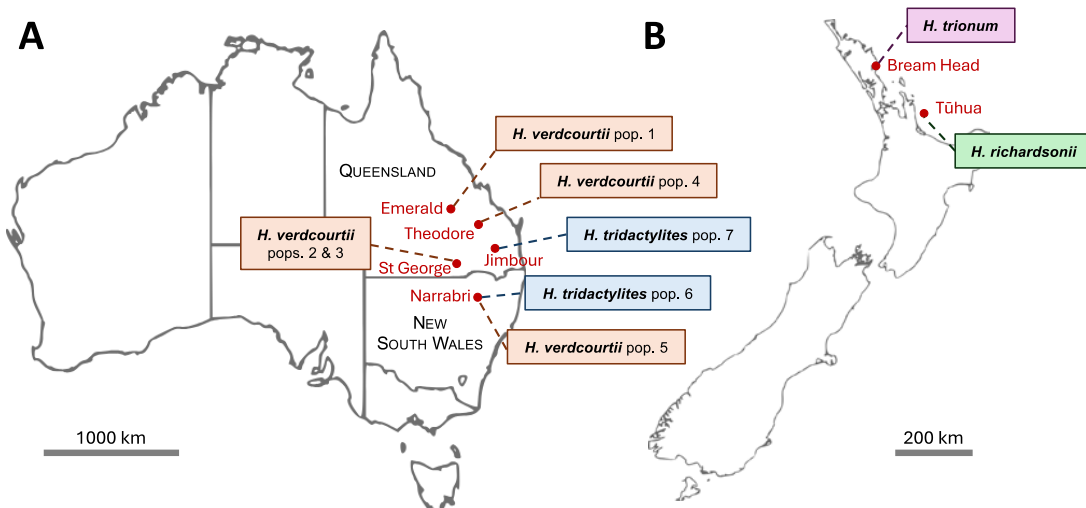


Fig. S1. Geographical location of *Hibiscus* species/populations used in this study. Wildtype *H. trionum* and *H. richardsonii* were isolated from Bream Head and Mayor Island (Tūhua), New Zealand, respectively. *H. verdcourtii* and *H. tridactylites* populations were isolated from various sites in Queensland and New South Wales, Australia (Craven et al., 2011).

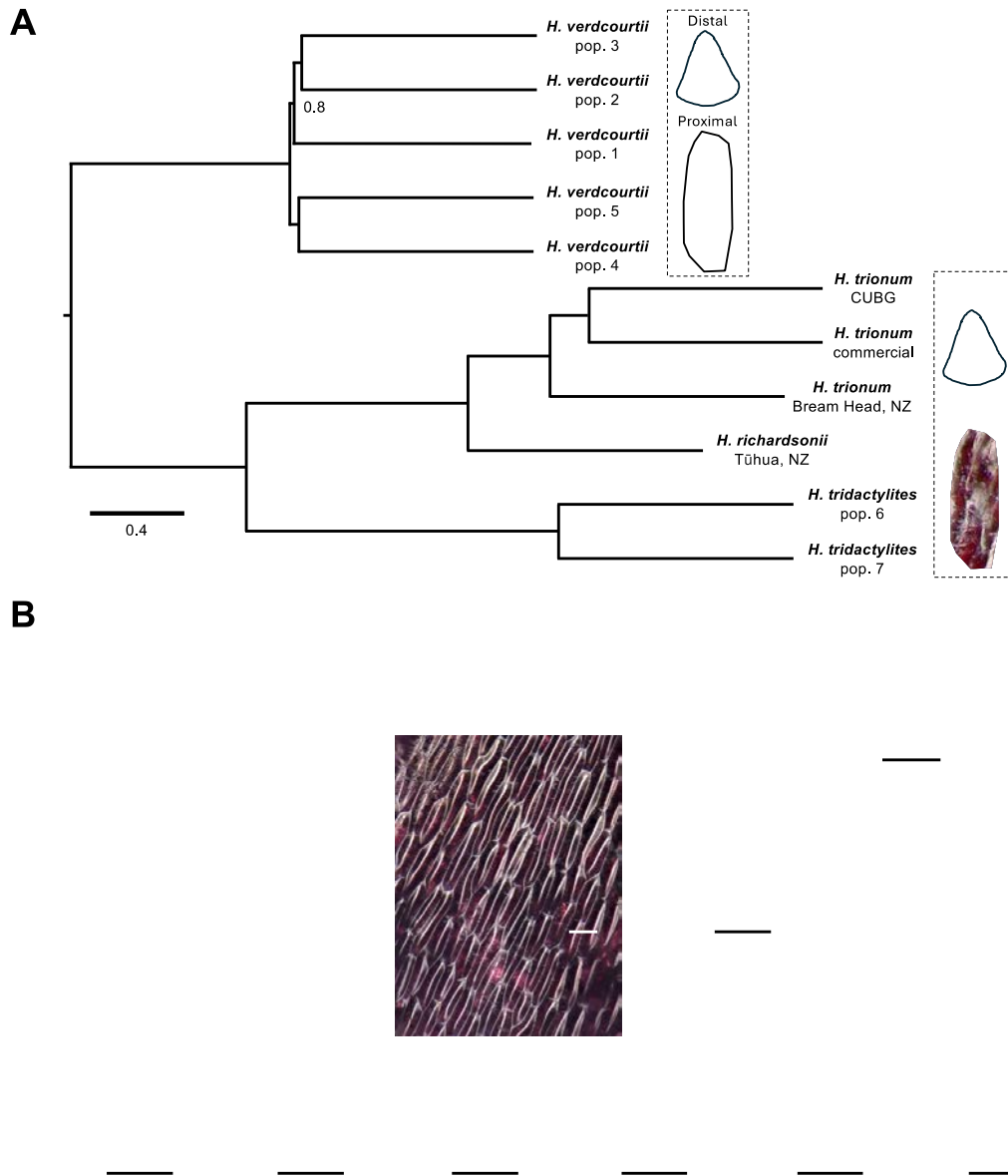
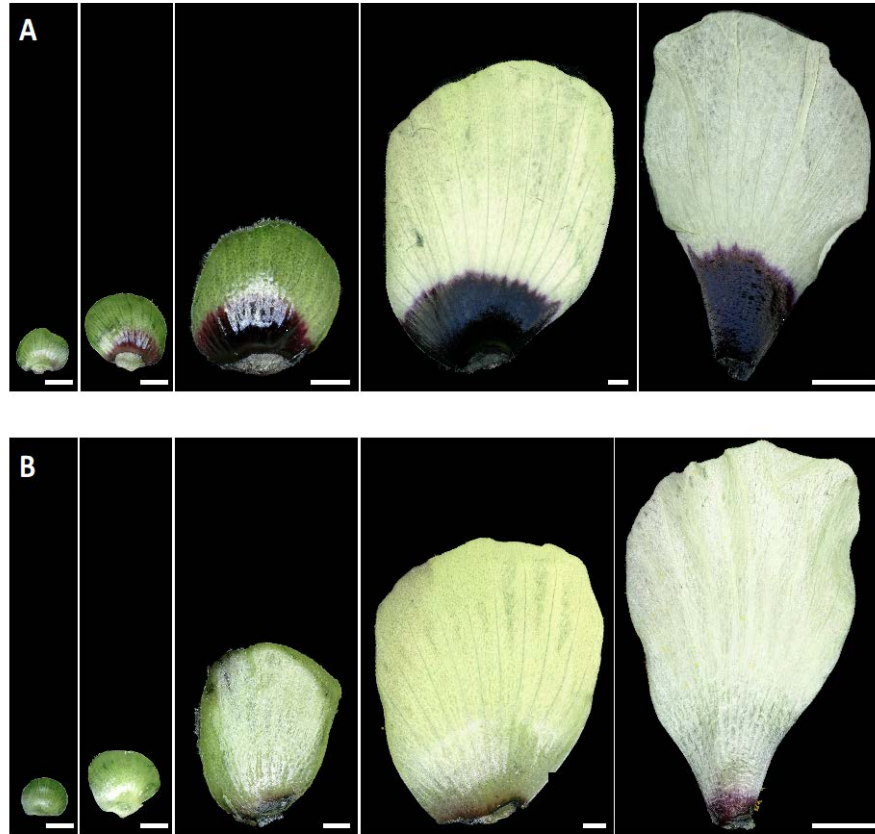


