Genetic basis for a rare floral mutant in an Andean species of Solanaceae[1]

Rachel A. Coburn[2], Randi H. Griffin[2,3], and Stacey D. Smith[2,4,5]

[2]University of Nebraska-Lincoln, 1104 T Street, Manter Hall, Lincoln, Nebraska 68588 USA; [3]Duke University, 130 Science Drive, Biological Sciences Building 108, Durham, North Carolina 27708 USA; and [4]University of Colorado Boulder, C127 Ramaley Hall, Campus Box 334, Boulder, Colorado 80309 USA

- *Premise of the study:* White forms of typically pigmented flowers are one of the most common polymorphisms in flowering plants. Although the range of genetic changes that give rise to white phenotypes is well known from model systems, few studies have identified causative mutations in natural populations.
- *Methods:* Here we combine genetic studies, in vitro enzyme assays, and biochemical analyses to identify the mechanism underlying the loss of anthocyanin pigment production in the naturally occurring white-flowered morph of *Iochroma calycinum* (Solanaceae).
- *Key results:* Comparison of anthocyanin gene sequences revealed a putative loss-of-function mutation, an 11 amino-acid deletion in dihydroflavonol 4-reductase (DFR), in the white morph. Functional assays of *Dfr* alleles from blue and white morphs demonstrated that this deletion results in a loss of enzymatic activity, indicating that the deletion could be solely responsible for the lack of pigment production. Consistent with this hypothesis, quantitative PCR showed no significant differences in expression of anthocyanin genes between the morphs. Also, thin layer chromatography confirmed that the white morph continues to accumulate compounds upstream of the DFR enzyme.
- *Conclusions:* Collectively, these experiments indicate that the structural mutation at *Dfr* underlies the rare white flower morph of *I. calycinum*. This study is one of only a few examples where a flower color polymorphism is due to a loss-of-function mutation in the coding region of an anthocyanin enzyme. The rarity of such mutations in nature suggests that negative consequences prevent fixation across populations.

Key words: anthocyanins; delphinidin; dihydroflavonol 4-reductase; flavonol; flower color; *Iochroma*; pleiotropy; quercetin; Solanaceae.

Many species of flowering plants exhibit naturally occurring color polymorphisms (Warren and Mackenzie, 2001; Rausher, 2008). The study of these polymorphisms has provided insight into a wide range of fundamental evolutionary questions (Clegg and Durbin, 2000), from the relative importance of natural selection and genetic drift (Schemske and Bierzychudek, 2007) to how variation is maintained within species (Gigord et al., 2001; Irwin, 2003). Because of its evolutionary lability, flower color has also become an important model for investigating the predictability of evolution, and in particular, whether certain genes are hotspots for phenotypic evolution (Stern and Orgogozo, 2009; Streisfeld and Rausher, 2011).

White forms of typically purple-, blue-, or pink-flowered species are one of the most common and well-studied classes of flower color polymorphisms (Richards, 1986; Rausher, 2008). Surveys spanning many angiosperm families indicate that these white forms lack the anthocyanin pigmentation present in the colored morphs (Warren and Mackenzie, 2001; Whittall et al., 2006; Rausher, 2008). This absence of pigmentation may be limited to the flowers (Tiffin et al., 1998; Dick et al., 2011) or characterize the entire plant (Les et al., 1989; Wu et al., 2013). Given the extensive knowledge of the flavonoid pathway that produces anthocyanins and its broad conservation across land plants (Winkel-Shirley, 2001; Grotewold, 2006; Campanella et al., 2014), it is possible to pinpoint the types of genetic changes that could result in the origin of a white-flowered morph. For example, mutations in any of the transcription factors that regulate the pathway (bHLH, MYB, and WD40) (Mol et al., 1998; Koes et al., 2005) could lead to the loss of expression of the structural genes (e.g., *Chi*, *F3h*; Fig. 1) and thus produce a

[1]Manuscript received 8 September 2014; revision accepted 22 December 2014.

The authors thank J. Stone, D. Pilson, and members of the Smith laboratory for advice as well as two anonymous reviewers for helpful suggestions on the manuscript. They also thank the Ministero del Ambiente of Ecuador for providing permits to conduct research (031-IC-FLO-DBAP-MA and 05-2012-IC-FLO-DBAP-MA) and the National Herbarium of Ecuador (QCNE) and the Herbarium of the Pontificia Universidad Católica (QCA) for assistance with fieldwork. This publication was made possible by grants from the National Science Foundation (DEB 1355518), the National Center for Research Resources (5P20RR016469), and the National Institute for General Medical Science (NIGMS-INBRE 8P20GM103427), a component of the National Institutes of Health (NIH). The contents are the sole responsibility of the authors and do not represent the views of NIGMS, NIH, or NSF.

[5]Author for correspondence (e-mail: stacey.d.smith@colorado.edu)

doi:10.3732/ajb.1400395

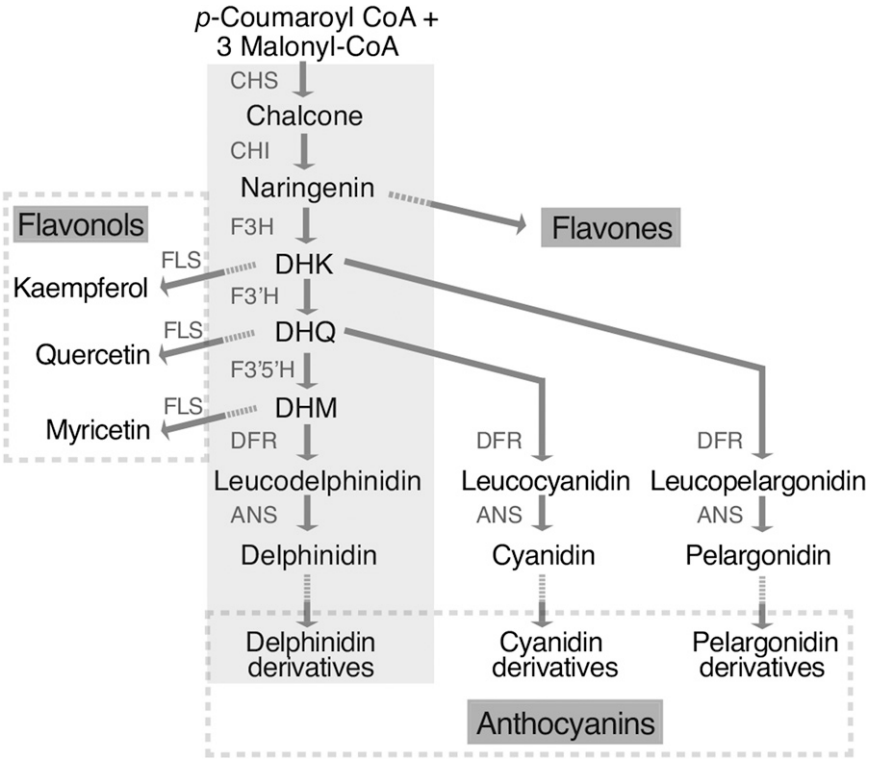

Fig. 1. Flavonoid biosynthetic pathway. Black text indicates substrates (DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin); gray text indicates enzymes (CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase). Products of this pathway include flavonols, flavones, and pigmented anthocyanins. Although the pathway can produce pelargonidin (red)- and cyanidin (purple)-derived anthocyanins, blue-flowered *Iochroma* species are pigmented with only blue delphinidin-derived anthocyanins.

white flower (de Vetten et al., 1997; Spelt et al., 2002; Morita et al., 2006). Equally, mutations in the structural genes themselves, whether in the *cis*-regulatory regions or in coding sequences, could cause the loss of pigmentation (e.g., Martin et al., 1985; van Houwelingen et al., 1999).

