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Precise spatio-temporal regulation of the anthocyanin biosynthetic pathway leads to petal spot formation in *Clarkia gracilis* (Onagraceae)

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Summary

- Petal spots are widespread in Angiosperms and are often implicated in pollinator attraction. *Clarkia gracilis* petals each have a single red-purple spot that contrasts against a pink background. The position and presence of spots in *C. gracilis* are determined by the epistatic interaction of alleles at two, as-yet-unidentified loci.
- We used HPLC to identify the different pigments produced in the petals, and qualitative and quantitative RT-PCR to assay for spatio-temporal patterns of expression of different anthocyanin pathway genes.
- We found that spots contain different pigments from the remainder of the petal, being composed of cyanidin/peonidin-based, instead of malvidin-based anthocyanins. Expression assays of anthocyanin pathway genes show that *Dfr2* has a spot-specific expression pattern and acts as a switch for spot production. Co-segregation analyses implicate the gene products of the *P* and *I* loci as trans-regulators of this switch. Spot pigments appear earlier in development due to early expression of *Dfr2* and *F3′h1*. Pigments in the background appear later, due to later expression of *Dfr1* and *F3′5′h1*.
- The evolution of this spot production mechanism appears to have been facilitated by duplication of the *Dfr* gene and to have required substantial reworking of the anthocyanin pathway regulatory network.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phenotypes and sections of petals used for HPLC analyses.

Fig. S2 Phenotypes, age, and sections of petals used for qualitative RT-PCR analyses.

Fig. S3 Stages of *C. gracilis* petal development.

Fig. S4 Genotypes at the P and I loci and expected phenotypes.

Fig. S5 Phylogenetic trees of anthocyanin pathway genes in section Rhodanthos.

Fig. S6 *Dfr2-A/B* expression and *Actin* control in *C. gracilis* plants derived from seeds collected in natural populations.

Fig. S7 Alignment of the six different *Dfr* sequences found in *C. gracilis*.

Table S1 Voucher informatio

Table S2 Primers used in qualitative and quantitative PCR assay

Table S3 Primers used and expected sizes for *Dfr2* genotype assays for P cros

Table S4 Primers used and expected sizes for *Dfr2* genotype assays for I cros

Notes S1 Text files containing alignments used for phylogenetic analyse

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Keywords

Clarkia; *Dfr*; flower color; petal spot; pigment pattern

Introduction

A major objective of evolutionary developmental biology ('EvoDevo') is to elucidate the genetic changes that result in the evolution of novel characters. One unresolved issue is whether novel characters can arise through changes in the activity of genes that are already active in developmental programs or if they require recruitment of additional genes into those programs (Keys *et al.*, 1999). In addition, while the evolution of novel morphologies is sometimes associated with gene duplication (Xiao *et al.*, 2008, Parker *et al.*, 2009), it is unclear how frequently duplication contributes to the emergence of novel traits. Because of the apparent simplicity and ecological relevance of wing spotting patterns, the developmental control of evolutionarily novel spots in animals, particularly butterflies, has been used to address these issues (Nijhout 1980, Carroll *et al*, 1994, Keys *et al.* 1999, Beldade *et al*. 2002, Reed & Serfas, 2004). These investigations have tentatively indicated that new wing spots have arisen by the modification of existing developmental programs.

In plants, petals with large spots— discrete depositions of visible pigments that contrast with the background coloration of the flower—occur in the families Liliaceae, Orchidaceae, Asteraceae, Papaveraceae, Fabaceae, and many others, indicating that they have evolved independently numerous times. Moreover, field studies have frequently demonstrated that spots are important in mediating interactions with pollinators (Jones, 1996; Johnson & Midgley, 1997; Van Kleunen *et al.*, 2007; Goulson *et al.*, 2009). Nevertheless, the genetic and developmental control of petal spots, and more generally, of petal color patterning, have seldom been investigated. Studies in model species such as petunia and snapdragon have clarified the genetic control of some patterns, namely vein-associated pigmentation (Schwinn *et al.*, 2006; Shang *et al.*, 2011) or variation in pigment intensity in different regions of the corolla (Jackson *et al.*, 1992; Schwinn *et al.*, 2006; Albert *et al.*, 2011). However, our general understanding of how floral pigment patterns develop and are regulated at the molecular level, specifically in relation to petals spots, remains poor. In this report, we describe experiments that begin to elucidate the biochemical, genetic and developmental basis of petal spots in the California wildflower, *Clarkia gracilis.*

Clarkia gracilis (Onagraceae) is native to northern California, Oregon and Washington. It is the only polyploid in section *Rhodanthos*. Based on cytological analyses, (Abdel-Hameed & Snow, 1972) suggested that this allotetraploid derived from hybridization between two diploid species, one closely related to *C. amoena* ssp. *huntiana* and one related to *C. lassenensis* and *C. arcuata*. However, the parentage of *C. gracilis* has not been tested adequately by modern molecular systematic methods.

Clarkia gracilis is composed of four different subspecies that have somewhat overlapping ranges: *C. g.* ssp. *albicaulis*, *C. g.* ssp. *gracilis*, *C. g.* ssp. *sonomensis*, and *C. g.* ssp. *tracyi*. The subspecies differ in their floral morphologies, particularly with respect to the presence and position of petal spots. Subspecies *albicaulis* and *tracyi* have a single spot at the base of each petal, ssp. *sonomensis* has a single spot in the center of each petal, and ssp. *gracilis* lacks petal spots (Fig. 1). In all cases, mature petal spots are dark reddish-purple concentrations of pigment on a pale pink background. However, there is some variation within some subspecies: *C. g. sonomensis* and *C.g. albicaulis* can be found in either spotted or unspotted morphs.

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The ecological significance of petal spots in *C. gracilis* has been studied in some detail. In mixed populations of central spotted and unspotted *C. gracilis* ssp. *sonomensis*, spotted plants have higher fitness, producing as much as 32% more seed than their unspotted counterparts in some growing seasons and sites (Jones, 1996). This difference might reflect pollinator preference, since petal spots were also shown to influence pollinator foraging behavior (Jones, 1996). In another species of *Clarkia*, *C. xantiana*, the maintenance of both spotted and unspotted morphs has been attributed to pollinator-based negative frequency-dependent selection (Eckhart et al., 2006).

Previous investigations have established that *C. gracilis* petal spots are under simple genetic control. Gottlieb & Ford (1988) identified two independent loci that interact epistatically to give rise to all of the morphs present in this species complex. One locus, *P*, has co-dominant alleles that determine the position of the spot within the petal: the P^B allele causes the production of basal spots, and the P^C allele causes the production of central spots. The other locus, *I,* affects the presence of basal spots: the I^A allele (or *I*, in (Gottlieb & Ford, 1988)) suppresses basal spots, while the I^P allele (*i*, in Gottlieb & Ford, 1988) allows basal spots to form. Neither allele at the *I* locus affects the formation of central spots. Gottlieb & Ford (1988) determined that *C. gracilis* ssp. *gracilis* generally has a $P^BP^BI^AI^A$ genotype, whereas spotted plants of ssp. *sonomensis* often have the genotype, $P^CP^CI^PI^P$. However, a variable dominance relationship was also found between I^A and I^P suggesting that additional modifiers or alternative alleles are present in different backgrounds.