Fig. S2. Cell shape and texture across the adaxial petal epidermis of the 11 different accessions from the Trionum Complex used in this study (see Fig. 1 and Fig. S1). Unless stated, all nodes are perfectly supported. Branch lengths are shown in coalescent units. All accessions display tabular cells in the proximal petal region and smooth conical cells in the distal region. The tabular cells in the proximal region are covered with a striated cuticle, except for the five *H. verdcourtii* accessions that display smooth tabular cells in the proximal region. Scale bar = 50uM.



1454 **Fig. S3.** Comparison of pigmentation throughout petal development in (A) *H. trionum* CUBG and
 1456 (B) *H. richardsonii*. Images show adaxial epidermis of petal primordia from Stage 1 (S1) to Stage
 5 (S5) as described in Moyroud *et al.*, 2022. Scale bar = 1mm in S1-S4 and 5mm in S5.

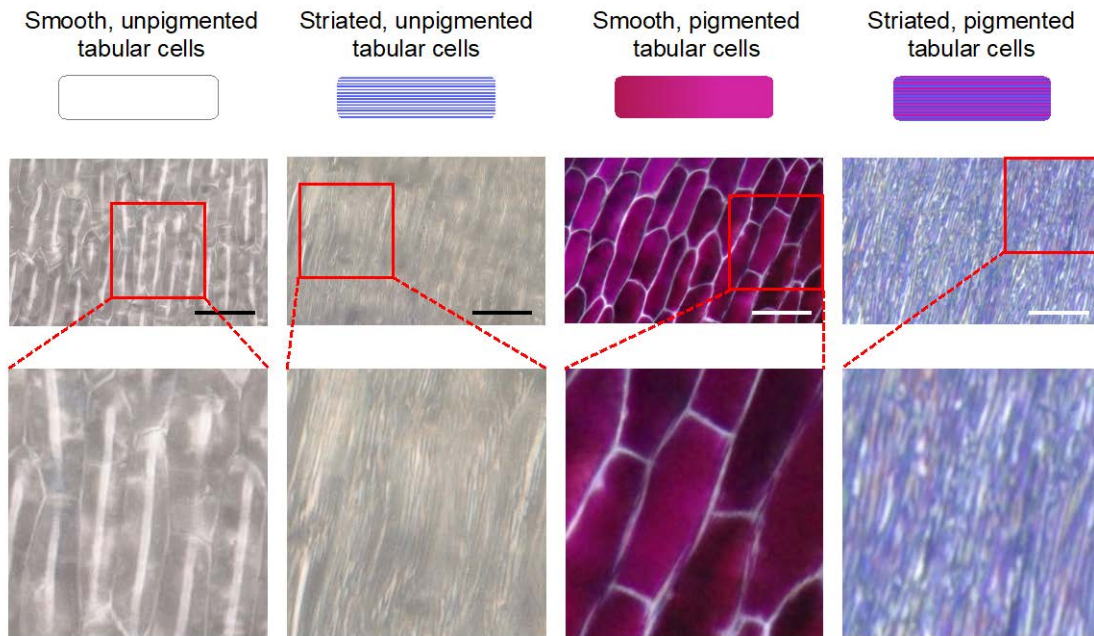


Fig. S4. Light microscopy images of *H. trionum* CUBG and *H. richardsonii* boundary cell types (middle and bottom rows). Cartoon symbols (top row) are used to describe F1 and F2 boundary phenotypes in Fig. 3. Scale bars = 50uM.