Although a wide range of mutations may potentially result in a white-flowered phenotype, their pleiotropic effects on flavonoid production may limit the extent to which these mutations persist in natural populations. In addition to their function in floral pigmentation and pollinator signaling (Schemske and Bradshaw, 1999; Streisfeld and Kohn, 2007), anthocyanins and related flavonoids play important roles in plant physiology. For example, flavonols are involved in male fertility (Mo et al., 1992; Pollak et al., 1993) and auxin transport (Kuhn et al., 2011), while both flavonols and anthocyanins have been implicated in response to UV stress (Chalker-Scott, 1999; Ryan et al., 2002). Thus, mutations that disrupt flavonoid production may result in negative consequences for plant fitness beyond their effects on flower color. Mutations in upstream genes (*Chs*, *Chi*; Fig. 1), affecting all classes of flavonoids, carry the greatest potential for negative pleiotropic effects and would be expected to be rare in nature (Coberly and Rausher, 2003). By contrast, mutations in downstream genes (*Dfr* and *Ans*; Fig. 1) could alter anthocyanin production without affecting flavones and flavonols and thus may contribute more commonly to flower color polymorphisms (Wu et al., 2013). Similarly, regulatory changes (in *cis* or *trans*) can allow floral specific loss of pigmentation with few consequences for flavonoid production in other tissues (Streisfeld and Rausher, 2009; Smith and Rausher, 2011; Streisfeld et al., 2013).

Despite this strong theoretical framework for investigating the genetic basis of white-flowered morphs, we have only a handful of cases in which the causative mutations have been identified in natural populations (*Ipomoea purpurea*: Habu et al., 1998; Chang et al., 2005; *Parrya nudicaulis*: Dick et al., 2011; *Mimulus lewisii*: Wu et al., 2013). These studies identified changes in both *cis*-regulatory (Dick et al., 2011) and coding regions (Habu et al., 1998; Chang et al., 2005; Wu et al., 2013) affecting three different loci (*Chs*, *Dfr*, and *Myb*). Given the large number of loci that could potentially result in a white-flowered morph (Fig. 1), expanding these studies to additional cases may reveal a broader spectrum of causative mutations. On the other hand, if mutations affecting particular loci have strongly negative consequences, the pool of loci underlying color polymorphisms may be limited. Here we focus on *Iochroma calycinum* Benth. (Solanaceae), a blue-flowered Andean shrub that exhibits rare white morphs. Combining analysis of anthocyanin gene sequences, comparative expression studies, in vitro assays of gene function, and flavonoid biochemistry, this study seeks to identify the genetic changes responsible for the loss of pigmentation in the white *I. calycinum*. Specifically, we aimed to distinguish between changes in gene expression and gene function as causative factors and, if possible, to determine the underlying mutation. Understanding the spectrum of changes that give rise to such polymorphisms is important not only for characterizing the number of routes to novel phenotypes,

but also the extent to which the predictability of genetic evolution commonly observed above the species level (e.g., Smith and Rausher, 2011) extends to variation within populations.

MATERIALS AND METHODS

***Study system*—**The genus *Iochroma* (Solanaceae) consists of approximately 25 species distributed throughout the Andes of South America and is best known for its diversity of flower forms and colors (Smith and Baum, 2006). *Iochroma* and allied genera fall into the Physaleae clade of the large Solanoid radiation (Olmstead et al., 2008). Like several other lineages of Physaleae, *Iochroma* includes a number of species with inflated calyxes, including *I. calycinum*. However, *I. calycinum* is the only species of *Iochroma* in which the calyx matches the deep blue color of the corolla (Fig. 2A). This species is native to northern Ecuador and Colombia and is principally pollinated by hummingbirds (Smith et al., 2008). Like many *Iochroma* species, the population sizes are small, on the order of 25 to 50 individuals. The common Spanish name of the species is teta de vaca (cow's teat plant) because its inflated calyx swells with viscous fluid before anthesis. These fleshy fluid-filled calyces have been hypothesized to deter florivores and nectar robbers (Lagerheim, 1891; Endress, 1996).

Field surveys and herbarium studies revealed that white *I. calycinum* mutants exist, albeit in low frequencies. While the common morph has a dark blue corolla and calyx, the white morph appears to produce no anthocyanin pigments in the vegetative or floral tissues. One white morph was sampled from the cloud forests around Chiriboga, Ecuador (0.22602°S, 78.76962°W), and another was observed in herbarium collections (QCA) from that same site. No other white morphs were observed among the roughly 150 specimens examined at other herbaria (MO, F, QCNE) or among over 75 plants seen in the field, suggesting that albino-flowered *I. calycinum* plants are extremely rare in nature. Blue morphs were sampled in and around the Reserva Rio Guajalito in Chiriboga, Ecuador (0.24577°S, 78.80903°W). Floral and leaf tissue of the blue morphs and the white morph were collected in silica gel for DNA sequencing and thin layer chromatography, and floral buds (ca. 1 cm long) were collected in RNAlater (Qiagen, Valencia, CA) for gene expression studies. Sampling at this bud size (roughly equivalent to *Petunia* bud stage 5) captures the expression of both upstream and downstream genes as corollas are just beginning to show color (Pollak et al., 1993; Smith and Rausher, 2011). Herbarium vouchers for the blue (*Smith 471*) and white (*Smith 510*) morphs were deposited at MO and QCA (Appendix 1).

***Sequence analysis of anthocyanin structural genes*—**Given the structure of the flavonoid pathway (Fig. 1), there are seven enzymes required for producing the delphinidin-derived anthocyanin pigments that are found in blue-flowered *Iochroma* species (Smith and Rausher, 2011). White flowers could potentially arise by structural (coding sequence) mutations at any of these loci (*Chs*, *Chi*, *F3h*, *F3′h*, *F3′5′h*, *Dfr*, and *Ans*). The two hydroxylating enzymes *F3′h* and *F3′5′h* may appear to be weak candidate genes for white flowers because loss of function at these loci should convert a blue delphinidin-producing flower to a red pelargonidin-producing flower (Smith and Rausher, 2011). However, in all Solanaceae examined to date, DFR shows some substrate specificity, usually exhibiting lower activity on DHK, the precursor of red pelargonidin pigments, relative to other substrates (Johnson et al., 2001; Des Marais and Rausher, 2008; Smith et al., 2013). Thus, depending on the specificity of downstream enzymes, a mutation at *F3′h* or *F3′5′h* could result in a white flower (van Houwelingen et al., 1998; Brugliera et al., 1999).