The identity of the pigments underlying this phenotype can provide important clues as to the genes that may be involved in spot formation. Species in the genus *Clarkia* produce anthocyanin pigments, mostly derivatives of malvidin and cyanidin, although not all species produce these pigments (Dorn & Bloom, 1984; Soltis, 1986). Production of pelargonidin and derivatives is rare in the genus, being known only in *C. unguiculata* (Robinson & Robinson, 1931), suggesting that the biochemical pathway branch leading to pelargonidin production is inactive in most species (Fig. 2). In *C. gracilis* flowers, the anthocyanidins malvidin, cyanidin and delphinidin (a precursor of malvidin) have been reported (Soltis, 1986).

Our objectives in this investigation were to identify genes associated with spot formation in *C. gracilis* and characterize their pattern of regulation. Specifically, we sought to answer the following questions:

1. What pigments are produced in *C. gracilis* flowers? Do pigments in spots differ from the rest of the petal? Do pigments in central spots differ from those in basal spots?
2. Is transcriptional regulation of specific anthocyanin biosynthetic genes involved in petal spot formation? If so, does any gene correspond to the spot position *P* locus?

Materials and Methods

Plant growth and crosses

Seeds from *C. gracilis* (Piper) A. Nelson & J. F. Macbride were germinated in vermiculite at 15°C in the dark, and transferred to a 1:1 metromix:peatmoss mixture once seedlings emerged. Seedlings were then transferred to 5-inch pots and were grown in a glasshouse (20–24°C) under natural light. Plants were periodically randomized to avoid possible light-induced changes in flower color.

Several individuals from various populations were grown, and only those from monomorphic (with respect to spots) populations were used as the parents for crosses. Two independent crosses were carried out to separate the *P* and *I* loci. For the '*P*' cross, a central-

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spotted *C. g. sonomensis* was crossed to a basal-spotted *C. g. albicaulis*. For the '*I*' cross, an unspotted *C. g. gracilis* was crossed to a basal-spotted *C. g. albicaulis*. Voucher information is available in Supporting Information Table S1. Pollen recipient flowers were emasculated several days before full stigma receptivity to avoid self-pollination. Stigmas were pollinated by touching them with a dehiscing anther from the designated sire. F1 individuals were selfed to obtain F2 progeny.

Pigment extraction and High Performance Liquid Chromatography (HPLC) analyses

Anthocyanins were extracted from F2 individuals derived from the '*P*' cross mentioned above as well as from *C. gracilis* individuals derived from several natural populations. Seven F2 individuals were examined: two central spotted, two basal spotted, and three double-spotted. Five to ten F1 individuals (all double spotted) were pooled into one sample. Individuals from natural populations included three *C. g.* ssp. *albicaulis*, and six *C. g.* ssp. *sonomensis* plants (three spotted and three unspotted, see Table S1 for voucher information). Each sample contained either the top, center, or base of 4–5 adult flowers per plant (Fig. S1).

Anthocyanidins were extracted as in Harborne (1984). Pigments were separated using HPLC and identified using the retention times of commercially available standards (delphinidin, pelargonidin, cyanidin, peonidin, malvidin, and petunidin, Polyphenols Laboratory, Sandnes, Norway) that were run using the same conditions as the unknown samples (previously described in Smith & Rausher, 2011).

DNA extraction, gene amplification and sequencing

Genomic DNA from *C. gracilis* ssp. for gene isolations and phylogenetic analyses was extracted from fresh plant tissue using the CTAB method (Doyle & Doyle, 1987). Genomic DNA from *C. amoena* ssp. *huntiana*, *C. lassenensis*, *C. arcuata*, *C. franciscana*, *C. rubicunda*, and *C. breweri* were kindly provided by Kenneth Sytsma (University of Wisconsin-Madison; voucher information in Table S1). We identified the two homeologs of *C. gracilis* genes (arbitrarily called *A* and *B*) by including sequences from other species in section *Rhodanthos* in phylogenetic analyses. This was facilitated by *C. amoena* and *C. lassenensis* (the putative parents of *C. gracilis*) not being sister taxa, allowing for the homeologs present in *C. gracilis* to be identified more easily.

We used sequences available in Genbank to design degenerate primers to isolate partial sequences corresponding to the anthocyanin biosynthetic genes (*F3h*, *F3′h*, *F3′5′h*, *Dfr* and *Ans*). Primers and conditions used for each gene/species are available upon request. PCR bands corresponding to predicted amplicon sizes were transformed into *E. coli* cells. At least 10 colonies per ligation were sequenced, and additional clones were sequenced as needed. Sequencing reactions used the BigDye Terminatorv. 3.1 system following the manufacturer's protocol (Applied Biosystems) and electrophoresis was performed at the University of Wisconsin-Madison Biotechnology Center.

Sequence and Phylogenetic analyses

Sequencherv. 4.7 (Gene Codes Corp.) was used to edit, assemble and align sequence fragments with further manual alignment in MacClade v. 4.05 (Maddison & Maddison, 2000). To confirm gene identity, sequences were submitted to the Basic Local Alignment Search Tool at the National Center for Biotechnology Information. All phylogenetic analyses were performed in PAUP* v. 4.0b10 using maximum likelihood (ML, Swofford, 2002). Potential PCR recombinants were excluded from the analyses. For all datasets, the GTR model with estimated base frequencies was assumed. Support values were obtained by

performing 1000 replicates of ML bootstrapping. Only coding sequences were used in analyses (alignments of included sequences provided in Notes S1.

mRNA expression analyses

Total RNA was extracted from petals using RNeasy Plant Mini kit (Qiagen), followed by a DNAse step (MBI Fermentas) and cDNA synthesis using ImpromII Reverse Transcription System (Promega), or Superscript III First Strand Synthesis (Invitrogen), all according to respective manufacturer's protocol.

For qualitative gene expression experiments, F2 plants of three different phenotypes, central, basal, and double spotted, from the *P* locus cross, and of two different phenotypes (basal spotted or unspotted) from the *I* locus cross, were used. Each RNA sample consisted of either the top, base, or center of three to four flower buds from each plant and three plants for each phenotype. Buds were collected at a stage of development when spot pigments were first appearing (or in equivalently sized petals of unspotted plants), but before background color was visible (Fig. S2).

For quantitative gene expression experiments, plants of three different phenotypes (central-, basal-, and non-spotted), derived from the P and I crosses, were used. Buds representing four different stages of development were collected, with three replicates of each stage. 'Stage' was based on the emergence and quantity of pigments, because petal length proved to be an unreliable marker for age, as flower size and maturation times varied greatly, within and between individuals. Change in floral pigmentation has been used previously in *Clarkia* spp. to determine floral developmental stage (Pichersky *et al.*, 1994). In *C. gracilis*, color develops in the following order: no color, central spot appears (if present), basal spot appears (if present), background color appears (Fig. S3).

Quantitative assays of gene expression were carried out using Clontech's SYBR Advantage qPCR premix and run in a Stratagene Mx3000P instrument. Dissociation curves and sequences of qPCR products were obtained to verify amplification of only the desired product. Absolute quantification of mRNA transcript levels was determined using a standard curve, followed by normalization to *Actin*. qPCR runs were performed in duplicate. Primers used for RT-PCR and qPCR (Table S2) flanked introns, with the exception of *F3h* and *Ans*. PCR products were sequenced to confirm that the desired copy was being amplified.