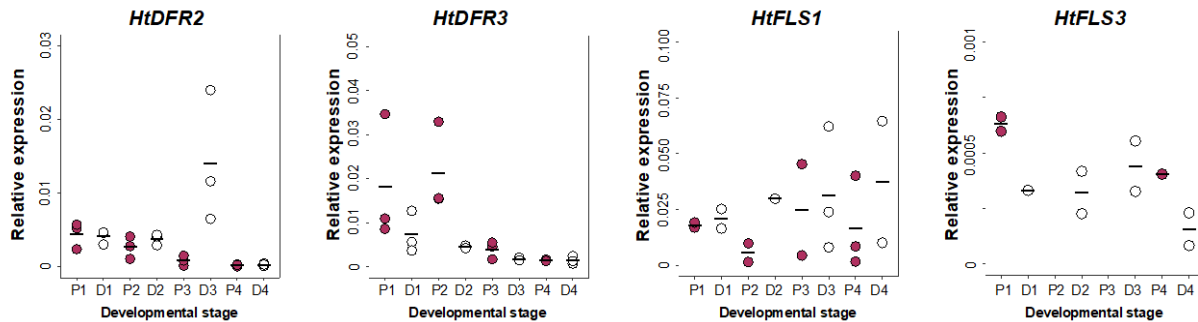


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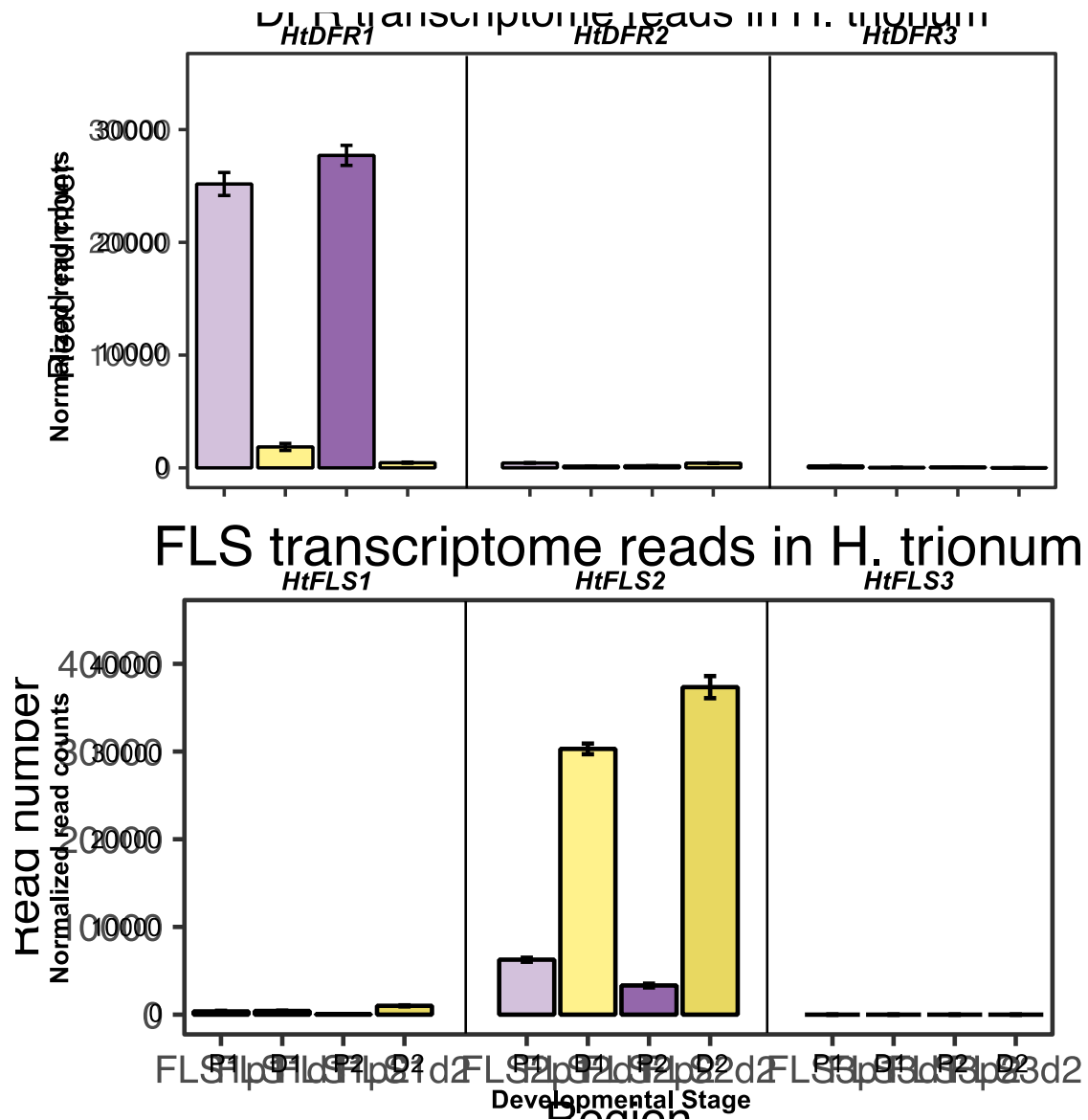


Fig. S6. Normalized read counts from RNAseq data for *HtDFR* homologs and *HtFLS* homologs in proximal (P1 and P2) and distal (D1 and D2) petal tissue of *H. trionum* CUBG at stage 1 and stage 2 (before and after bullseye pigmentation emergence, respectively). The y-axis shows the normalized read counts in Transcripts Per Million (TPM). Error bars = +/- SD with n = 5 independent biological replicates.