To identify putative loss-of-function mutations in the white morph, we amplified each of the seven genes from one blue and one white individual using custom primers (Appendix S1, see Supplemental Data with the online version of this article) designed from an unpublished *Iochroma cyaneum* 454 floral transcriptome. Although one of these genes (*Chs*) is commonly present in multiple copies in Solanaceae, only one or a few copies are florally expressed (Koes et al., 1989; Van der Meer et al., 1990; Morita et al., 2012), facilitating primer design from the *Iochroma* transcriptome. Polymerase chain reactions were carried out with iProof High-Fidelity DNA Polymerase following manufacturer's protocols (Bio-Rad, Hercules, California, USA [CA]), and the products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Direct sequencing was performed using the BigDye Terminator v3.1 Sequencing Kit (Invitrogen, Carlsbad, CA), and Sanger sequencing was conducted at Duke University's Genome Sequencing and Analysis Core (Durham, North Carolina, USA). Chromatograms were edited and analyzed using the programs Four Peaks v.1.7.1 (Griekspoor and Groothuis, 2007) and MacClade 4.0 (Maddison and Maddison, 2005). Full-length coding sequences were deposited into GenBank (Appendix 1), and the alignments and trees for each gene were deposited in TreeBase under study number S16161 (http://www.treebase.org).

Gene trees were estimated for each of the seven genes from both the white and blue *I. calycinum* morphs to verify orthology with known anthocyanin loci from model systems. Coding sequences from five closely related species (*Petunia hybrida*, *Nicotiana benthamiana*, *Solanum lycopersicum*, *Solanum tuberosum*, and *Iochroma cyaneum*) were retrieved from GenBank, http://solgenomics.net, or transcriptomic data in the case of *I. cyaneum* (Appendix 1). Sequences were aligned manually in MacClade 4.08a (Maddison and Maddison, 2005), and maximum likelihood trees were estimated using a heuristic search in the program PAUP* version 4.0b10 (Swofford, 2002) with a GTR+G model and tree-bisection-reconnection (TBR) branch swapping. The resulting gene trees were rooted with *Petunia* sequences as the outgroup.

***Assay of DFR function*—**Our sequence analyses identified a large deletion in the coding region of *Dfr* in the white morph. To determine whether this mutation affects DFR activity we performed in vitro assays of protein function using sequences from both color morphs. The *Dfr* gene from each morph was directionally cloned into the pENTR-SD/D-TOPO vector and recombined into the pDEST14 overexpression vector using the Gateway kit (Invitrogen, Carlsbad, CA). The vectors were transformed into strain BL21* (DE3) of *Escherichia coli* (Invitrogen). Overnight cultures of 5 mL LB-AMP_{100} were used to inoculate 200 mL cultures and grown at 37°C until reaching OD_{600} 0.5. To induce expression, 500 µL of 200 mM isopropyl-β-D-thiogalactopyranoside was added to the culture and incubated for 3 h at 28°C. Expression of the transgene was verified with qualitative real-time PCR using 2.5 ng of cDNA template synthesized from pelleted cells from 15 mL of postexpression culture. A functional assay was performed on the crude protein extract following Des Marais and Rausher (2008), and dihydroflavonols (DHK, DHQ, and DHM) were used as the substrates. The resulting leucoanthocyanidins were heated at 95°C for 30 min in 95% butanol–5% HCl v/v to convert the substrates into colored anthocyanidin products (delphinidin, cyanidin, and pelargonidin). These were quantified by measuring peak absorbance in a Beckman DU-800 spectrophotometer. Absorbance values were converted into µmoles by comparison with a standard dilution series. The percentage of substrate converted was calculated by dividing the µmoles of anthocyanidin produced by the µmoles of dihydroflavonol substrate provided.

***Quantification of anthocyanin gene expression*—**Gene expression was measured to determine whether the loss of pigment in the white morph could be due to expression level differences in pathway genes. Total RNA was extracted from RNAlater-preserved floral buds of the white morph and five blue-flowered individuals using the Spectrum kit (Sigma-Aldrich, St. Louis, Missouri, USA). An on-column DNase digestion (Qiagen) was performed to remove gDNA from extracted RNA. First strand cDNA synthesis was done with 0.5 µg total RNA in a 20 µL reaction using SuperScript II Reverse Transcriptase following manufacturer's instructions (Invitrogen). The resulting cDNA was diluted to 2.5 ng/µL for quantitative real-time PCR (qPCR). Each 20 µL qPCR reaction contained 2.5 ng of the cDNA template, 10 µL DyNAmo SYBR Green mix (Invitrogen), 0.4 µL 1× 6-carboxy-X-rhodamine passive reference dye, 0.4 µL of forward primer (10 µM), and 0.4 µL of reverse primer (10 µM). The qPCR primers for *Chi*, *Chs*, and *F3h* were designed as a part of this study; those for the remaining genes were those of Smith and Rausher (2011) (online Appendix S2). Elongation factor (EF1-α) was used as a reference gene. The qPCR reactions were run on an ABI 7500 Fast machine under the following conditions: 95°C for 10 min; followed by 40 cycles of 95°C for 20 s and 55°C for 1 min; and a dissociation step to verify primer specificity. The qPCR products were additionally run on a 2% high resolution agarose gel to verify that only one product was amplified. Three to six replicate reactions were run for each cDNA and primer combination. Efficiency for each gene was determined as described by Peirson et al. (2003). We calculated relative expression levels following Pfaffl (2001) to quantify the variation across loci and across blue flowered individuals and determine whether the white individual showed significantly lower expression of any of the structural genes. Differences in relative expression across individuals and morphs were analyzed using a linear mixed model with REML model fitting in JMP Pro 11 (SAS, Cary, North Carolina, USA), treating color as a fixed effect and individual as a nested random effect.

***Identification of anthocyanidins and related flavonoids*—**Floral and vegetative flavonoids (including anthocyanidins) were identified using thin layer chromatography to establish any differences between the blue and white morphs. Flavonoids were extracted from 0.025 g of dried flower or leaf tissue by soaking