Genotyping F2 individuals from P and I crosses at the *Dfr2-A* and *Dfr2-B* loci

Thirty-two and 31 F2 plants from the P cross were genotyped for *Dfr2-A* and *Dfr2-B*, respectively. Homeolog specific primers were designed to amplify a portion of exon 2 and intron 2 (*Dfr2-A*), and a portion of intron 2 (*Dfr2-B*). Fifty plants from the F2 generation from the I cross were genotyped at *Dfr2-A* and *Dfr2-B* by amplifying intron 3 and partial sequences of exon 3 and 4. The same *Dfr2* primers used for qPCR analyses were used. All alleles were identified using fragment length polymorphisms, with the exception of alleles of *Dfr2-B* for the P cross, which were genotyped by the presence of a SNP using restriction enzyme digestion (Tables S3 and S4). Fragment length runs were completed in an ABI 3730XL DNA Analyzer at the Duke Genome Sequencing & Analysis Core Resource, visualized using the GeneMapper software (Applied Biosystems) and scored by eye. Fisher's exact test was used to test for possible co-segregation between spot presence and *Dfr2-A/B* alleles.

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Results

Crosses confirm the two-locus model and provide segregating families for further analysis

Although the genetics of petal spot inheritance had already been determined, we established populations of individuals with known genotypes to facilitate genetic and molecular analyses and to confirm previous genetic results. We developed two separate segregating F2 populations for the two independent loci, thus removing the epistatic interaction that could mask the genotype at the *P* locus.

Segregation patterns (Table 1) confirmed those previously reported (Gottlieb & Ford, 1988; Jones, 1996). In the *P*-cross (see Materials and Methods), 100% of F1 progeny were double-spotted. Segregation in the F2 population did not deviate significantly from the expected 1:2:1 ratio of basal spotted:double spotted:central spotted plants (chi-square test, $P = 0.74$) consistent with a model of a single locus with co-dominant alleles. According to this model $P^B P^B$ plants have basal spots, $P^C P^C$ plants have central spots, and $P^B P^C$ plants have both a central and a basal spot.

In the *I*-cross (see Materials and Methods), all F1 individuals had basal spots, suggesting that the suppressing I^A allele was recessive to the spot-permitting allele I^P. In the F2 population, we saw variation in spot intensity, from a strongly defined spot to an ill-defined ‘smudge’, to unspotted petals. Because it was difficult to distinguish spots from smudges, plants showing any color at the base were coded as ‘basal spotted’ for statistical analyses. As shown in Table 1, using this scoring, a 3:1 pattern of spotted:unspotted plants could not be rejected (chi-square test, $P = 0.77$), suggesting that the I^P allele isolated is generally (but variably) dominant to the I^A allele. Genotypes for *P* and *I* and expected phenotypes under this genetic model are presented in Fig. S4.

It should be noted that, while in our crosses the I^P allele appears to be dominant, previous work has shown that alleles at the *I* locus can switch from dominant to recessive in different backgrounds, possibly due to other modifiers (Gottlieb & Ford, 1988). Further genetic studies are necessary to fully understand the phenotypic expression of this allele in different *C. gracilis* populations and subspecies.

HPLC analyses show that spots contain different pigments from the rest of the petal

While the pigment composition of entire *C. gracilis* petals had been previously determined through chromatography (Soltis, 1986), it was unclear whether the spots contained the same pigments as the background color. Using HPLC, we analyzed anthocyanidins present in mature petals from several *C. gracilis* individuals, spanning all spot phenotypes (see Materials and Methods). In all cases, irrespective of subspecies or spot phenotype, the distribution of anthocyanidins in the petal followed the same pattern. Petals of *C. gracilis* were found to include the anthocyanidins malvidin, cyanidin, and peonidin (Fig. 3, Table 2). These results contrast with a published report where delphinidin was found instead of peonidin (Soltis, 1986). The discrepancy between this study and Soltis (1986) may be due to the different methodologies used (HPLC versus thin-layer chromatography).

Cyanidin and peonidin were present in all petal sections that included spots and were never detected in petal sections that lacked spots (Fig. 3, Table 2). Likewise, petal sections that lacked spots, only having background color, contained only malvidin. The central regions of central-spotted flowers sometimes contained small amounts of malvidin, consistent with the presence of some background color in the central sector. In contrast, the basal regions of basal-spotted flowers contained very little or no malvidin, consistent with the almost complete lack of background color near the petal base (Fig. S1).

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Given these findings, we conclude that, regardless of spot position, anthocyanins in *C. gracilis* petal spots are derived from cyanidin and peonidin, two similar anthocyanidins that differ in that peonidin has a methyl substitution on the B-ring. In contrast, the background anthocyanins present in the petal appears to be malvidin-based, an anthocyanidin that has two methyl substitutions on the B-ring, and derives from a different branch of the anthocyanin pathway (Fig. 2).

Clarkia gracilis carries multiple copies of anthocyanin biosynthetic genes

In the anthocyanin pathway the branch-point between cyanidin/peonidin and malvidin occurs after the F3H-mediated synthesis of DHK (Fig. 2). This points to the regulation or action of *F3′h* and/or *F3′5′h* as possible determinants of spot formation. Another possible candidate for the synthesis of different pigments is *Dfr*. Numerous studies show that single or very few amino acid changes in DFR can lead to changes in substrate specificity that redirect flux down a different branch of the pathway (Johnson *et al.*, 2001; Fischer *et al.*, 2003; Shimada *et al.*, 2005; DesMarais and Rausher, 2008). Consequently, we focused on documenting whether differences in the patterns of regulation of *F3′h*, *F3′5′h* and *Dfr* are associated with the different petal spot phenotypes of *C. gracilis*. In addition, we also examined *F3h* and *Ans*, which are involved in earlier and later steps of pigment biosynthesis, respectively, to serve as controls and provide a more comprehensive understanding of patterns of expression of anthocyanin biosynthetic genes.

We conducted PCR amplification of partial gene sequences using multiple degenerate and specific primers, designed to conserved regions of the genes encoding the targeted enzymes, and cloned numerous PCR products in an attempt to identify all homologous gene copies in *C. gracilis* and, when possible, in related diploid species. We analyzed sequences for the presence of stop codons to assess gene functionality and used phylogenetic analyses to confirm gene identity (Fig. S5). However, it should be noted that we obtained only partial sequences for most genes from most species.

Two copies of *F3h, F3′h,* and *F3′5′h* and three copies of *Dfr* were found in diploid species in Sect. *Rhodanthos,* while a single copy of *Ans* was found. For *F3′h* and *F3′5′h*, only one copy appeared to be functional in the diploids: one copy of each gene had a premature stop codon or a frameshift mutation. For *F3h*, one copy from *C. amoena* and one from *C. arcuata* appeared to be divergent (as evidenced by longer branches, see Fig. S5). There were no indications from the sequences to suggest that any *Dfr* genes lack function (Fig. S5). We focused our efforts on isolating the putative functional or non-divergent copies of these genes from *C. gracilis,* and recovered one copy (two homeologs) corresponding to the putative functional *F3h* and *F3′h*, and all three copies (and 2 homeologs for each copy) of *Dfr*. We isolated both homeologs of the functional copy of *F3′5′h*, and one homeolog of the nonfunctional *F3′5′h* gene, which was confirmed to have an early stop codon. We isolated a single sequence corresponding to *Ans*, but is unclear whether only one homeolog exists, or whether both homeologs are present but do not differ in sequence within the short fragment obtained.

Spatial expression of *Dfr2* shows a tight correlation to spot location in *C. gracilis* petals

If spot formation depends on location-specific expression of any of the anthocyanin biosynthetic genes, it follows that such gene(s) would be expressed mainly in areas where spots are formed. To examine spatial expression patterns, we performed RT-PCR on RNA extracted from three different petal sections (top, middle and base) from young buds, from plants with central (P^CP^C), basal (P^BP^B), or double-spotted (P^BP^C) flowers. Copy-specific primers were used for all of the putatively functional genes, with homeolog-specific primers being used in *C. gracilis* when that proved possible.