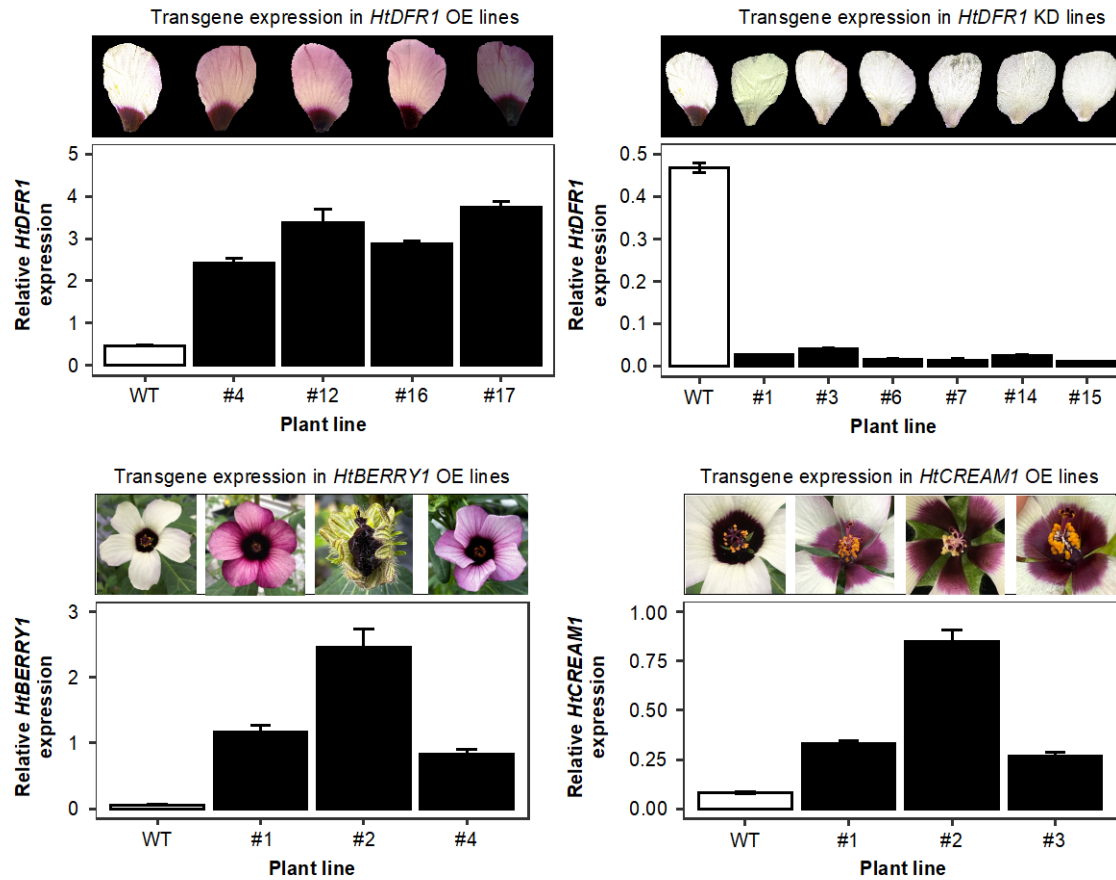


Fig. S7. Transgene expression level in various *HtDFR1*, *HtBERRY1* and *HtCREAM1* *H. trionum* CUBG lines. In *HtBERRY1* OE line #2, severe defects to floral development were observed (reduced growth of all floral organs and sterility of reproductive organs). Floral development was normal in other lines and sterility appeared to correlate with the strength of ectopic pigmentation suggesting constitutive overexpression of *HtBERRY1* and/or abnormal production of anthocyanin levels were associated with sterility. Each column indicates an average of three biological replicates, error bars represent +/- SD. Expression values are relative to *HtACTIN1* expression levels. WT = *H. trionum* CUBG.