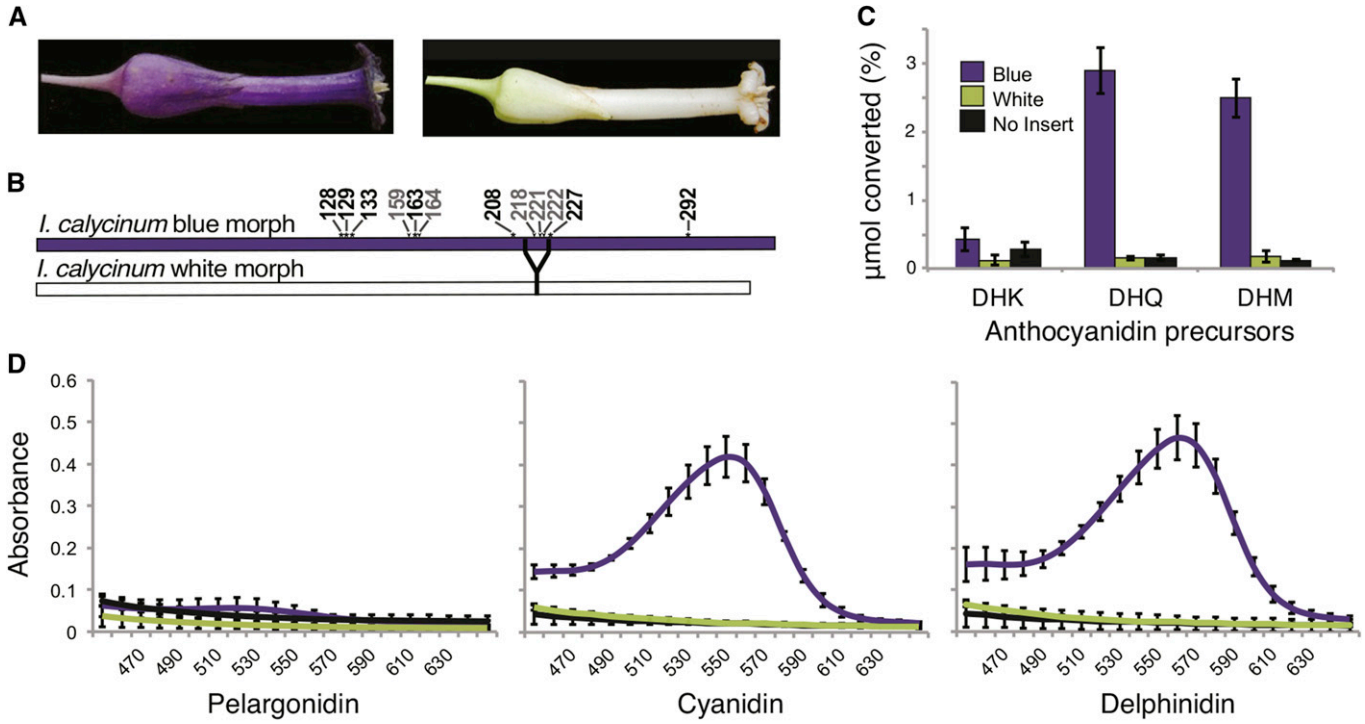

Fig. 2. Relationship of *Dfr* structure and function to flower color. (A) Floral phenotype of blue and white morph of *Iochroma calycinum*. (B) Diagram of structural mutation in *Dfr* associated with white morph. Residues important for substrate binding (black numbers) and active site conformation (gray numbers) are based on the crystal structure of grape (Petit et al., 2007). The deletion in the white allele is indicated by the vertical black bar. (C) Percentage µmoles of the substrates DHK, DHQ, and DHM converted into pelargonidin, cyanidin, and delphinidin, respectively, by the blue morph DFR, white morph DFR, and the vector without an insert. The absorbance curves for these products are shown in (D). Peak absorbance for each product was converted to percentage µmoles using standard dilution measurements. Error bars represent ±1 SD. (D) Absorbance curves for pelargonidin, cyanidin, and delphinidin, respectively, from the functional assays of the DFR proteins from the blue and white morphs of *I. calycinum*. The shape of these curves traces the characteristic absorbance spectrum for each compound. Line colors follow (C). See Fig. 1 caption for abbreviations.

overnight in 2 mL 2N HCl and refluxed at 95°C for 1 h to convert the glycosylated compounds (e.g., anthocyanins) to their corresponding aglycones (e.g., anthocyanidins; Harborne, 1998). Flavonols were separated by washing twice with 0.5 mL ethyl acetate, and anthocyanidins were extracted by washing once with 0.15 mL isoamyl alcohol. Both layers were evaporated to dryness, eluted in 50 µL methanol 1% HCl, and spotted onto cellulose plates for chromatography. Plates were developed for 4 to 6 h in forestal solvent (30 : 3 : 10 acetic acid–12 N HCl–$H_2$0), and compounds were identified by comparison of R_f values with standards. Standards for delphinidin, malvidin, petunidin, myricetin, quercetin, and kaempferol were obtained from Extrasynthese (Genay, France).

RESULTS

***Sequence variation between blue and white morphs*—**Full coding regions were obtained for seven structural anthocyanin pathway genes (*Chs*, *Chi*, *F3h*, *F3′h*, *F3′5′h*, *Dfr*, and *Ans*) from both morphs of *I. calycinum*. Phylogenetic analyses suggest that these sequences are orthologous with the functionally characterized enzymes from model systems. At the amino acid level, the sequences from *I. calycinum* were 77–95% similar to sequences from other Solanaceae. Moreover, the topology of the gene trees for each locus mirror the species relationships, with *Iochroma* more closely related to *Solanum* than to *Nicotiana* or *Petunia* (online Appendix S3). Although, like other Solanaceae, *Iochroma* has multiple genomic copies of *Chs* (S. D. Smith, unpublished data), we isolated a single florally expressed copy that is most similar to *CHS-A*, the dominant copy expressed in *Petunia* flowers (Koes et al., 1986). This pattern suggests that the floral-specific activity of this "A" copy may be broadly conserved across Solanaceae.

We examined sequence variation in these structural genes to identify differences that could underlie the flower color polymorphism, such as frameshifts, premature stop codons, or insertion-deletion (indel) events. Comparing the sequences from the white and blue morphs of *I. calycinum* across all loci, we observed 14 synonymous mutations, five nonsynonymous mutations, and one deletion in *Dfr* (online Appendix S4). These nonsynonymous changes could potentially reduce enzyme function and account for the loss of pigment production; however, in each case, the amino acid variant present in the white morph allele was also present in one or more pigment-producing outgroup species (*P. hybrida*, *N. benthamiana*, *S. lycopersicum*, *S. tuberosum*, and *I. cyaneum*). Moreover, since none of the genes from the white morph had more than one nonsynonymous difference compared with the blue morph, negative interactions among mutations at the same locus in the white morph would also not explain the phenotype. By contrast, the *Dfr* deletion was unique to the white morph and would be predicted to affect function given its position in the protein. The 11 amino-acid deletion spans position 216–226 in the previously elucidated grape (*Vitis vinifera*) crystal structure, and this area includes multiple sites important for the conformation of the active site for the enzyme (Fig. 2B; Petit et al., 2007). If this mutation were responsible for the loss of pigmentation, we would expect it to be absent in (or at least heterozygous) in all pigmented individuals. A PCR screen of five additional blue

individuals confirmed that all lacked the deletion while the white individual was homozygous for the deletion (online Appendix S5).

***Nonfunctional DFR in white* I. calycinum—**In vitro assays comparing the function of blue and white alleles of *Dfr* indicate that the 11 amino-acid deletion results in a loss of enzymatic activity. A functional *Dfr* should convert dihydroflavonols into leucoanthocyanidins (which are converted to anthocyanidins during the assay protocol). The allele from the blue-flowered *I. calycinum* was able to convert all three dihydroflavonol precursors into colored anthocyanidins, while the white allele was nonfunctional (Fig. 2C, D). The pattern of activity for the blue morph copy was similar to other blue-flowered *Iochroma* species previously examined (Smith and Rausher, 2011; Smith et al., 2013) where activity is low on DHK (the precursor of red anthocyanins) and high on DHQ and DHM (the precursors of purple and blue anthocyanins). By contrast, the deletion-carrying allele from the white morph of *I. calycinum* showed negligible activity on any substrate and was not statistically different from the vector without an insert (Fig. 2C). Given that the white allele of *Dfr* shows no putative loss-of-function mutations other than the deletion, these results suggest that the disruption of the active site caused by the deletion is sufficient to eliminate enzymatic activity.