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As shown in Fig. 4, the gene encoding the most upstream enzyme examined in the anthocyanin pathway, *F3h,* and one of the two copies of the gene encoding the next enzyme, *F3′h1-A*, showed consistent, robust expression in all sections of all petals. *F3′h1-B* transcript was not found in floral tissue, despite multiple attempts using several primers, suggesting it is either not expressed or limited to other tissues. Genes encoding enzymes further down the pathway, such as *F3′5′h*, *Dfr1,* and *Ans* (hereafter, 'downstream genes') showed variable expression that did not correlate with genotype or spot location. This variation most likely reflects slight differences in the age of petals. For instance, the most consistent pattern among the downstream genes is that their levels tend to be lower in central spotted petals than in either basal-spotted or double spotted petals. This is likely due to the age of the floral buds. Central spots appear earlier in development than basal spots, which means that central spotted flowers were harvested at a slightly earlier stage of development than the other genotypes (Fig. S3). This is significant because it suggests that, at this early stage of development, downstream genes are not fully and consistently activated.

The two homeologs of one gene, *Dfr2*, had a pattern that was strikingly distinct from all the other genes surveyed, a pattern that is correlated with the presence of the spot (Fig. 4a). *Dfr2* appeared to be expressed at high levels in sections of the petal that contained a spot, but not in unspotted sectors. In central-spotted individuals *Dfr2* was expressed at high levels only in the center of the petal, but was expressed only at the base of basal-spotted petals. Similarly, double-spotted individuals showed high expression of *Dfr2* in both central and basal sections of the petal. The low levels of expression that were sometimes seen in sections adjacent to the spot can be attributed to imprecise sectioning of the buds, since they are only a few millimeters in length when dissected. The sole exception to this pattern is that one central spotted individual, showed a brighter band for *Dfr2* in the top as opposed to the central section of the petal (Fig. 4a). This could be partly due to initial RNA quality, as the top section for that petal yielded a brighter band than the central sections for the other genes surveyed, including *Actin*. The spot-specific pattern of expression of *Dfr2* in petals (and lack in unspotted petals) was confirmed in nine other *C. gracilis* plants grown from seed derived from wild populations (3 unspotted and 2 central spotted *C. g. sonomensis*, 3 basal spotted *C. g. albicaulis,* and 1 basal spotted *C. g. tracyi*, Fig. S6). Therefore we conclude that *Dfr2* (and only this gene) shows a spot-specific expression pattern.

The role of *Dfr2* in spot formation was further evaluated by examining the expression of this gene in individuals segregating for the *I* locus. In basal-spotted plants (I^PI^P or I^PI^A), *Dfr2* is expressed at high levels in the basal section whereas unspotted siblings (I^AI^A) show very low or no *Dfr2* expression (Fig. 4b). This corroborates the pattern seen in F2s from the *P* locus cross, suggesting that *Dfr2* is necessary for spot production. It also indicates that suppression of the basal spot by the *I* locus is genetically upstream of *Dfr2.*

Dfr2 deviates from other downstream genes in showing expression early in petal development

The variable expression of genes encoding downstream enzymes suggested the possibility that temporal regulation plays a role in petal spot development. This seems plausible since petal spots, especially central spots, are visible several days before the appearance of background color (Fig. S3), implying that genes required for spot development might be expressed earlier than genes that are only required for the production of background color. To investigate this possibility, we used qPCR to obtain an expression time-course for *Dfr* and for the functional copies of *F3′h1*, *F3′5′h1*, which are situated at the branch-point between cyanidin/peonidin and malvidin biosynthesis in developing buds. We assayed all three copies of *Dfr* and both homeologs for all genes except the *B* copy of *F3′h1,* which did not appear to be expressed in petals.

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As shown in Fig. 5, two genes, *F3′h1-A* and *Dfr2*, are expressed early in development, having high expression levels at the first time points (as the spot appears) and declining expression in more mature buds (which are developing background color). Although *F3′h1-A* shows a similar pattern in all three phenotypes, *Dfr2* expression is reduced or absent in flowers that lack spots, consistent with the results obtained from non-quantitative PCR (Fig. 4). In contrast to *F3′h1-A* and *Dfr2*, *F3′5′h1* and *Dfr1* were expressed later in petal development, having low expression levels in early time points, but higher expression later in development.

Finally, one gene *Dfr3* shows no clear pattern, being expressed at very low levels in all tissues except for a peak in late, central-spotted flowers. The latter could be an experimental artifact (note error bars). The success in amplifying *Dfr3* in RT-PCR experiments confirms that this gene is expressed, but the level of expression appears to be substantially lower than the other *Dfr* gene copies, indicating that this gene may lack a function in pigment production in the petal.

Co-segregation analysis suggests that the *P* and *I* loci correspond to trans-regulators of *Dfr2*

Qualitative and quantitative gene expression data indicate that cyanidin/peonidin-based petal spots in *C. gracilis* correlate with the early and localized expression of *Dfr2*, coupled with the early expression of *F3′h1-A*. Under the assumption that localized, early expression of *Dfr2* is the proximate cause of petal spot development, then the *P* and *I* loci must act by modulating the expression of *Dfr2*, and likely correspond to upstream regulators of *Dfr2*. However, it is possible that either *P* or *I* could correspond to *Dfr2* itself if it had undergone a cis-regulatory change that resulted in altered expression.

To verify whether *Dfr2* alleles co-segregate with spot location and/or presence, we genotyped several F2 individuals from both the P and I crosses, for both homeologs of *Dfr2* (Table 3). The genotypes at neither of the *Dfr2* homeologs co-segregated perfectly with spot location in the *P* cross, suggesting that the *P* locus does not encode *Dfr2*, but rather is a *trans*-regulator of *Dfr2*. However, the p-value for co-segregation between *Dfr2-B* and *P* approached significance ($P = 0.0843$), suggesting that perhaps there is some genetic linkage between *P* and *Dfr2-B*. Larger numbers of offspring would be necessary to confirm this hypothesis. Similarly, neither of the *Dfr2* homeologs co-segregated with the absence/presence of basal spots in F2s from the I locus cross, suggesting that *I* is also a *trans* regulator of *Dfr2*.

Discussion

A model for the development of pigmentation patterns in *C. gracilis* petals

Based on the results presented here we propose a model for petal pigment development in *C. gracilis* that can explain the production of cyanidin/peonidin-based anthocyanins in spots and malvidin-based anthocyanins in the background (Fig. 6). This model invokes transcriptional regulation of anthocyanin biosynthetic genes as a function of time and space. Although post-translational regulation may occur, it is not necessary to explain the observed data. For this model, we assume that *Dfr3* is expressed at such low levels that it plays no significant role in petal pigmentation. Likewise we will ignore the putatively non-functional copies of *F3′h* and *F3′5′h*.

According to the model (Fig. 6), most of the core enzyme-coding genes of the anthocyanin pathway (*Chs*, *Chi*, *F3h*, *F3′h*, *Ans*, and *Uf3gt*) are activated early in development and expressed throughout petal tissue. In addition, *Dfr2* is activated early but is expressed only in the area destined to become a pigmented spot, implying that it is under different

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regulatory control. By contrast, neither *F3′ 5′ h1* nor *Dfr1* are expressed at this time. Because *Dfr* is required for pigment production, the spatial restriction of *Dfr2* explains why pigment is produced only as a spot. Further, because *F3′ 5′ h1* is not activated early, only anthocyanins derived from dihydroquercetin (DHQ) (i.e., cyanidin and peonidin) are produced in the spot domain.