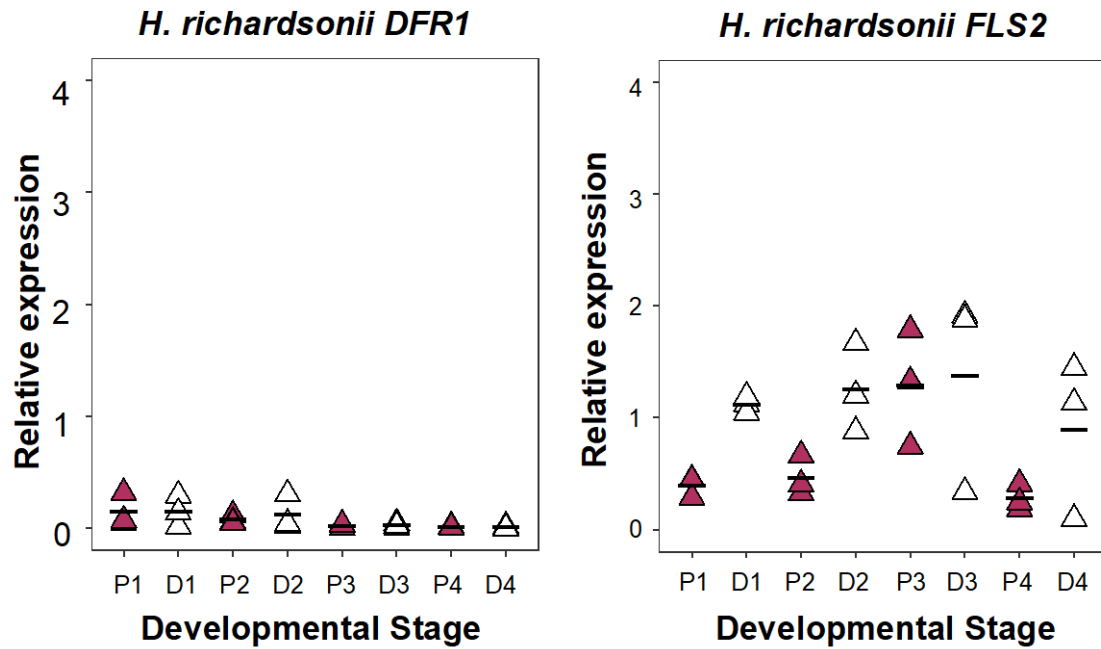


Fig. S8. Expression of *HrDFR1* and *HrFLS2* throughout flower development in proximal (P1 to P4) and distal (D1 to D4) petal tissue of *H. richardsonii*. Three biological replicates were extracted per stage. Each data point indicates an average of three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative *HrACTIN1* expression levels.

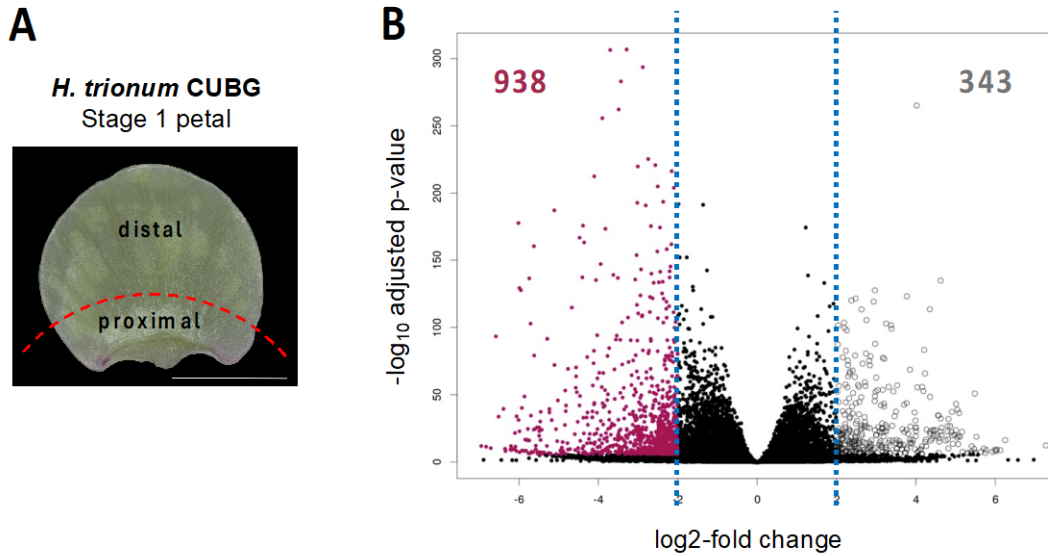


Fig. S9. Identification of differentially expressed genes between the proximal and distal regions of stage 1 petal primordia in *H. trionum* CUBG. **(A)** Image of a dissected *H. trionum* CUBG stage 1 petal showing the two regions used for RNA extraction and transcriptome analysis: region above the red dotted line = distal region, region below the red dotted line = proximal region. **(B)** Volcano plot displaying genes identified in the transcriptome analysis of stage 1 petals. Genes preferentially expressed in the proximal region (>4x difference compared to distal region) are represented by purple dots while genes preferentially expressed in the distal region (>4x difference compared to proximal region) are represented by white dots. The blue lines represent the log₂-fold threshold used for differential expression cut-off. Genes considered not differentially expressed between proximal and distal regions are depicted with black dots. Data associated with complete differential gene expression analysis and transcript assembly are provided as Supplementary Data 1 and the complete phylogeny is provided as Supplementary Data 2.