Expression differences between blue and white morphs— Comparisons of gene expression levels between the white morph and five randomly selected blue morphs revealed no significant association between gene expression and color. We observed about a 5-fold difference in expression across individuals and genes; however, in all cases the expression levels for the white sample fell within the range of the blue samples (Fig. 3). Looking within genes, levels of gene expression varied roughly 3-fold across individuals, a range similar to that observed for intraspecific expression variation in other studies (Streisfeld and Rausher, 2009; Cooley et al., 2011; Smith and Rausher, 2011) and which may reflect slight differences in the age of the buds sampled or simply developmental noise. When white and blue samples were compared, expression levels for the white individual were relatively low for *Dfr* and *Ans*, but equivalent to or higher than the blue individuals for the other five loci. Even for *Dfr* and *Ans*, the average expression was similar to at least one of the blue individuals, and results from linear mixed models showed no significant effect of color on expression (P = 0.16–0.76 across genes). In addition, the differences in expression observed across the *I. calycinum* individuals are minor relative to the changes that affect flower color in other cases (24- to 200-fold; Streisfeld and Rausher, 2009, 2011; Des Marais and Rausher, 2010; Dick et al., 2011; Smith and Rausher, 2011). Overall, these results indicate that differences in gene expression are not likely to be responsible for the white morph of *I. calycinum*. In addition, the pattern of expression variation suggests transcription factors did not play a role in the lack of pigmentation because, in that case, we would expect to see multiple genes coordinately downregulated in white flowers (Whittall et al., 2006).

Effects of* Dfr *loss of function on flavonoid production— Thin layer chromatography (TLC) was conducted on floral and leaf tissue of blue and white *I. calycinum* to determine whether anthocyanidins and related flavonoids were being produced. If the *Dfr* deletion is the only change in the pathway, we would predict that anthocyanidin production would be lost while upstream products (e.g., flavonols) would not be affected (Fig. 1). The TLC analysis confirmed the presence of the two anthocyanidins (delphinidin and malvidin, a delphinidin derivative) in blue-flowered *I. calycinum* and the lack of these compounds in

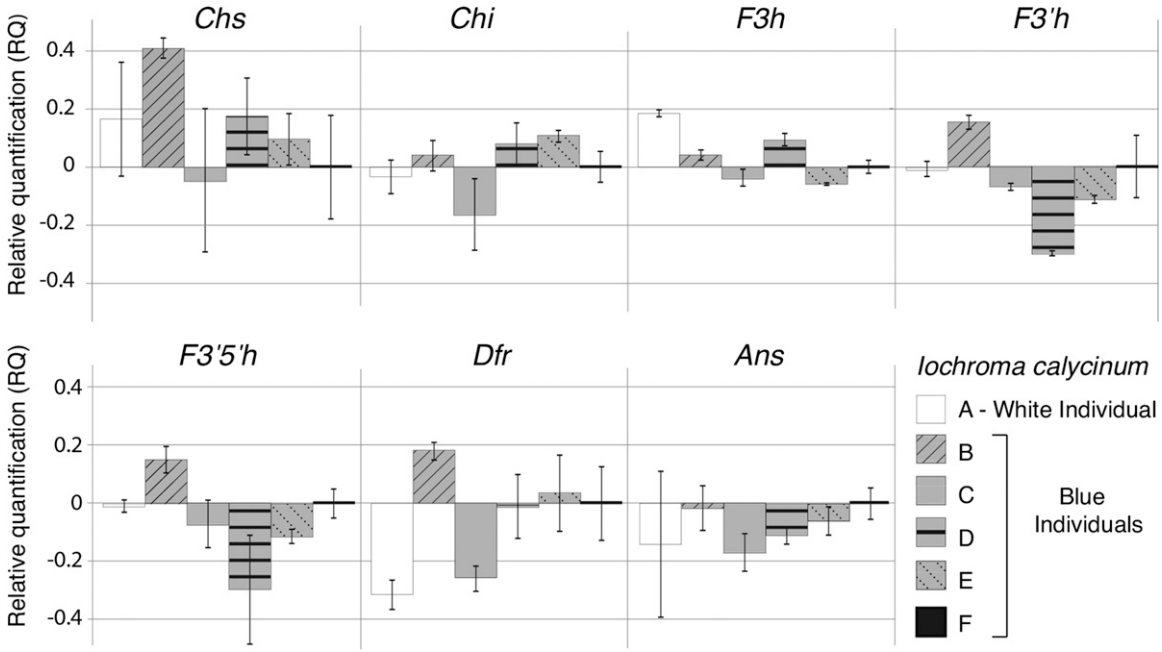

Fig. 3. Anthocyanin gene expression differences between white individual and five blue individuals of *Iochroma calycinum*. Expression was measured in floral bud tissue for *Chs*, *Chi*, *F3h*, *F3′h*, *F3′5′h*, *Dfr*, and *Ans* (see Fig. 1 caption for gene abbreviations), and mean relative quantification values were calculated (±1 SE). Expression values are shown relative to individual F, which was randomly chosen among the blue individuals. The RQ values correspond to a $\log_{10}$-fold change in expression in comparison to individual F.

the white morph (online Appendix S6). These delphinidin-derived pigments are also responsible for blue and purple colors in other *Iochroma* and related species (Smith and Rausher, 2011; S. D. Smith, unpublished data). The flowers of the blue morph also produced the flavonol quercetin, and consistent with our prediction, this compound is also present in the flowers of the white morph. None of these compounds were detected in leaf tissue (Appendix S6).

DISCUSSION

The goal of this project was to determine the genetic basis for the lack of pigmentation in the white morph of the normally blue-flowered *I. calycinum*. Analysis of the coding regions of anthocyanin structural genes revealed a single putative loss of function mutation (an 11-amino-acid deletion in DFR) distinguishing the blue and white morphs. Assays of DFR function using alleles cloned from each morph confirmed that this deletion causes a loss of enzyme activity and thus blocks anthocyanin production. We did not observe any significant differences in gene expression between the blue and white morphs, suggesting that this structural mutation alone underlies the loss of flower color. Consistent with the downstream position of DFR in the pathway (Fig. 1), we found that the white morph continues to make upstream pathway products (flavonols) despite the loss of pigmentation.

Functional evolution of DFR and flower color variation—Like the other enzymes of the flavonoid biosynthetic pathway, DFR and its role in pigment production are deeply conserved, dating back to the common ancestor of land plants (Rausher, 2006; Campanella et al., 2014). This enzyme is responsible for reducing dihydroflavonols such as DHK, DHQ, and DHM to form leucoanthocyanidins, which in turn are converted into anthocyanin pigments. Although DFR has the potential to act on multiple substrates, functional analyses commonly reveal substrate specialization, where the enzyme preferentially reduces particular compounds (Johnson et al., 2001; Halbwirth et al., 2003; Xie et al., 2004; Des Marais and Rausher, 2008; Leonard et al., 2008). Previous studies demonstrated that *Dfr* copies from blue-flowered *Iochroma* species show higher activity on DHQ and DHM, the precursors of purple and blue anthocyanins than on DHK, the precursor of red anthocyanins (Smith et al., 2013). This study shows that the blue-flowered *I. calycinum* shares the same pattern of substrate specificity, which has also been documented in *Petunia* (Johnson et al., 2001).

These comparative functional studies of DFR together with the recently published crystal structure from *V. vinifera* (Petit et al., 2007) have clarified the regions of the enzyme that are involved in function and specificity. Sites important for specificity fall into two clusters in the gene, one around position 133 in *V. vinifera* and another around position 227 (Petit et al., 2007). Although early studies emphasized the role of the first cluster in underlying species differences in pigmentation (Beld et al., 1989; Johnson et al., 2001), recent studies suggest that the second cluster may be equally important in functional evolution (Fischer et al., 2003; Smith et al., 2013). Both areas are part of the substrate-binding site and interact with the B-ring hydroxyl groups that distinguish the classes of dihydroflavonols. The present study confirms the key role of the second cluster in DFR function. The 11 amino-acid deletion found in the *Dfr* allele from the white morph spans positions 216 to 226 in *V. vinifera* (Fig. 2B), and the effect of this mutation is to eliminate DFR activity on dihydroflavonols (Fig. 2C). Additional studies would be needed to determine whether this effect is due to a loss of substrate binding specifically or the overall deformation of the active site.