At a later stage of development, the core enzymes are still active. This may represent continuous expression of these genes, or a second wave of expression. At this point, *F3′ h1-A* and *Dfr2* expression has ceased. Activation of both *F3′ 5′ h1* and *Dfr1* throughout the developing petal means that the complete set of enzymes is present, causing the background pigment to be deposited. The expression of *F3′ 5′ h1* causes this pigment to be derived via DHM rather than DHK or DHQ, explaining the production of malvidin in the background. Thus, the difference in pigment type between background and spot, as well as the spatial restriction of the spot, is explained by the different temporal expression of *F3′ h1-A/Dfr2* versus *F3′ 5′ h1/Dfr1*.

Regulation of enzyme-coding genes

In most species that have been examined, the core enzyme genes of the anthocyanin pathway (those depicted in Fig. 2) are coordinately regulated by common sets of transcription factors (Cone et al., 1986; Paz-Ares *et al.*, 1986, 1987; Ludwig *et al.,* 1989; Goodrich *et al.*, 1992; de Vetten *et al.*, 1997; Elomaa *et al.*, 1998; Quattrocchio *et al.*, 1998, 1999; Walker, 1999; Borevitz *et al.*, 2000; Spelt *et al.*, 2000, 2002; Carey *et al.*, 2004; Schwinn *et al.*, 2006; Gonzalez *et al.*, 2008; Lin-Wang *et al.*, 2010; Niu *et al.*, 2010; Albert *et al.*, 2011). In some taxa, such as *Ipomoea* and maize, all core genes are expressed at similar times in development and are activated by a single set of transcription factors (Cone *et al.*, 1986; Paz-Ares *et al.*, 1987; Ludwig *et al.*, 1989; Morita *et al.*, 2006). In other species, such as *Petunia* and *Antirrhinum*, genes are activated in two blocks, with those coding for upstream enzymes activated first, and those coding for downstream enzymes activated later, perhaps by a different set of transcription factors (Martin *et al.*, 1991; Goodrich *et al.*, 1992; Jackson *et al.*, 1992; Quattrocchio *et al.*, 1998; Spelt *et al.*, 2000; Schwinn *et al.*, 2006).

The pattern of regulation exhibited by *C. gracilis* differs from these canonical patterns. Although we have not demonstrated it directly, the production of anthocyanins in the petal implies that upstream and downstream genes, represented by *F3h* and *Ans,* are expressed from the time anthocyanins are first produced in spots to the time they are last produced in the remainder of the petal—although the existence of sub-blocks within these genes cannot be ruled out. However, *Dfr*, *F3′ h*, and *F3′ 5′ h* exhibit more restricted temporal distributions, with *Dfr2* and *F3′ h1-A* being expressed only in the early stages of development and *Dfr1* and *F3′ 5′ h1* being expressed only later in development. This pattern suggests that factors regulating these genes differ from those regulating the remaining core enzymes, and that the factors regulating *Dfr2* and *F3′ h1-A* differ from those regulating *Dfr1* and *F3′ 5′ h1.* Moreover, two patterns indicate that *Dfr2* is regulated differently from all other enzyme coding genes. First, only *Dfr2* exhibits a spatially restricted expression domain, confined to the regions that develop spots. Second, variation at the *P* and *I* loci affect only expression of *Dfr2*. Because neither of these loci cosegregates with *Dfr2*, they likely correspond to transcription factors or cofactors that are unique to this gene. These patterns imply that the production of spots has required a substantial reworking of the anthocyanin regulatory network. We next discuss the evolutionary implications of this reworking.

Possible substrate specificity in different copies of DFR

DFR2 and DFR1 appear to only encounter one substrate, DHQ or DHM, respectively, and, while the model we propose for formation of cyanidin/peonidin-based spots does not require

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evolution of substrate specificity among the different copies of DFR, it is possible that it has occurred. In *C. gracilis*, the two different copies differ in numerous sites, including in the active site, where they differ by nine amino acid substitutions. One such substitution, at residue 133 (Fig. S7), has been implicated in substrate specificity in other species (Shimada et al., 2005; DesMarais and Rausher, 2008; Johnson et al., 2001). Possible evolution of substrate specificity could be explored by biochemical characterization of these enzymes combined with more fine-scale temporal expression studies.

Evolutionary implications

Although the progenitors of *C. gracilis* have petal spots, as do other species in the section *Rhodanthos*, our analysis of spot formation in *C. gracilis* sheds light on aspects of how petal spots originally evolved in this group. Two major types of regulatory change appear to have been required for the evolution of spots. The first was a spatial restriction of the expression domain of *Dfr2*. It is reasonable to assume that pigment deposition throughout the petal was the ancestral state because species that are situated in the phylogeny as sister to the rest of the genus, namely *C. breweri*, *C. concinna*, and *C. pulchella*, are all pink and unspotted. Although it is unclear when the duplication of *Dfr* occurred, it is likely that initially both paralogs exhibited the same broad spatial expression pattern. Restriction of *Dfr2* expression to the areas of spot formation subsequently occurred. Because *Dfr2* seems no longer to be activated by the anthocyanin transcription factors that activate the other core anthocyanin enzymes, we suspect its *cis*-regulatory region evolved to respond to a new set of activators that are expressed at the time and position at which spot formation initiates. The gene products of the *P* and *I* loci are candidates for these new activators, since they influence expression of *Dfr2* but not expression of the other core enzyme-coding genes.

The second major type of regulatory change necessary for the evolution of cyanidin-based spots separated the timing of expression of *Dfr2* and *F3′h* on the one hand from *Dfr1* and *F3′5′h* on the other. By itself, divergence in spatial expression domains of the two *Dfr* paralogs might produce a spot of increased pigment intensity, which could explain the existence of *Clarkia* species that produce spots but only malvidin in their flowers (Soltis, 1986), but cannot account for cyanidin/peonidin spots on a malvidin background, as seen in *C. gracilis*. Our analyses suggest that temporal separation of the expression of *F3′h* and *F3′5′h* is largely responsible for the production of cyanidin/peonidin-based anthocyanin in spots.

In most plant species that have been examined, both *F3′h* and *F3′5′h* are active simultaneously and result in the production of malvidin/delphinidin derivatives (Gerats *et al.*, 1982; Tornielli *et al.* 2009, Hopkins & Rausher 2011). We therefore presume this was the pattern in ancestors of *C. gracilis* that lacked spots (only malvidin is produced in the unspotted flowers of *C. breweri* and *C. concinna,* Soltis, 1986). Cyanidin, peonidin and their derivatives can only be produced if *F3′5′h* activity is greatly reduced or eliminated. *C. gracilis* has apparently achieved this by delaying the expression of *F3′5′h* so that, at the time of spot development, only *F3′h* is expressed. By contrast, at the time of background pigment development, only *F3′5′h* is expressed, allowing the accumulation of malvidin derivatives. Finally, restriction of the expression of *Dfr1* to the late stage of development was required to prevent cyanidin- and peonidin-based anthocyanins from being synthesized in the petal background.