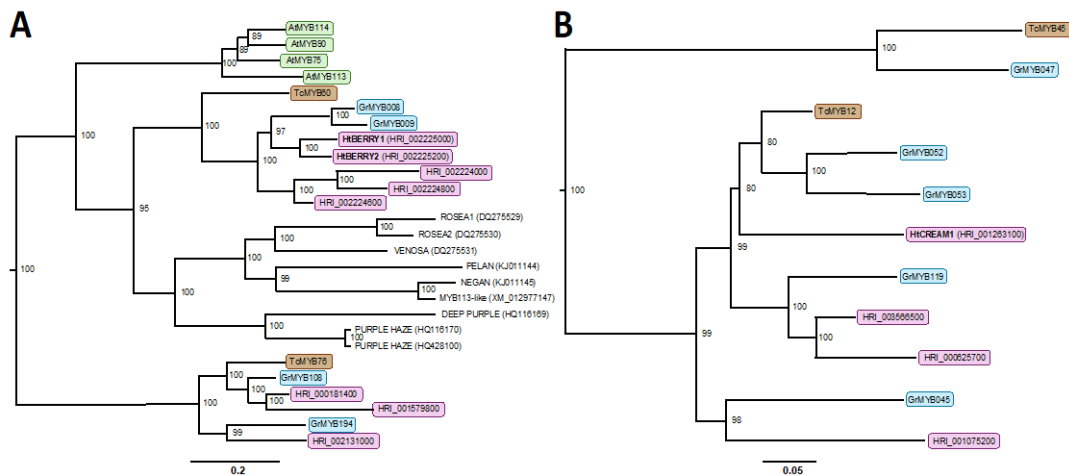


Fig. S10. Placement of *HtBERRY1*, *HtBERRY2* and *HtCREAM1* within the MYB family phylogenetic tree. **(A)** Phylogenetic tree depicting the position of *HtBERRY1* and *HtBERRY2* within the subgroup 6 R2R3-MYB cluster. **(B)** Phylogenetic tree depicting homology relationships between *HtCREAM1* and its three *H. trionum* paralogs and R2R3-MYBs from cotton and cocoa. *H. trionum* CUBG genes are highlighted in purple, cotton (*Gossypium raimondii*) genes are highlighted in blue, cocoa (*Theobroma cacao*) genes are highlighted in brown and *Arabidopsis thaliana* genes are boxed in green. Ultrafast Bootstraps values are given next to each node. Scale bar represents number of changes per site. Subgroup 6 R2R3-MYBs from other species, known to regulate anthocyanin production in the flowers of other species have also been included in (A). *Antirrhinum majus*: ROSEA1, ROSEA2, VENOSA; *Mimulus lewisii*: PELAN, NEGAN; *Mimulus guttatus*: MYB113-like; *Petunia hybrida*: DEEP PURPLE, PURPLE HAZE. The trees presented in A and B are part of a larger R2R3-MYB phylogenetic analysis we conducted, including all known R2R3-MYBs from *A. thaliana*, *G. raimondii* and *T. cacao*. The sequences used for this analysis are provided a Supplementary Data 3 and the complete phylogeny is provided as Supplementary Data 4.

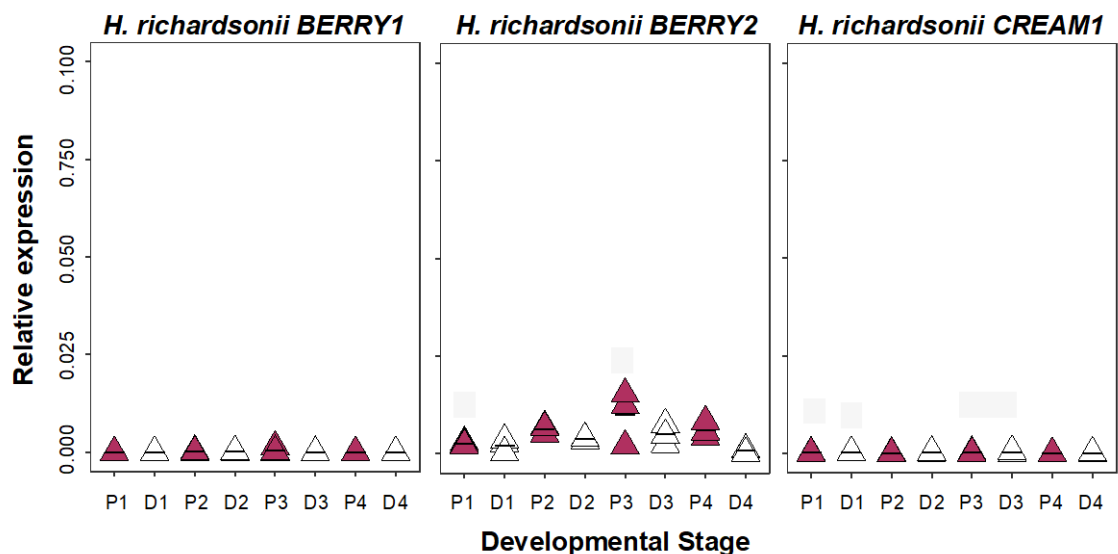


Fig. S11. Expression of *HrBERRY1*, *HrBERRY2* and *HrCREAM1* throughout development in proximal (P1 to P4) and distal (D1 to D4) petal tissue of *H. richardsonii*. Three biological replicates were extracted per stage and each data point indicates an average of three technical replicates, horizontal bars indicate mean relative expression values. Expression values are relative to *HrACTIN1* expression levels.