Possible consequences of loss of DFR function—Our surveys of natural populations and herbarium collections suggest that white-flowered individuals of *I. calycinum* are rare (only found in and around the town of Chiriboga, Ecuador). The rarity of this form could relate to direct effects of the loss-of-function mutation on plant–pollinator interactions. For example, pollinators may discriminate against white flowers because of innate preferences (Launau and Maier, 1995; Weiss, 1997) or longer handling times (Waser and Price, 1983). Innate preferences seem unlikely to play a major role in this system because the hummingbird pollinators of *I. calycinum* are known to visit a wide array of flower colors, including white, in *Iochroma* and in other Andean genera (Snow and Snow, 1980; Dziedzioch et al., 2003; Smith et al., 2008). White-flowered morphs of *Delphinium nuttalianum* have been found to incur longer handling times due to the loss of nectar guides (Waser and Price, 1983, 1985). The tubular blue flowers of *I. calycinum* are uniform in color (without apparent nectar guides), and the white form should present a strong contrast against the green background, arguing against inefficient foraging as an explanation.

An alternative explanation for the rarity of white is that the loss of DFR function has negative consequences beyond its effects on floral pigmentation. Since *Dfr* is a single-copy gene in Solanaceae (Des Marais and Rausher, 2008), any mutations in the coding region will affect its function in any tissue where it is expressed. However, we observed no anthocyanins in vegetative tissue (Appendix S6), suggesting that there may be little DFR activity outside the flower. The other major class of flavonoids that requires DFR function, condensed tannins (derived from leucoanthocyanidins), are not present in Solanaceae (Eich, 2008). Even though the sampled blue-flowered *I. calycinum* did not express anthocyanins in vegetative tissue, the ability to do so may still have adaptive significance. Anthocyanins are often expressed in vegetative tissues when plants are stressed, e.g., by temperature extremes or drought (Chalker-Scott, 1999), and several studies suggest that this response is adaptive (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Coberly and Rausher, 2003). *Iochroma* are long-lived shrubs, likely to experience environmental stresses over their lifetimes, and the inability to produce anthocyanins might limit the potential for white morphs to survive to flowering (typically 2–3 yr) and beyond.

Finally, the low frequency of the white morph may be due to neutral (nonselective) factors. For example, if the white allele arose only recently, it may still be at low frequency even if it were strongly favored by selection. Genetic drift could also play a role in shaping the frequency of color morphs, although no studies to date have found flower color variation to be neutral (reviewed in Rausher, 2008). There are multiple population genetic approaches for testing for selection against the white allele of *I. calycinum* (e.g., Slatkin, 2000), which could be used to examine the neutral hypothesis in future studies.

The genetic basis of within-species flower color polymorphisms—Despite the large number of pigmented species that exhibit white-flowered morphs (e.g., Epling and Dobzhansky, 1942; Waser and Price, 1981; Brown and Clegg, 1984; Levin

and Brack, 1995), relatively little is known about the exact genetic cause for these losses of pigmentation. To date, there are four cases in which the genetic basis of white-flowered morphs has been identified, and these involve an array of mutations affecting both coding and *cis*-regulatory regions of structural genes and transcription factors. The white morph of the arctic mustard *Parrya nudicaulis* is due to a *cis*-regulatory mutation in *Chs* (Dick et al., 2011), and a transposon insertion in the coding region of this gene causes white flowers in *Ipomoea purpurea* (Habu et al., 1998; Coberly and Rausher, 2003). White morphs of *I. purpurea* have also been associated with a loss-of-function allele (containing two deletions) at the *Ipmyb1* locus that encodes an R2R3-MYB transcription factor (Chang et al., 2005). Finally, a frameshift mutation causing a premature stop codon in DFR is responsible for white flowers in *Mimulus lewisii* (Wu et al., 2013). This study thus represents the second case of a loss-of-function mutation in DFR contributing to intraspecific flower color variation, although here the mutation is in-frame and likely acts by disrupting the active site. Overall, this is the third instance in which a loss-of-function mutation in a structural gene underlies white-flowered forms within species.

The wide variety of mutations involved in segregating flower color variation within populations stands in stark contrast with the pattern observed for fixed differences between species. In all cases (*Petunia axillaris*, several *Antirrhinum* species), evolutionary transitions from pigmented to white flowers have involved mutations in the R2R3-MYB transcription factors that regulate anthocyanin biosynthesis (Quattrocchio et al., 1999; Schwinn et al., 2006; Hoballah et al., 2007). This genetic convergence has been hypothesized to relate to the specificity of this class of transcription factors. Among this large gene family, one subgroup is specialized for regulating anthocyanins (Dubos et al., 2010), and different copies within this subgroup are often tissue-specific (Ramsay and Glover, 2005; Schwinn et al., 2006). Thus, mutations in the R2R3-MYBs have the potential to alter floral pigment production with minimal pleiotropic consequences for the production of other classes of flavonoids or flavonoid production in other tissues (Streisfeld and Rausher, 2011).

This contrast between the genetic basis of segregating flower color morphs and species-level differences suggests that a small and predictable subset of the mutations that give rise to white flowers within populations, namely those that affect R2R3-MYB genes, are preferentially fixed over longer evolutionary timescales (Stern and Orgogozo, 2008; Streisfeld and Rausher, 2011). Understanding the mechanism responsible for this predictability will require additional exploration of the genes underlying segregating white morphs and the selection acting on those variants. Cases where the nature of selection acting on white-flowered plants is already known (e.g., Kay, 1976; Waser and Price, 1983; Levin and Brack, 1995) would be ideal systems for these genetic studies. Given the diverse roles of flavonoids in plants (Harborne and Grayer, 1993; Winkel-Shirley, 2001), we expect that sources and strength of selection acting on mutations at different pathway genes will also be diverse. Indeed, strong purifying selection acting on some pathway loci (e.g., *Chi* and *F3h*) may explain why naturally occurring loss-of-function mutants have yet to be found. Targeting populations with exceptionally rare white morphs, as in the case of *I. calycinum*, may provide the best approach for discovering these "missing" mutants and characterizing the spectrum of genetic variants segregating in nature.

LITERATURE CITED

Beld, M., C. Martin, H. Huits, A. R. Stuitje, and A. G. M. Gerats. 1989. Flavonoid synthesis in *Petunia hybrida*- partial characterization of dihydroflavonol-4-reductase genes. *Plant Molecular Biology* 13: 491–502.

Brown, B. A., and M. T. Clegg. 1984. Influence of flower color on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea. Evolution* 38: 796–803.

Brugliera, F., G. Barri-Rewell, T. A. Holton, and J. G. Mason. 1999. Isolation and characterization of a flavonoid 3′-hydroxylase cDNA clone corresponding to the *Ht1* locus of *Petunia hybrida. Plant Journal* 19: 441–451.

Campanella, J., J. Smalley, and M. Dempsey. 2014. A phylogenetic examination of the primary anthocyanin production pathway of the Plantae. *Botanical Studies (Taipei, Taiwan)* 55: 10.

Chalker-Scott, L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* 70: 1–9.

Chang, S. M., Y. Lu, and M. D. Rausher. 2005. Neutral evolution of the nonbinding region of the anthocyanin regulatory gene *Ipmyb1* in *Ipomoea. Genetics* 170: 1967–1978.