Our analysis of the genetic control of spot pattern formation has revealed that the gene *Dfr2* acts as a switch for spot production. Activating this gene completes the anthocyanin pathway and allows pigment deposition in spots. The evolution of this switch mechanism appears to have been facilitated by duplication of the ancestral *Dfr* gene, which allowed one paralog to evolve to serve as a spot regulator. Based on the broad occurrence of petal spots

in *Clarkia*, we infer that the *Dfr* gene duplication occurred early in the radiation of the genus. Subsequent evolutionary remodeling of the regulatory network for both copies of *Dfr*, as well as for *F3′h* and *F3′5′h*, resulted in the novel ability to finely regulate the spatial pattern of contrasting colors in the petals of many *Clarkia* species. Further work in this group, particularly temporal and spatial expression assays of anthocyanin pathway genes in petals from species throughout the genus will help to illuminate the order of events that led to the evolution of petal spot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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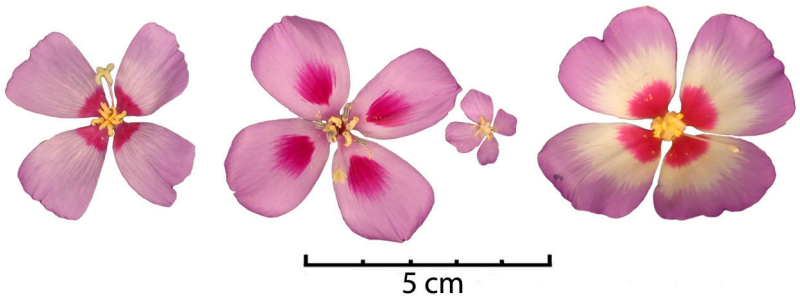

Fig. 1.
Flowers of subspecies in the *C. gracilis* species complex. From left: *C. g.* ssp. *tracyi*, *C. g.* ssp. *sonomensis*, *C. g.* ssp. *gracilis*, and *C. g.* ssp. *albicaulis*.

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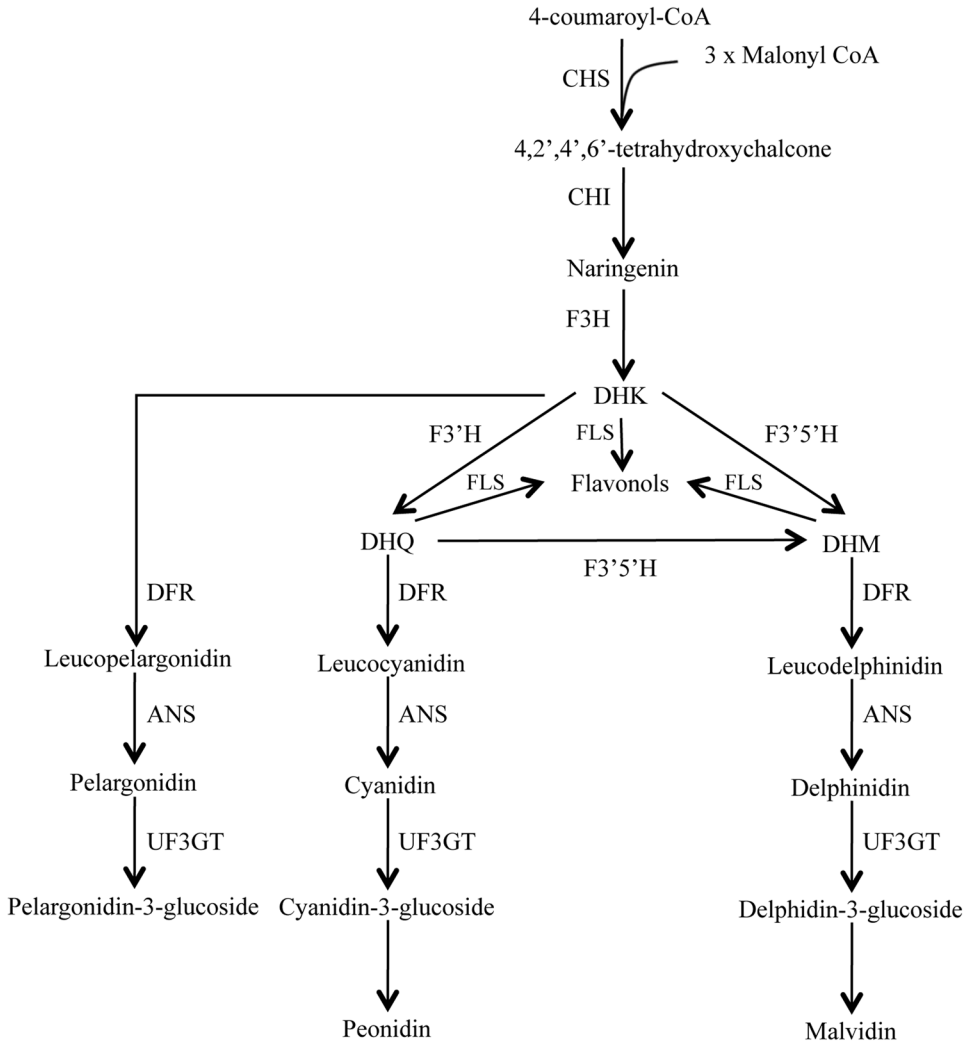

Fig. 2.

Simplified schematic representation of the anthocyanin biosynthetic pathway. Abbreviations for enzymes: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3 hydroxylase; F3′H, flavanoid 3′ hydroxylase; F3′5′H, flavonoid 3′-5′ hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; FLS, flavonol synthase; UF3GT, UDP-flavonoid-3-glucosyl-transferase. Dihydroflavonols: DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin.

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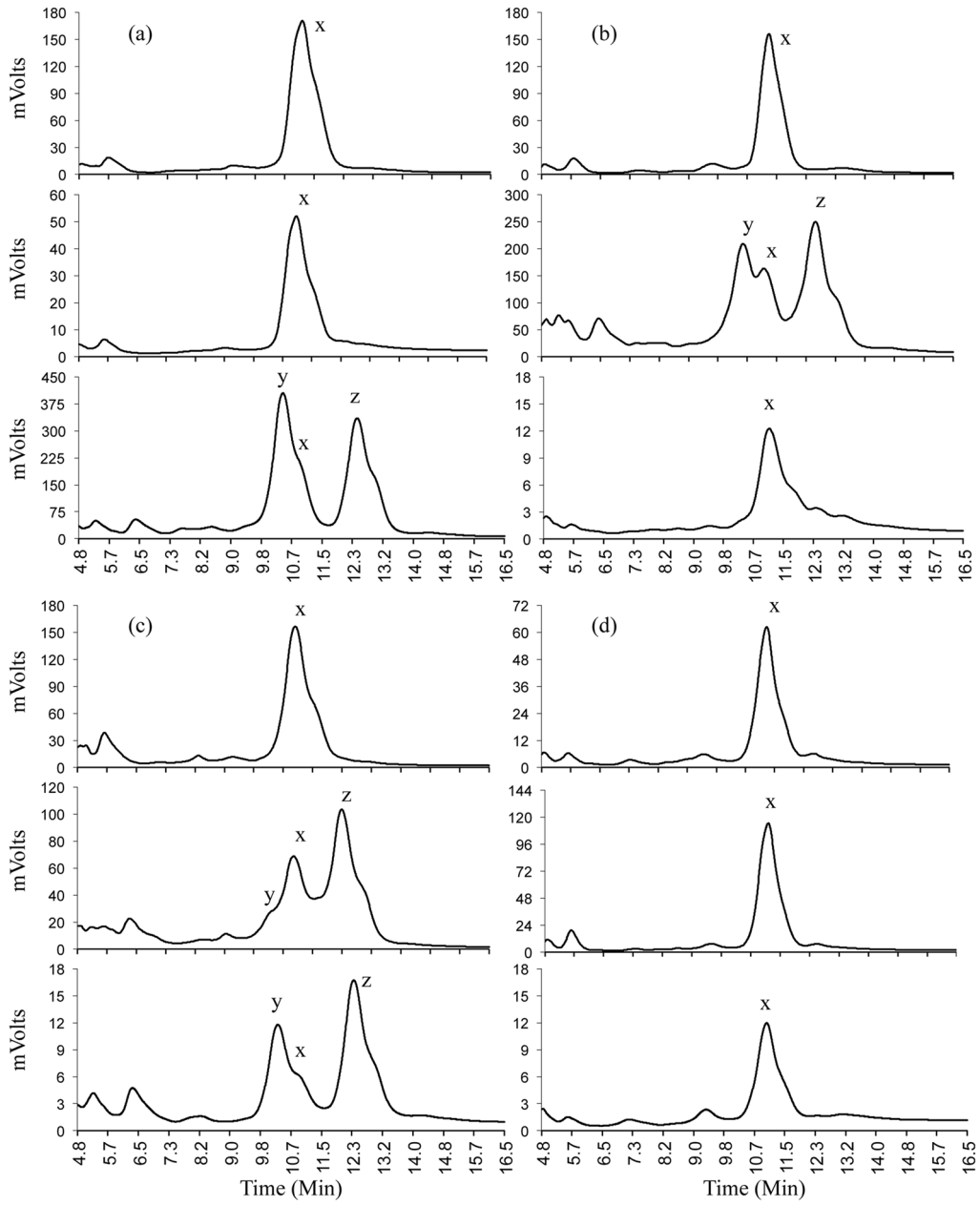