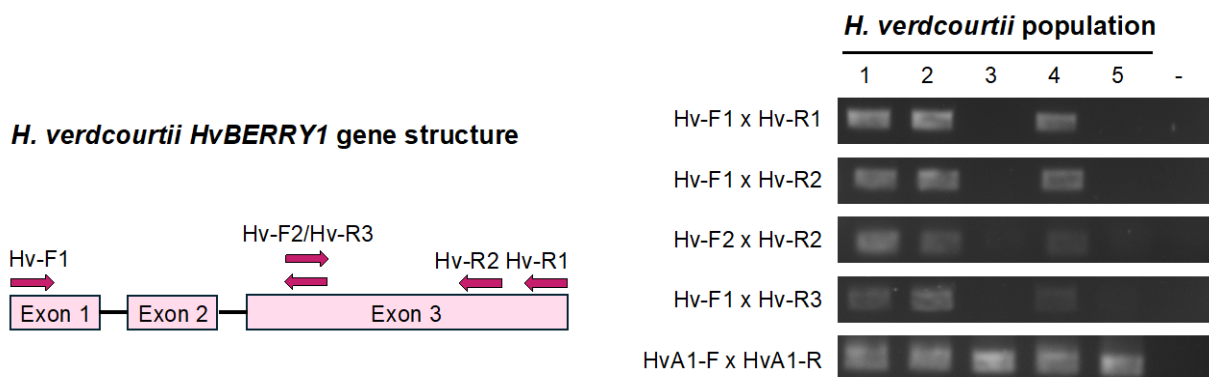


Fig. S12. Predicted gene structure of *HvBERRY1* in *H. verdcourtii* with additional allele-specific primers. *HvBERRY1* can be amplified from *H. verdcourtii* pops. 1, 2 and 4 but not from pop 3 or 5. Hv-F2 and Hv-R1 primers were used to genotype the F2 population as described in Fig. 8B. (-) = no template control.

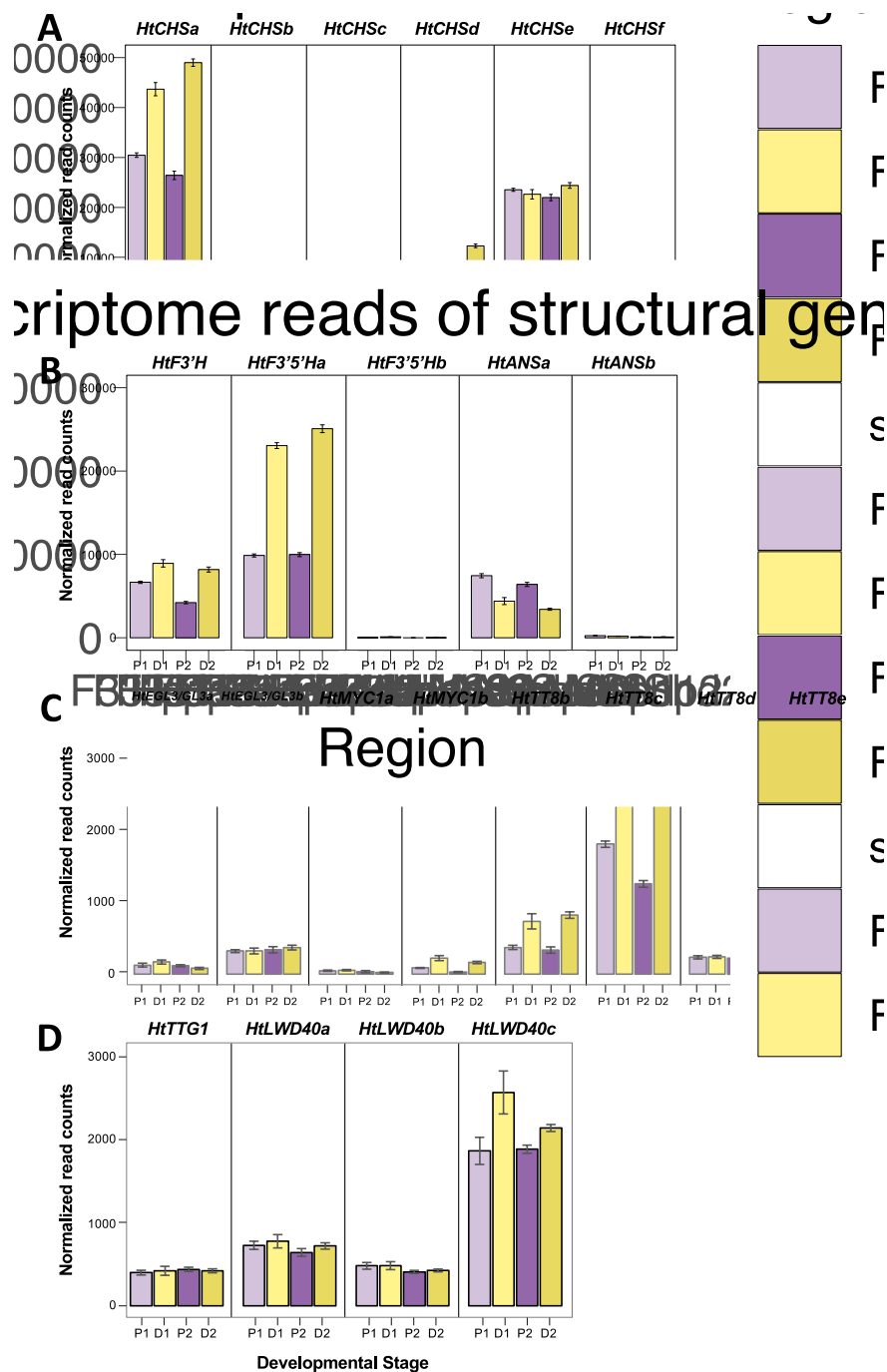


Fig. S13. Normalized read counts for flavonoid-related structural genes, subgroup IIIf bHLH and *TTG1/LWD40* homologs in *H. trionum* CUBG Stage 1 and 2 petal tissue. **(A)** *CHS* orthologs, **(B)** *HtF3'H*, *HtF3'5'Ha*, *HtF3'5'Hb*, *HtANSa*, *HtANSb* **(C)** subgroup IIIf *bHLH* and **(D)** *TTG1/LWD40* genes expressed in proximal (P1 and P2) and distal (D1 and D2) petal tissue of *H. trionum* CUBG at stage 1 and stage 2 (before and after bullseye pigmentation emergence, respectively). The y-axis shows the normalized read counts in Transcripts Per Million (TPM). Error bars = +/- SD with n = 5 independent biological replicates.