Clegg, M. T., and M. L. Durbin. 2000. Flower color variation: A model for the experimental study of evolution. *Proceedings of the National Academy of Sciences, USA* 97: 7016–7023.

Coberly, L. C., and M. D. Rausher. 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: Amelioration of heat stress. *Molecular Ecology* 12: 1113–1124.

Cooley, A. M., J. L. Modliszewski, M. L. Rommel, and J. H. Willis. 2011. Gene duplication in *Mimulus* underlies parallel floral evolution via independent trans-regulatory changes. *Current Biology* 21: 700–704.

de Vetten, N., F. Quattrocchio, J. Mol, and R. Koes. 1997. The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. *Genes & Development* 11: 1422–1434.

Des Marais, D. L., and M. D. Rausher. 2008. Escape from adaptive conflict after duplication in an anthocyanin pathway gene. *Nature* 454: 762–765.

Des Marais, D. L., and M. D. Rausher. 2010. Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in *Ipomoea. Evolution* 64: 2044–2054.

Dick, C. A., J. Buenrostro, T. Butler, M. L. Carlson, D. J. Kliebenstein, and J. B. Whittall. 2011. Arctic mustard flower color polymorphism controlled by petal-specific downregulation at the threshold of the anthocyanin biosynthetic pathway. *Public Library of Science One* 6: e18230.

Dubos, C., R. Stracke, E. Grotewold, B. Weisshaar, C. Martin, and L. Lepiniec. 2010. MYB transcription factors in *Arabidopsis. Trends in Plant Science* 15: 573–581.

Dziedzioch, C., A. D. Stevens, and G. Gottsberger. 2003. The hummingbird plant community of a tropical montane rain forest in southern Ecuador. *Public Library of Science Biology* 5: 331–337.

Eich, E. 2008. Solanaceae and Convolvulaceae: Secondary metabolites: Biosynthesis, chemotaxonomy, biological and economic significance (a handbook). Springer, Berlin, Germany.

Endress, P. K. 1996. Diversity and evolutionary biology of tropical flowers. Cambridge University Press, Cambridge, UK.

Epling, C., and T. Dobzhansky. 1942. Genetics of natural populations. VI: Microgeographic races of *Linanthus parryae. Genetics* 27: 317–332.

Fischer, T. C., H. Halbwirth, B. Meisel, K. Stich, and G. Forkmann. 2003. Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductase from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. *Archives of Biochemistry and Biophysics* 412: 223–230.

Gigord, L. D., M. R. Macnair, and A. Smithson. 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. *Proceedings of the National Academy of Sciences, USA* 98: 6253–6255.

Griekspoor, A., and T. Groothuis. 2007. 4Peaks, version 1.7.1. Website http://www.mekentosj.com/4peaks/.

Grotewold, E. 2006. The genetics and biochemistry of floral pigments. *Annual Review of Plant Biology* 57: 761–780.

Habu, Y., Y. Hisatomi, and S. Iida. 1998. Molecular characterization of the mutable *flaked* allele for flower variegation in the common morning glory. *Plant Journal* 16: 371–376.

Halbwirth, H., S. Martens, U. Wienand, G. Forkmann, and K. Stich. 2003. Biochemical formation of anthocyanins in silk tissue of *Zea mays*. *Plant Science* 164: 489–495.

Harborne, J. B. 1998. Phytochemical methods: A guide to modern techniques of plant analysis, 3rd ed. Chapman and Hall, London, UK.

Harborne, J. B., and R. J. Grayer. 1993. Flavonoids and insects, 589–618. Chapman and Hall, London, UK.

Hoballah, M. E., T. Gubitz, J. Stuurman, L. Broger, M. Barone, T. Mandel, A. Dell'Olivo, et al. 2007. Single gene-mediated shift in pollinator attraction in *Petunia. Plant Cell* 19: 779–790.

Irwin, R. E. 2003. Impact of nectar robbing on estimates of pollen flow: Conceptual predications and empirical outcomes. *Ecology* 84: 485–495.

Johnson, E. T., S. Ryu, H. K. Yi, B. Shin, H. Cheong, and G. Choi. 2001. Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant Journal* 25: 325–333.

Kay, Q. O. N. 1976. Preferential pollination of yellow-flowered morphs of *Raphanus raphanistrum* by *Pieris* and *Eristalis* spp. *Nature* 261: 230–232.

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

Koes, R. E., C. E. Spelt, and J. N. Mol. 1989. The chalcone synthase multigene family of *Petunia hybrida* (V30): Differential, light-regulated expression during flower development and UV light induction. *Plant Molecular Biology* 12: 213–225.

Koes, R. E., C. Spelt, H. J. Reif, P. J. van den Elzen, E. Veltkamp, and J. N. Mol. 1986. Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. *Nucleic Acids Research* 14: 5229–5239.

Kuhn, B. M., M. Geisler, L. Bigler, and C. Ringli. 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. *Plant Physiology* 156: 585–595.

Lagerheim, G. 1891. Zurbiologie de *Iochroma macrocalyx. German Botanical Society* 9: 348–351.

Launau, K., and E. J. Maier. 1995. Innate color preferences of flower visitors. *Journal of Comparative Physiology, A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 177: 1–19.

Leonard, E., Y. Yan, J. Chemler, U. Matern, S. Martens, and M. A. G. Koffas. 2008. Characterization of dihydroflavonol 4-reductases for recombinant plant pigment biosynthesis applications. *Biocatalysis and Biotransformation* 26: 243–251.

Les, D. H., R. Whitkus, F. A. Bryan, and L. E. Tyrrell. 1989. Biochemical basis of floral color polymorphism in a heterocyanic population of *Trillium sessile* (Liliaceae). *American Journal of Botany* 76: 23–29.

Levin, D. A., and E. Brack. 1995. Natural selection against white petals in *Phlox. Evolution* 49: 1017–1022.

Maddison, D. R., and W. P. Maddison. 2005. MacClade 4: Analysis of phylogeny and character evolution, version 4.08a. Website http://macclade.org.

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

Mo, Y., C. Nagel, and L. P. Taylor. 1992. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proceedings of the National Academy of Sciences, USA* 89: 7213–7217.

Mol, J., E. Grotewold, and R. Koes. 1998. How genes paint flowers and seeds. *Trends in Plant Science* 3: 212–217.

Morita, Y., R. Saito, N. Tannikawa, K. Kuchitsu, T. Ando, M. Yoshikaawa, Y. Habu, et al. 2012. Tandemly arranged *chalcone synthase A* genes contribute to the spatially regulated expression of siRNA and the natural bicolor floral phenotype in *Petunia hybrida. Plant Journal* 70: 739–749.

Morita, Y., M. Saitoh, A. Hoshino, E. Nitasaka, and S. Iida. 2006. Isolation of cDNAs for R2R3-MYB, bHLH, and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. *Plant & Cell Physiology* 47: 457–470.

Olmstead, R. G., L. Bohs, H. A. Migid, E. Santiago-Valentin, V. F. Garcia, and S. M. Collier. 2008. A molecular phylogeny of the Solanaceae. *Taxon* 57: 1159–1181.

Peirson, S. N., J. N. Butler, and R. G. Foster. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Research* 31: e73.