Fig. 3.

Anthocyanidins found in *C. gracilis* flowers. HPLC traces showing pigments in different sections of petals. Each panel (a–d) shows traces for sections of the petal (top, center, and base, in that order) for one of the four phenotypic classes: Basal-spotted (a); central-spotted (b); double-spotted (c), and; unspotted (d). Peaks corresponding to malvidin, cyanidin, and peonidin (indicated x, y, and z, respectively) were identified by comparison to known standards.

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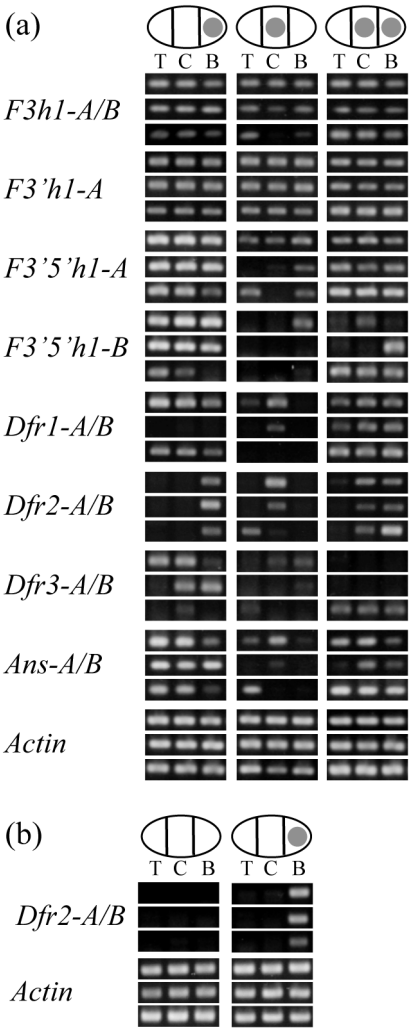

Fig. 4.

Spatial expression of anthocyanin biosynthetic genes in *C. gracilis* petals. (a) Expression patterns for three individual plants for each of the *P*-locus phenotypes. The same nine individuals were used for all genes, and they are placed in the same position for each panel. Ovals represent petals, and filled circles represent spot locations. T, C, and B refer to top, central, and basal sections of the petals. *F3h*, flavanone 3 hydroxylase; *F3′h*, flavanoid 3′ hydroxylase; *F3′5′h*, flavonoid 3′-5′ hydroxylase; *Dfr*, dihydroflavonol-4-reductase; *Ans*, anthocyanidin synthase; (b) Expression patterns for *Dfr2* in three spotted and three unspotted plants from a F2 population segregating for I.

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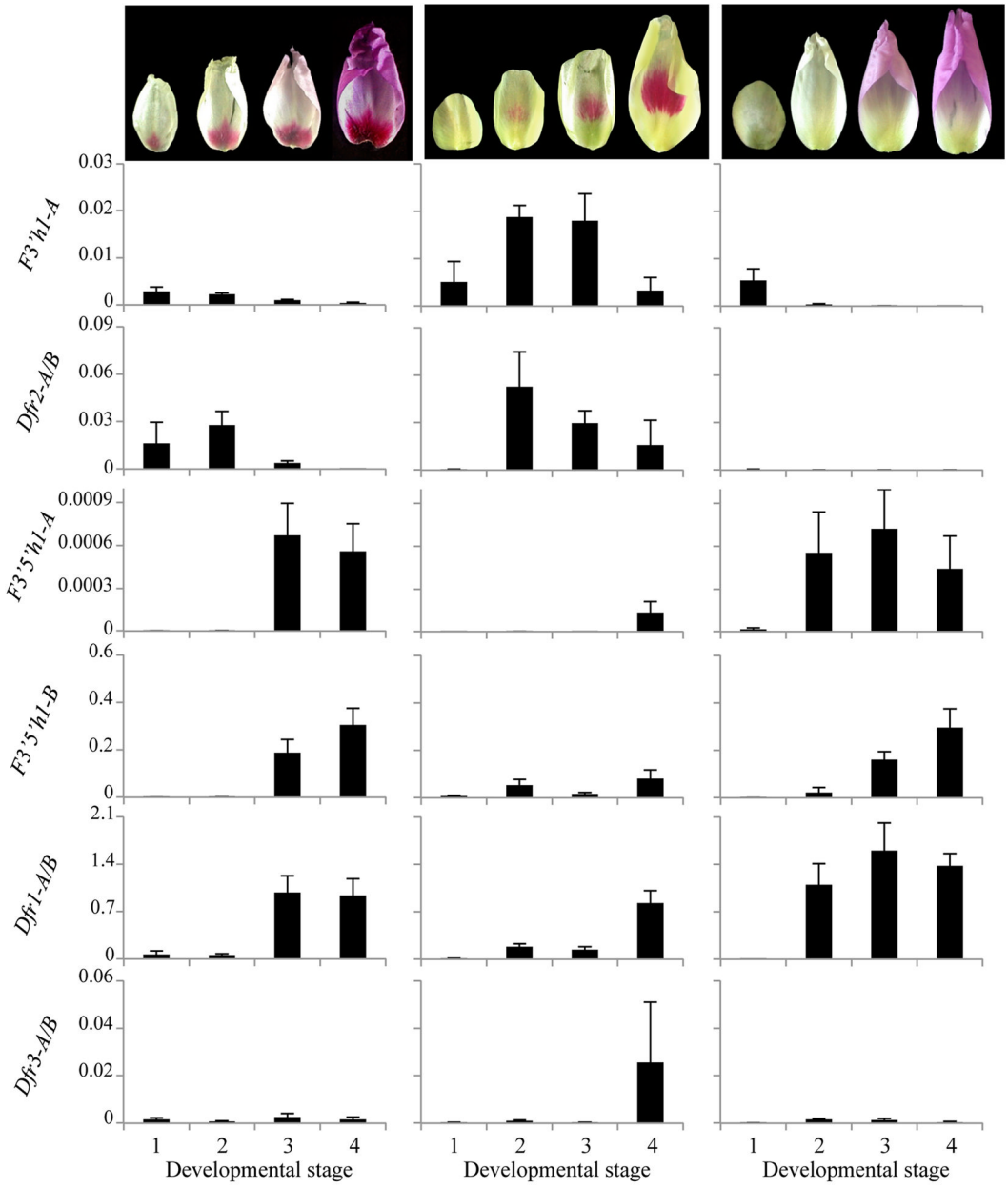

Fig. 5.