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Species	Description	Provenance	Voucher
<i>H. trionum</i> L.	Wildtype	Cambridge University Botanic Garden collection	CGE00046422
<i>H. trionum</i> L.	Diploid New Zealand naturalised race	Bream Head, New Zealand	AK253689, CGE00046417
<i>H. trionum</i> L.	Commercial	Netherlands	CGE00080883
<i>H. richardsonii</i> Sweet ex Lindl.	Wildtype	Mayor Island (Tūhua), New Zealand	AK251841, CGE00046420
<i>H. verdcourtii</i> Craven	Population 1	Emerald, Queensland	CGE00046415
<i>H. verdcourtii</i> Craven	Population 2	St. George, Queensland	CGE00046414
<i>H. verdcourtii</i> Craven	Population 3	St. George, Queensland	CGE00080882
<i>H. verdcourtii</i> Craven	Population 4	Theodore, Queensland	CGE00046413
<i>H. verdcourtii</i> Craven	Population 5	Narrabri, NSW	CGE00046366
<i>H. tridactylites</i> Lindl.	Population 6	Narrabri, NSW	CGE00046364
<i>H. tridactylites</i> Lindl.	Population 7	Jimbour, Queensland	CGE00046363

1574 **Table S1.** *Hibiscus* species used in this study.

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Line	Plant Background	Construct	Description
AF29.1	<i>H. trionum</i>	pAF29	<i>HtCREAM1</i> overexpression
AF52.1	<i>H. trionum</i>	pAF52	<i>HtBERRY1</i> overexpression
VT9.17	<i>H. trionum</i>	pVT9	<i>HtDFR1</i> overexpression
VT9.6	<i>H. trionum</i>	pVT9	<i>HtDFR1</i> knockdown

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Table S2. *H. trionum* transgenic lines generated in this study.

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Gene	Sequence	Purpose
HtDFR1-qF	TGCCGCCTAGCTTGATTACC	qPCR
HtDFR1-qR	CACGAACTGCCCTTGCCTAATA	qPCR
HtDFR2-qF	ACCTCCTTTAGTGGTTGGTCC	qPCR
HtDFR2-qR	TCCCAGTGATGGGAGAAAGTG	qPCR
HtDFR3-qF	TGGGACTTCTGCAACGAGAATAA	qPCR
HtDFR3-qR	ATCGGAGGCAGTGGAACATAAA	qPCR
HtFLS1-qF	GGAAGAGAAAGAGGTGTACGCTAAG	qPCR
HtFLS1-qR	CAGCCCAGTTCTTCTTCCCATTAA	qPCR
HtFLS2-qF	GAATACGCTAAGCACATGCATGG	qPCR
HtFLS2-qR	CATGTCGTCCCCACCTAGAG	qPCR
HtFLS3-qF	GCGCCCTCGTTGTTTCATATTG	qPCR
HtFLS3-qR	TCCGAGTTCTCTCCTTGTCTAC	qPCR
HtBERRY1-qF	GGTAGACTGCCTGGAAGAACAG	qPCR
HtBERRY1-qR	GATGAGGTTTCGGGTTTCGAGTTA	qPCR
HtBERRY2-qF	GGTAGACTGCCAGGAAGAACAT	qPCR
HtBERRY2-qR	TGGATGAGGTTTCGGGTTTGA	qPCR
HtCREAM1-qF	GGACCGGGTTTCTTTCTGCTTAT	qPCR
HtCREAM1-qR	GTCATCTCCACTTGCTGGTACAC	qPCR
HrBERRY1-qF	GGTAGACTGCCTGGAAGAACAG	qPCR
HrBERRY1-qR	GATGAGGTTTCGGGTTTCGAGTTA	qPCR
HrBERRY2-qF	GGTAGACTGCCAGGAAGAACAT	qPCR
HrBERRY2-qR	TGGATGAGGTTTCGGGTTTGA	qPCR
HrCREAM1-qF	GGACCGGGTTTCTTTCTGCTTAT	qPCR
HrCREAM1-qR	GTCATCTCCACTTGCTGGTACAC	qPCR
HtBERRY1-F	TTTGAGGGTTAGTGTTAAACAGCTACG	genotyping
HtBERRY1-R	AAACTTCTGGCTATAAGTTGAACACATTC	genotyping
HrBERRY1-F	CCTCGGTAACAGGTAACCTCATTAG	genotyping
HrBERRY1-R	GGTCGAGTATGGTCCAATGG	genotyping
HvBERRY1-F1	ATGGAAAGGCCATCTTTAAGTGTGA	genotyping
HvBERRY1-F2	GATCAATCCTTCCGACAGTAACTCAAACC	genotyping
HvBERRY1-R1	CTATAGGTTGAACACATTCCAGAACTCTGC	genotyping
HvBERRY1-R2	TTGGTTGAACCTGGTTGTTGATCA	genotyping
HvBERRY1-R3	GGTTTGAGTTACTGTCGGAAGGATTGATC	genotyping
HvACTIN1-F	CCCAGATCATGTTTGAGACCTT	genotyping
HvACTIN1-R	ACCGGAATCCAGCACAATAC	genotyping

Table S3. Primer sequences used in this study.

Vector	Recombinant DNA	Reference
pSG55	Modified pCAMBIA1300 with 2xp35S and pUBQ10::eYFPmyr	This study
pAF29	2xp35SS::HtCREAM1 in pSG55	This study
pAF52	2xp35SS::HtBERRY1 in pSG55	This study
pVT9	2xp35SS::HtDFR1 in pSG55	This study

Table S4. Plant expression vectors generated for this study.