Petit, P., T. Granier, B. L. d'Estaintot, C. Manigand, K. Bathany, J. M. Schmitter, V. Lauvergeat, et al. 2007. Crystal structure of grape dihydroflavonol 4-reductase, a key enzyme in flavonoid biosynthesis. *Journal of Molecular Biology* 368: 1345–1357.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29: e45.

Pollak, P. E., T. Vogt, Y. Mo, and L. P. Taylor. 1993. Chalcone synthase and flavonol accumulation in stigmas and anthers of *Petunia hybrida. Plant Physiology* 102: 925–932.

Quattrocchio, F., J. Wing, K. van der Woude, E. Souer, N. de Vetten, J. Mol, and R. Koes. 1999. Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell* 11: 1433–1444.

Ramsay, N. A., and B. J. Glover. 2005. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends in Plant Science* 10: 63–70.

Rausher, M. D. 2006. The evolution of flavonoids and their genes. *In* E. Grotewold [ed.], The science of flavonoids, 175–213. Springer, New York, New York, USA.

Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* 169: 7–21.

Richards, A. J. 1986. Plant breeding systems. G. Allen & Unwin, London, UK.

Ryan, K. G., E. E. Swinny, K. R. Markham, and C. Winefield. 2002. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. *Phytochemistry* 59: 23–32.

Schemske, D. W., and P. Bierzychudek. 2001. Perspective: Evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. *Evolution* 55: 1269–1282.

Schemske, D. W., and P. Bierzychudek. 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: Was Wright right? *Evolution* 61: 2528–2543.

Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences, USA* 96: 11910–11915.

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

Slatkin, M. 2000. Allele age and a test for selection on rare alleles. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 355: 1663–1668.

Smith, S. D., and D. A. Baum. 2006. Phylogenetics of the florally diverse Andean clade Iochrominae (Solanaceae). *American Journal of Botany* 93: 1140–1153.

Smith, S. D., and M. D. Rausher. 2011. Gene loss and parallel evolution contribute to species difference in flower color. *Molecular Biology and Evolution* 28: 2799–2810.

Smith, S. D., S. J. Hall, P. R. Izquierdo, and D. A. Baum. 2008. Comparative pollination biology of sympatric and allopatric Andean *Iochroma* (Solanaceae). *Annals of the Missouri Botanical Garden* 95: 600–617.

Smith, S. D., S. Wang, and M. D. Rausher. 2013. Functional evolution of an anthocyanin pathway enzyme during a flower color transition. *Molecular Biology and Evolution* 30: 602–612.

Snow, D. W., and B. K. Snow. 1980. Relationships between hummingbirds and flowers in the Andes of Colombia. *Bulletin of the British Museum (Natural History). Zoology, London* 38: 105–139.

SPELT, C., F. QUATTROCCHIO, J. MOL, AND R. KOES. 2002. ANTHOCYANIN1 of *Petunia* controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *Plant Cell* 14: 2121–2135.

STERN, D. L., AND V. ORGOGOZO. 2008. The loci of evolution: How predictable is genetic evolution? *Evolution* 62: 2155–2177.

STERN, D. L., AND V. ORGOGOZO. 2009. Is genetic evolution predictable? *Science* 323: 746–751.

STREISFELD, M. A., AND J. R. KOHN. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus. Journal of Evolutionary Biology* 20: 122–132.

STREISFELD, M. A., AND M. D. RAUSHER. 2009. Altered *trans*-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus. Molecular Biology and Evolution* 26: 433–444.

STREISFELD, M. A., AND M. D. RAUSHER. 2011. Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* 65: 629–642.

STREISFELD, M. A., W. N. YOUNG, AND J. M. SOBEL. 2013. Divergent selection drives genetic differentiation in an R2R3-MYB transcription factor that contributes to incipient speciation in *Mimulus aurantiacus. Public Library of Science Genetics* 9: e1003385.

SWOFFORD, D. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer, Sunderland, Massachusetts, USA.

TIFFIN, P., R. E. MILLER, AND M. D. RAUSHER. 1998. Control of expression patterns of anthocyanin structural genes by two loci in the common morning glory. *Genes & Genetic Systems* 73: 105–110.

VAN DER MEER, I. M., C. E. SPELT, J. N. MOL, AND A. R. STUITJE. 1990. Promoter analysis of the chalcone synthase (*chsA*) gene of *Petunia hybrida*: A 67 bp promoter region directs flower-specific expression. *Plant Molecular Biology* 15: 95–109.

VAN HOUWELINGEN, A., E. SOUER, J. MOL, AND R. E. KOES. 1999. Epigenetic interactions among three *dTph1* transposons in two homologous chromosomes activate a new excision-repair mechanism in *Petunia. Plant Cell* 11: 1319–1336.

VAN HOUWELINGEN, A., E. SOUER, K. SPELT, D. KLOOS, J. MOL, AND R. KOES. 1998. Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida. Plant Journal* 13: 39–50.

WARREN, J., AND S. MACKENZIE. 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytologist* 151: 237–241.

WASER, N. M., AND M. V. PRICE. 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii. Evolution; International Journal of Organic Evolution* 35: 376–390.

WASER, N. M., AND M. V. PRICE. 1983. Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii. Nature* 302: 422–424.

WASER, N. M., AND M. V. PRICE. 1985. The effect of nectar guides on pollinator preference: Experimental studies with a montane herb. *Oecologia* 67: 121–126.

WEISS, M. R. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Animal Behaviour* 53: 1043–1052.

WHITTALL, J. B., C. VOELCKEL, D. J. KLIEBENSTEIN, AND S. A. HODGES. 2006. Convergence, constraint and the role of gene expression during adaptive radiation: Floral anthocyanins in *Aquilegia. Molecular Ecology* 15: 4645–4657.

WINKEL-SHIRLEY, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology* 126: 485–493.

WU, C. A., M. A. STREISFELD, L. I. NUTTER, AND K. A. CROSS. 2013. The genetic basis of a rare flower color polymorphism in *Mimulus lewisii* provides insight into the repeatability of evolution. *Public Library of Science One* 8: e81173.

XIE, D. Y., L. A. JACKSON, J. D. COOPER, D. FERREIRA, AND N. L. PAIVA. 2004. Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from *Medicago truncatula. Plant Physiology* 134: 979–994.

APPENDIX 1. Vouchers and gene accession information. Vouchers are deposited at the University of Wisconsin-Madison Herbarium (WIS) and the Missouri Botanical Garden (MO).

Taxon; *Voucher*; Description; Location; GenBank accessions: *Chs*, *Chi*, *F3h*, *F3′h*, *F3′5′h*, *Dfr*, *Ans*.

Solanaceae. ***Iochroma cyaneum*** (Lindl.) M. L. Green; *Smith 265* (WIS); Treelet, 2 m, flowers blue; Cultivated at University of Wisconsin-Madison Greenhouses; KJ094348, KJ094350, KJ094352, KJ094353, KJ094354, GU595064 (from Smith and Rausher [2011]), KJ094351. ***I. calycinum*** Benth.; *Smith 471* (MO); Treelet, 2 m, flowers blue; Reserva Rio Guajalito, Chiriboga, Ecuador; KJ094360, KJ094355, KJ094347, KJ094344, KJ094346, JN593314 (from Smith et al. [2013], KJ094356. ***I. calycinum*** Benth.; *Smith 510* (MO); Treelet, 2 m, flowers white; Vicinity of Chiriboga, Ecuador; KF955587, KJ094342, KJ094358, KJ094343, KJ094345, KJ094357, KJ094359.