Quantitative analysis of gene expression of anthocyanin biosynthetic genes through *C. gracilis* petal development. Photos above the graphs show a representative petal for each of the four developmental stages defined for the purpose of this experiment. From L to R: basal, central, and unspotted. All buds are shown in the upright position (apex at the top, base at the bottom). From left to right, basal spotted plants: spot first appearing, spot well defined, background beginning to show, mature petal. Central spotted plants: no color, spot first appearing, spot well formed, background color beginning to appear. Unspotted plants: young colorless, older colorless, background first appearing, mature petal. Y-axes show expression of genes normalized to *Actin*. *F3′h*, flavanoid 3′ hydroxylase; *F3′5′h*, flavonoid 3′-5′ hydroxylase; *Dfr*, dihydroflavonol-4-reductase. Columns represent averages across three replicates (except last time point for central spotted, n=2), error bars indicate standard error.

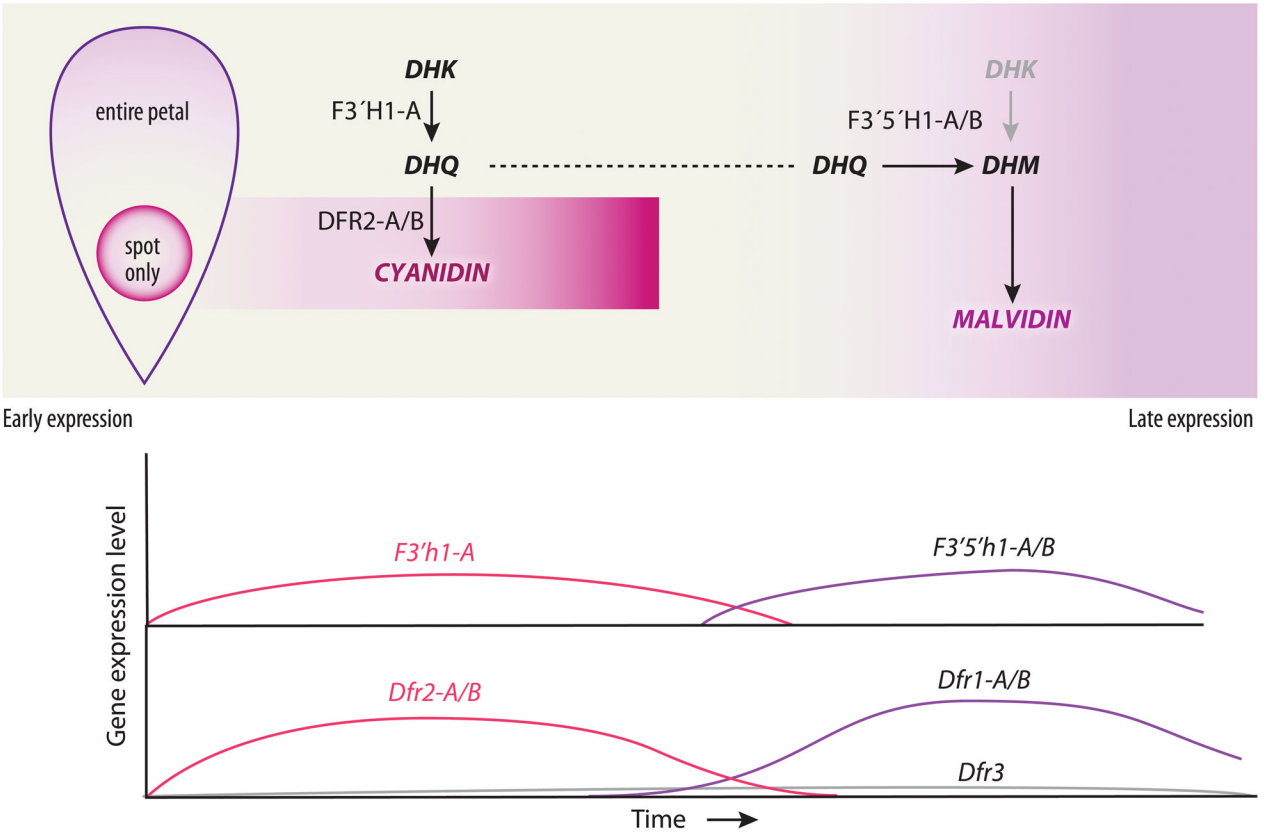

Fig. 6.

Model of pigment pattern development in *C. gracilis* flowers. Early pigment production in spots is explained by *F3′h* expression throughout the petal and *Dfr2* expression in spots, which leads to dihydroquercetin (DHQ) production throughout the petal and cyanidin/peonidin production in spots only. Later in development expression of *F3′5′h* and *Dfr1* converts DHQ (and any residual dihydrokaempferol (DHK)) in the petal background to dihydromyricetin (DHM) and then malvidin. *F3′h*, flavanoid 3′ hydroxylase; *F3′5′h*, flavonoid 3′-5′ hydroxylase; *Dfr*, dihydroflavonol-4-reductase.

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Table 1

Segregation for presence versus absence and position of petal spot in two separate crosses, in F1 and F2 generations of *C. gracilis* plants

		Spot Position			
	n	**Central**	**Basal**	**Double**	**Absent**
P cross					
F1	23		-	23	-
F2	82	18	23	41	-
I cross					
F1	7	-	7	-	-
F2	101	-	77	-	24

P cross refers to central-spotted *C. g. sonomensis* basal-spotted *C. g. albicaulis* cross, while *I* cross refers to unspotted *C. g. gracilis* basal-spotted *C. g. albicaulis* cross.

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Table 2

Pigment composition in *C. gracilis* petals with different spot phenotypes

		Phenotype			
		Basal spot	**Central spot**	**Central & Basal spot**	**No spot**
Petal section	Top	0.171 (±0.050), 5	0.132 (±0.026), 3	0.205 (±0.102), 3	0.137 (±0.033), 2
	Center	0.227 (±0.078), 2	3.524 (±1.599), 5	2.717 (±0.179), 3	0.143 (±0.026), 2
	Base	4.55 (±1.020), 5	0.494*, 1	5.316 (±1.091), 3	0.355 (±0.115), 2

Numbers refer to the ratio of cyanidin+peonidin: malvidin (mean ± standard deviations), followed by number of individuals sampled. Amount of each pigment was inferred from the average peak height between 10.4–10.5 min. for cyanidin, 11.1–11.2 min. for malvidin, and 12.3–12.4 min. for peonidin.

*All but one central-spotted individuals had spots that extended close to the petal base (on the abaxial, but not the adaxial surface), impeding dissection of spot-free tissue. Therefore, only one individual was used.

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Table 3

Analysis of co-segregation of alleles at the *DFR2-A* and *DFR2-B* loci with phenotypes obtained from the *I* and *P* crosses

		***I* Cross**			***P* Cross**			
		Phenotype			**Phenotype**			
Homeolog	**Genotype**	**S**	**U**	***P***	**B**	**D**	**C**	***P***
Dfr2-A	A1A1	11	2		3	5	1	
	A1A2	19	5	0.591	5	8	5	0.9314
	A2A2	12	1		2	2	1	
Dfr2-B	B1B1	10	4		3	1	0	
	B1B2	18	3	0.2365	0	6	2	0.0843
	B2B2	14	1		6	8	5	

Different alleles of *Dfr2-A/B* were identified using SNPs and indels in introns 2 or 3.

Abbreviations: S = spotted; U = unspotted; B = basal-spotted; D = double-spotted; C = central spotted. *P* are from Fisher's exact test. *Dfr*, dihydroflavonol-4-reductase.